University of Alberta

Skeletal and Cardiac Muscle Energy Metabolism in Health and Disease

by

Miranda Mei-Yiu Sung

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Dedication

This thesis is dedicated to my wonderful family. To my parents – thank you for always believing in me and for all your unconditional love and support. I couldn't have done it without you. Thanks to God for His abundant grace and provision that sustained me throughout my PhD and made this achievement possible.

Abstract

Several lines of evidence show that obesity results in marked alterations in energy metabolism that contribute to the pathophysiology of several cardiovascular and metabolic disorders. As aging is an important risk factor for the development of these obesity-related diseases, this thesis demonstrated that there are physiological and metabolic changes that predispose middle-aged mice to developing insulin resistance and cardiomyopathy. We show that overall metabolic rate is reduced with aging and this is associated with impaired skeletal muscle activation of AMP-activated protein kinase (AMPK), a key regulator of energy metabolism. Furthermore, middle-aged mice fed a high fat diet develop more dramatic insulin resistance, as well as cardiac hypertrophy, than young mice. We show that these effects are due in part to increased skeletal muscle and heart expression of CD36, a key fatty acid transport protein. Indeed, ablation of CD36 can prevent middle-aged mice from developing these conditions. Moreover, increased CD36 levels in the heart and skeletal muscle were correlated with accumulation of potentially toxic lipid derivatives. These data support a link between lipotoxicity and skeletal muscle insulin resistance and cardiac hypertrophy. In addition to the role of AMPK in energy metabolism, it is also a known negative regulator of cardiac hypertrophy and we show that AMPK activation is markedly reduced in the hypertrophic middle-aged heart following high fat diet. Therefore, reduced AMPK activity may create a permissive environment for the activation of pro-hypertrophic signaling pathways in the

middle-aged heart. While calorie reduction is beneficial to combat the effects of obesity, reduced caloric intake also has been shown to be cardioprotective against myocardial ischemia-reperfusion injury. In this thesis we also show that promoting glucose metabolism may contribute to improved ischemic tolerance and functional recovery of hearts from mice following short-term calorie restriction. Together, these studies suggest that perturbations in energy metabolism are important contributing factors to the etiology of a number of cardiovascular and metabolic disorders and that strategies aimed at optimizing energy metabolism may have significant therapeutic potential in the treatment of these conditions.

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Abbreviations

ACC	acetyl CoA carboxylase
ACSL	long chain acyl-CoA synthetase
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide 1-β-ribofuranoside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AS160	Akt substrate of 160 kDa
ATP	adenosine 5'-triphosphate
BPM	beats per minute
BSA	bovine serum albumin
BW	body weight
CACT	carnitine-acylcarnitine translocase
CaMK	calmodulin-dependent protein kinase
cAMP	cyclic AMP
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoA	coenzyme A
COX	cyclooxygenase
CPT	carnitine palmitoyl transferase
CR	calorie restriction
CS	citrate synthase
CTE	cytosolic thioesterase
CV	calorific value
CVD	cardiovascular disease
DAG	diacylglycerol
2-DG	2-deoxyglucose

DGAT1	diacylglycerol acyltransferase 1
DN	dominant negative
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor-2
EF	ejection fraction
Erk	extracellular signal-regulated kinase
ETC	electron transport chain
FA	fatty acid
FABP	fatty acid binding protein
FACS	fatty acyl CoA synthetase
FAT/CD36	fatty acid translocase
FATP	fatty acid transport protein
F1,6BP	fructose 1,6-bisphosphate
F2,6BP	fructose 2,6-bisphosphate
F6P	fructose-6-phosphate
$FAD/FADH_2$	flavin adenine dinucleotide
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FDG	fluorodeoxyglucose
FS	fractional shortening
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT	glucose transporter
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
GC	gas chromatography
GP	glycogen phosphorylase
GS	glycogen synthase
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate

β-HAD	β-hydroxyacyl CoA Dehydrogenase
HF	high fat
HNE	4-hydroxy-2-nonenal
HOMA-IR	homeostasis model assessment of insulin resistance
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HR	heart rate
HW	heart weight
I.P.	intraperitoneal
I/R	ischemia-reperfusion
IR	insulin receptor
IRS	insulin receptor substrate
IVRT	isovolumic relaxation time
IVS	interventricular septum
3-KAT	L-3-ketoacyl CoA thiolase
KD	kinase dead
kDa	kilodalton
KO	knockout
LCACoA	long chain acyl CoA
LCAD	long chain acyl CoA dehydrogenase
LF	low fat
LPL	lipoprotein lipase
LPM	liters per minute
LV	left ventricular
LVEDD	LV end-diastolic diameter
LVESD	LV end-systolic diameter
LVPW	LV posterior wall
MAPK	mitogen-activated protein kinase
MCAD	medium-chain acyl-CoA dehyodrogenase
MCD	malonyl CoA decarboxylase

MI	myocardial infarction
mRNA	messenger ribonucleic acid
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
mTOR	mammalian target of rapamycin
NAD+/NADH	nicotinamide adenine dinucleotide
PBS	phosphate-buffered saline
PDH	pyruvate dehydrogenase
PDHP	pyruvate dehydrogenase phosphatase
PDK	pyruvate dehydrogenase kinase
PFK-1/2	phosphofructokinase-1/2
PGC-1α	peroxisome proliferator-activated receptor coactivator-1α
PH	pleckstrin homology
P_i	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidylinositol (4,5) bisphosphate
PIP3	phosphatidylinositol (3,4,5) trisphosphate
PKA	protein kinase A
PKB/ Akt	protein kinase B
PKC	protein kinase C
PP	protein phosphatase
PPAR	peroxisome proliferator-activated receptor
PSP	peak systolic pressure
RISK	reperfusion injury salvage kinase
RER	respiratory exchange ratio
ROS	reactive oxygen species
RICTOR	rapamycin insensitive companion of mTOR
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

SEM	standard error of the mean
SH2	src homology 2
SHR	spontaneously hypertensive rat
SIRT	sirtuin
SNARE	soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor
SPT-1	serine palmitoyl transferase-1
SSO	sulfo-N-succinimidyl oleate
T2D	type 2 diabetes
TCA	tricarboxylic acid
TG	triglyceride
TL	tibia length
TNF	tumor necrosis factor
TSC-2	tuberous sclerosis complex-2
UCP	uncoupling protein
UDP	uridine diphosphate
UDPG-PPL	UDP-glucose pyrophosphorylase
VAMP	vesicle associated membrane protein
VLCAD	very-long chain acyl CoA dehydrogenase
VLDL	very low-density lipoprotein
VCO_2	volume of carbon dioxide
VO_2	volume of oxygen
WISK	wortmannin-sensitive and insulin-stimulated protein kinase
WT	wild-type
WW	wet weight

CHAPTER 1.

Introduction

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Introduction

Cardiovascular Disease and Obesity

Cardiovascular disease (CVD) is the leading cause of death amongst Canadians accounting for nearly 31% of all mortality (30% of mortality in male and 31% of mortality in females) [1]. The most recent statistics from 2006 indicate that CVD was responsible for 69,019 deaths in Canada or the equivalent of one Canadian dying every seven minutes, and is estimated to cost the Canadian economy more than $22.2 billion dollars every year in physician costs, hospitalization costs, and lost wages [1]. Therefore, this adds to the urgency of understanding the underlying mechanisms responsible for the development and progression of CVD with the goal of improving treatment to reduce associated morbidity and mortality, as well as alleviate the tremendous burden these chronic diseases exert on the healthcare system. While there are numerous forms of CVD and there are multiples causes of these diseases, abnormal cardiac energy metabolism is known to be a key contributor to the etiology of many of these diseases, including cardiac hypertrophy, diabetic cardiomyopathy, myocardial infarction and ischemic heart disease (reviewed in [2]).

One of the major independent risk factors for developing CVD is obesity [3, 4]. With the prevalence of obesity increasing in almost all populations and age groups across the world, it has been described by the World Health Organization and American Heart Association as a global epidemic [3]. Unlike more traditional epidemics in history involving famine or infection, obesity is a problem of surplus. Traditionally, obesity has been defined as an excess body fat in relation to lean mass, which is associated with increased risk of morbidity and mortality, as well as reduced life expectancy [5, 6]. In the United States, statistics published earlier this year show that close to 68% of the population is considered overweight or obese, with similar statistics observed in Canada [7, 8]. It has been well established by several population cohort studies, in particular the Framingham Heart Study, that obesity is associated with a greater incidence of CVD, including coronary artery disease, heart failure and premature death [4, 9]. In addition, it is valuable to highlight that obesity is commonly associated with a cluster of co-morbidities as it also greatly increases the risk for developing insulin resistance, metabolic syndrome and type 2 diabetes (T2D), and these in turn increase the risk for CVD [10, 11]. Therefore, it is imperative to have a thorough understanding of the different mechanisms underlying the pathogenesis linking obesity to the development of insulin resistance/T2D and CVD. While the etiology of these diseases is complex and multifactorial, changes in energy metabolism have been proposed to be important contributors to the pathogenesis of these cardiovascular and metabolic disorders. As such, the goal of this thesis will be to investigate the mechanisms by which alterations in fatty acid and

glucose metabolism contribute to the development of obesity-related skeletal muscle insulin resistance and cardiomyopathy. In particular, since the aging population is the fastest growing segment of the population and the incidence of obesity, T2D and CVD all increase dramatically with advancing age [12, 13], studies in this thesis will focus on exploring the pathogenesis of obesity-related pathologies in the highly clinically relevant context of aging. Investigations herein will aim to determine whether alterations in energy metabolism that may occur in the normal process of aging may predispose middle-aged individuals to developing insulin resistance and cardiomyopathy. While the initial two studies presented in this thesis address how perturbations in energy substrate metabolism contribute to disease development and the negative effects of caloric excess, the last chapter will look at the other side of the coin using the novel dietary strategy of caloric restriction to modulate energy metabolism in order to benefit the heart. In this introductory chapter, energy metabolism mainly in the heart and skeletal muscle will be discussed, followed by a review of the role of altered fatty acid metabolism in the development of skeletal muscle insulin resistance and cardiac dysfunction. In addition, the dietary strategy of calorie restriction will be introduced and the beneficial effects of calorie restriction on the cardiovascular system will be discussed, as well as the mechanisms by which this may occur.

Energy Metabolism

The heart has a very high energy demand and as a result must produce considerable energy in the form of adenosine 5'-triphosphate (ATP). Indeed, the human heart produces between 3.5 to 5 kg of ATP per day in order to sustain proper contractile function and ionic homeostasis [14]. Under normal physiological conditions, the healthy adult heart derives >95 % of its ATP from mitochondrial oxidative phosphorylation, while the remainder is generated from glycolysis and GTP formation in the tricarboxylic acid (TCA) cycle [15]. Although the heart can utilize many different substrates to generate ATP, the normal healthy adult heart has a preference for fatty acids as a fuel substrate and obtains 50-70% of its ATP from the oxidation of fatty acids [16, 17].

Unlike the heart which is continuously contracting throughout life to pump blood throughout the body, skeletal muscle transitions through periods of rest and contraction (exercise). Under normal physiological conditions, similar to the heart, skeletal muscle displays a metabolic flexibility to switch between fatty acid and carbohydrate oxidation [18]. In the post-prandial state when glucose availability is high, skeletal muscle preferentially utilizes glucose as a fuel substrate. However, under conditions of fasting and exercise, there is an increased reliance on fatty acid oxidation which spares glucose utilization during fasting and delays consumption of glycogen during exercise [18-20]. The degree of substrate (carbohydrate or fatty acid) utilization also varies based on the skeletal muscle fiber type, diet as well as the duration and intensity of exercise and physical

training of the individual [19]. Carbohydrates (mainly glucose) and fatty acids are the preferred metabolizable substrates for both the heart and skeletal muscle and their metabolism will be discussed in more detail in the following sections.

Carbohydrate Metabolism

Glucose Uptake

The majority of glucose that is metabolized by the heart comes from glucose that is taken up from the circulation by facilitative glucose transporters (GLUT). While GLUT1 is largely found at the plasma membrane and is responsible for basal glucose uptake, GLUT4 translocates from intracellular stores to the sarcolemmal membrane in response to insulin stimulation, contraction or ischemia [21, 22]. As the intracellular concentration of glucose is normally low, glucose transporters facilitate glucose movement down its concentration gradient [22]. In the absence of insulin-stimulation, the majority (> 90%) of GLUT4 is located primarily in intracellular organelles, including the *trans*-Golgi network, recycling endosomes and insulin-responsive GLUT4-storage vesicles [23, 24]. It is likely that the microtubule network and actin cytoskeleton play a crucial role in GLUT4 trafficking, potentially by directing the movement of GLUT4-containing vesicles to the plasma membrane [25].

In the heart, several factors aside from circulating concentrations of glucose and insulin [26, 27] can influence glucose uptake, including availability of other energy substrates [28], catecholamine stimulation [29], AMP-activated protein kinase (AMPK) activation [30-32], workload of the heart [33], exercise [34] and

hypoxia/ischemia [21, 30, 35, 36]. The mechanisms behind modification of glucose uptake by these factors may include regulation of transcription of GLUTs, translocation of GLUTs to the plasma membrane from intracellular stores, or endocytosis of GLUTs and their recycling back to stores. In skeletal muscle, the two most physiological stimuli for increased glucose uptake and GLUT4 translocation are insulin stimulation and contraction (exercise) [37, 38]. The mechanism behind exercise-stimulated GLUT4 translocation appears to be distinct from that of insulin-stimulated pathways, and may involve the activation of AMPK, calmodulin-dependent protein kinases (CaMK) and/or protein kinase C (PKC) [38]. Once inside the cell, glucose is rapidly converted to glucose 6-phosphate (G6P) by hexokinase and effectively removed from the transmembrane gradient. From this point, G6P can be metabolized by multiple pathways that will be described in the following sections.

Glycolysis

Glycolysis converts one molecule of glucose into two molecules of pyruvate and produces a net gain of two molecules of ATP and two molecules of NADH. The reactions involved in this process are depicted in Figure 1-1. Rates of glycolysis can be affected by cardiac work [39] and oxygen supply and/or ischemia [40, 41]. Regulation of the glycolytic pathway occurs at multiple enzymatic steps including hexokinase, phosphofructokinase-1 (PFK-1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and pyruvate kinase.

As mentioned above, upon entry into the cell glucose is rapidly phosphorylated to G6P by the actions of hexokinase and this represents the first

irreversible step of glycolysis. In heart and skeletal muscle, the accumulation of G6P can in turn feedback and inhibit the hexokinase reaction, thereby decreasing glycolytic flux. Since the K_m of hexokinase is very low, the phosphorylation of glucose can occur even under very low concentrations of glucose, thus maintaining the glucose concentration gradient across the plasma membrane and ensuring sustained facilitative glucose transport into the cell [42].

Phosphoglucose isomerase catalyzes the next step in the glycolytic pathway converting G6P to fructose-6-phosphate (F6P), which is then converted to fructose 1,6-bisphophate (F1,6BP) by PFK-1 in a largely irreversible reaction that commits glucose to the glycolytic pathway. PFK-1 is under the regulation of multiple factors, making this a rate-limiting step of glycolysis. While ATP is a co-factor for PFK-1 activity, when cellular ATP levels are high ATP can bind to an allosteric site on PFK-1 and reduce the enzyme's affinity for F6P. Other negative allosteric effectors of PFK-1 include citrate, protons and phosphocreatine [41]. Whereas when energy levels are low, AMP, inorganic phosphate (P_i) and F6P act as positive allosteric regulators of PFK-1 [43]. Furthermore, F6P can be metabolized by 6-phosphofructo-2-kinase (PFK-2) into fructose 2,6-bisphosphate (F2,6BP), and although F2,6BP is not an intermediate in the glycolytic pathway it is a potent allosteric activator of PFK-1 by increasing the affinity of PFK1 for F6P and reducing the inhibitory effects of ATP on this enzyme [41, 43]. In the heart, PFK-2 activity is positively regulated by phosphorylation mediated by a number of different enzymes, including Akt, protein kinase A (PKA), AMPK and wortmannin-sensitive and insulin-stimulated protein kinase (WISK) in response to

increased workload, adrenaline or insulin, thus leading to the activation of PFK-2 and ultimately an increased rate of glycolysis [44-47]. Unlike in the heart, the skeletal muscle isoform of PFK-2 does not appear to be regulated by phosphorylation, but rather by the availability of F6P [43, 46].

The next major regulatory step in the glycolytic pathway is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which catalyzes the NAD^+-dependent oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate and produces one molecule of NADH. Next 1,3-bisphosphoglycerate undergoes several conversion steps as outlined in Figure 1.1. Pyruvate kinase catalyzes the final step of glycolysis by converting phosphoenolpyruvate to pyruvate, and producing two molecules of ATP per molecule of glucose metabolized. In both the heart and skeletal muscle, ATP as well as acetyl-Coenzyme A (CoA) and long-chain fatty acids can allosterically inhibit pyruvate kinase activity [42]. Negative allosteric inhibition of pyruvate kinase by ATP allows for parallel regulation with PFK-1, which is also inhibited by high ATP concentrations. Furthermore, F1,6BP, the product of the PFK-1 reaction discussed earlier, can allosterically activate pyruvate kinase by feed forward stimulation, which is advantageous in contracting muscle to enhance glycolytic flux.

Glycolysis normally contributes only a small proportion to the total ATP produced in the heart, however the contribution of glycolysis to ATP production is greater under conditions of ischemia or cardiac hypertrophy [48, 49]. In skeletal muscle, the contribution of glycolysis varies depending on skeletal muscle fiber type whereby fast-twitch type II b/d and IIx fibers are highly glycolytic and have

low fatigue resistance as compared to slow-twitch type I fibers and fast-twitch type IIa fibers, which have high mitochondrial density and oxidative capacity [50].

Glycogen Metabolism

Alternatively, G6P produced by hexokinase in the initial step of glycolysis can be stored within the cell in the form of glycogen, a compact, highly-branched polymer of glucose molecules that acts as an energy reserve to be utilized in times of need (i.e. fasting, exercise) [51, 52]. The pathway begins with the conversion of G6P into glucose 1-phosphorate (G1P) by the actions of phosphoglucomutase, followed by conversion of G1P to uridine disphosphate (UDP)-glucose mediated by UDP-glucose pyrophosphorylase (UDPG-PPL). Glycogen synthesis is mediated by the actions of three enzymes namely glycogenin, glycogen synthase (GS) and branching enzymes, which add glucosyl units creating tiers of glycogen until steric hindrance prevents any further addition of glucose by GS. While glycogen phosphorylase (GP) is the rate limiting enzyme in cytosolic glycogen degradation or glycogenolysis, and catalyzes the phosphorolysis of the α-1,4-glycosidic linkages on the outer chain of glycogen leading to the production of G1P and shortened glycogen [51, 52]. The G1P released by GP-mediated glycogenolysis is converted back into G6P by the actions of phosphoglucomutase, and this G6P can then re-enter the glycolytic pathway (as described in detail above). Since glycogen-derived G6P bypasses the hexokinase reaction of glycolysis, 3 molecules of ATP are generated as opposed to the 2 molecules of ATP normally produced from metabolism of glucose taken up from the blood.

Glucose Oxidation

The majority of pyruvate formed from glycolysis enters the mitochondria where it is decarboxylated by the pyruvate dehydrogenase (PDH) complex to produce acetyl CoA, CO_2, and NADH. Acetyl CoA then enters the TCA cycle for complete oxidation, where it produces reducing equivalents that act as electron donors for the ETC, generating the proton motive force that drives ATP production during mitochondrial oxidative phosphorylation.

The PDH complex is a highly organized multienzyme complex found in the mitochondrial matrix and is the rate limiting step of glucose oxidation that links glycolysis to the oxidative pathway of the TCA cycle. Since the PDH complex plays such an important role in maintaining glucose homeostasis, carbon flux through the PDH complex is tightly regulated by transcriptional controls, as well as short-term reversible phosphorylation/dephosphorylation control catalyzed by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatases (PDHP), respectively [53, 54]. The E1 subunit of the PDH complex catalyzes the irreversible and rate-limiting step of the PDH complex, and is the target of PDK and PDHP, whereby PDK-mediated phosphorylation at three specific serine residues on the α subunit of E_1 results in inhibition of PDH complex activity [53, 55]. In addition, a further level of regulation occurs at the level of PDK, where PDK activity is inhibited by high concentrations of pyruvate (produced from glycolysis or circulating lactate) and high levels of ADP, NAD^+, CoA and P_i (indicative of a low energy state), which would result in increased PDH complex activity [56, 57]. High levels of acetyl CoA and NADH, products from both the

PDH complex reaction and fatty acid β-oxidation, can activate PDK activity. In this manner, high levels of acetyl CoA and NADH produced from accelerated rates of fatty acid oxidation can lead to potent allosteric inhibition of PDH complex activity, in what is generally known as the "Randle cycle" of fatty acid-induced inhibition of glucose oxidation [55, 58, 59]. This will be discussed in more detail in the following section entitled "The Randle Cycle."

Insulin Signaling and Control of Glucose Metabolism

Circulating insulin acts to maintain blood glucose levels within the normal physiological range via its actions on peripheral tissues such as skeletal muscle, liver and adipose tissue [60]. In general, insulin stimulates glucose uptake in muscle and adipose tissue, while inhibiting hepatic glucose production or gluconeogenesis. Skeletal muscle is responsible for up to 75% of glucose disposal [61]. The insulin signaling cascade begins when insulin binds to the insulin receptor (IR) on the cell surface of target tissues. The IR is a heterotetrameric complex consisting of two extracellular α-subunits containing the insulin binding sites, and two transmembrane β-subunits, which possess intrinsic tyrosine kinase activity. In the basal state, the α-subunit allosterically inhibits the tyrosine kinase activity of the β-subunit. Binding of insulin to the α-subunit induces the auto-transphosphorylation of the IR, whereby one β-subunit phosphorylates the other at multiples tyrosine residues on the activation loop of the kinase domain, resulting in full catalytic activity of the kinase [25, 62]. The activated IR then phosphorylates tyrosine residues on a variety of intracellular scaffolding-type

substrates, one of the most important being the insulin receptor substrate (IRS) family of proteins. Of the four IRS isoforms identified, both IRS1 and IRS2 are known to play key roles in insulin signaling and insulin-stimulated glucose uptake in skeletal muscle. Indeed, both IRS1 and IRS2 knockout (KO) mice display peripheral insulin resistance [63-66]. While IRS proteins do not have intrinsic catalytic activity, insulin-stimulated tyrosine phosphorylation of IRS by the activated IR generates 'docking sites' that allow IRS proteins to interact with downstream signaling molecules that contain Src homology 2 (SH2) domains, which recognize specific phosphotyrosine motifs on the IRS proteins [62].

While there are multiple downstream effectors of IRS proteins, activation of phosphatidylinositol-3-kinase (PI3K) is essential in insulin-stimulated glucose uptake and GLUT4 translocation [60]. Interaction of phosphorylated IRS-1/2 with the p85 regulatory subunit of PI3K stimulates the activation of the p110 catalytic subunit of PI3K. At the membrane, PI3K primarily catalyzes the phosphorylation of phosphatidylinositol (4,5) bisphosphate (PIP_2) at the 3' position to produce phosphatidylinositol (3,4,5) trisphosphate (PIP_3) [25, 67]. Insulin-stimulated increases in PIP_3 lead to the membrane recruitment and activation of phosphoinositide-dependent kinase (PDK)-1 through its pleckstrin homology (PH) domain which binds with high affinity to PIP_3.

From this point in the insulin signaling cascade, PDK1 can activate two parallel pathways that result in increased GLUT4-mediated glucose uptake. In the first pathway, atypical protein kinase (PKC)ζ is recruited to the membrane through a hydrophobic motif which shares similarity to the PDK1-binding sites

found in other proteins [68]. PDK1-mediated phosphorylation of PKCζ at threonine 410 of the activation loop of the kinase domain is essential for PKC activation [69-71]. Although several lines of evidence support a key role for atypical PKCs, namely PKC λ and ζ, in insulin-stimulated glucose transport and GLUT4 translocation to the membrane in both adipocytes and skeletal muscle, the precise mechanisms that operate downstream of PKC that directly contribute to GLUT4 translocation are incompletely understood [72-76]. A recent study in muscle cells has suggested that PKCζ may participate in cytoskeletal actin remodelling which creates a scaffold for GLUT4-containing vesicles to interact with target-SNARE proteins on the plasma membrane [77]. Interestingly, PKCζ has been shown to co-localize with the GLUT4 compartment and induce serine phosphorylation of vesicle-associated membrane protein (VAMP)2/ synaptobrevin, which is associated with increased glucose uptake in rat skeletal muscle [78].

In the second pathway of insulin-stimulated glucose uptake, PDK1 activates protein kinase B (PKB)/Akt by phosphorylation of PKB/Akt at threonine 308 in the activation loop of the kinase domain. Full activation of PKB/Akt requires phosphorylation at both threonine 308 and a second site, serine 473, in the hydrophobic loop of the carboxy terminal [79]. The identity of the kinase that phosphorylates PKB/Akt at serine 473 has long been elusive, however, recent studies have suggested that the kinase responsible is the TORC2 protein complex, comprised of the mammalian target of rapamycin (mTOR) and rapamycin insensitive companion of mTOR (RICTOR) [79, 80]. Similar to PDK1, PKB/Akt

is recruited to the plasma membrane via its PH domain that binds to PIP_3. There is substantial evidence from gene silencing and overexpression models showing that PKB/Akt, in particular the Akt2 isoform, plays an essential role in promoting GLUT4 translocation to the membrane and glucose transport [81-84].

Fatty Acid Metabolism

Circulating Lipids and Fatty Acid Uptake

The heart and skeletal muscle have a limited potential for fatty acid synthesis and therefore rely heavily upon fatty acids supplied from the lipolysis of endogenous triglyceride (TG) stores or from exogenous circulating sources in the blood. Hydrophobic long chain fatty acids are present in the circulation either complexed with albumin or esterified in the lipid core of very-low density lipoproteins (VLDLs) and chylomicrons [85]. Upon arrival at target tissues, albumin-bound fatty acids are capable of dissociating from albumin, however, esterified TG-associated fatty acids found in chylomicrons and VLDL must first be hydrolyzed by lipoprotein lipase (LPL) at the luminal surface of the endothelium in order to release the non-esterified fatty acids prior to tissue utilisation [86, 87]. By a mechanism that is not completely understood, long chain fatty acids are transported across the endothelium. Once in the interstitial space, fatty acids are again bound to albumin for translocation through the aqueous environment to reach the plasma membrane of target myocytes [85].

Free fatty acids can enter the skeletal muscle or cardiac myocyte either by passive diffusion via a flip-flop mechanism of the fatty acids across the lipid

bilayer or by protein facilitated transport [88, 89]. The three major fatty acid transport proteins identified to date, include fatty acid translocase (FAT)/ CD36, the plasma membrane isoform of fatty acid binding protein ($FABP_{pm}$), and fatty acid transport protein (FATP) 1-6 [88]. $FABP_{pm}$ is bound peripherally to the outer leaflet of the plasma membrane and is proposed to bind both saturated and unsaturated long chain (≥14-C) fatty acids and concentrate them at the membrane surface for uptake by either passive diffusion or facilitated transport via FAT/CD36 or FATP 1-6 [88, 90]. Interestingly, inhibition of both FAT/CD36 and $FABP_{pm}$ has been shown not to produce an additive effect on FA uptake in the heart and skeletal muscle, suggesting that these two proteins may act together to facilitate long chain fatty acid transport across the plasma membrane [91]. The proposed theory for the mechanism of long chain fatty acid transport by FAT/CD36 and FATP 1-6 is that these transmembrane proteins can either act as acceptor molecules to concentrate fatty acids at the membrane or potentially the proteins themselves facilitate fatty acid transport across the phospholipid bilayer [88].

Fatty Acid Translocase (FAT)/ CD36

Of the three fatty acid transporters identified, FAT/CD36 is one of the most well-studied and has been shown to play an integral role in transport of fatty acids across the plasma membrane. The tissue distribution of FAT/CD36 show that it is highly expressed in tissues with a high metabolic capacity for fatty acids, such as heart, adipose tissue and skeletal muscle of rodents [92] and humans [93]. There is strong evidence for a key role for FAT/CD36 in mediating fatty acid uptake in

both heart and skeletal muscle. Indeed, studies in FAT/CD36 deficient mice have shown that FAT/CD36 is responsible for 50-80% of the fatty acids taken up by the heart and 40-75% of fatty acid uptake in skeletal muscle [94]. Previous work from our lab and others have found that inhibition of CD36-mediated fatty acid uptake via genetic deletion results in a marked 40-60% reduction in fatty acid oxidation in the heart, as well as decreased levels of TG esterification, suggesting that the degree of FAT/CD36-mediated fatty acid uptake also dramatically impacts fatty acid catabolism [95, 96]. Similar results have been observed in skeletal muscle, where fatty acid oxidation rates are severely blunted in oxidative muscle from FAT/CD36 null mice as compared to wild-type (WT) mice in both the basal and AICAR-stimulated states (26% and 60% lower net rates in null vs. WT, respectively) [97]. Moreover, patients with a genetic FAT/CD36 deficiency have markedly lower myocardial fatty acid uptake of a non-metabolizable radioactive fatty acid analogue [98-102], which is consistent with FAT/CD36 playing an essential role in regulating *in vivo* myocardial fatty acid catabolism in humans.

FAT/CD36, also known as glycoprotein IV, is a member of the class B scavenger receptor protein family and is expressed in numerous different cell types aside from cardiac and skeletal myocytes, including platelets and macrophages [88, 103]. The protein is anchored to the plasma membrane by two transmembrane hydrophobic regions at the carboxy and amino termini that are separated by a large extracellular hydrophilic region [104]. FAT/CD36 is heavily glycosylated (10 predicted N-linked glycosylation sites in the extracellular loop) which results in the observed molecular weight of 78-94 kDa by SDS-PAGE

despite a theoretical molecular weight of 53 kDa as predicted by the cDNA sequence [92, 105]. Glycosylation of FAT/CD36 has been shown to be necessary for proper trafficking to the plasma membrane [105]. Both the N- and C- terminal cytoplasmic tails of FAT/CD36 are also known to be palmitoylated, and this is thought to be necessary for intracellular trafficking and localization of FAT/CD36 to the plasma membrane, as deletion of either region results in retention in golgi-like or endoplasmic reticulum-like organelles [106-108]. Interestingly, FAT/CD36 has been shown to localize with cholesterol- and sphingolipid-rich lipid rafts, as well as in caveolae [106, 109, 110]. Co-localization of FAT/CD36 with caveolin-3, the predominant isoform found in the heart and skeletal muscle, suggests that caveolins may participate in targeting FAT/CD36 to the plasma membrane or may either directly or indirectly assist in fatty acid transport [109-111]. However, recent studies in caveolin-3 null mice did not reveal any alterations in cardiac fatty acid uptake or oxidation [112], therefore, a role for caveolins in mediating fatty acid uptake is still under investigation.

The function of FAT/CD36 in long chain fatty acid uptake is largely regulated by intracellular translocation and transcriptional control. Approximately 50% of FAT/CD36 in the heart [113] and skeletal muscle [114] is estimated to be stored in intracellular storage compartments, and must be translocated to the plasma membrane in order to actively participate in the transport of fatty acids. Physiological stimuli, including contraction, exercise and insulin, have been shown to induce the translocation of FAT/CD36 from intracellular depots to the plasma membrane in the heart [113, 115, 116] and

skeletal muscle [114, 117]. Indeed, short-term exercise and muscle contraction is associated with an increase in sarcolemmal FAT/CD36 to likely facilitate increased fatty acid transport and oxidation to meet the elevated energy demands with exercise [114, 116, 118]. The mechanisms of contraction and insulin inducing FAT/CD36 translocation may involve activation of the PI3K/Akt pathway and AMPK pathway, respectively [88]. Alternatively, endurance exercise training and/or chronic electrical stimulation experimentally is known to shift fuel selection at rest and during exercise towards fatty acid oxidation, and this elevated fatty acid uptake is associated with an increase in total FAT/CD36 expression [118, 119]. Similar to other proteins involved in lipid metabolism, expression of FAT/CD36 is under the transcriptional control of nuclear peroxisome proliferator-activated receptors (PPAR) [120].

Intracellular Fatty Acid Activation

Once in the cytosol, long chain fatty acids may either bind to the cytosolic form of FABP for transport through the cytosol or they are rapidly esterified and converted into fatty acyl-CoA esters in a two-step reaction mediated by long chain acyl-CoA synthetase (ACSL), also known as fatty acyl-CoA synthetase (FACS) [121, 122]. In a process known as 'vectorial acylation' it has been proposed that the rapid esterification of fatty acids essentially traps fatty acids inside the cell, in a similar fashion to trapping of glucose by conversion to G6P by the actions of hexokinase, thus maintaining the fatty acid concentration gradient across the plasma membrane. The fatty acyl-CoAs can enter several different pathways, including undergoing further esterification in the synthesis of TGs and

phospholipids or the fatty acid moiety can be transferred to carnitine and taken up by the mitochondria for β-oxidation [16, 123]. In addition, fatty acyl-CoAs in the cytosol may be hydrolyzed by cytosolic thioesterase 1 (CTE1), which acts to cleave the CoA moiety. The released non-esterified fatty acid can once again be esterified by ACSL isoforms found in different subcellular compartments, for example, at the mitochondria or in peroxisomes, and may channel these fatty acids into distinct pathways [124]. However, the concerted actions of ACSL and CTE1 can create a futile cycling of fatty acids at the expense of ATP and could contribute to impaired myocardial efficiency during diseased states, such as diabetic cardiomyopathy.

Mitochondrial Fatty Acid Uptake and the AMPK-ACC-Malonyl CoA Axis

Fatty acyl-CoA esters can be transported across the mitochondrial membrane via a carnitine–dependent shuttle system, consisting of three proteins namely carnitine palmitoyl transferase 1 (CPT1), carnitine:acylcarnitine translocase (CACT) and carnitine palmitoyl transferase 2 (CPT2) [125]. In brief, the first step involves CPT1 on the outer mitochondrial membrane catalyzing the conversion of long chain acyl-CoA ester to long chain acylcarnitine, which is then translocated across the inner mitochondrial membrane by CACT in exchange for carnitine. In the final step, CPT2, an enzyme found on the matrix side of the inner mitochondrial membrane, converts long chain acylcarnitines back to their respective long chain acyl-CoA esters where they then undergo β-oxidation in the mitochondrial matrix [125].

CPT1 is the rate-limiting enzyme controlling fatty acid uptake into the mitochondria, and as described above, catalyzes the conversion of activated fatty acids to long chain acylcarnitines and directs fatty acids to the β-oxidation pathway [126]. Regulation of CPT1 activity occurs both at the transcriptional level and more acutely via allosteric inhibition by endogenous malonyl CoA [125, 126]. Indeed, CPT1 mRNA levels are increased in response to fatty acid-induced PPARα activation in both heart and skeletal muscle [127, 128]. Malonyl CoA is a potent allosteric inhibitor of CPT1 and thus a key regulator of fatty acid transport into the mitochondria and subsequent β-oxidation in multiple tissues, including skeletal muscle [129-131] and heart [132]. Interestingly, total tissue concentrations of malonyl CoA in heart and skeletal muscle are estimated to be 10- to 100-fold higher (~5 μmol/L) than the IC_{50} for CPT1 inhibition (50-100 nmol/L) by malonyl CoA [133, 134], strongly suggesting that a significant amount of malonyl CoA must be sequestered in intracellular compartments and inaccessible to CPT1, otherwise fatty acid oxidation would constantly be inhibited. The turnover of malonyl CoA in the heart is rapid with a $t_{1/2}$ of ~1.25 min [135], therefore malonyl CoA concentrations are tightly regulated by the balance between its synthesis and degradation.

Malonyl CoA is synthesized primarily by the enzyme acetyl CoA carboxylase (ACC) which catalyzes the carboxylation of acetyl CoA to form malonyl CoA [134, 136]. Two mammalian isoforms of ACC exist, ACC-α and ACC-β, with cytosolic ACC-α being the most abundant in lipogenic tissues, such as liver and adipose tissue, while mitochondrial localized ACC-β is the

predominant isoform found in oxidative tissue such as the heart and skeletal muscle [134, 137, 138]. This tissue specific isoform distribution suggests that ACC-α provides malonyl CoA more selectively for *de novo* lipogenesis, while malonyl CoA produced by ACC-β may be more involved in the regulation of fatty acid oxidation [126, 134]. Indeed, several studies have demonstrated that ACC activity is inversely correlated with fatty acid oxidation in the heart [137, 139-141] and skeletal muscle [131, 142, 143]. Furthermore, ACC-β deficient mice have markedly increased fatty acid oxidation rates in skeletal muscle and a concomitant reduction in malonyl CoA levels, confirming that ACC-β is an important regulator of fatty acid oxidation in skeletal muscle [144, 145]. Interestingly, a recent study in muscle-specific ACC-β KO mice showed similar rates of fatty acid oxidation and malonyl CoA levels between WT and KO mice [146]; however, this may have been the result of compensatory reductions in skeletal muscle malonyl CoA decarboxylase (MCD) and CPT1 in these mice.

The activity of ACC is regulated by allosteric effectors and phosphorylation and dephosphorylation control. Long chain fatty acyl CoAs can allosterically inhibit ACC, which may be an important regulatory mechanism during fasting to permit fatty acid oxidation when circulating fatty acids levels are elevated [134]. Phosphorylation of ACC-α and -β by AMPK is a key determinant of ACC activity in the heart and skeletal muscle [147-150], as phosphorylation leads to an almost complete loss of ACC activity [132, 151, 152]. Furthermore, both ACC isoforms have been found to co-purify with the α2 isoform of the catalytic subunit of AMPK [151], suggesting a tight association between ACC and AMPK in the

heart. Several lines of evidence show an inverse relationship between AMPK activity and ACC activity, whereby increases in activation of AMPK are associated with reductions in ACC activity and malonyl CoA levels, and subsequently increased fatty acid oxidation [126, 134]. AMPK mediated regulation of ACC activity is particularly important in the setting of exercise in skeletal muscle [143, 153, 154] or myocardial ischemia [132, 152, 155, 156], where increases in the ADP/ATP and AMP/ATP ratio lead to activation of AMPK and increases in fatty acid utilization.

Malonyl CoA levels are also regulated by the rate of its degradation by MCD, which catalyzes the decarboxylation of malonyl CoA back into acetyl CoA. In humans, MCD is highly expressed in the heart and skeletal muscle [157]. Interestingly, MCD activity has been shown to be in 50-fold excess over that of ACC in the heart [132, 158], suggesting that MCD activity must be tightly controlled and largely inaccessible to intracellular malonyl CoA. Several conditions where fatty acid oxidation is elevated are also associated with increased MCD activity in the heart, including fasting, diabetes, maturation of the newborn heart and ischemia-reperfusion (I/R) injury [132, 158-161]. Similarly, in skeletal muscle increased MCD activity is positively correlated with increased fatty acid oxidation rates [153, 161-163]. Moreover, a direct role for MCD in regulating skeletal muscle lipid and glucose metabolism was confirmed in human skeletal muscle myotubes where knockdown of MCD using small interfering-RNA inhibited fatty acid oxidation while reciprocally increasing glucose metabolism [164].

In the past few years, evidence has suggested that FAT/CD36 also resides on the outer mitochondrial membrane and may regulate skeletal muscle fatty acid transport into the mitochondria for subsequent oxidation [165-167]. Contrary to this, some studies have failed to show co-localization of FAT/CD36 with the mitochondria [168, 169]. While initial studies showed that the putative FAT/CD36 inhibitor sulfo-N-succinimidyl oleate (SSO) could inhibit fatty acid oxidation by ~80% in isolated mitochondria [166], these results have recently been disputed as SSO may reduce fatty acid oxidation via indirect effects on the mitochondrial respiratory chain [170]. Moreover, isolated mitochondria from cardiac and skeletal muscle from WT and FAT/CD36-null mice have similar rates of fatty acid oxidation [171], thus arguing against an important role for FAT/CD36 in directly regulating mitochondrial fatty acid uptake and oxidation.

Fatty Acid Oxidation

Fatty acid β-oxidation occurs primarily in the mitochondria, with a smaller proportion occurring in peroxisomes. In brief, peroxisomes are responsible for oxidation of very long chain fatty acids, and although peroxisomes possess all the enzyme machinery to fully oxidize fatty acids, they only partially oxidize fatty acids and the products are then further metabolized in the mitochondria [172]. Only mitochondrial β-oxidation will be discussed in depth in this thesis. Once in the mitochondrial matrix, long chain acyl CoAs undergo β-oxidation where one cycle of fatty acid oxidation results in the shortening of the chain length of the fatty acyl CoA moiety by two carbons, as well as the production of acetyl-CoA and the reduced forms of NADH and flavin adenine dinucleotide ($FADH_2$)

(Figure 1-2). The metabolism of fatty acyl CoA esters involves the sequential enzymatic actions of acyl CoA dehydrogenase, enoyl CoA hydratase, L-3-hydroxylacyl CoA dehydrogenase, and L-3-ketoacyl CoA thiolase (3-KAT). In the final reaction, 3-KAT splits 3-ketoacyl-CoA to produce acetyl CoA and a fatty acyl CoA that is shortened by two carbons [42, 52]. This sequence of reactions continues until the original fatty acyl CoA has been completely metabolized to form acetyl CoA. Various isoforms exist of each of the four β-oxidation enzymes that have different chain-length specificities, for example, medium chain acyl CoA dehydrogenase (MCAD) and very-long chain acyl CoA dehydrogenase (VLCAD). Importantly, each of these enzymes is subject to feedback inhibition by the products of the enzymatic reactions, such as $FADH_2$ and NADH. In particular, 3-KAT can be inhibited by an accumulation of acetyl CoA, which is an important regulatory mechanism in times of low energetic demand where a decrease in ETC and TCA cycle activity leads to the accumulation of acetyl CoA, NADH, and $FADH_2$ that then can feedback and inhibit β-oxidation enzymes. Furthermore, increased levels of acetyl CoA and NADH coming from the PDH complex can also directly inhibit fatty acid oxidation. Therefore, the rate of β-oxidation is controlled by energy demand and substrate supply. In addition, β-oxidation enzymes are under a high degree of transcriptional regulation mainly by nuclear PPAR and peroxisome proliferator activated receptor γ coactivator-1 (PGC1) α (reviewed in [173]).

The Tricarboxylic Acid Cycle

The acetyl CoA produced from both glucose and fatty acid oxidation then enter a common pathway, known as the TCA cycle for further metabolism. Acetyl CoA undergoes a series of reactions (Figure 1-3) resulting in the production of one molecule of GTP, three molecules of NADH and one molecular $FADH_2$, which will subsequently be utilized by the ETC to produce several molecules of ATP during oxidative phosphorylation. The regeneration of a molecule of oxaloacetate in the final reaction allows the TCA cycle to continue [42].

The rate of flux through the TCA cycle is under tight regulation and governed by the availability of substrate for citrate synthase (oxaloacetate and acetyl CoA) and feedback inhibition by accumulating products. Activity of the TCA cycle increases with workload, primarily due to changes in the $NADH/NAD^+$ ratio in the mitochondria, which regulates the activity of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase of the TCA cycle [14]. During times of energy sufficiency, a high ratio of $NADH/NAD^+$ acts to inhibit these enzymes and limit flux through the TCA cycle. Citrate, as well as ATP, can feedback and inhibit citrate synthase activity [42].

Oxidative Phosphorylation

The reducing equivalents or hydrogen carriers NADH and $FADH_2$, produced from the metabolism of acetyl CoA in the TCA cycle in the mitochondrial matrix, are oxidized in the ETC for ATP synthesis, in a process known as oxidative phosphorylation. There are four complexes of the respiratory

chain, namely NADH-ubiquinone oxidoreductase (Complex I); succinate dehydrogenase (Complex II); ubiquinone-cytochrome c oxidoreductase (Complex III); and cytochrome oxidase (Complex IV), and these are embedded in the inner mitochondrial membrane. Together, the oxidation of NADH produces the energy to pump 10 protons from the mitochondrial matrix into the intermembrane space, while oxidation of $FADH_2$ will result in production of 6 protons. Since the inner mitochondrial membrane is impermeable to protons, this leads to proton accumulation in the intermembrane space making the mitochondrial matrix more negatively charged as compared to the intermembrane space [174]. ATP synthase couples the flow of protons down the electrochemical gradient back into the mitochondrial matrix with ATP synthesis. Usually oxidation and phosphorylation of ADP are tightly coupled; therefore the rate of mitochondrial respiration can be controlled by the availability of ADP. However, uncoupled respiration or 'proton leak' does exist, whereby protons are transported across the inner mitochondrial membrane that are not coupled to ATP production, as evidenced in isolated mitochondria which even in the absence of ADP continue to consume oxygen. Uncoupling proteins (UCP) are a mechanism by which protons can re-enter the mitochondrial matrix and bypass ATP synthase, and act to dissipate the electrochemical gradient and lower the mitochondrial membrane potential [175].

The Randle Cycle

An inverse relationship exists where high rates of fatty acid oxidation are known to inhibit glucose oxidation and vice versa, in a 'glucose-fatty acid' cycle also known as the Randle cycle [58]. Elevated fatty acid oxidation results in an

increase in the mitochondrial ratios of NADH/NAD^+ and acetyl-CoA/CoA, and as mentioned earlier NADH and acetyl-CoA can allosterically inhibit the PDH complex, the rate limiting enzyme in the glucose oxidation pathway. Furthermore, increases in these ratios also stimulate PDK, the enzyme responsible for phosphorylating and inhibiting PDH activity [59]. Accumulation of citrate produced from the TCA cycle as a result of increased fatty acid oxidation can exit the mitochondria via the tricarboxylate carrier and inhibit PFK-1 of the glycolytic pathway [176, 177]. On the reciprocal side, accumulation of acetyl CoA produced from increased glucose oxidation can be a substrate for ACC for the synthesis of malonyl CoA and subsequent inhibition of CPT1, the key regulatory step controlling fatty acid transport into the mitochondria. Almost 50 years later, the Randle cycle continues to be an area of great research interest and is known to play vital roles in the etiology of both skeletal muscle insulin resistance and myocardial ischemia, which will be discussed further in following sections.

AMP-Activated Protein Kinase

AMPK is a cellular fuel gauge that is ubiquitously expressed in mammalian cells that turns on ATP-generating (catabolic) pathways and turns off ATP-consuming (anabolic) pathways in response to energetic stress (i.e. increase in AMP/ATP ratio and creatine/ phospho-creatine ratio) (reviewed in [148, 178]). AMPK is a key regulator of energy metabolism in the heart and its activation is linked to increased rates of fatty acid oxidation [132, 137, 139, 152, 156] and

glycolysis [46], as well as increased glucose uptake [179]. AMPK is a heterotrimeric enzyme composed of a catalytic α subunit and β and γ regulatory subunits [178, 180]. Each subunit has at least two isoforms (α_1/α_2; β_1/β_2; $\gamma_1/\gamma_2/\gamma_3$) with varying tissue distribution [178, 181-183]. While the α_1-subunit is ubiquitously expressed, the α_2 subunit is predominantly expressed in the heart and skeletal muscle and accounts for a greater proportion of AMPK activity over the α_1 subunit [184-187]. In a similar fashion, the β_1 subunit is widely expressed but the β_2 subunit is mainly expressed in the heart and skeletal muscle [188, 189]. For the γ-subunit, the γ_1 isoform is ubiquitously expressed and in the heart the majority of AMPK activity is associated with AMPK complexes that contain the γ_1-subunit [184], whereas the γ_3 isoform is only expressed in skeletal muscle [190]. The β regulatory subunit is thought to act as a scaffolding protein and the glycogen binding site may be important in targeting of the AMPK complex. The γ regulatory subunit is able to bind ATP and AMP, thus conferring the AMP sensitivity of the complex and allows AMPK to detect the energetic status of the cell. As the name suggests, AMP is thought to bind to the two tandem Bateman domains of the γ-subunit of AMPK to induce a conformational change in the enzyme which allows phosphorylation of residue threonine 172 within the "activation loop" of the kinase domain of the α catalytic subunit leading to the activation of AMPK [191, 192]. Furthermore, the conformational change inhibits dephosphorylation of residue threonine 172 by protein phosphatase 2C [192, 193]. Several upstream AMPK kinases have been identified so far, including LKB1

[194], calcium-calmodulin-dependent protein kinase kinase β [195] and TGF-beta activated kinase-1 [196].

AMPK Regulation of Energy Metabolism

As mentioned previously, AMPK has a number of effects on both fatty acid and glucose metabolism. AMPK is capable of increasing fatty acid availability and uptake via recruitment of LPL to the coronary lumen [197] and expression and translocation of FAT/CD36 from intracellular depots to the plasma membrane [116]. Moreover, AMPK activation also increases fatty acid oxidation through the phosphorylation (serine 79) and inhibition of ACC. As previously mentioned, ACC is responsible for synthesis of malonyl CoA, therefore AMPK-mediated inhibition of ACC reduces malonyl CoA levels and relieves the inhibition on CPT-1, thus accelerating fatty acyl CoA entry into the mitochondria for oxidation [132, 151, 152]. Therefore, AMPK is a critical regulator of fatty acid utilization via its multiple actions to promote fatty acid delivery, fatty acid transport and fatty acid oxidation. AMPK regulates glucose utilization at several different steps in the heart and skeletal muscle including at the level of glucose uptake by promoting GLUT4 expression and translocation to the sarcolemma [30, 31], though not in glycogen replete hearts [198]. Furthermore, AMPK activation promotes glycolysis by the direct phosphorylation and activation of PFK-2 [46, 199], as well as potentially regulates glycogen metabolism [200-202]. In addition to acute metabolic effects, AMPK is also known to directly and indirectly regulate the transcription of several genes involved in glucose and lipid metabolism and mitochondrial biogenesis via the modulation of a number of transcription factors

and co-activators, including PGC1α and PPARs [203, 204]. Indeed, activation of AMPK has been shown both *in vitro* and *in vivo* to be associated with increased fatty acid oxidation and expression of PPARα target genes (i.e. CPT1) involved in lipid transport, storage and oxidation [205].

Regulation of Cardiac Hypertrophy by AMPK

Cardiac hypertrophy is characterized by an increase in cardiac myocyte cell size and myocardial remodelling [344] and is associated with several changes at the molecular level, including alterations in gene expression, increased mRNA translation to increase protein synthesis and altered cardiac energy metabolism [345-347]. The precise role that AMPK plays in the development of hypertrophy is still under debate. Indeed, mutations in the γ subunit of AMPK that result in AMPK activation are associated with cardiac hypertrophy [206-208], as well increased AMPK activity has been observed in a model of pressure overload-induced hypertrophy in rat hearts [209]. However, studies from our lab and others have shown that activation of AMPK can in fact inhibit cardiac hypertrophy [210-212]. A necessary mediator of cardiac myocyte growth is enhanced protein synthesis, and AMPK is known to phosphorylate and inhibit enzymes involved in translation initiation and peptide-chain elongation, including eukaryotic elongation factor-2 (eEF2) kinase and tuberous sclerosis complex-2 [213, 214]. Furthermore, AMPK can regulate the activity of mTOR, a kinase that plays a central role in cell growth and proliferation [215, 216], as well as indirectly modulate the downstream targets of mTOR, including p70S6 kinase (p70S6K) [217] and 40S ribosomal protein S6 [218]. Previous studies from our laboratory

have shown that Akt can negatively regulate AMPK in the heart, therefore one mechanism by which Akt may promote hypertrophic growth in the heart is to inhibit AMPK to create a permissive environment for enhanced protein synthesis [219]. Indeed, pharmacological activation of AMPK with metformin and AICAR can prevent Akt-induced cardiac myocyte hypertrophy, which is associated with decreased p70S6K phosphorylation and increased phosphorylation of eEF2, and reduced protein synthesis [211]. It has been shown in adiponectin-deficient mice which have diminished AMPK activity, that these mice are more susceptible to pressure overload-induced hypertrophy [212]. Moreover, increased oxidative stress and reduced activation of the LKB1/AMPK pathway is linked to hypertrophy in spontaneously hypertensive rat hearts [220]. Taken together, these data suggest that AMPK plays an important role in the regulation of cardiac hypertrophy; however, further studies are required to confirm whether reduced AMPK activity increases the susceptibility to hypertrophic growth stimuli in other models.

Interestingly, it has been shown that there are age-associated reductions in the activation of AMPK in skeletal muscle by exercise and pharmacological activators [221]. As AMPK is a key regulator of energy metabolism and hypertrophic growth, these data suggest that reductions in AMPK activation may contribute to the development of several age-related diseases. Studies in this thesis were undertaken to elucidate the potential role of age-associated alterations in AMPK and energy substrate metabolism and their contribution to the

development of skeletal muscle insulin resistance and T2D, as well as obesity-related cardiomyopathy, in the middle-aged population.

Skeletal Muscle Insulin Resistance

Insulin Resistance and Type 2 Diabetes

Insulin resistance is typically defined as a state of reduced responsiveness of insulin-sensitive tissues to normal circulating levels of insulin. It is generally accepted that insulin resistance is a characteristic feature of T2D and precedes the development of β-cell dysfunction and frank T2D by nearly a decade [222-225]. Furthermore, insulin resistance in the offspring of individuals with T2D is one of the best predictors for the development of T2D in the future [226]. Apart from its fundamental role in the pathophysiology of T2D, insulin resistance is also linked to a wide-array of other metabolic and CVDs, including metabolic syndrome, hypertension and atherosclerosis [11, 227, 228]. While the pathogenesis of T2D is complex and multifactorial, the three primary defects associated with T2D are: 1) decreased glucose uptake into the skeletal muscle and adipose tissue, 2) increased hepatic glucose production secondary to reduced ability of insulin to inhibit gluconeogenesis and 3) impaired pancreatic β-cell insulin secretion [222, 229, 230]. While insulin resistance is known to precede T2D, not all obese, insulin-resistant individuals develop T2D. The key event that appears to trigger the transition from the insulin resistant state into overt glucose intolerance and T2D is

β-cell failure, characterized by loss in β-cell mass and deterioration of β-cell function (i.e. glucose-stimulated insulin secretion) [231].

Early during the course of insulin resistance the β-cell is able to compensate for peripheral insulin resistance in skeletal muscle and adipose tissue by increasing insulin secretion in order to maintain normal blood glucose levels [223]. Hepatic insulin resistance also contributes to fasting and/or postprandial hyperglycaemia as a result of reduced hepatic glucose uptake and failure of insulin to suppress gluconeogenesis and hepatic glucose production [232]. In addition to reduced glucose uptake in adipose tissue, insulin resistance in this tissue also results in increased lipolysis contributing to elevated circulating plasma fatty acid levels and hyperlipidemia, which further negatively impacts insulin responsiveness and promotes ectopic lipid accumulation in peripheral tissues, as well as adversely effects pancreatic β-cell function [223]. Over time hypersecretion of insulin by the β-cell can result in progressive β-cell failure resulting in worsening glucose intolerance and ultimately the progression to overt T2D [231, 233].

As skeletal muscle serves as the primary site of insulin-stimulated glucose uptake and accounts for roughly 70-80% of postprandial glucose disposal [234, 235], defects in glucose clearance by this tissue play a major role in impaired glucose homeostasis in patients with T2D. Although the precise mechanisms that cause skeletal muscle insulin resistance are not fully understood, there has been significant progress identifying the molecular signaling pathways that are impaired in the insulin resistant state and potential culprits. Multiple factors have

been implicated in the pathogenesis of this disease including oxidative stress [236, 237], inflammation involving cytokines such as tumor necrosis factor (TNF)-α [238, 239], adipokine (leptin, adiponectin) resistance [240, 241] and intracellular skeletal muscle lipid accumulation [242-245]. The latter theory has received a great deal of attention in regards to the development of skeletal muscle insulin resistance and will be discussed in depth in this thesis.

Intramuscular Lipid Metabolite Accumulation and Insulin Resistance

One of the prevailing theories regarding the mechanism of skeletal muscle insulin resistance involves the intracellular accumulation of potentially damaging fatty acid intermediates, such as TG, long chain acyl CoA (LCACoA), diacylglycerol (DAG) and ceramide, which are known to interfere at multiple points within the insulin signaling cascade to impair insulin-stimulated glucose uptake [244, 246, 247]. Indeed, a strong inverse correlation exists between intramyocellular lipid accumulation and skeletal muscle insulin sensitivity under conditions of both acute and chronic lipid exposure, suggesting that excessive fatty acid storage plays a critical role in mediating skeletal muscle insulin resistance [248-250]. As mentioned previously, multiple lipid species have been implicated as potential mediators of insulin resistance in skeletal muscle, however, it remains unclear which lipid intermediate is primarily responsible for impairing insulin sensitivity. Numerous studies have attempted to address this topic using genetic models of obesity and T2D in rodents, as well as diet-induced obesity models, but so far no consensus has been reached as each study shows accumulation of different lipid species. This may indicate that lipid-induced

insulin resistance can be mediated by several different fatty acid intermediates or that the role of each species varies depending on the diabetic model. Regardless, these lipid intermediates appear to converge on similar targets in the insulin signaling cascade to impair insulin responsiveness of skeletal muscle.

As described above, insulin binding to its receptor results in the initiation of a complex cascade whereby tyrosine phosphorylation of IRS-1 and -2 is essential in activation of downstream target PI3K and subsequent GLUT4 translocation [62]. However, it has been shown in multiple cell types and tissues that phosphorylation of specific serine and/or threonine sites on IRS-1 and -2 impairs insulin signaling by inhibiting insulin receptor-mediated tyrosine phosphorylation [251]. Much investigation has focused on novel PKC isoforms as being the primary serine/threonine kinase responsible for mediating insulin resistance [252]. Indeed, PKC activation has been observed during acute lipid infusion in humans and rodents [251, 253, 254], as well as in diabetic patients and animal models of high fat (HF) feeding [255-257]. It is well established that classical and novel PKC isoforms are activated by lipid intermediates, mainly DAG, therefore making this pathway an ideal candidate in skeletal muscle insulin resistance [252, 258]. Moreover, PKC θ KO mice are protected against fat-induced insulin resistance in skeletal muscle following acute lipid infusion [259]. Similar protection to HF diet-induced insulin resistance has also been observed in PKC δ and ε KO mice [260], supporting a role for novel PKC isoforms as important mediators of lipid-induced insulin resistance. In addition to inhibition of IRS, ceramides are capable of inhibiting Akt/PKB by two separate mechanisms.

Firstly, ceramides can directly activate PP2A to dephosphorylate Akt/PKB [261-263], and secondly they can activate PKCζ which phosphorylates residue threonine 34 in the PF domain of Akt/PKB and prevents its translocation to the plasma membrane [264, 265]. Furthermore, LCACoAs have been found to inhibit hexokinase, the first enzyme of glycolysis, in a concentration-dependent manner in both human and rodent skeletal muscle, which may also be an alternative route by which LCACoAs can directly regulate glucose metabolism and impair insulin-stimulated glucose uptake [266]. Although elevated intramuscular LCACoA levels are strongly correlated with insulin resistance, since increased levels of CoA esters are often accompanied by increases in DAG and ceramides, it is not clear whether the fatty acyl CoA esters themselves interfere with insulin signaling or act as a precursor for DAG and ceramide synthesis.

Mitochondrial Dysfunction in the Development of Skeletal Muscle Insulin Resistance

While it is generally accepted that the intramuscular accumulation of lipids contributes to obesity-related insulin resistance, the precise mechanism by which these lipids build-up is the topic of much debate. There are traditionally two schools of thought on this topic, lipids accumulate as a result of either impaired fatty acid utilization and/or increased fatty acid delivery and uptake that exceeds the capacity for its oxidation. A growing body of evidence suggests that lipid accumulation and insulin resistance develop secondary to reduced mitochondrial fatty acid oxidation [246, 267-273]. Indeed, using ^{31}P-MRS Shulman *et al.* found that reductions in mitochondrial substrate oxidation and ATP production were

associated with intramyocellular lipid accumulation in young, lean insulin-resistant offspring of parents with T2D [274, 275]. This same group also proposed that impaired mitochondrial function and oxidative capacity observed in children of patients with T2D may be the result of an inherited reduction in mitochondrial content and/or oxidative protein expression, which predisposes these individuals to intramuscular lipid accumulation and impaired insulin signaling [271]. These findings have contributed to the proposal that accelerating fatty acid oxidation in skeletal muscle may be a beneficial approach to ameliorate the accumulation of these potentially harmful lipid intermediates, as well as restore insulin sensitivity [276]. Indeed, studies have shown that increasing fatty acid oxidation by the overexpression of CPT1 or inhibition of ACC improves insulin action in rodent models of lipid-induced insulin resistance [255, 277]. Moreover, pharmacological inhibition of CPT1 by etomoxir impaired insulin sensitivity when given alone, as well as exacerbated HF diet-induced insulin resistance in rodents [278]. However, this proposal of mitochondrial dysfunction being responsible for insulin resistance and T2D is in disagreement with the Randle cycle described previously. Promoting fatty acid oxidation as a potential therapeutic approach may lead to a reciprocal reduction in glucose metabolism via inhibition of PDH and PFK-1, as well the subsequent accumulation of G6P which would then inhibit hexokinase activity and reduce glucose uptake [279]. Based on this rationale, accelerating fatty acid oxidation would actually impair insulin-stimulated glucose metabolism, therefore further exacerbating peripheral insulin resistance and accelerate the progression of T2D. Based on recent *in vivo* studies in healthy volunteers [280],

Shulman and colleagues have proposed as a solution to this problem that potentially the Randle cycle does not operate in skeletal muscle [268].

While several studies support a role for mitochondrial dysfunction in the development of insulin resistance and T2D, there are also a growing number of studies showing that mitochondrial dysfunction does not precede insulin resistance and T2D [281-285]. Even under the assumption that mitochondrial function is in fact impaired in the insulin resistant and/or diabetic state, the debate continues as to whether a decreased capacity for mitochondrial fatty acid oxidation is a cause or correlate of insulin resistance. To the contrary, several reports now suggest that skeletal muscle insulin resistance results from excessive fatty acid oxidation and ensuing mitochondrial stress [222, 285, 286]. Recently, Koves *et al.* [286] demonstrated that obesity-induced insulin resistance may be due to metabolic overload in skeletal muscle where high rates of mitochondrial fatty acid oxidation exceed the capacity of the TCA cycle leading to incomplete oxidation of fatty acids and ensuing mitochondrial stress. Therefore, it remains to be clearly determined whether alterations in fatty acid oxidation may directly contribute to the development of skeletal muscle insulin resistance and studies in this thesis will attempt to address this topic in a physiological model of diet-induced insulin resistance.

Increased Fatty Acid Uptake Leading to Intramuscular Lipid Accumulation

Alternatively, lipid intermediates may accumulate in skeletal muscle when fatty acid uptake far exceeds its capacity for oxidation. Particularly in the setting of obesity, this is mediated in part by an increase in delivery of fatty acids to the

muscle and/or an increase in the efficiency of protein-facilitated fatty acid uptake [88]. High circulating levels of plasma fatty acids are known to be associated with insulin resistance [287, 288] and lowering plasma fatty acids concentrations improves insulin sensitivity in diabetic and non-diabetic subjects [289, 290]. Indeed, several studies have demonstrated a marked increase in the rates of fatty acid transport into skeletal muscle of diabetic rodents [291-293] and humans with T2D [294]. Most studies agree that this is the result of an increase in the expression of one or more fatty acid transporters (FAT/CD36, FATP-1 and -4, and/or $FABP_{pm}$) in insulin-resistant skeletal muscle [292, 295, 296] and/or the permanent translocation of transport proteins (i.e. FAT/CD36) from intracellular depots to the plasma membrane [88, 293, 294, 297]. Together, these findings strongly suggest that a chronic imbalance between lipid uptake and utilization is associated with the accumulation of lipid intermediates and development of insulin resistance. Indeed, genetic ablation of FAT/CD36 and FATP1 has been found to protect against HF diet-induced insulin resistance and metabolic syndrome in rodents [298, 299], thus suggesting that inhibition of fatty acid uptake is a potential strategy for preventing intramuscular lipid accumulation and obesity-related insulin resistance.

Age-Associated Skeletal Muscle Insulin Resistance and Type 2 Diabetes

Aging is a well-recognized risk factor for the development of insulin resistance and T2D. The high prevalence of T2D in the elderly population makes it one of the most common chronic diseases in the world, affecting nearly 50 million people aged 60 years of age and older in developed countries. Given the

growing size of the aging population and the increased propensity towards physical inactivity and obesity, the prevalence of T2D is expected to increase dramatically in the next 5-10 years [300]. Therefore, it is vital to understand the metabolic alterations that occur in the normal process of aging and how/if middle-aged individuals are more susceptible to the development of insulin resistance and T2D.

Similar to obesity-induced insulin resistance, it has been proposed that age-associated glucose intolerance arises from impairments in mitochondrial function and oxidative capacity, which subsequently leads to the accumulation of intracellular lipid metabolites and disruption of insulin signaling in muscle. [267, 301]. Several lines of evidence suggest that skeletal muscle mitochondrial function declines with advanced age in both rodents and humans [302, 303]. Petersen *et al.* [301] showed that in comparison to matched young control subjects that healthy lean elderly subjects have severe skeletal muscle insulin resistance associated with intramyocellular lipid accumulation and a ~40% reduction in basal mitochondrial oxidative and phosphorylation activity. This is consistent with other studies demonstrating reductions in maximal ATP synthesis rates and muscle oxidative capacity with aging in humans [304-308]. The decline in mitochondrial function with advanced age has been attributed to increased oxidative damage to mitochondrial DNA [304, 309], reduced mitochondrial DNA abundance [310, 311], reduced mitochondrial protein expression [308, 311] and reduced mitochondrial enzyme activity [304, 308]. However, at the same time, some studies have failed to show age-associated changes in muscle metabolism

and mitochondrial function [312-314]. Therefore, whether mitochondrial dysfunction and impaired oxidative metabolism play a causative role in aging-related insulin resistance is still under investigation. Studies detailed in this thesis aim to examine whether skeletal muscle energy substrate metabolism is altered in the normal process of aging and whether these changes may predispose aged rodents to the development of age- and obesity-related insulin resistance and metabolic disease.

Cardiac Lipotoxicity

Cardiac Dysfunction in Obesity and Type 2 Diabetes

Lipotoxic cardiomyopathy is a condition that arises due to excessive accumulation of detrimental fatty acid intermediates (TG, ceramide, DAG, LCACoA) within the cardiac myocyte that results in impaired cardiac function [315-317]. Several studies have shown that intramyocardial lipid accumulation is deleterious to the heart leading to numerous cardiac pathologies including increased cardiac myocyte apoptosis, myocardial fibrosis, left ventricular (LV) hypertrophy, LV dilatation, contractile dysfunction and impaired diastolic filling [318-320]. Similar to skeletal muscle, it has been proposed that when fatty acid supply and uptake is in excess of the heart's capacity to oxidize fatty acids this results in cardiac steatosis. Cardiac steatosis and lipotoxic cardiomyopathy are observed in conditions including obesity, insulin resistance and T2D. A close relationship exists between obesity and T2D with both conditions associated with

insulin resistance. High circulating levels of fatty acids and TG are observed in obesity and T2D in both rodents and humans, and this is usually present in parallel with intramyocardial lipid accumulation [287, 321-323]. Therefore, it has been proposed that the exposure of the heart to high levels of fatty acids can cause the build-up of potentially toxic lipid derivatives and TG in the cardiomyocyte and likely plays a causal role in the development of obesity-related cardiac dysfunction [320].

Since obesity is a complex disorder and does not occur in isolation from other metabolic disorders, other factors than alterations in cardiac energy metabolism and cardiac lipotoxicity also likely contribute to obesity-related cardiomyopathy. In order to test the hypothesis that perturbations in lipid uptake and metabolism in the cardiomyocyte directly contributes to cardiac dysfunction, several mouse models were developed with targeted cardiac-specific gene overexpression of fatty acid handling proteins (i.e. FATP, LPL, ACSL). Common to all these mouse models was a phenotype characterized by excessive lipid uptake resulting in cardiac steatosis and impaired systolic function, even in the absence of obesity or systemic metabolic disturbances such as hyperglycaemia [319, 324, 325]. Interestingly, perturbed myocardial fatty acid handling in these mice recapitulates the cardiac phenotype observed in diabetic cardiomyopathy. Thus these studies highlight the fact that excessive lipid accumulation is pathological to the heart, and suggest that cardiac steatosis observed in the setting of obesity and T2D is not just a correlate but likely exerts direct adverse effects on cardiac function.

Increased Myocardial Fatty Acid Uptake in Obesity and Diabetes

As mentioned above, in the setting of obesity and T2D the heart is exposed to high circulating levels of plasma free fatty acids which are implicated as a major contributor to the deleterious effects of T2D on cardiac structure and function. Indeed, fatty acid uptake has been shown to be increased in hearts from obese Zucker rats, *db/db* mice and rodents fed a HF diet [293, 326-329]. Increased myocardial fatty acid uptake in obesity and T2D is attributed in part due to high plasma fatty acids but also to the increased expression and/or sarcolemmal content of fatty acid transport proteins, most commonly FAT/CD36 and $FABP_{pm}$ [88, 293, 322, 328, 330]. Increased expression of FAT/CD36 may be the result of increased fatty acid-induced activation of PPAR family of nuclear receptor, in particular PPAR-α, which regulates the gene expression of several key proteins involved in lipid uptake and metabolism in the heart [120]. Activation of the PPAR pathway by fatty acids creates a link between supply of fatty acids and oxidation. Indeed, the PPAR-α transcriptional pathway has been shown to be activated in hearts from type 1 and type 2 diabetics [331]. Moreover, heart-specific overexpression of PPAR-α recapitulates the diabetic cardiac phenotype with an increase in FAT/CD36 expression, marked lipid accumulation and ventricular dysfunction [331].

Interestingly, it is often levels of FAT/CD36 that are upregulated in models of diabetes and cardiac lipotoxicity. Moreover, our laboratory has previously shown that FAT/CD36 expression is dramatically upregulated in the aged WT heart promoting myocardial lipotoxicity and impaired contractile performance

[332]. Together these findings suggest that FAT/CD36 may play a central role in the accumulation of potentially toxic lipid intermediates in the heart and that modulation of FAT/CD36-mediated fatty acid uptake may be an exciting new approach for preventing the onset and/or treating age- and obesity-related impairments in cardiac function.

Myocardial Fatty Acid Oxidation in Obesity and Diabetes

Some controversy continues to exist as to whether the accumulation of detrimental lipids in the heart in obesity and T2D is primarily the result of a chronic oversupply of fatty acids that exceeds the capacity for its oxidation in the heart or whether impaired mitochondrial fatty acid oxidation also plays a role. Some studies have suggested that reduced mitochondrial fatty acid oxidation in the insulin-resistant and diabetic heart contributes to the development of lipotoxicity [333-335]. Indeed, Young *et al.* showed in obese Zucker rats that myocardial fatty acid β-oxidation is impaired following an overnight fast, and this was associated with marked intramyocardial lipid deposition and contractile dysfunction [333]. A similar phenotype is also observed in humans with inherited defects in mitochondrial fatty acid oxidation [336, 337]. Further support for this idea has come from a recent report in ACC2 deficient mice (ACC2-/-), which display elevated cardiac fatty acid oxidation rates but interestingly also exhibit increased myocardial glucose oxidation rates and insulin stimulated 2-deoxyglucose (2-DG) uptake as compared to WT controls [335]. While this would suggest that promoting fatty acid oxidation would be beneficial to the insulin-resistant and diabetic heart in order to reduce excessive lipid deposition, the

authors have acknowledged that the reported glucose oxidation rates in this study were 10-20 fold lower than what is typically observed in the literature. Interestingly, the findings in the ACC2-/- mice [335] are in opposition with the Randle glucose-fatty acid cycle, which has been clearly shown by numerous studies to be operational in the heart [41, 338, 339]. Accelerating fatty acid oxidation may lead to a reciprocal inhibition of glucose metabolism, and thus would not be a rational strategy for improving glucose metabolism and contractile function in the insulin-resistant, diabetic heart [59].

In contrast to reports implicating reduced rates of cardiac fatty acid oxidation in the development of insulin resistance and lipotoxicity, several studies in rodents [340-343] and humans [344, 345] have shown that the heart has an increased reliance on fatty acids as a fuel source and that fatty acid β-oxidation rates are accelerated in the setting of obesity, insulin resistance and diabetes. Studies in insulin-resistant obese JCR:LA rats have demonstrated that fatty acid β-oxidation rates are not impaired following an overnight fast, and that fatty acids account for >60% of ATP production in these hearts, which was similar to values obtained from the lean control rats [346, 347]. In agreement with this, Aasum and colleagues have demonstrated that fatty acid oxidation is increased in hearts from leptin receptor deficient *db/db* mice, as well as in mice subjected to HF diet-induced obesity, which is likely the most relevant model to human pathophysiology involving weight gain, increased fat mass and development of insulin resistance [340, 348-350]. These findings have also been confirmed in other rodent models of obesity and diabetes, including leptin deficient *ob/ob* mice

[321, 341, 351]. Moreover, cardiac specific overexpression of PPAR-α mimics a diabetic phenotype where these mice display dramatically elevated levels of cardiac fatty acid oxidation with a concurrent reduction in glucose oxidation and glucose uptake, associated with LV hypertrophy and dysfunction [331]. This is further supported by elegant human studies by Petersen and colleagues, using positron emission tomography and ^{11}C-palmitate, showing that uptake and oxidation of fatty acids is increased in obese young women and subjects with type 1 diabetes [344, 345]. Taken together, there is considerable evidence suggesting that cardiac fatty acid β-oxidation is increased in obesity and T2D, as opposed to a reduction in fatty acid β-oxidation. Accelerated cardiac fatty acid oxidation and the concomitant decrease in glucose oxidation in the setting of obesity likely directly contribute to myocardial insulin resistance and decreased insulin-stimulated glucose metabolism.

Interestingly, while fatty acid oxidation is enhanced in hearts from obese and insulin resistant animals most of these models are also characterized by intramyocardial lipid accumulation. This suggests that despite accelerated oxidation of fatty acids in these hearts that fatty acid uptake is far in excess of its capacity to be utilized, therefore resulting in storage of fatty acids in the cardiomyocyte [316]. Indeed, Severson *et al.* attributed the elevated fatty acid utilization (oxidation and esterification) in type 2 diabetic *db/db* hearts to an increase in plasma membrane content of FAT/CD36 and $FABP_{pm}$, which results in increased fatty acid uptake [326]. This further reinforces the important role of fatty acid transporters in the pathogenesis of obesity-related cardiomyopathy. In

the debate over whether fatty acid oxidation is increased or decreased in obesity and diabetes, fatty acid oxidation is potentially elevated early on in the progression of insulin resistance and T2D prior to the onset of contractile dysfunction and may decline over time due to mitochondrial damage. Associated with elevated fatty acid oxidation rates for energy production in the heart are reductions in myocardial efficiency which may be due to increases in myocardial oxygen consumption, metabolic futile cycling and uncoupled mitochondrial respiration dependent upon activation of UCPs [175, 321, 338, 350, 352, 353]. Increased fatty acid oxidation has been proposed to increase the delivery of reducing equivalents to the ETC, resulting in the increased production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, and lipid peroxidation, which can lead to a vicious cycle of further uncoupled respiration and mitochondrial damage [175, 354-356]. Together these series of events may contribute to the compromised myocardial energetics and decline in cardiac function commonly observed in the later stages of obesity and diabetes.

Age-Related Changes in Cardiac Function and Energy Metabolism

Age is a significant risk factor for the development of a number of CVDs, such as LV hypertrophy and heart failure [12, 357, 358]. The incidence and prevalence of these CVDs is known to increase dramatically with advancing age, and since the number of individuals aged 65 years of age or older is expected to double to 60 million in the United States by the year 2030, this will undoubtedly increase the burden of age-related diseases on the health care system [359]. While atherosclerosis and hypertension are major contributing factors to aging-related

cardiac dysfunction, independent of these are also progressive alterations in cardiovascular structure and deterioration of cardiac function that are intrinsically associated with the normal process of aging, which can predispose or negatively impact clinical cardiac disease outcomes for this population. Epidemiological data from the Framingham Heart Study and the Baltimore Longitudinal Study of Aging show that in healthy populations of elderly subjects that there is an age-dependent increase in the prevalence of LV hypertrophy, a decline in diastolic function, a decline in exercise capacity and increased incidence of atrial fibrillation [12]. As both studies were focused on individuals without hypertension or signs of CVD, these changes are likely due to cardiac aging *per se* and not secondary to co-existing morbidities. Advanced age is associated with several cardiovascular abnormalities, including endothelial dysfunction, arterial stiffening, cardiac interstitial fibrosis and cardiomyocyte apoptosis [12, 357, 360-364]. These cardiac changes are considered part of the normal process of aging that are then modifiable by environmental influences, including diet and physical activity. Although the mechanisms responsible for age-related CVD are likely numerous, derangements in the pattern of myocardial energy metabolism is thought to play an important role in the progressive decline of cardiac function with age [17]. Under normal physiological conditions, mitochondrial β-oxidation of fatty acids accounts for approximately 50-75% of acetyl CoA-derived ATP in the healthy adult heart [16, 17]. However, it has been shown that fatty acid utilization is dramatically reduced in the aged rodent and human heart [365-368]. Furthermore, McMillin *et al.* showed in the senescent rat heart that fatty acids

were unable to suppress glucose oxidation, suggesting that with aging there is a loss of myocardial metabolic flexibility and substrate switching [367]. Indeed, our laboratory has recently shown using the *ex vivo* isolated working mouse heart model that both glucose and fatty acid oxidation were reduced by 2.5- and 4-fold, respectively, in hearts from 50-52 week old mice as compared to young mice [332]. Although overall acetyl CoA-derived ATP production was markedly decreased in the aged heart, there was a greater percentage contribution of fatty acids to ATP production in these hearts as compared to young mice. These data suggest that overall mitochondrial function may decline with aging and thus contribute to impairments in the mitochondrial oxidation of glucose and fatty acids.

Similar to skeletal muscle, mitochondrial function has been proposed to decline with age and contribute to the decline in myocardial fatty acid oxidation, as well as potentially reduced glucose metabolism [369]. In support of the work of Koonen *et al.* [332], several studies have shown age-dependent impairments in mitochondrial function characterized by a reduction in mitochondrial oxidative capacity due primarily to decreased activity of complexes I, III and IV of the ETC [369-376]. Moreover, the activities of TCA cycle enzymes, such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, are found to be decreased in the aged rat heart [370]. In particular a decline in complex I activity may become strongly rate limiting to electron transfer and decrease the capacity of ATP production [369]. Furthermore, reduced complex I activity is negatively correlated with oxidative damage and

lipid peroxidation, suggesting that superoxide and ROS generation are elevated in the aged heart [369, 377]. Increased oxidative stress in the heart can create a vicious cycle of mitochondrial damage, reduced mitochondrial enzyme activity and further mitochondrial ROS generation [369].

While the effects of age on fatty acid oxidation are generally agreed upon, the effect on myocardial glucose oxidation is not completely clear. The findings of Koonen *et al.* and other groups suggest that overall oxidative metabolism is diminished in the aged heart [332, 378, 379]. Interestingly, substrate metabolism in the aged heart is poorly defined and there is a noticeable paucity of studies examining glucose metabolism, in particular glycolytic flux. Most of what is known regarding cardiac glucose metabolism with aging is extrapolated from gene and protein expression studies, which have produced conflicting results where some studies have shown increases in GLUT-4, PFK-1 and PDH [380, 381], while others have found age-associated declines in these very same enzymes [379, 382]. While the study by Koonen *et al.* [332] measured both fatty acid and glucose oxidation rates in the aged murine heart, rates of glycolysis was not measured and further studies will be required to fully characterize the alterations in energy substrate metabolism that occur with advancing age.

Myocardial Ischemia-Reperfusion Injury

Myocardial ischemia occurs when oxygen supply to the myocardium is insufficient to meet the energy demands of the heart. The consequences of

myocardial ischemia are dependent upon the degree of severity and nature of the ischemic insult and reperfusion of the myocardium. While the prompt restoration of blood flow (reperfusion) to the heart is necessary to salvage the myocardium and prevent tissue death, reperfusion itself can result in further damage which impairs cardiac contractile function, in a phenomenon known as 'reperfusion injury' [383]. A large proportion of I/R injury in patients results from acute MI, however I/R can also occur in other settings such as following open-heart surgery. Although myocardial I/R injury involves numerous complex biological and cellular mechanisms, there is clear evidence that a component of I/R injury is dependent upon the types of substrates metabolized by the heart for the generation of ATP during and following the ischemic period [149, 338]. The metabolic perturbations that occur as a consequence of ischemia and subsequent reperfusion of the myocardium, specifically in regards to fatty acid utilization will be discussed.

Alterations in Energy Metabolism During Myocardial Ischemia-Reperfusion

In the setting of acute MI and post-cardiac surgery, circulating levels of plasma fatty acids are significantly elevated in patients during and following ischemia [384]. This is primarily the result of increased catecholamine release and stimulation of adipose tissue lipolysis to release free fatty acids into the circulation [385]. During severe forms of ischemia (i.e. acute MI or cardiac surgery) the lack of oxygen results in a dramatic reduction in oxidative metabolism and ATP production. Under these ischemic conditions the heart relies more on anaerobic glycolysis for energy production; however, in the absence of

pyruvate oxidation these high rates of glycolysis lead to an accumulation of lactate and protons. This results in ATP being diverted away from myocardial contraction and towards re-establishing ionic homeostasis, which reduces cardiac function and efficiency [386, 387].

While overall oxidative metabolism is reduced during ischemia, fatty acid oxidation is responsible for a significant proportion of residual oxidative metabolism that occurs in the ischemic heart [15, 388]. This is the result of high circulating levels of plasma free fatty acids and changes in subcellular control of fatty acid oxidation. Myocardial ischemia is associated with the rapid activation of AMPK and subsequently the phosphorylation and inhibition of ACC, which results in reduced malonyl CoA levels [132, 152]. As described earlier in this chapter, a reduction in malonyl CoA levels relieves inhibition of CPT1 allowing for mitochondrial fatty acid entry and subsequently fatty acid β-oxidation.

Upon reperfusion of the myocardium following ischemia, both the activation of AMPK and plasma fatty acid levels remain elevated and contribute to the rapid recovery of myocardial fatty acid oxidation rates observed during reperfusion. However, these high rates of fatty acid oxidation can dramatically inhibit glucose oxidation via the Randle cycle and lead to an uncoupling of glycolysis from glucose oxidation, which further exacerbates intracellular acidosis, as well as result in Na^+ and Ca^{2+} overload and impairs recovery of post-ischemic cardiac function and efficiency [338]. Therefore, it has been proposed that directly or indirectly promoting glucose oxidation may improve cardiac

function and be a novel therapeutic approach to lessen ischemic damage to the heart [149, 389-392].

Interestingly, there continues to be controversy as to whether activation of AMPK is beneficial or detrimental to the ischemic heart. Indeed, activation of AMPK may be an adaptive response of the heart to increase ATP production during I/R by stimulating glycolysis; however, activating AMPK also increases mitochondrial fatty acid oxidation at the expense of glucose oxidation, which may increase post-ischemic contractile dysfunction. While an earlier study by Russell *et al.* [179] showed that cardiac function was impaired during aerobic perfusion and ischemia in AMPKα_2 KD (K45R) transgenic mice, more recent studies performed in AMPKα_2 KO [393] and AMPK α_2 DN mice [394] have demonstrated that recovery of post-ischemic contractile function is not impaired in these mice. Therefore, the role of AMPK in the ischemic myocardium remains unclear and requires further investigation. In the next section of this chapter, the novel cardioprotective strategy of calorie restriction will be discussed and the proposed role of AMPK in mediating these beneficial effects.

Caloric Restriction

Moderate caloric restriction (CR), a reduction in caloric intake by 20-40%, has been shown to be an effective strategy to extend the life span of a number of different species [395]. CR as its name implies is defined as a reduction in energy intake without malnutrition while maintaining adequate levels of nutrients. While

the precise mechanisms by which CR prolongs lifespan remain to be fully elucidated, the current theory suggests that CR is a mildly stressful condition that provokes a survival response that helps the organism to endure a more severe stress by altering metabolism and improving cellular defences [396]. Recent studies suggest that one of the key mechanisms by which CR promotes lifespan in lower animals is the induction of the yeast protein silent information regulator 2 (Sir2), a member of the sirtuin family of NAD^+-dependent histone deacetylases [396-398]. The mammalian homologues of Sir2 are designated SirT (1-7), and potentially also mediate lifespan extension in mammals. SirT1 is the sirutin family member sharing the most homology to the yeast Sir2 and is under extensive investigation. Indeed, it has recently been shown that extension of lifespan by CR is abolished in transgenic mice that lack SirT1 [399].

In addition to its notable effects on longevity, CR has numerous beneficial effects on the cardiovascular system and reduces CVD risk. Studies in humans and experimental animals have shown that CR attenuates vascular dysfunction [400], LV hypertrophy [401, 402], diastolic dysfunction [401, 403] and myocardial ischemic injury [404-409]. Together these data strongly suggest that short-term CR could be a potential new treatment for and/or prevent the development of CVD. While multiple mechanisms are likely involved in the protective effects of CR on the cardiovascular system, some of the more prominent theories include reductions in oxidative stress [401, 410, 411] and inflammation [401, 411, 412]. Shinmura and colleagues have proposed that the cardioprotective effects of short-term CR are largely mediated by the adipokine

known as adiponectin [409]. In the aforementioned study, a 5-week restriction of caloric intake by 40% in mice led to an almost 2-fold increase in serum levels of adiponectin and this was associated with a significant reduction in infarct size following ischemia and improved post-ischemic functional recovery. Furthermore, CR-induced cardioprotection was partially abrogated in adiponectin antisense transgenic mice, suggesting that adiponectin is a key mediator of the improved ischemic tolerance afforded by short-term CR. Adiponectin is thought to be essential in mediating these protective effects via activation of the AMPK signaling pathway. Indeed, pharmacological inhibition of AMPK activity or genetic ablation of AMPK attenuates the beneficial metabolic [413, 414], anti-inflammatory [415], vascular/ angiogenic [416, 417] and cardioprotective effects [409] of adiponectin. Several studies have shown that adiponectin confers significant protection against myocardial ischemia [409, 418]; however, some debate exists as to whether AMPK is the central mediator of cardioprotective effects of adiponectin and/or CR. Interestingly, a recent study performed in mice with a cardiomyocyte-specific overexpression of a mutant AMPKalpha2 subunit (AMPK-DN) found that these mice retained a significant proportion of the cardioprotective effects of adiponectin against myocardial I/R injury [419]. Furthermore, Ouedraogo *et al.* [420] demonstrated that exogenous administration of adiponectin could suppress hyperglycaemia-induced superoxide production in cultured endothelial cells and that this effect was not inhibited by pre-treatment with AMPK inhibitors, suggesting that adiponectin reduces oxidative stress via an AMPK-independent mechanism likely involving cAMP/PKA dependent pathway.

The contribution of AMPK to cardiomyocyte injury appears to be largely dependent upon the type of cardiac injury and the specific model. For example, Shibata and colleagues demonstrated that adiponectin mediated activation of AMPK is important in preventing cardiomyocyte and cardiac fibroblast apoptosis induced by hypoxia-reoxygenation, whereas cyclooxygenase (COX)-2 and not AMPK was found to be the major contributor to adiponectin-induced protection against lipopolysaccharide-induced TNF-α production [421]. While AMPK activation during the ischemic insult may be a beneficial adaptive response by the ischemic heart to promote glucose utilization (glucose uptake and glycolysis) and ATP production when oxygen supply is limited [149, 179, 422], the activation of AMPK during reperfusion following ischemia may be potentially deleterious. Activation of AMPK during and following ischemia may result in accelerated fatty acid oxidation rates at the expense of glucose oxidation leading to intracellular acidosis and impaired recovery of cardiac function [132, 152]. Due to the lack of specific pharmacological inhibitors of AMPK, it has been difficult to assess the direct effect of AMPK activity on post-ischemic recovery of mechanical function. Recent studies in AMPK transgenic mice have been performed to better address this question however they have produced conflicting results. Contrary to the report by Russell *et al.* [179] which showed that cardiac function was impaired during low-flow ischemia in AMPKα_2 knockdown mice, Folmes *et al.* [394] demonstrated that hearts from AMPKα_2 DN mice were neither energetically nor functionally compromised during I/R. Therefore, the role of AMPK in mediating myocardial I/R injury remains inconclusive and may differ

depending on substrate availability and the balance of glucose and fatty acid metabolism. Based on this rationale, studies in this thesis were undertaken to further elucidate the contribution of AMPK activation to the cardioprotective effects of CR against I/R injury.

Hypothesis and Objectives

General Hypothesis

Coordinated energy metabolism is vital for the proper functioning of both skeletal muscle and the healthy heart. Alterations in fatty acid and glucose metabolism in these muscles are known to be important contributors in the pathogenesis of numerous cardiovascular diseases and metabolic disorders, such as myocardial I/R injury, diabetic cardiomyopathy and insulin resistance/diabetes. As such, strategies that optimize energy substrate metabolism are being investigated as potential therapeutic approaches for the prevention and/or treatment of these diseases. Since the prevalence of CVD and metabolic disorders are highest in the aging population, we hypothesize that intrinsic metabolic alterations associated with advanced age increases the susceptibility for the development of insulin resistance and cardiomyopathy. Excessive fatty acid uptake into muscle appears to play a central role in the etiology of insulin resistance and cardiomyopathy, thus strategies to limit fatty acid utilization may prove to be protective against these conditions and act to indirectly improve glucose metabolism.

Specific Hypotheses

The specific hypotheses of this thesis are described in detail within the individual chapters pertaining to the experimental results.

Specific Aims

1. To determine how aging impacts systemic and skeletal muscle metabolism, and whether advanced age increases the susceptibility to development of obesity-induced insulin resistance. Furthermore, we sought to investigate whether the inhibition of FAT/CD36-mediated fatty acid uptake could prevent the detrimental effects of obesity on skeletal muscle insulin sensitivity.

2. To elucidate how aging effects the development of HF diet-induced cardiomyopathy and cardiac hypertrophy, as well as to determine how increased myocardial FAT/CD36 expression may contribute to altered fatty acid handling and cardiac dysfunction.

3. To investigate the mechanisms by which short-term CR improves ischemic tolerance of the heart including alterations in energy substrate metabolism and the potential contribution of AMPK and other molecular signaling pathways in these cardioprotective effects.

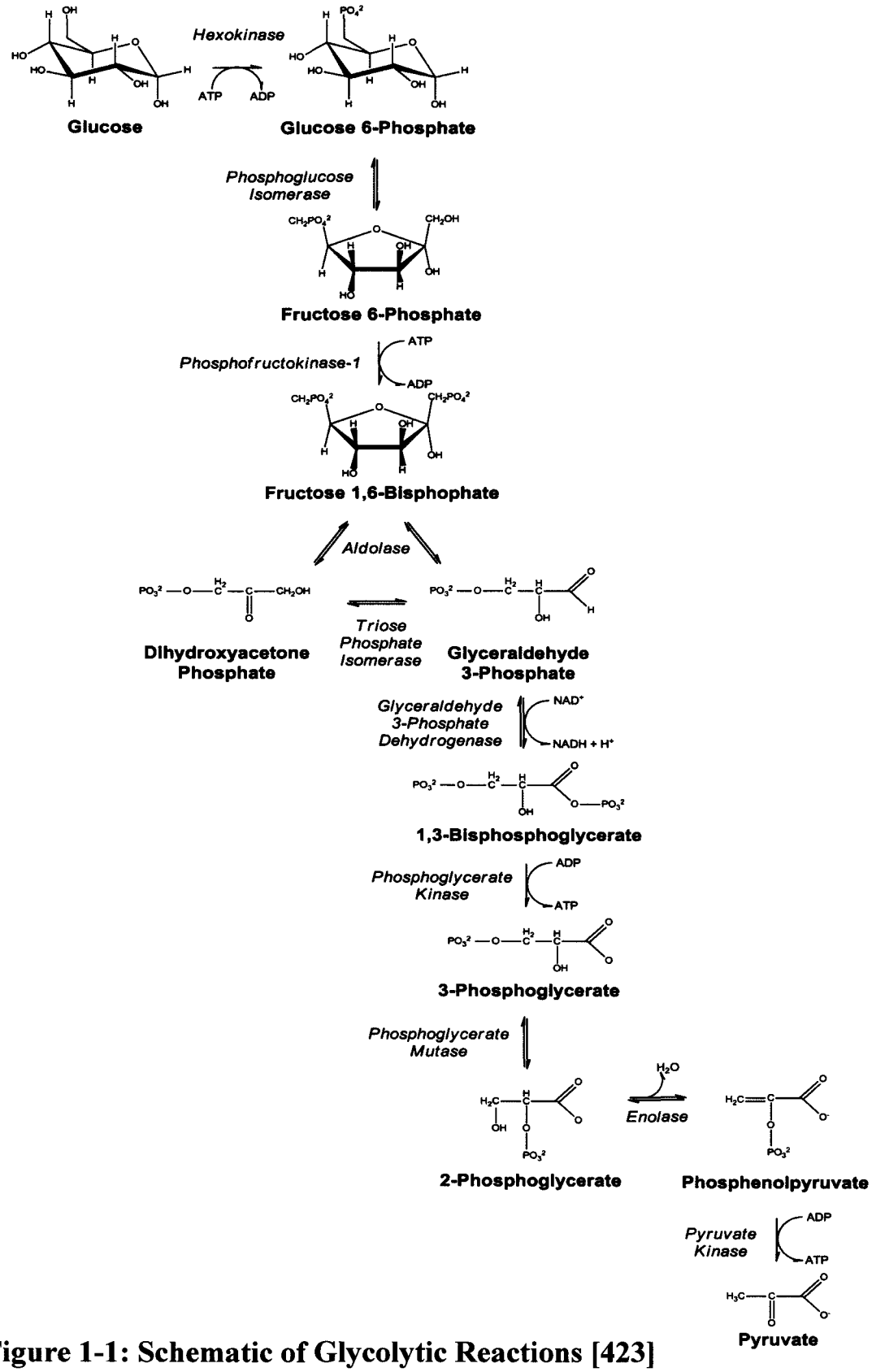

Figure 1-1: Schematic of Glycolytic Reactions [423]

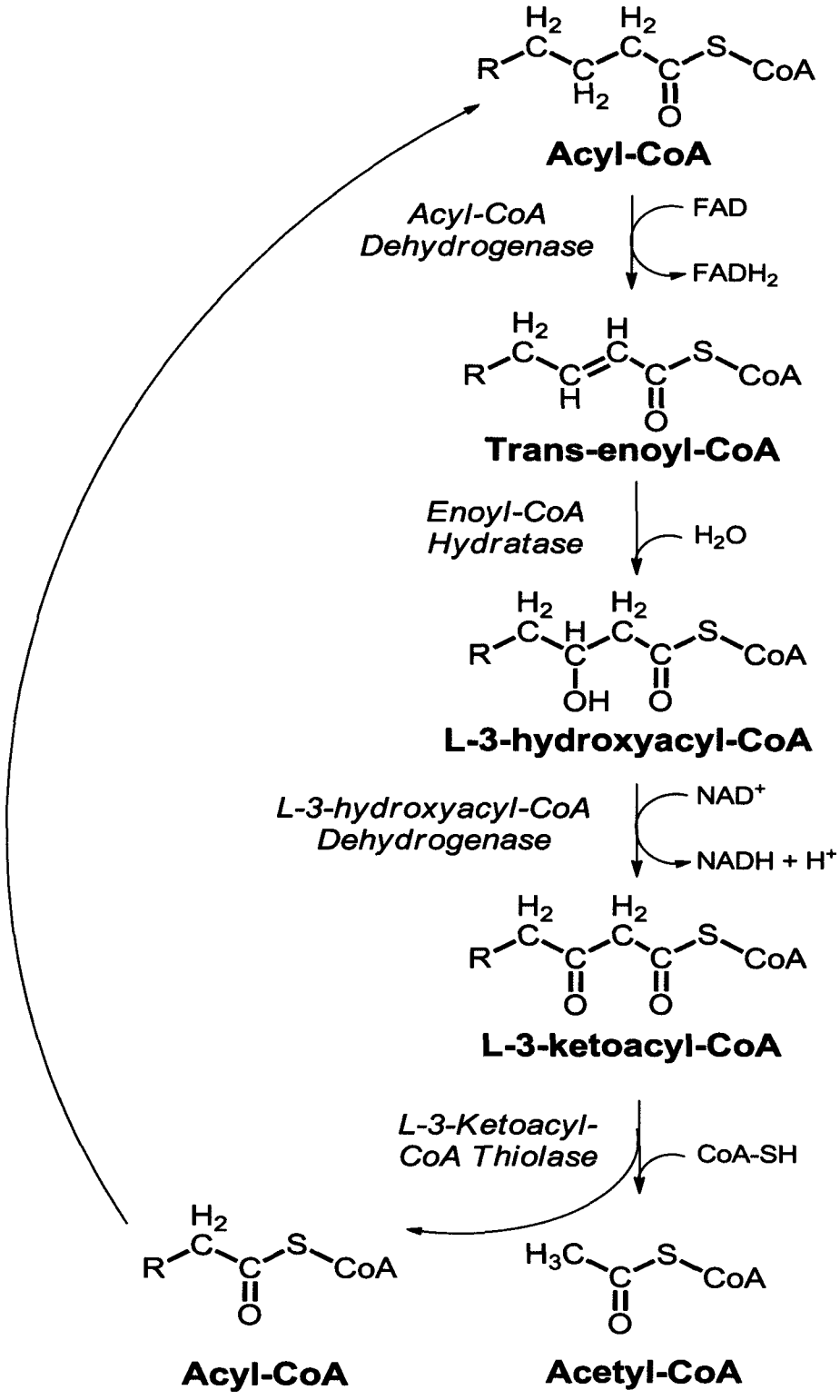

Figure 1-2: Schematic of β-oxidation reactions (adapted with permission from [423])

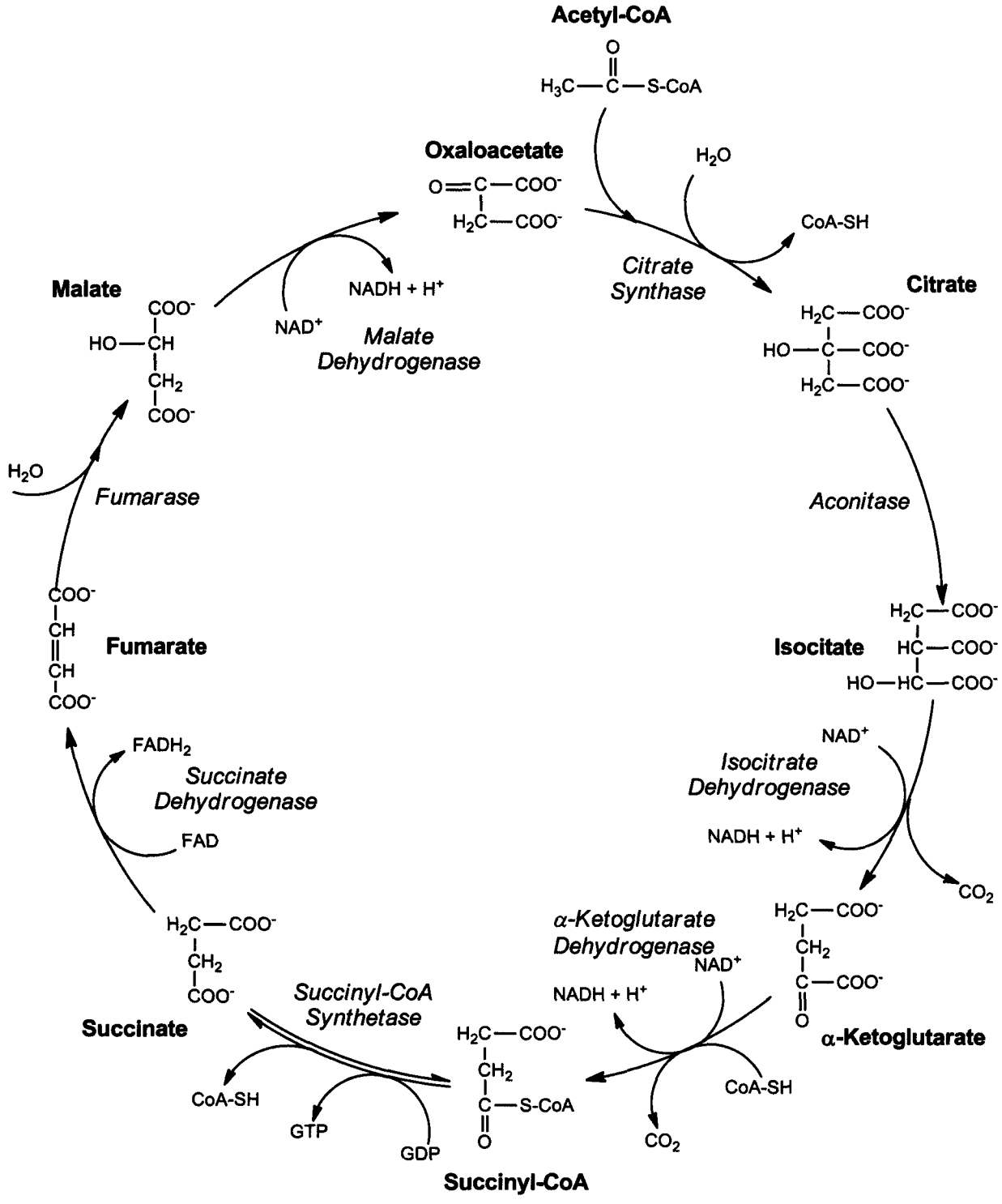

Figure 1-3: Schematic of TCA cycle reactions (adapted with permission from [423])

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CHAPTER 2.

Materials and Methods

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Materials

Primary antibodies used in this thesis were anti-phospho-ACC (Ser79), anti-ACSL1, anti-phospho Akt (Ser473), anti-Akt, anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho glycogen synthase kinase (GSK)3 (Ser9), anti-GSK3β, anti-mTOR, anti-phospho-mTOR (Ser2448), anti-phospho-p70 S6 kinase (Thr421/Ser424 and Thr389), anti-eEF2, anti-phospho-eEF2 (Thr56), anti-PDH, anti-phospho S6 ribosomal protein (Ser240/244), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (Erk1/2), and anti-tubulin were all purchased from Cell Signaling Technology (Danvers, Massachusetts). HRP-labelled anti-CD36 antibody was purchased from Novus Biologicals (Littleton, Colorado). Rabbit anti-MCD antibody was generated in house by Dr. Jason Dyck (University of Alberta; Edmonton, Alberta). Separate anti-phospho-PDHE1α antibodies targeted at phosphorylation sites Ser293, Ser232 and Ser300 were purchased from Calbiochem (Gibbstown, New Jersey). Peroxidase-labelled streptavidin was purchased from Kirkegaard and Perry Labs (Gaithersburg, Maryland). Anti-actin antibody and horseradish peroxidase (HRP)-labelled secondary antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz,

California). Human recombinant insulin (Novolin™ ge Toronto) was obtained through the University of Alberta Hospital stores from Novo Nordisk Canada (Mississauga, Ontario). Glucose analysis kit and protease inhibitor cocktail was purchased from Sigma-Aldrich (St. Louis, Missouri). Phosphatase inhibitor cocktail set IV was purchased from Calbiochem (Gibbstown, New Jersey). Radiochemicals [U-^{14}C]glucose, D-[5-^{3}H]glucose and [9,10-^{3}H-(*N*)]palmitic acid were obtained from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). Trans-Blot® Transfer Medium (pure nitrocellulose) was purchased from Bio-Rad Laboratories (Richmond, California). FUJI Medical X-ray films were purchased from Mandel Scientific (Guelph, Ontario). Western Lightning® Chemiluminescence Reagents Plus kit was obtained from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). Ecolite™ Aqueous Counting Scintillation fluid was obtained from MP Biomedicals (Solon, Ohio). Bovine serum albumin (BSA fraction V, fatty acid free) used in perfusion buffers was purchased from Equitech-Bio Inc. (Kerrville, Texas) while BSA (fraction V) for other purposes was purchased from Sigma Aldrich (St. Louis, Missouri). Unifilter P81 96-well filterplates were obtained from Whatman (Florham Park, New Jersey). Hyamine hydroxide (1 mol/L in methanol solution) was obtained from NEN Research Products (Boston, Massachusetts). HR-series non-esterified free fatty acid (NEFA) determination kits were obtained from Wako Pure Chemicals Industries, Ltd (Osaka, Japan). Insulin (mouse) ultrasensitive enzyme-linked immunosorbent assay kits were purchased from Alpco Diagnostics (Salem, New Hampshire). Quantikine mouse adiponectin/Acrp30 immunoassay kit was

purchased from R&D Systems (Minneapolis, Minnesota). For the analysis of adenine nucleotides by high performance liquid chromatography (HPLC), a Supelcosil™ LC-18-T Super Guard cartridge, 5 μm particle size, 2 x 4 mm and a Supelcosil™ LC-18-T, 5 μm particle size, 250 x 4.6 mm column were obtained from Supelco/Sigma-Aldrich (St. Louis, Missouri). For the measurement of LCACoA esters by HPLC, a C18 guard cartridge, 4 x 3 mm and a Luna, 5 μm particle size, C18(2) 100 A, 250 x 2 mm column were obtained from Phenomenex (Torrance, California). Rodent diets were purchased from Research Diets (New Brunswick, New Jersey). ACCU-CHEK Aviva blood glucose test strips were purchased from Roche Diagnostics (Laval, Quebec). Blood was collected from the tail vein using Microvette CB 300 K2E tubes coated in EDTA dipotassium salt from Sarstedt (Nümbrecht, Germany). All other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri).

Methods

Animals

The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with the Canadian Council of Animal Care guidelines. Male C57BL/6 mice were commercially supplied from The Jackson Laboratory.

Generation and Breeding of Fatty Acid Translocase/ CD36 Deficient Mice

Fatty acid translocase (FAT)/CD36 deficient mice were generated in the laboratory of Dr. Maria Febbraio (Lerner Research Institute; Cleveland, Ohio) [1]. Construction of the CD36 targeting vector was carried out in the plasmid pPNT, which contains *neo* and thymidine kinase for selection of homologous recombinants. Two fragments flanking exon 3 of murine CD36, which contained the translation start site, were subcloned into pPNT in a transcription orientation opposite to the neo^r cassette. Exon 3, which encodes the first 40 amino acids of CD36 and contains the putative N-terminal transmembrane domain, was deleted in the homologous recombined allele. CJ7 embryonic stem cells were electroporated with the linearized targeting vector. Embryonic stem cells were cultured and the G418-resistant clones were screened by Southern blot analysis to select targeted cells. Selected stem cells were then injected into C57BL/6 blastocysts to produce chimeras. Male chimeras were bred with C57BL/6 females, and offspring were screened for the presence of the mutated CD36 gene by Southern blot analysis. Offspring heterozygous for the CD36 mutation were interbred, and mice homozygous for the CD36 disrupted allele were identified by Southern blot. Heterozygous mice were backcrossed 6X with wild-type (WT) C57BL/6 mice in order to obtain mice with a genetic background > 99% similar to the C57BL/6 background.

Diet-Induced Obesity Feeding Protocol

Mice were *ad libitum* fed either a low fat (LF; 10kcal% fat from lard; Research Diets, Inc., D12450B) or high fat (HF; 60kcal% fat from lard; Research Diets, Inc., D12492) diet to induce weight gain and obesity. Animals were fed this diet for 12 weeks at which time insulin resistance was present, as determined by glucose and insulin tolerance tests.

Caloric Restriction Protocol

C57BL/6 mice (8 weeks of age) were individually housed and maintained on a 12:12 hr dark-light cycle (0800:2000 dark) with free access to food and water for a 2-week acclimatization period. Daily food consumption (g) was measured during the last week of acclimatization and the average total daily food intake and body weight was used to calculate amount of food allotted for each individual mouse on a restricted diet. Mice (10 weeks of age) were randomly assigned into groups and fed *ad libitum* an AIN93M standard chow diet (Research Diets, Inc., D10012M) for 5 weeks (control) or a caloric restriction (CR) diet. CR mice received 90% of the average baseline caloric intake of the control mice for three weeks (Research Diets, Inc., D07101103) followed by 40% of the average caloric intake for the final two weeks of the experiment (Research Diets, Inc., D01092702). CR mice were fed once daily at the same time each day. CR diets were enriched in vitamins, minerals and salts such that restricted animals were not deficient compared to the control animals.

In Vivo Ultrasound Echocardiography

Transthoracic echocardiography was performed on mildly anaesthetized mice (isoflurane) to measure *in vivo* cardiac function with a Vevo 770 high-resolution echocardiography imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Ontario) as previously described [2]. M-mode echocardiographic images were used for measurements of LV wall thickness, LV end-diastolic diameter (LVEDD), and LV end-systolic diameter (LVESD). LV ejection fraction (EF) and fractional shortening (FS) were calculated as a measure of systolic cardiac function.

Isovolumic relaxation time (IVRT) was determined with pulsed-wave tissue Doppler. IVRT was calculated as the time from closure of the aortic valve to initiation of the early (E) filling wave (in msec).

% EF was calculated with the following equation:

$$\text{EF (\%)} = (\text{LV end diastolic volume [LVEDV]} - \text{LV end systolic volume}) / \text{LVEDV} \times 100$$

% FS was calculated with the following equation:

$$\text{FS (\%)} = (\text{LVEDD} - \text{LVESD}) / \text{LVEDD} \times 100$$

In vivo Metabolic Analysis

Metabolic and behavioural measurements, including O_2 consumption (VO_2), CO_2 production (VCO_2), food intake, heat production and locomotor activity were measured by indirect calorimetry using the Comprehensive Lab Animal

Monitoring System (Oxymax/CLAMS; Columbus Instruments, Colombus, Ohio). Mice were individually housed in separate metabolic cages with free access to food and water. Following an initial 24 hr acclimatization period, mice were monitored every 13 min for 24 hr to complete a full 12 hr dark (active)/12 hr light (inactive) cycle. The following equations were used to calculate VO_2 and VCO_2. The VO_2 was calculated by taking the difference between the input oxygen flow and the output oxygen flow. Similarly, the VCO_2 was calculated by taking the difference between the output and input carbon dioxide flows.

$VO_2 = ViO_2i - V_oO_{2o}$ where V_i and V_o are the input and output ventilation rates expressed as litres per minute (LPM) and O_{2i} and O_{2o} are oxygen fractions at the input and output

$VCO_2 = V_oCO_{2o} - V_iCO_{2i}$ where V_i and V_o are the input and output ventilation rates expressed as litres per minute (LPM) and CO_{2i} and CO_{2o} are carbon dioxide fractions at the input and output

The respiratory exchange ratio (RER) is the ratio of carbon dioxide production to oxygen consumption and as such has no unit value attached. The RER ratio is used to estimate the percent contribution of fat and carbohydrate to whole-body *in vivo* energy metabolism in mice. The RER ratio was calculated before any unit conversion, weight normalization, or effective mass correction.

$$RER = VCO_2/VO_2$$

Heat production was calculated using a derived 'calorific value (CV)' based on the observed RER values, and this CV is then used with VO_2 to calculate heat production. Heat production was calculated before the application of any normalization for weight or correction and therefore reflects the exact heat of the subject. In some cases, heat production was normalized to body weight manually during analysis.

$$\text{Heat production} = CV \times VO_2$$

$$CV = 3.815 + 1.232 \times RER$$

Cumulative food intake, as well as amount of food eaten in each bout, was monitored using Mettler Toledo balances. Feeders are designed to prevent spillage of food and prevent foraging. Locomotor activity was monitored by dual axis detection (X, Z axis) using IR photocell technology. Interruption of an IR beam is considered one activity "count." The height of these beams is placed such that they intersect the animal midway vertically. Placement of the IR photocell at a height above the animal detects rearing or jumping (Z-axis). Total activity was calculated by adding Z-counts (rearing or jumping) to total counts associated with ambulatory movement and stereotypical behaviour (grooming, scratching).

Isolated Working Mouse Heart Perfusion

C57BL/6 mice were anaesthetized with sodium pentobarbital (60 mg/kg body weight) administered by intraperitoneal (i.p) injection and hearts were excised and immersed in ice-cold Krebs-Henseleit bicarbonate solution containing 118 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 5.9 mmol/L KCl, 5 mmol/L EDTA pH = 7.4,

1.2 mmol/L $MgSO_4 \bullet 7H_2O$, 2.5 mmol/L $CaCl_2 \bullet 2H_2O$ and 5 mmol/L glucose. The aorta was cannulated and perfused with Krebs-Henseleit solution (37 °C) at a hydrostatic pressure of 60 mmHg. Hearts were then trimmed of excess tissue and the opening to the left atrium was cannulated. After equilibration in the Langendorff mode, hearts were switched to the working mode by clamping off the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. Mouse hearts were perfused in the working ejecting mode with modified Krebs–Henseleit solution containing 5 mmol/L [5-^{3}H/U-^{14}C]glucose, 1.2 mmol/L [9,10-^{3}H-(*N*)]palmitic acid pre-bound to 3% fatty acid–free albumin and in the presence of 50 μU/mL insulin as described previously [3, 4]. Spontaneously beating hearts were aerobically perfused at a constant left atrial preload pressure of 11.5 mmHg and a constant aortic afterload pressure of 50 mmHg. The perfusate was re-circulated, and pH was adjusted to 7.4 by gassing the perfusate in a glass oxygenator with a gas mixture with a composition of 95% O_2 and 5% CO_2. Isolated hearts were aerobically perfused for 30 min, subjected to 18 min of global, no-flow ischemia, followed by 40 min of aerobic reperfusion. At the end of perfusion, hearts were rapidly frozen in liquid N_2 with Wollenberger tongs, and tissue stored at -80 °C.

Measurement of Mechanical Function in Isolated Working Mouse Hearts

Heart rate (beats per min, BPM) and aortic pressure (mmHg) were measured with a Gould P21 pressure transducer (Harvard Apparatus; Holliston,

Massachusetts) connected to the aortic outflow line. Cardiac output and aortic flow (mL/min) were measured with Transonic T206 ultrasonic flow probes in the preload and afterload lines, respectively. Coronary flow (mL/min) was calculated as the difference between cardiac output and aortic flow. Cardiac power was calculated with the following equation: cardiac power (mWatts) = (cardiac output x peak systolic pressure x 2.22)/ 1000. Data were collected utilizing an MP100 system from AcqKnowledge (BIOPAC Systems, Inc USA; Goleta, California).

Measurement of Glycolysis, Glucose Oxidation and Palmitate Oxidation Rates in Isolated Perfused Hearts

Glycolysis, glucose oxidation rates and palmitate oxidation rates were measured by perfusing hearts with [5-^{3}H/U-^{14}C]glucose and [9,10-^{3}H-(*N*)]palmitic acid, respectively [3, 5]. Total myocardial production of $^{3}H_2O$ and $^{14}CO_2$ was determined at 10 min intervals during the 30 min and 40 min aerobic perfusion periods. Rates of glycolysis from [5-^{3}H]glucose were measured by separating $^{3}H_2O$ in perfusate samples from other radiolabelled substrate (i.e. [5-^{3}H]glucose) using a Dowex column. Glucose oxidation rates were determined by quantitative measurement of $^{14}CO_2$ production from the metabolism of [U-^{14}C]glucose, where $^{14}CO_2$ is released as a gas in the oxygenation chamber and $^{14}CO_2$ trapped in the perfusate buffer as $NaHCO_3$. The gaseous form of $^{14}CO_2$ is trapped by using 1 mol/L hyamine hydroxide solution and collected by continuously bubbling outflow gases from the perfusion system into 15 mL of hyamine hydroxide [6]. The $^{14}CO_2$ trapped in the perfusate as $NaHCO_3$ is released by addition of 9N H_2SO_4 to 1 mL of perfusion buffer in a sealed 25 mL test tube. Acidification of

the perfusate samples releases $^{14}CO_2$ which is trapped in the center well on filter paper saturated with hyamine hydroxide. Fatty acid oxidation rates were measured by collection of ^3H_2O derived from [9,10-^{3}H-(*N*)]palmitic acid [3]. ^3H_2O in perfusate samples was separated from [9,10-^{3}H-(*N*)]palmitic acid by a vapour transfer method. Briefly, 500 µL of water is added into a 5 mL scintillation vial and a lidless microfuge tube is placed inside the scintillation vial. 200 µL of perfusate sample is added to the microcentrifuge tube and the scintillation vial is capped. Vials are stored at 50 °C for 24 hr and then stored at 4 °C for 24 hr. The microcentrifuge tube is removed, and scintillation fluid added and vials were counted for radioactivity in the liquid scintillation counter.

Calculation of Tricarboxylic Acid Cycle (TCA) Acetyl CoA Production

The contribution of glucose and palmitate to acetyl CoA production entering the TCA cycle was calculated from the rates of palmitate oxidation and glucose oxidation, as measured in isolated perfused working mouse hearts. This calculation is based on oxidation of 1 mole of glucose producing 2 moles of acetyl CoA and 1 mole of palmitate producing 8 moles of acetyl CoA.

Glucose Tolerance Tests

Mice were individually housed and fasted for 6 hr and then subsequently injected intraperitoneally with glucose (2 g glucose/kg body weight). Blood glucose levels (mmol/L) were monitored in blood collected from the tail vein at time 0 min (baseline) just prior to glucose injection and at 10, 20, 30, 60, 90 and

120 min after the glucose injection using an ACCU-CHEK Aviva glucometer (Roche Diagnostics; Laval, Quebec) and test strips (0.6 μL blood sample).

Insulin Tolerance Tests

Mice were individually housed and fasted for 6 hr and then injected intraperitoneally with human recombinant insulin (Novo Nordisk Canada, Inc; 0.3 U insulin/kg body weight in young mice and 0.7 U insulin/kg body weight in aged mice on LF or HF diet). Blood glucose levels (mmol/L) were measured in blood collected from the tail vein at time 0 min (baseline) just prior to insulin injection and at 20, 30, 60, 90 and 120 min after the insulin injection using an ACCU-CHEK Aviva glucometer (Roche Diagnostics; Laval, Quebec) and test strips (0.6 μL blood sample).

Insulin Signaling Studies *in vivo*

To assess the status of insulin signaling pathways, mice were fasted overnight (16 hr), and then administered intraperitoneal injections of saline or human recombinant insulin (10 U insulin/kg body weight). Mice were sacrificed by cervical dislocation 10 min post-injection, and gastrocnemius muscle was rapidly removed, freeze-clamped in liquid nitrogen and stored at -80 $^{\circ}$C until processing for immunoblot analysis of various proteins in the insulin signaling pathway.

Analysis of Plasma Free Fatty Acids

NEFA concentrations were determined in plasma collected from fed and overnight fasted (16 hr) mice using a HR Series NEFA-HR free fatty acid determination kit (Wako Diagnostics, Germany). Blood was taken from the tail vein and collected into potassium-EDTA coated tubes and kept on ice. Whole blood was centrifuged for 10 min at 4,000 rpm at 4 °C and plasma collected and stored at -80 °C until time of analysis. The enzymatic method employed by Wako for the quantitative determination of plasma FFA levels involves acylation of coenzyme A (CoA) by the FAs present in 5 μL of plasma by the addition of acyl-CoA synthetase (ACS). The acyl-CoA produced is then oxidized by acyl-CoA oxidase to generate hydrogen peroxide, which in the presence of HRP allows for the oxidative condensation of 3-methy-N-ethyl-N(β-hydroxyethyl)-aniline (MEFA) with 4-aminoantipyrine to form a purple colored adduct which can be measured colorimetrically at 550 nm.

Analysis of Plasma Insulin

Insulin concentrations were determined in plasma from fed and overnight fasted mice using the commercially available insulin (mouse) ultrasensitive enzyme-linked immunosorbent assay kit (ALPCO Diagnostics; Salem, New Hampshire). Mouse monoclonal antibodies specific for insulin are immobilized to the 96-well plate and 5 μL of each plasma sample was added to each well with 75 μL of HRP enzyme labelled monoclonal antibody conjugate, and the 96-well plate was incubated for 2 hr at room temperature on an orbital microplate shaker at

~700-900 rpm. After incubation, the plate was thoroughly washed 6X with wash buffer, and then 100 μL of a provided substrate was added to each well to start the reaction, which was terminated after 30 min via addition of 100 μL of stop solution to each well. Plasma insulin levels (ng/mL) were measured by reading the optical density of the plate in a microplate reader at a wavelength of 450 nm.

Calculation of the Homeostasis Model Assessment of Insulin Resistance

The homeostasis model assessment of insulin resistance (HOMA-IR), an accepted surrogate marker of insulin sensitivity and resistance [7], was calculated taking into account both plasma glucose and insulin levels using the following equation:

$$\text{HOMA} = [\text{fasting glucose (mg/dL)} \times \text{fasting insulin (}\mu\text{U/mL)}]/22.5$$

Citrate Synthase Activity Assay

Frozen gastrocnemius muscle (5-10 mg) was homogenized in homogenization buffer containing 50 mmol/L Tris HCl (pH 8 at 4 °C), 1 mmol/L EDTA, 10% glycerol (w/v), 0.02% Brij-35 (w/v), 1 mmol/L dithiothreitol (DTT), and protease inhibitor (Sigma Aldrich; St. Louis, Missouri) and phosphatase inhibitor (Calbiochem; Gibbstown, New Jersey) cocktails. Samples were homogenized for 30 sec, left on ice for 10 min and then centrifuged at 10,000 x *g* for 20 min at 4 °C. The resulting supernatant was brought to a final dilution of 1/50, and 2 μL of the diluted sample was pipetted directly into a 96-well plate. Each well was brought up to a final volume of 190 μL with the addition of 184 μL of assay buffer (100 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L $MgCl_2$), 2 μL

of 5,5'-dithiobis-(2-nitrobenzic acid) (DTNB), and 2 μL of 30 mmol/L acetyl CoA. The reaction was initiated by the addition of 10 μL of 10 mmol/L oxaloacetic acid to each well, and using a spectrophotometer kinetic plate reader the absorbance was measured at 412 nm for a total of 2 min with readings taken at 30 sec intervals. The change in absorbance ($\Delta Absorbance_{412}$) was calculated by subtracting the readings after addition of 10 mmol/L oxaloacetic acid from baseline readings made prior to addition of oxaloacetic acid, and then multiplied by 60 to obtain a citrate synthase (CS) activity rate per min ($\Delta Absorbance_{412}$/min). CS activity was expressed as "μmol/mg wet weight /min" and was calculated with the following equation:

CS activity (μmol/mg wet weight tissue /min) = ($\Delta Absorbance_{412}$/min x 0.2 mL (volume of reaction) / (13.6 (ε for TNB) x 0.552 (pathlength for 96-well plate) x (mg of tissue in reaction))

β-Hydroxyacyl CoA Dehydrogenase Activity Assay

Frozen gastrocenemius muscle (5-10 mg) was homogenized in buffer containing 50 mmol/L Tris HCl (pH 8 at 4 °C), 1 mmol/L EDTA, 10% glycerol (w/v), 0.02% Brij-35 (w/v), 1 mmol/L dithiothreitol (DTT), and protease inhibitor (Sigma Aldrich; St. Louis, Missouri) and phosphatase inhibitor (Calbiochem; Gibbstown, New Jersey) cocktails. Samples were homogenized for 30 sec, left on ice for 10 min and then centrifuged at 10,000 x *g* for 20 min at 4 °C. The resulting supernatant was brought to a final dilution of 1/20, and 10 μL of the diluted sample was pipetted directly into a 96-well plate. Each well was brought up to a

final volume of 190 μL with the addition of 160 μL of 50 mmol/L imidazole (pH 7.4) and 20 μL of 1.5 mmol/L NADH. The reaction was initiated by the addition of 10 μL of 2 mmol/L acetoacetyl CoA and using a spectrophotometer kinetic plate reader the absorbance was measured at 340 nm for a total of 5 min with readings taken at 30 sec intervals. The absorbance of the 96-well plate was also read at 340 nm for 5 min prior to the addition of acetoacetyl CoA in order to obtain a baseline reading. The change in absorbance ($\Delta Absorbance_{340}$) was calculated by subtracting the readings after addition of 2 mmol/L acetoacetyl CoA from baseline readings made prior to addition of acetoacetyl CoA, and then multiplied by 60 to obtain a β-hydroxyacyl CoA Dehydrogenase (βHAD) activity rate per min ($\Delta Absorbance_{340}$/min). βHAD activity was expressed as "μmol/mg wet weight /min" and was calculated with the following equation:

βHAD Activity (μmol/mg wet weight /min) = ($\Delta Absorbance_{340}$/min x 0.2 mL (volume of reaction)) / (6.22 (ε for NADH disappearance) x 0.552 (pathlength for 96-well plate) x (mg of tissue in reaction))

Metabolic Profiling

Metabolic profiling of gastrocnemius muscle involved quantitative determination of metabolites (acylcarnitines, organic acids and amino acids) by gas chromatography–mass spectrometry (GC/MS) based on the dilution of stable-isotope-labelled internal standards (D3-C14:0, D3-C16:0, D3-C18:0, and 13C1-C18:1; CDN isotopes, Pointe-Claire, Quebec, and Isotec, St. Louis, Missouri), using a Trace Ultra GC coupled to a Trace DSQ MS (Thermo Fisher Scientific;

Austin, Texas). Frozen powdered gastrocnemius muscle (~50 mg) was homogenized in 300 μL ice-cold deionized H_2O in Potter-Elvehjem tubes. The lysate samples are diluted to a final concentration of 20:1 (tissue mass: volume) ratio with deionized H_2O. Lysates are subjected to one freeze-fracture cycle in liquid N_2 and thawed on ice and sonicated with a small pencil-tip probe at ~3 watts for 5 x 1 sec pulses. The sonicated lysate was then centrifuged at 14,000 x *g* for 15 min at 4 °C and supernatant divided into aliquots for determination of organic acids, acylcarnitines/amino acids and free/total carnitine. Acylcarnitine measurements in muscle lysates were made using flow injection tandem MS as previously described [8]. The data were acquired using a Micromass Quattro MicroTM system equipped with a model 2777 autosampler, a model 1525 HPLC solvent delivery system, as well as a data system controlled by MassLynx 4.0 operating system (Waters; Millford, Massachusetts). Organic acids were quantified from the lysate as previously described [9], employing Trace Ultra GC coupled to Trace DSQ MS operating under Excalibur 1.4 (Thermo Fisher Scientific; Austin, Texas).

Determination of Tissue Glycogens

Frozen gastrocenemius muscle or ventricular tissue (~ 5 mg) was boiled in 30% KOH at 12:1 (w/v) for 1 hr. Following the addition of 40 μL of 2% Na_2SO_4 and 400 μL absolute ethanol (EtOH), the samples were placed in the -20 °C freezer overnight. The next day samples were centrifuged at 3500 rpm for 5 min at 4 °C and the pellet washed with 66% EtOH. Glycogen in the pellet was converted

to glucose by boiling samples with 200 μL 2N H_2SO_4 for 3 hr. 100 μL of 1 mol/L MOPS (pH 6.8) was added to samples and samples were adjusted to a pH of 6.8-7. Samples were centrifuged at 3500 rpm for 5 min at 4 °C and the resulting supernatant collected for glucose determination. The glucose concentration in the supernatants was quantified using a Sigma Aldrich (St. Louis, Missouri) glucose analysis kit and standardized to tissue pellet mass.

Determination of Tissue Triglycerides

Triglyceride (TG) levels were determined in tissue homogenates prepared from either heart or gastrocnemius muscle (0.5-0.8 mg of protein). Tissue was powdered and lysed by homogenization using a Polytron® Homogenizer in lysis buffer (20 mmol/L Tris-HCL (pH 7.4), 50 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L Na pyrophosphate, 0.25 mol/L sucrose) supplemented with protease inhibitor (Sigma Aldrich; St. Louis, Missouri) and phosphatase inhibitor (Calbiochem; Gibbstown, New Jersey) cocktails. Homogneates were not centrifuged prior to analysis and protein concentration was determined using Bradford protein reagent. After phospholipid digestion with phospholipase C for 2 hr at 30 °C, tridecanoin (20 ng) was added as an internal standard, and lipids were extracted. The amount of TG was determined by gas-liquid chromatography.

Determination of Tissue Long Chain Acyl CoA Species

Approximately 5-7 mg of frozen ventricular tissue or gastrocnemius muscle was sonicated in 200 μL of ice cold 100 mmol/L KH_2PO_4 for 20 sec followed by the addition of 200 μL of cold n-propanol and sonicated for another 20 sec. Next

25 μL of saturated ammonium sulfate and 400 μL of acetonitrile were added successively to the homogenate, and the sample vortexed for 5 min and then centrifuged at 2,100 x *g*. The top layer was transferred to a microcentrifuge tube and evaporated under N_2 gas. The solid residue was mixed with 100 μL of 0.5 mol/L chloroacetaldehyde reagent and then centrifuged at 21,000 x *g* for 5 min to form acyl etheno-CoA esters. The resultant supernatant was then heated at 85 °C for 20 min and stored at -20 °C until HPLC analysis [10].

Identification and quantification of the major LCACoA molecular species (C16:0, C18:0, and C18:1) was performed by HPLC. In brief, 5-20 μL of sample was run through a C18 Guard Cartridge and a Luna column of phenylhexyl coated silica particles maintained at 40°C on a Beckman System Gold HPLC. Flow rate was set at 0.4 mL/min and the analyte was detected utilizing a Perkin Elmer LS-5 fluorescence spectrophotometer with an excitation wavelength of 230 nm and an emission wavelength of 420 nm. The mobile phase consisted of buffer A (90% (v/v) acetonitrile, 1% (v/v) acetic acid) and buffer B (90% (v/v) acetonitrile). Peaks were integrated utilizing the Beckman System Gold software.

Determination of Tissue Ceramides

Ceramides were extracted from approximately 5-7 mg of frozen ventricular tissue or gastrocnemius muscle and measured by HPLC [11]. In brief, ceramides was extracted from tissue in 1 mL of a 1:1:1 chloroform-methanol-1 N HCl in the presence of 0.3 mL saline solution. The resulting organic phase was separated and dried under N_2 gas. Next 0.5 mL of 1 mol/L KOH in 90% (v/v) methanol was

added to the solid residue and samples were heated at 90 °C for 1 hr to deacylate ceramide into sphingosine. Samples were then extracted with 1 mol/L HCl in methanol, chloroform, and 1 mol/L aqueous NaCl. The resulting organic phase was dried under N_2 gas, redissolved in methanol, and derivatized to *o*-phthalaldehyde to generate a fluorescent compound that was separated by HPLC and quantified by fluorescence spectrometry.

Determination of Adenine Nucleotides

Approximately 20-25 mg of frozen ventricular tissue was homogenized for 30 sec with a Polytron® Homogenizer in 300 μL of 6% (v/v) perchloric acid and 2 mmol/L DTT. After homogenization the samples were left on ice for 30 min and then centrifuged at 12,000 x *g* for 5 min. The resulting supernatant was collected and volumes recorded, and EGTA was added to a final concentration of 0.5 mmol/L. Samples were then neutralized to a pH to 7.6-7.8 using 0.5 mol/L K_2CO_3, to ensure stability of the adenine nucleotides. Neutralized perchloric acid extracts were left on ice for 10 min and then centrifuged at 10,000 x *g* for 2 min, and the resultant supernatant stored at -80 °C until analysis by HPLC [12].

100 μL of each sample was run through a Supelcosil™ LC-18-T guard cartridge and a Supelcosil™ LC-18-T column on a Beckman System Gold HPLC. Flow rate was set at 1.5 mL/min and analyte detection occurred at an absorbance of 260 nm on a Beckman System Gold model 168 diode array detector. The mobile phase consisted of buffer A (35 mmol/L K_2HPO_4, 6 mmol/L tetrabutylammoniumhydrogensulfate, pH = 6.0) and buffer B (a mixture of buffer

A and acetonitrile in a ratio of 1:1 (v/v)). Peaks were integrated utilizing the Beckman System Gold software.

Tissue Homogenization and Immunoblot Analysis

Overnight fasted mice were anaesthetized with sodium pentobarbital, and tissues, including heart, liver and gastrocnemius muscle, were rapidly removed and freeze-clamped in liquid nitrogen and stored at −80 °C. Hearts were rapidly removed and rinsed in ice cold 1X PBS (pH 7.4) to remove blood and excess tissue around the heart trimmed prior to being freeze-clamped in liquid N_2. Tissue was powdered using a motor and pestle chilled in liquid N_2. Tissue (20-50 mg) was lysed by mechanical homogenization (30 sec) in ice cold lysis buffer (20 mmol/L Tris-HCL (pH 7.4), 50 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L Na pyrophosphate, 0.25 mol/L sucrose) added at a 10:1 ratio of buffer to tissue, supplemented with protease inhibitor (Sigma Aldrich; St. Louis, Missouri) and phosphatase inhibitor cocktails (Calbiochem; Gibbstown, New Jersey). Homogenates were centrifuged for 10 min at 1000 x g at 4 °C and the supernatant was collected and pellet discarded. Protein concentration was determined by the Bradford Protein Assay (Bio-Rad; Richmond, California) with samples assayed in triplicate. Equal amounts of protein (20 μg) were loaded onto 5 or 8% bis-acrylamide gels and separated by SDS-PAGE, and transferred onto nitrocellulose membranes at 90 V for 2.5 hrs. Membranes were incubated for 1 hr at room temperature in 5% milk-TBST blocking solution and incubated overnight at 4 °C with their respective primary antibodies diluted in 5% BSA-TBST buffer.

Membranes were washed 3 x 5 min with 1X TBST buffer with constant agitation, and incubated with the appropriate HRP-conjugated secondary antibodies diluted in 5% milk-TBST for 1 hr at room temperature. Membranes were washed 3 x 5 min with 1X TBST buffer and the immune-complex was visualized using the Amersham Pharmacia enhanced chemiluminescence Western blotting detection system.

Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). For the comparison of two group at multiple timepoints, a Two-way repeated measures analysis of variance (ANOVA) was used for the determination of statistical significance. For comparison of two groups, a two-tailed Student's unpaired t-test was employed. For the comparison of more than two groups, a One-way ANOVA was employed. For the comparison of two groups affected by two factors, a Two-way ANOVA was employed. A Bonferroni post-hoc analysis was employed after ANOVA. A value of $p < 0.05$ is considered significant.

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CHAPTER 3.

Alterations in Skeletal Muscle Fatty Acid Handling Predispose Middle-aged Mice to Diet-induced Insulin Resistance

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* These authors contributed equally to this work.

My role in this work involved performing diet experiments in young and aged mice (except those noted below). Experiments, data analysis and writing of the manuscript were performed in direct collaboration with post-doctoral fellow Debby Koonen. Timothy Koves, Olga Ilkayeva and Deborah Muoio performed the GC/MS experiments, René Jacobs performed the triglyceride assays, Debby Koonen performed analysis of metabolic cage data and the citrate synthase and β-hydroxyacyl-CoA dehydrogenase assays, and Cindy Kao and Carrie-Lynn Soltys performed some of the immunoblots.

CHAPTER 3.

Alterations in Skeletal Muscle Fatty Acid Handling Predispose Middle-aged Mice to Diet-induced Insulin Resistance

Abstract

Although advanced age is a risk factor for type 2 diabetes, a clear understanding of the changes that occur during middle-age that contribute to the development of skeletal muscle insulin resistance is currently lacking. Therefore, we sought to investigate how middle-age impacts skeletal muscle fatty acid handling and to determine how these alterations may contribute to the development of high fat diet-induced insulin resistance. Systemic and skeletal muscle insulin resistance were studied in young and middle-aged wild-type (WT) and CD36 knockout (KO) mice fed either a standard or a high fat diet for 12 weeks. Molecular signaling pathways, intramuscular lipid accumulation, as well as *in vivo* mitochondrial substrate flux were analyzed in skeletal muscle from young and middle-aged mice. Middle-aged mice fed a standard diet demonstrated an overall decline in whole-body metabolic rate, and this was associated with an increase in intramuscular triglycerides and a modest impairment in glucose utilization and insulin-stimulated Akt activation in muscle. When middle-aged

mice were fed a high fat diet for 12 weeks they were more susceptible to the effects of high fat feeding and developed more severe insulin resistance when compared to that of young mice. However, limiting skeletal muscle fatty acid uptake and excessive lipid accumulation in middle-aged CD36 KO mice prevented the high fat diet-induced reduction in metabolic rate and development of insulin resistance. Taken together, our data provide insight into the mechanisms by which aging becomes a risk factor for the development of insulin resistance. Our data also demonstrate that limiting skeletal muscle fatty acid uptake is an effective approach for treating and/or preventing the development of age-associated insulin resistance and metabolic disease during exposure to a high fat diet.

Introduction

Over the past several decades the prevalence of type 2 diabetes (T2D) has increased dramatically, largely due to the obesity epidemic brought about by the move to a more sedentary lifestyle and consumption of foods high in dietary fat [1]. With the total number of people affected by T2D estimated to increase to 366 million worldwide by the year 2030, T2D is by far one of the main health challenges of the 21st century [2]. Although it is widely accepted that skeletal muscle insulin resistance is a major determinant in both the onset and progression of T2D [3], the precise cause and mechanism responsible for decreased insulin action in skeletal muscle remains to be fully understood. That said, in obesity-related insulin resistance, increased fatty acid (FA) availability has been suggested to be one of the major contributors to this disorder [4]. The prevailing theory is that exposure of skeletal muscle to excessive lipid supply leads to increased FA uptake and intramuscular lipid accumulation in the form of long chain acyl CoA (LCACoA), triglyceride (TG), ceramide and/or diacylglycerol (DAG) [5, 6]. Accumulation of these lipid intermediates is thought to directly or indirectly interfere with insulin signalling in skeletal muscle, thereby leading to insulin resistance [7].

Multiple mechanisms may contribute to intramuscular lipid accumulation including increased FA transport [8-10] and/or diminished mitochondrial uptake and oxidation of FAs [11-13]. It is generally believed that skeletal muscle insulin resistance develops secondary to impaired mitochondrial FA oxidation [11-13]. In

humans [14-18] and rodents [19, 20], skeletal muscle mitochondria from subjects with insulin resistance and/or T2D have been found to be reduced in number and/or have diminished oxidative capacity. Based on this evidence, it has been proposed that impaired mitochondrial function plays a critical role in the pathogenesis of insulin resistance [21]. Contrary to this, a growing number of studies have shown that lipid accumulation is not associated with skeletal muscle insulin resistance [6, 22, 23], and that skeletal muscle mitochondrial capacity is actually increased in models of insulin resistance [24-28]. Furthermore, it has recently been proposed that the cause of skeletal muscle insulin resistance may not result from impaired FA oxidation, but rather excessive skeletal muscle mitochondrial FA oxidation and ensuing mitochondrial stress [28, 29]. While it is not known which of these two processes is most relevant in the pathogenesis of skeletal muscle insulin resistance, it is clear that excessive uptake of FAs into the skeletal muscle plays a central role in diet-induced insulin resistance.

As advanced age is a significant risk factor in the etiology of T2D [30, 31], the accompanying loss of mitochondrial function commonly observed with normal aging has been proposed to contribute to the high incidence of T2D in the elderly population [32]. Similar to the pathogenesis of obesity-related insulin resistance discussed above, skeletal muscle oxidative enzyme activity and mitochondrial content have been shown to decline with age [32-35]. However, clear evidence of a functional impairment in mitochondria in aging and T2D remains inconclusive [21, 28, 36]. A clear understanding of the physiological and metabolic changes that occur during the onset of middle-age and the influence

that this may have on the development of insulin resistance is currently lacking. Based on this rationale, the study herein was designed to investigate how middle-age impacts whole-body glucose utilization, FA handling and intramuscular lipid accumulation within skeletal muscle. As aging acts as a risk factor for many diseases, we hypothesize that aging alters whole-body and skeletal muscle energy metabolism, potentially via impaired mitochondrial function, and subsequently increases the susceptibility for the development of diet-induced obesity and insulin resistance. A better understanding of how aging impacts the development of insulin resistance and T2D is particularly important given the growing size of the middle-aged population in the western world, and the continuing obesity epidemic which will undoubtedly create a significant burden on the health care system [37].

Materials and Methods

Animals

The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with Canadian Council of Animal Care guidelines.

CD36 Deficient Mice

CD36 WT and deficient mice (C57BL/6) were supplied by Dr. Maria Febbraio (Lerner Research Institute, Cleveland, Ohio) and generated in her laboratory as previously described in Chapter 2.

In vivo Metabolic Analysis

Metabolic and behavioural measurements, including O_2 consumption (VO_2), CO_2 production (VCO_2), food intake, heat production and locomotor activity were measured by indirect calorimetry, as previously described in Chapter 2.

Analysis of β-hydroxyacyl CoA Dehydrogenase and Citrate Synthase Activity

β-hydroxyacyl CoA dehydrogenase (β-HAD) activity was determined by measuring the disappearance of NADH, and citrate synthase (CS) activity was determined, using oxaloacetate as a substrate, in freshly-made homogenates prepared from frozen gastrocnemius muscle collected from mice following an overnight (16 hr) fast.

Determination of Skeletal Muscle Triglyceride, Long-chain Acyl CoA and Ceramide Levels

TGs, LCACoAs and C-18 ceramides were extracted from frozen powdered gastrocnemius muscle of fasted (16 hr) mice and assayed as described previously in Chapter 2.

Glucose Tolerance Tests

Mice were fasted for 6 hr starting at the onset of the dark phase (active) and then injected intraperitoneally with glucose (2 g glucose/kg body weight), and blood glucose levels (mmol/L) monitored over 120 min, as described previously in Chapter 2.

Insulin Tolerance Tests

Mice were fasted for 6 hr and then injected intraperitoneally with human recombinant insulin (0.3 U insulin/kg body weight in young mice and 0.7 U insulin/kg body weight in aged mice) and blood glucose levels (mmol/L) monitored over 120 min, as described previously in Chapter 2.

Analysis of Plasma Parameters

Blood was collected from fed and overnight (16 hr) fasted mice via the tail vein and blood glucose, plasma free fatty acids and plasma insulin levels were assayed as described in the Methods chapter (Chapter 2).

Determination of the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)

The homeostasis model assessment of insulin resistance (HOMA-IR), used as a surrogate marker of insulin resistance, was calculated using the equation:

HOMA = [fasting glucose (mg/dL) x fasting insulin (μU/mL)]/22.5.

Immunoblot Analysis

Tissue was homogenized, homogenates were run on SDS-PAGE gels and membranes probed with antibodies as described in Chapter 2. Signals were visualized using chemiluminescence and densitometry performed using Image J software.

Metabolic Profiling

Frozen gastrocnemius muscle from overnight fasted mice was prepared as described in Chapter 2. Acylcarnitine measurements were made using flow injection tandem mass spectrometry (MS/MS) and organic acid measurements were made by gas chromatography (GC) as previously described in the Methods chapter.

Statistical Analysis

All data are presented as means ± SEM. For comparison of two groups, a Student's unpaired t-test was used. For comparison of two groups over timecourse experiments, two-way ANOVA with repeated measures on time followed by a Bonferroni multiple comparisons post-hoc test was performed. For comparisons of levels of acylcarnitines and organic acids between aged CD36 WT and KO mice, main effects of genotype and diet as well as genotype x diet interactions of each metabolite were detected by two-way ANOVA. A value of $p < 0.05$ is considered significant.

Results

Depressed metabolic rate in middle-aged mice compared to young mice fed a standard laboratory diet

As expected, middle-aged C57BL/6 mice (52-58 weeks) had significantly greater body weight when compared to that of young (12-14 weeks) mice (Fig. 3-1A; BW: 35.93 ± 1.94 vs. 27.32 ± 0.98 g, for middle-aged and young mice, respectively, $p < 0.05$). As an age-related decline in resting metabolic rate and energy expenditure has been proposed to contribute to the development of insulin resistance, we determined if metabolic rate is depressed in middle-aged mice fed a standard laboratory diet (4 kcal% fat). To address this, C57BL/6 mice of 12-14 (young) or 52-58 (middle-aged) weeks of age were analyzed using indirect calorimetry. As mice aged, whole-body substrate utilization was altered as indicated by reductions in respiratory exchange ratio ($RER=VCO_2/VO_2$; Fig. 3-1B). Whereas young mice use more carbohydrates as an energy substrate (RER closer to 1), middle-aged mice use a greater proportion of FA throughout the day, as indicated by a lower RER value (Fig. 3-1B). In addition, significant reductions in oxygen consumption (VO_2; Fig. 3-2A) and carbon dioxide production (VCO_2; Fig. 3-2B) were observed during both the dark (active) and light (inactive) phase in middle-aged mice as compared to young mice. Consistent with this, heat production (Fig. 3-3A) was reduced by 15-20% (dark and light phase) in middle-aged mice as compared to young mice. Despite the increase in body weight, total activity (rearing, grooming, and ambulation) was similar between age groups (Fig.

3-3B). Taken together, these data indicate that middle-aged mice have a lower metabolic rate and energy expenditure (VO_2 and heat production) as compared to young mice, and that this may increase the susceptibility towards obesity and metabolic disease.

Mitochondrial content and function is not reduced in skeletal muscle from middle-aged mice

As skeletal muscle metabolism significantly contributes to whole-body basal metabolic rate, we addressed whether skeletal muscle metabolism was indeed depressed in middle-aged mice by determining the activities of two key enzymes involved in regulating mitochondrial metabolism. Interestingly, the activity of β-hydroxyacyl-CoA dehydrogenase (β-HAD; Fig. 3-4A) was elevated in muscle from middle-aged mice as compared to young mice, and the activity of citrate synthase (CS; Fig. 3-4B) followed a similar upward trend, suggesting that despite a decline in metabolic rate that β-oxidation and TCA cycle activity, respectively, were not directly compromised in middle-aged mice. Since it has been reported that skeletal muscle mitochondrial content and protein expression are diminished with advanced age [38-40], we determined protein expression of several mitochondrial oxidative phosphorylation complexes in gastrocnemius muscle. However, we found no difference in mitochondrial protein expression between muscle from young and aged mice (Fig. 3-4C), suggesting that mitochondrial content is not altered at middle-age, and is likely not a contributing factor to the observed decline in metabolic rate.

Age-induced reduction in AMP-activated protein kinase signaling and lipid accumulation in skeletal muscle is associated with the development of whole-body glucose intolerance

As AMP-activated protein kinase (AMPK) is a key regulator of glucose and lipid metabolism [41, 42], we next assessed whether skeletal muscle AMPK activation is altered in middle-age. Consistent with previous reports in aged rodents [43, 44], phosphorylation of AMPK (Thr 172), which is indicative of its activity, was significantly reduced in skeletal muscle from middle-aged mice as compared to levels in young mice (Fig. 3-5A and 3-5B), whereas total levels of AMPK remained unchanged (Fig. 3-5A and 3-5C). As well, the phosphorylation status of acetyl CoA carboxylase (ACC), the downstream target of AMPK that indirectly regulates FA entry into the mitochondria, was also significantly decreased in middle-aged skeletal muscle (Fig. 3-5D). Furthermore, intramuscular levels of free carnitine (C_0) were reduced in muscle from middle-aged mice (Fig. 3-6A), a finding that is consistent with previous studies showing a decline in carnitine reserve in insulin resistant states, including advanced age and diet-induced obesity [45]. As carnitine is an essential co-factor in the translocation of long chain FAs across the mitochondria for β-oxidation, an age-related carnitine insufficiency may result in perturbations in mitochondrial FA oxidation by limiting FA entry into the mitochondria.

Consistent with decreased energy expenditure, increased adiposity and potentially depressed FA metabolism in skeletal muscle from middle-aged mice, levels of intramuscular TG were significantly elevated in middle-aged mice as

compared to young mice (Fig. 3-6B). As lipid accumulation in the skeletal muscle of both rodents and humans has been proposed to be one of the primary causes of skeletal muscle insulin resistance [46, 47], we next investigated whether lipid accumulation is associated with impaired glucose sensitivity in middle-aged mice. Indeed, whole body glucose tolerance was modestly impaired in middle-aged mice (Fig. 3-7A and 3-7B). While fed plasma glucose and fasted insulin levels (Fig. 3-7C and 3-7D, respectively) were similar between groups, there was a trend towards increased fasting plasma glucose levels in aged mice (Fig. 3-7C). Furthermore, basal and insulin-stimulated Akt phosphorylation in skeletal muscle was decreased by 40 and 25%, respectively, in the muscle of middle-aged mice compared to young mice (Fig. 3-8A and 3-8B, respectively). Taken together, these data suggest that whole-body insulin sensitivity is modestly impaired with aging *per se* and may partly explain the high prevalence of insulin resistance and metabolic syndrome in the elderly population.

Aging increases the sensitivity to diet-induced obesity and metabolic disease

As we speculated that middle-aged mice are more susceptible than young mice to the development of obesity-related insulin resistance, we fed young and middle-aged mice a low fat (LF) or high fat (HF; 10 or 60 kcal% fat, respectively) diet for 12 weeks. As expected the HF-fed young mice displayed weight gain (Fig. 3-9A and 3-9B) and showed fasting hyperglycaemia (Fig. 3-9C) as compared to young mice fed a LF diet. Although young mice fed a HF diet displayed signs of whole-body glucose intolerance (Fig. 3-10A and 3-10B) and

impaired insulin sensitivity (Fig. 3-10C), surprisingly skeletal muscle Akt phosphorylation (Fig. 3-10D) and plasma insulin levels (Fig. 3-11B and 3-11C, left panel) remained relatively normal in these mice. In contrast, middle-aged mice fed a HF diet displayed greater weight gain than young mice at the end of the 12 weeks (Fig. 3-11A), and had dramatically elevated insulin levels during both the fed and fasted states (Fig. 3-11B and 3-11C, right panel). Moreover, the HF diet-induced hyperinsulinemia was associated with a modest increase in fasting glucose concentrations (Fig. 3-12A), as well whole-body glucose clearance (Fig. 3-12B and 3-12C) was significantly impaired in middle-aged mice fed a HF diet when compared to age-matched mice fed a LF diet. Although activation of insulin signaling pathways, as determined by phosphorylation status of Akt, does not appear impaired in skeletal muscle of young (Fig. 3-10D) or middle-aged mice (Fig. 3-13A) in response to a HF diet compared to those mice on LF diet, this is likely due to compensatory increases in levels of circulating insulin (Fig. 3-11B and 3-11C, right panel) observed in the respective HF groups in attempts to overcome peripheral insulin resistance. Despite this, HOMA-IR values, which are a surrogate marker for insulin resistance that take into account both glucose and insulin levels [48], were found to be significantly higher in the HF-fed middle-aged mice (Fig. 3-13B), suggesting that 12 weeks of HF feeding induces a more dramatic insulin resistance in middle-aged mice than in young mice and that advanced age increases the susceptibility to developing diet-induced insulin resistance.

Altered FA handling and lipid accumulation in skeletal muscle from insulin resistant middle-aged mice

As HF diet-induced insulin resistance in young rodents is associated with an increased efficiency of FA uptake into skeletal muscle [10], we next determined expression of CD36, a protein that facilitates FA transport across the plasma membrane, in skeletal muscle of middle-aged mice fed a HF diet. Consistent with previous reports in young mice [10, 49, 50], CD36 expression was found to be significantly elevated in skeletal muscle of middle-aged mice fed a HF diet compared to a LF diet (Fig. 3-14A). In accordance with increased CD36 protein expression and the important role of CD36 in FA transport, there was a 3-fold increase in intramuscular TG levels in HF-fed middle-aged mice compared to LF-fed mice (Fig. 3-14B), as well, lipid-derived intermediates including LCACoA esters (Fig. 3-14C) and ceramides (Fig. 3-14D) were also significantly elevated. Taken together, these data suggest that increased CD36-mediated FA uptake may contribute to lipid accumulation and impaired insulin sensitivity in skeletal muscle of middle-aged mice fed a HF diet.

Ablation of CD36 protects against diet-induced obesity and improves metabolic rate in middle-aged mice

As increased skeletal muscle FA uptake has been shown to contribute to lipid accumulation during diet-induced obesity and is linked to insulin resistance [8, 10, 50], we next addressed whether inhibition of FA transport into the skeletal muscle could alter the observed responses of middle-aged mice to a HF diet. To

investigate this we utilized the CD3 knockout (KO) mouse, which has skeletal muscle FA transport rates approximately 40-70% of WT mice [51, 52]. Interestingly, there was a striking difference in weight gain between middle-aged WT and CD36 KO mice following 12 weeks of HF feeding (Fig. 3-15A) with middle-aged CD36 KO mice accumulating 51% less weight than WT mice over the same period of time (Fig. 3-15B). While differences in caloric intake (Fig. 3-15C) or substrate preference (Fig. 3-16A) between groups could not account for this dramatic difference in weight gain, indirect calorimetry indicated that although RER appeared similar between HF-fed WT and CD36 KO mice, that in fact middle-aged CD36 KO mice fed a HF diet had significantly increased VO_2 (Fig. 3-16B) and VCO_2 (Fig. 3-16C) during both the dark (active) and light (inactive) phase as compared to HF-fed middle-aged WT mice. Despite that HF–fed middle-aged WT mice weighed nearly 20 g more than WT mice fed a LF diet, total activity was similar between the two WT groups (Fig. 3-17A). Interestingly, total activity of middle-aged CD36 KO mice was found to be 3-times greater than middle-aged WT mice (Fig. 3-17A). Although this increased activity in the middle-aged CD36 KO mice fed a HF diet could be attributed to the absence of obesity (Fig. 3-15B), heat production was also increased in middle-aged CD36 KO mice fed a LF diet as compared to LF fed middle-aged WT mice (Fig. 3-17B) and in HF fed middle-aged KO mice when normalized for body weight (Fig. 3-17C). Together these data suggest that energy expenditure and metabolic rate is improved in CD36 KO mice as compared to their age-matched WT counterparts,

and that limiting FA uptake via ablation of CD36 may protect against the development of HF diet-induced obesity.

Alterations in muscle metabolites and lipid balance correspond with protection against diet-induced insulin resistance

As energy expenditure and metabolic rate were significantly increased in middle-aged CD36 KO mice, we next sought to determine whether skeletal muscle metabolism was preserved in middle-aged CD36 KO mice fed a HF diet. To gain a more comprehensive metabolic assessment of skeletal muscle metabolism in middle-aged WT and CD36 KO mice fed a LF or HF diet, we used mass spectrometry to measure a broad range of intermediary metabolites, including acylcarnitines of various chain lengths and organic acids. Acylcarnitines are by-products of fuel catabolism that respond to changes in substrate availability and/or flux limitations at specific mitochondrial enzymes [29, 53, 54]. Middle-aged CD36 KO mice fed a LF diet had elevated levels of acetyl-carnitine (C2), and β-hydroxybutyryl-carnitine (C4OH) compared to their WT counterparts (Fig. 3-18A; Table 3-1). Whereas several short-chain acylcarnitine species, including C2 and C4OH, as well as propionyl-carnitine (C3) and succinyl-carnitine (C4DC) tended to increase in response to HF diet, these same metabolites trended downward in CD36 KO mice fed a HF diet (Fig. 3-18A; Table 3-1).

In addition, several long chain (LC) acylcarnitine species were reduced in muscle from WT mice fed a HF diet, while at the same time levels of hydroxylated acylcarnitine species (LCOH) were increased, resulting in a robust increase in the LC to LCOH acylcarnitine ratio (Fig. 3-19; Fig. 3-18B and 3-18C).

As LC acylcarnitines accumulate when their production by mitochondrial CPT1 exceeds flux through β-oxidation enzymes, such as long chain acyl CoA dehydrogenase (LCAD) and βHAD [55], this metabolite pattern is consistent with a diet-induced shift in flux limitation from LCAD to βHAD. Notably, levels of many LC and LCOH acylcarnitines were lower in the CD36 KO mice fed a HF diet compared to WT mice fed a HF diet (Fig. 3-19; Fig. 3-18B and 3-18C). The organic acid intermediates of the TCA cycle were less responsive to both diet and genotype, although subtle changes were detected in succinate, fumarate and citrate levels (Fig. 3-20A and 3-20B; Table 3-2). Although these metabolite measurements do not fully characterize mitochondrial substrate flux, together the data suggest that ablation of CD36 not only alters baseline mitochondrial and intermediary metabolism, but also significantly impacts the muscle response to elevated lipid exposure via a HF diet.

Ablation of CD36 and reduction in intramuscular lipid accumulation prevent the development of diet-induced insulin resistance

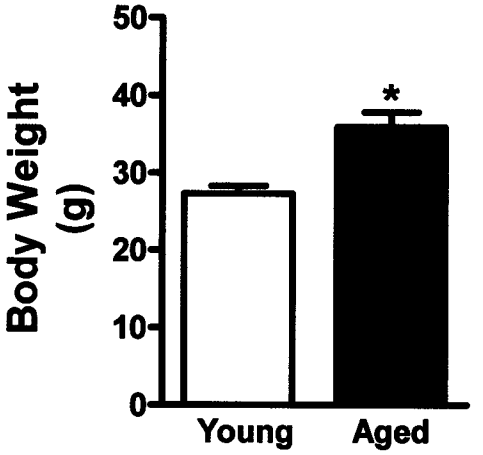
Despite the changes in muscle acylcarnitine levels, the activity of β-HAD was not altered in WT mice (data not shown). Interestingly, while aging was associated with a decline in AMPK activation, HF feeding did not appear to further impair AMPK activity in muscle from middle-aged WT mice on a HF diet as compared to LF fed WT mice (Fig. 3-21A). Moreover, middle-aged CD36 KO mice fed a HF diet were not protected from reductions in P-AMPK or P-ACC levels (Fig. 3-21A and 3-21B, respectively), suggesting that alternate mechanisms

are responsible for the improved metabolic phenotype observed in CD36 KO mice. Likely as a result of reduced tissue FA uptake in CD36 KO mice, plasma free FA levels were significantly elevated in HF-fed CD36 KO mice as compared to HF-fed WT mice (Fig. 3-22A). Despite high circulating levels of FAs, CD36 ablation was associated with a significant reduction in skeletal muscle TG (Fig. 3-22B) and LCACoA (Fig. 3-22C) levels. Interestingly, levels of intramuscular ceramides, which have been proposed to directly impair insulin signaling [46, 56], remained similar between HF-fed groups (Fig. 3-22D), suggesting that accumulation of lipid-derived intermediates other than ceramides, may contribute to impaired insulin sensitivity in response to HF feeding. Indeed, reduced intramuscular lipid accumulation in middle-aged CD36 KO mice was associated with both significantly lower fasting blood glucose levels (Fig. 3-23A) and fasting plasma insulin levels (Fig. 3-23B) compared to HF-fed middle-aged WT mice. Moreover, middle-aged CD36 KO mice on a HF diet displayed improved whole-body glucose utilization by glucose tolerance test (Fig. 3-23C and 3-23D), as well as dramatically improved insulin-induced glucose clearance (Fig. 3-24A) compared to HF-fed middle-aged WT mice, suggesting that whole-body insulin sensitivity and glucose utilization may be restored by preventing lipid accumulation in peripheral tissues, such as skeletal muscle. Interestingly, despite improved glucose utilization and reduced plasma insulin levels in these mice, phosphorylation status of Akt remained similar in skeletal muscle from middle-aged WT & CD36 KO mice on HF diet (Fig. 3-24B).

Figure 3-1: Body weights and respiratory exchange ratio from young and middle-aged mice fed a standard rodent diet

Body weight of young (12-14 weeks) and middle-aged (52-58 weeks) C57BL/6 mice **(A)**. Indirect calorimetry was performed to measure respiratory exchange ratio (RER; VCO_2/VO_2) in young and middle-aged mice over 24 hr for a complete 12 hr dark (active) and 12 hr light (inactive) cycle **(B)**. Values are means ± SEM of $n = 5–7$ mice in each group. * $p < 0.01$ vs. young mice as determined Student's unpaired t-test **(A)**, * $p < 0.05$ vs. young mice as determined by two-way ANOVA with a Bonferroni post-hoc test **(B)**.

A


Body Weight (g)
50
40
30
20
10
0
*
Young
Aged

B

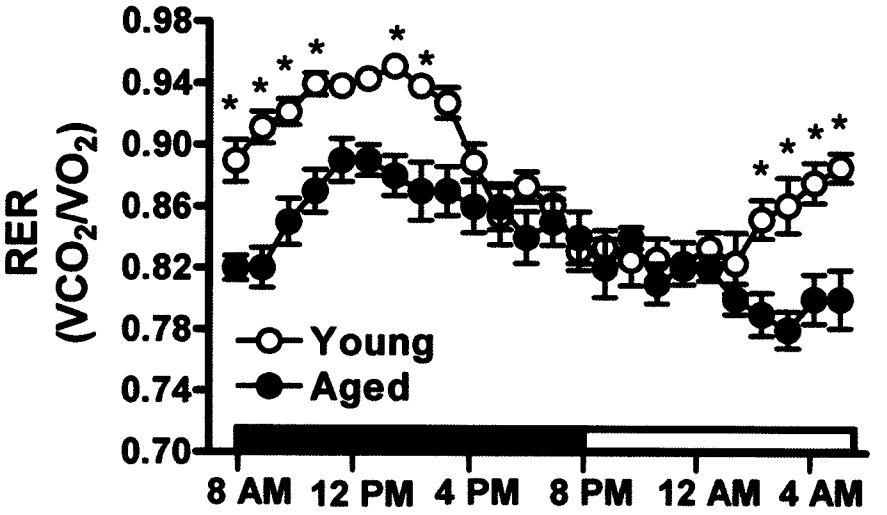
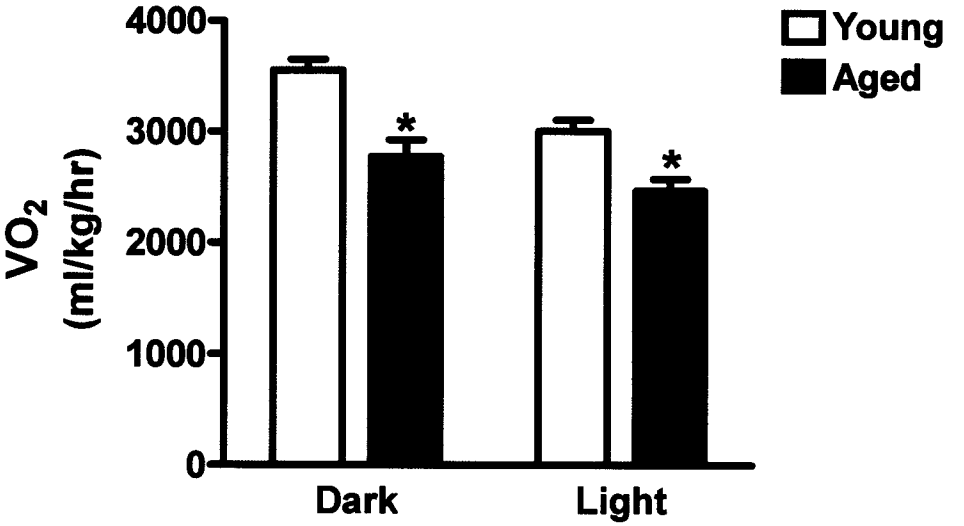

RER (VCO_2/VO_2)
0.98
0.94
0.90
0.86
0.82
0.78
0.74
0.70
Young
Aged
8 AM
12 PM
4 PM
8 PM
12 AM
4 AM

Figure 3-2: Reduced oxygen consumption and carbon dioxide production in middle-aged mice as compared to young mice at rest

Indirect calorimetry was performed on young (12-14 weeks) and middle-aged (52-58 weeks) C57BL/6 mice to measure rates of oxygen consumption (VO_2; **A)** and carbon dioxide production (VCO_2; **B)** over the course of 24 hr cycle during the dark (active) and light (inactive) phase and adjusted for total body weight. Values are means ± SEM of n = 5–7 mice in each group. * $p < 0.01$ vs. young mice in either dark or light phases as determined by Student's unpaired t-test.

A


4000
3000
2000
1000
0
VO2 (ml/kg/hr)
Young
Aged
*
*
Dark
Light

B

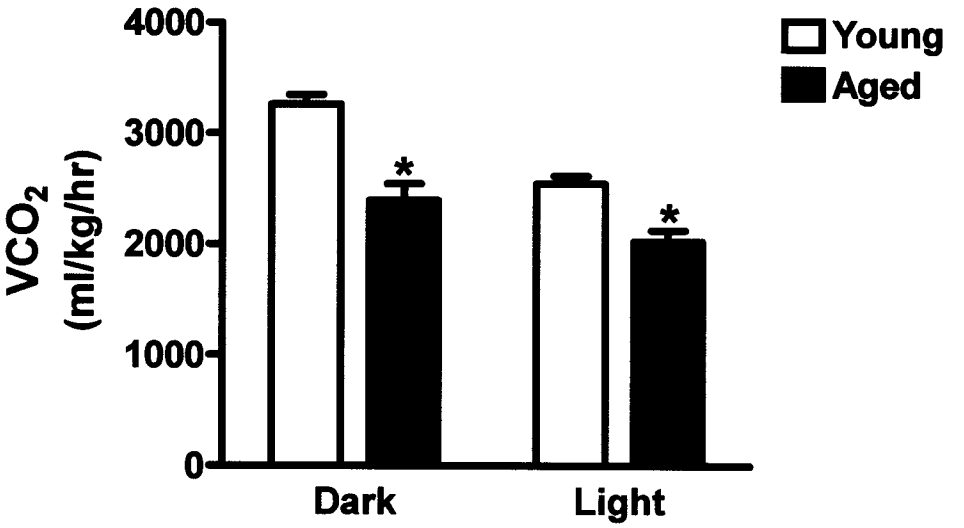
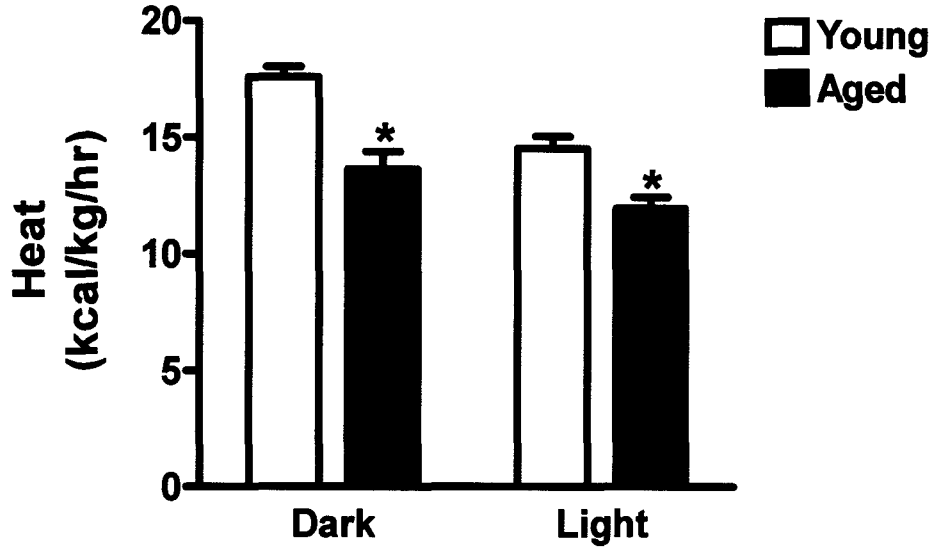

4000
3000
2000
1000
0
VCO2 (ml/kg/hr)
Young
Aged
*
*
Dark
Light

Figure 3-3: Heat production is decreased in middle-aged mice compared to young mice, whereas total activity levels were unchanged

Heat production was measured in young and middle-aged mice while being housed in enclosed metabolic cages for 24 hr cycle. Values were adjusted for total body weight and graphs represent the average over the dark (active) or light (inactive) phases **(A).** Total activity levels (represented as number of beam brakes) were measured as a sum of all locomotor activities (rearing, jumping, grooming/scratching) over the dark and light phases **(B).** Values are means ± SEM of n = 5–7 mice in each group. * $p < 0.01$ vs. young mice in either dark or light phases as determined by Student's unpaired t-test.

A


20
15
10
5
0
Heat
(kcal/kg/hr)
*
*
Young
Aged
Dark
Light

B

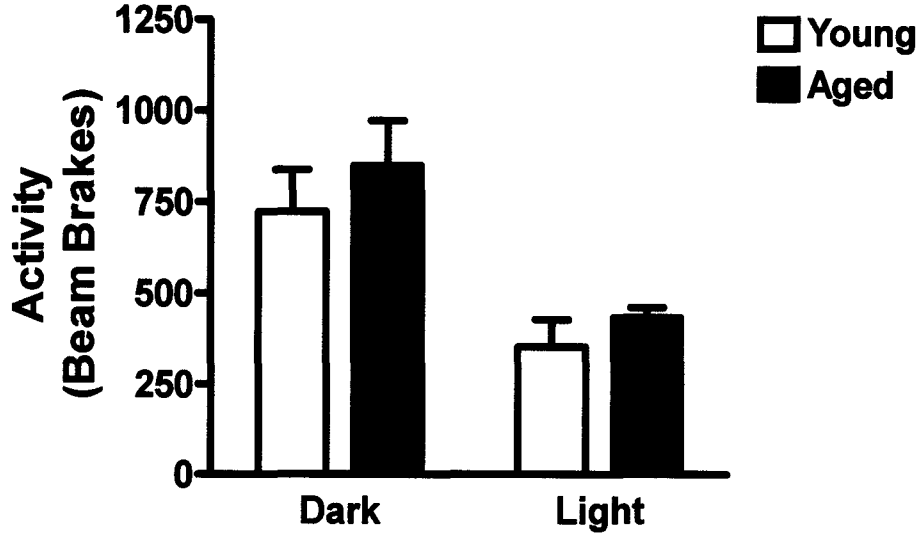

1250
1000
750
500
250
0
Activity
(Beam Brakes)
Young
Aged
Dark
Light

Figure 3-4: Mitohcondrial enzyme activity and content in skeletal muscle from young and middle-aged mice

Maximal *in vitro* enzyme activity of β-hydroxyacyl–CoA dehydrogenase (β-HAD; **A)** and citrate synthase (CS; **B)** of the β-oxidation pathway and TCA cycle, respectively, was determined in gastrocnemius muscle collected from overnight (16 hr) fasted young and middle-aged mice. Muscle homogenates were prepared from gastrocnemius muscle from young (12-14 weeks) and middle-aged (52-58 weeks) mice and subjected to SDS-PAGE. Immunoblot analysis was performed using a total oxidative phosphorylation (OXPHOS) complex antibody cocktail and immunoblots were normalized against tubulin as a control for protein loading **(C).** Representative immunoblots are shown. n = 5–7 mice in each group. * $p < 0.01$ vs. young mice by Student's unpaired t-test.

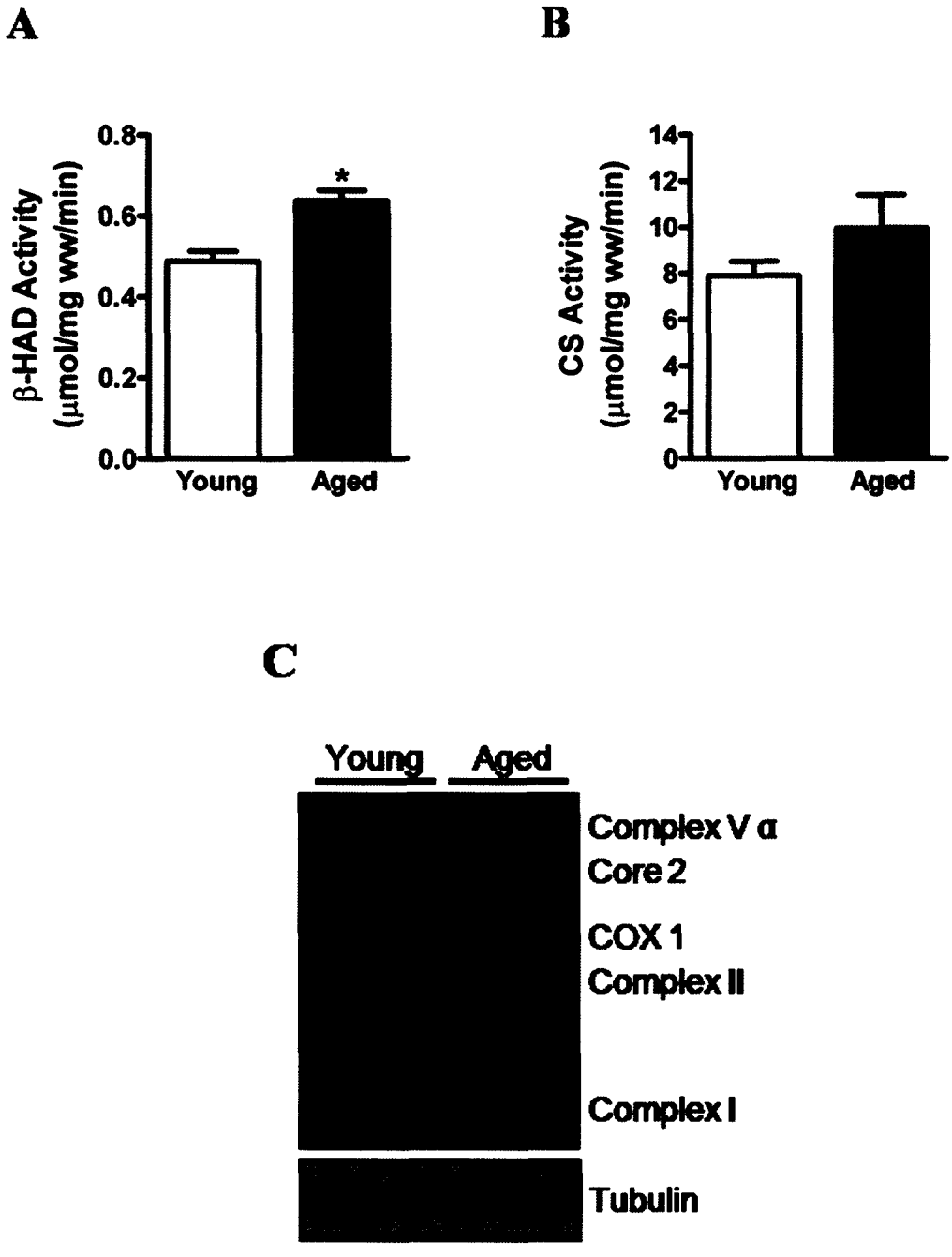

A
B
β-HAD Activity (μmol/mg ww/min)
0.8
0.6
0.4
0.2
0.0
*
Young
Aged
CS Activity (μmol/mg ww/min)
14
12
10
8
6
4
2
0
Young
Aged
C
Young
Aged
Complex V α
Core 2
COX 1
Complex II
Complex I
Tubulin

Figure 3-5: Reduced phosphorylation of AMPK and ACC in skeletal muscle from middle-aged mice compared to young mice

Muscle homogenates were prepared from gastrocnemius muscle from young (12-14 weeks) and middle-aged (52-58 weeks) mice and subjected to SDS-PAGE and immunoblot analysis. Representative immunoblots of AMPK are shown **(A).** Phosphorylation status of AMPKα at threonine 172 (T172) was detected using anti-phospho-AMPKα antibody and normalized against total protein levels of AMPKα **(B).** Total AMPKα levels were measured by densitometry and normalized against total protein levels of tubulin **(C).** Phosphorylation status of both ACC isoforms at serine 79 (S79) was detected using anti-phospho-ACC antibody and normalized against total levels of ACC **(D).** Values are means ± SEM of n = 5–7 mice in each group. * $p < 0.05$ vs. young mice as determined by Student's unpaired t-test.

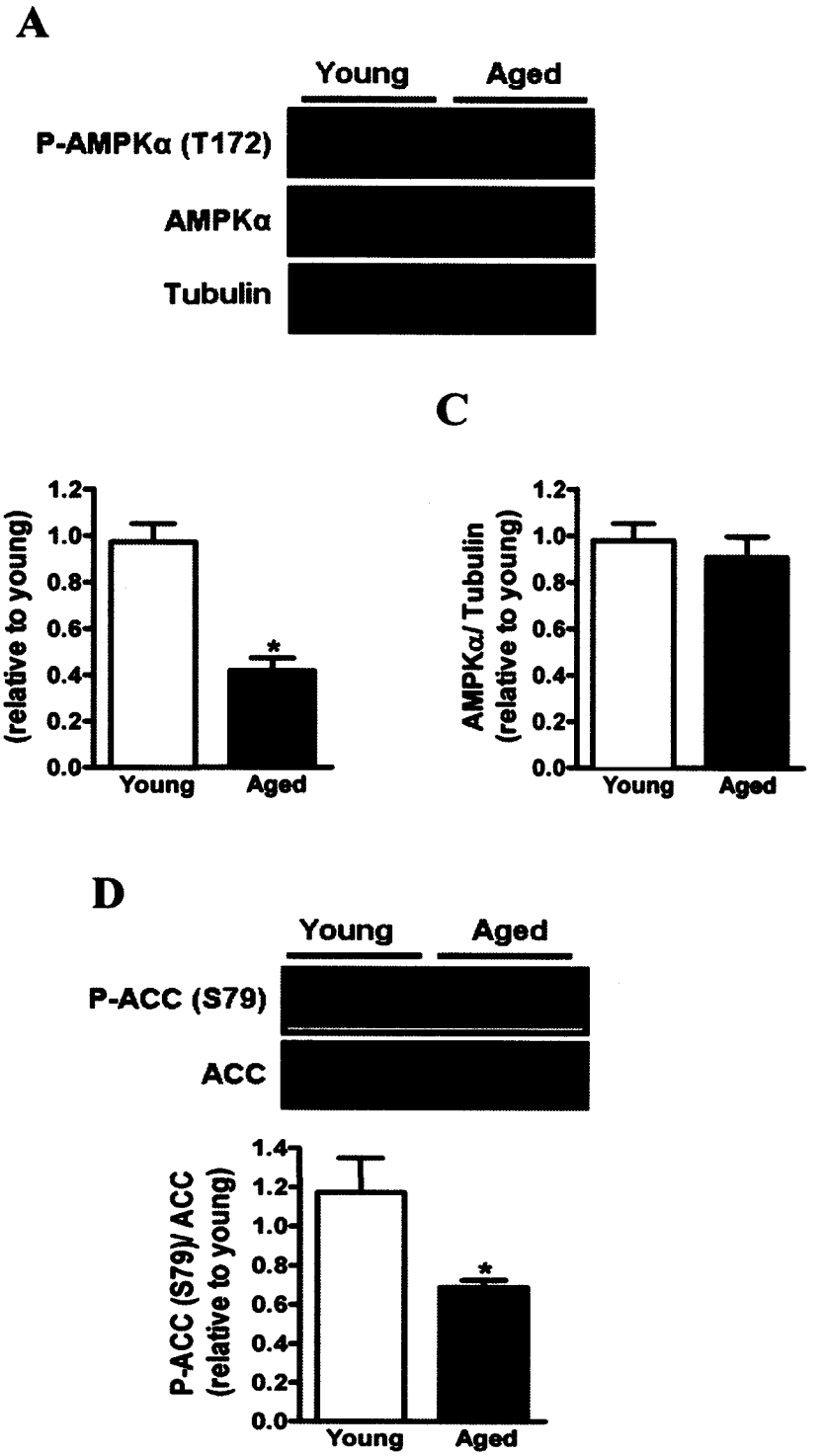
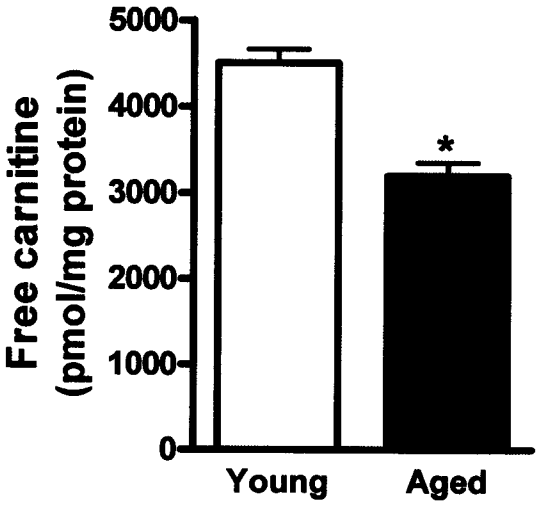

A
Young
Aged
P-AMPKα (T172)
AMPKα
Tubulin
B
P-AMPKα (T172)/AMPKα (relative to young)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Young
Aged
C
AMPKα/ Tubulin (relative to young)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Young
Aged
D
Young
Aged
P-ACC (S79)
ACC
P-ACC (S79)/ ACC (relative to young)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Young
Aged

Figure 3-6: Reduced free carnitine levels and elevated intramuscular triglyceride levels in skeletal muscle from middle-aged mice as compared to young mice

Free carnitine levels were measured by mass-spectrometry based metabolic profiling in gastrocnemius muscle from young and middle-aged mice after an overnight 16 hr fast and normalized to mg protein **(A).** Triglyceride (TG) content (μg/mg protein) was measured in gastrocnemius muscle collected from young and middle-aged mice after an overnight 16 hr fast **(B)**. Values are means ± SEM of *n* = 5–6 mice in each group. * $p < 0.05$ vs. young mice by Student's unpaired t-test.

A


Free carnitine
(pmol/mg protein)
5000
4000
3000
2000
1000
0
*
Young
Aged

B

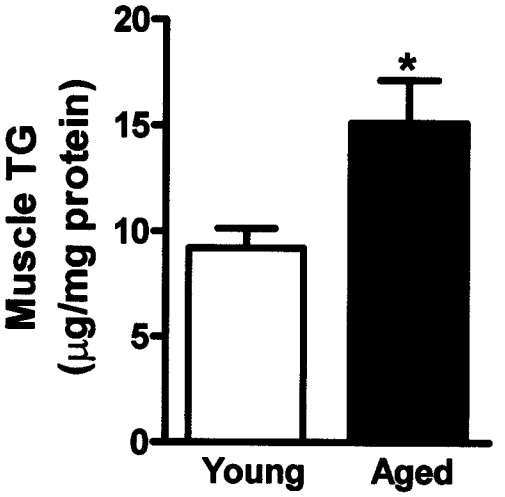

Muscle TG
(μg/mg protein)
20
15
10
5
0
*
Young
Aged

Figure 3-7: Modest impairment in glucose tolerance with aging associated with normal glucose and insulin levels

Young and middle-aged mice were administered a bolus i.p. dose of glucose (2 g glucose/kg body weight) for glucose tolerance tests and blood glucose was monitored over 120 min with the use of a glucometer **(A).** Area under the curve (AUC) of the glucose tolerance test **(B).** Blood glucose was measured in the fed and overnight (16 hr) fasted state in young and middle-aged mice **(C).** Plasma insulin was measured in young and middle-aged mice following a 16 hr fast **(D).** Values are means ± SEM of n = 10-16 mice in each group for **(A and B)**, n = 5-10 per group for **(C and D).** * $p < 0.05$ vs. young mice as determined by two-way repeated measures ANOVA with a Bonferonni post-hoc test in **(A),** * $p < 0.05$ vs. young mice as determined by Student's unpaired t-test.

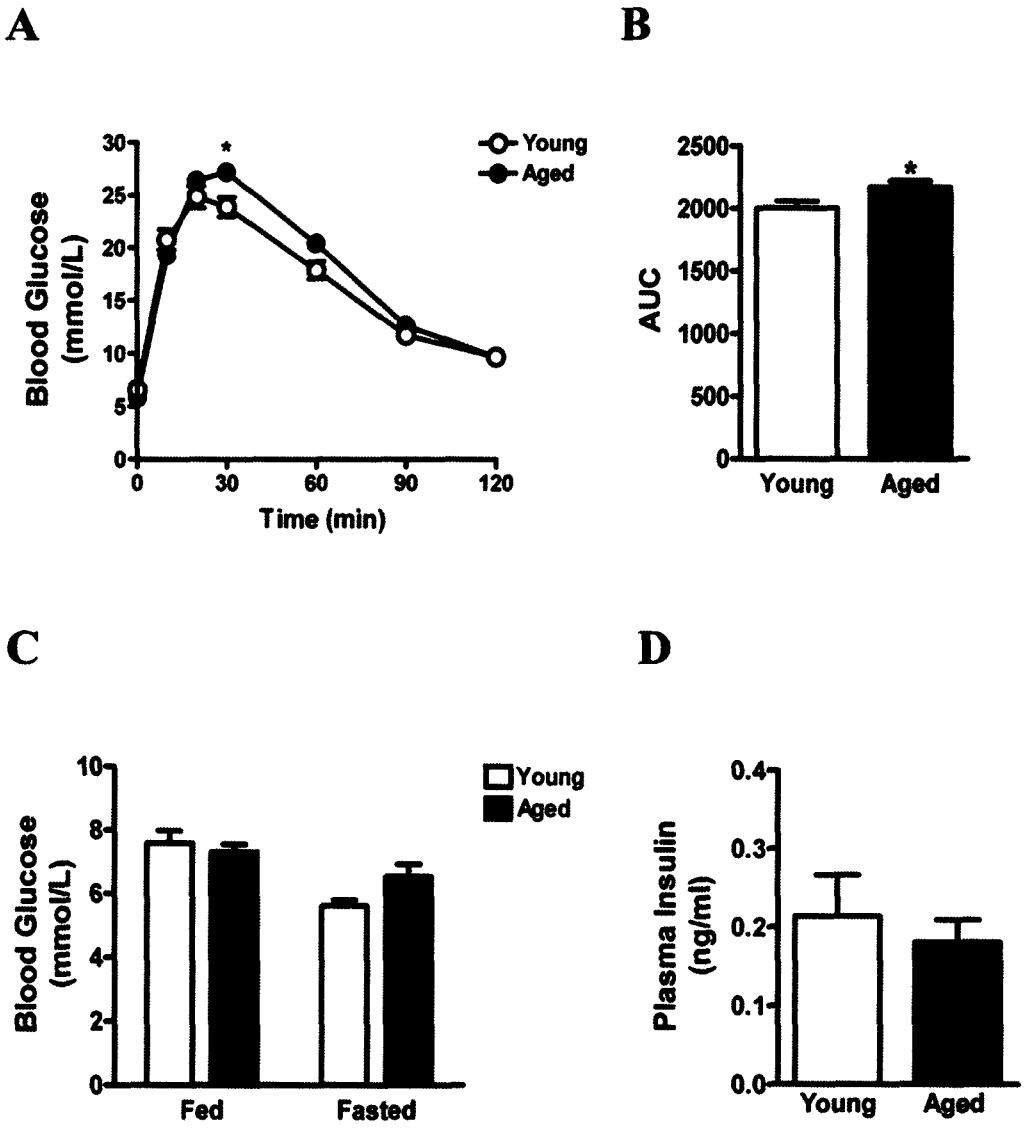
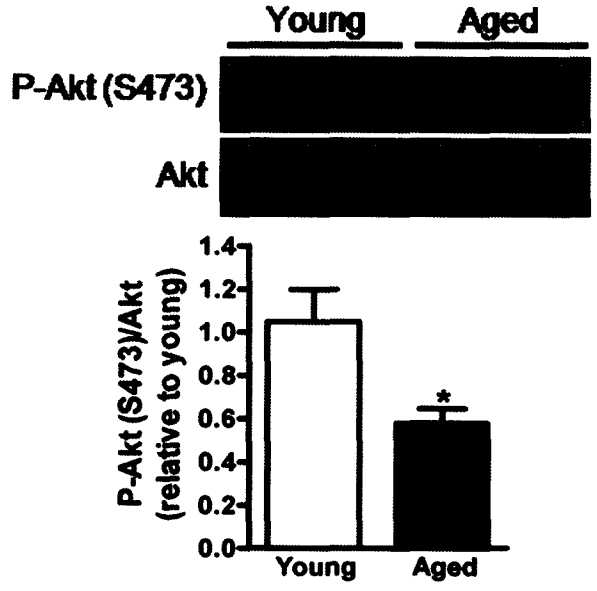

A
B
C
D
Young
Aged
Blood Glucose (mmol/L)
Time (min)
AUC
Fed
Fasted
Plasma Insulin (ng/ml)
*

Figure 3-8: Impaired Akt signaling in the fasted and insulin-stimulated state in skeletal muscle from young and middle-aged mice

Immunoblot analysis was performed on skeletal muscle homogenates from young and middle-aged mice. Phosphorylation status of Akt at serine 473 (S473) was measured using phospho-speficic antibodies in gastrocnemius muscle collected following an overnight (16 hr) fast **(A)** and 15 min following insulin stimulation (10 U insulin/kg body weight) in mice fasted for 6 hr **(B).** Representative immunoblots are shown above and densitometric measurements normalized to either total Akt or tubulin levels. Values are means ± SEM of n = 5-7 mice in each group. * $p < 0.05$ vs. young mice as determined by Student's unpaired t-test.

A


Young
Aged
P-Akt (S473)
Akt
P-Akt (S473)/Akt (relative to young)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Young
Aged

B

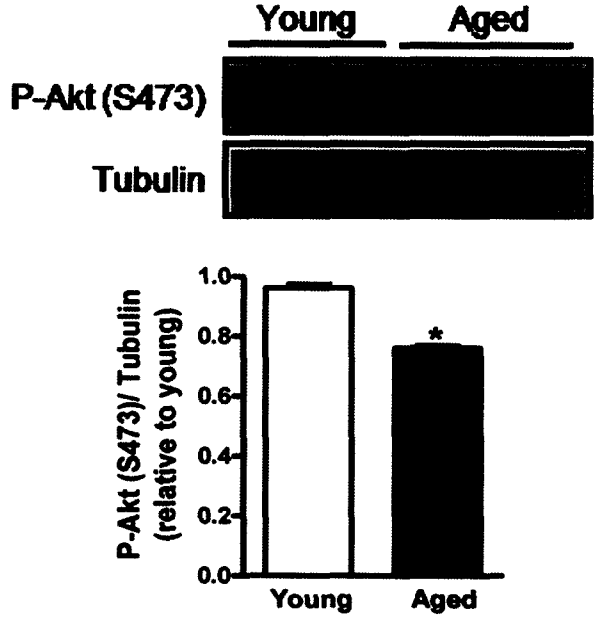

Young
Aged
P-Akt (S473)
Tubulin
P-Akt (S473)/ Tubulin (relative to young)
1.0
0.8
0.6
0.4
0.2
0.0
*
Young
Aged

Figure 3-9: Increased body weight and impaired fasting glucose homeostasis in young mice fed a high fat diet

Young (10-12 weeks) C57BL/6 mice were fed a LF (10 kcal% fat) or HF (60 kcal% fat) diet for 12 weeks. Absolute body weights **(A)** and weight gain **(B)** measured at the end of 12 weeks of feeding. Blood glucose levels were measured from blood collected from the tail vein of mice during both the fed and overnight fasted (16 hr) states **(C)**. $n = 6$ per group, * $p < 0.0001$ vs. LF-fed young mice **(A and B)**, * $p < 0.05$ indicates comparisons between LF- or HF-fed mice in the fed or fasted state **(C)**.

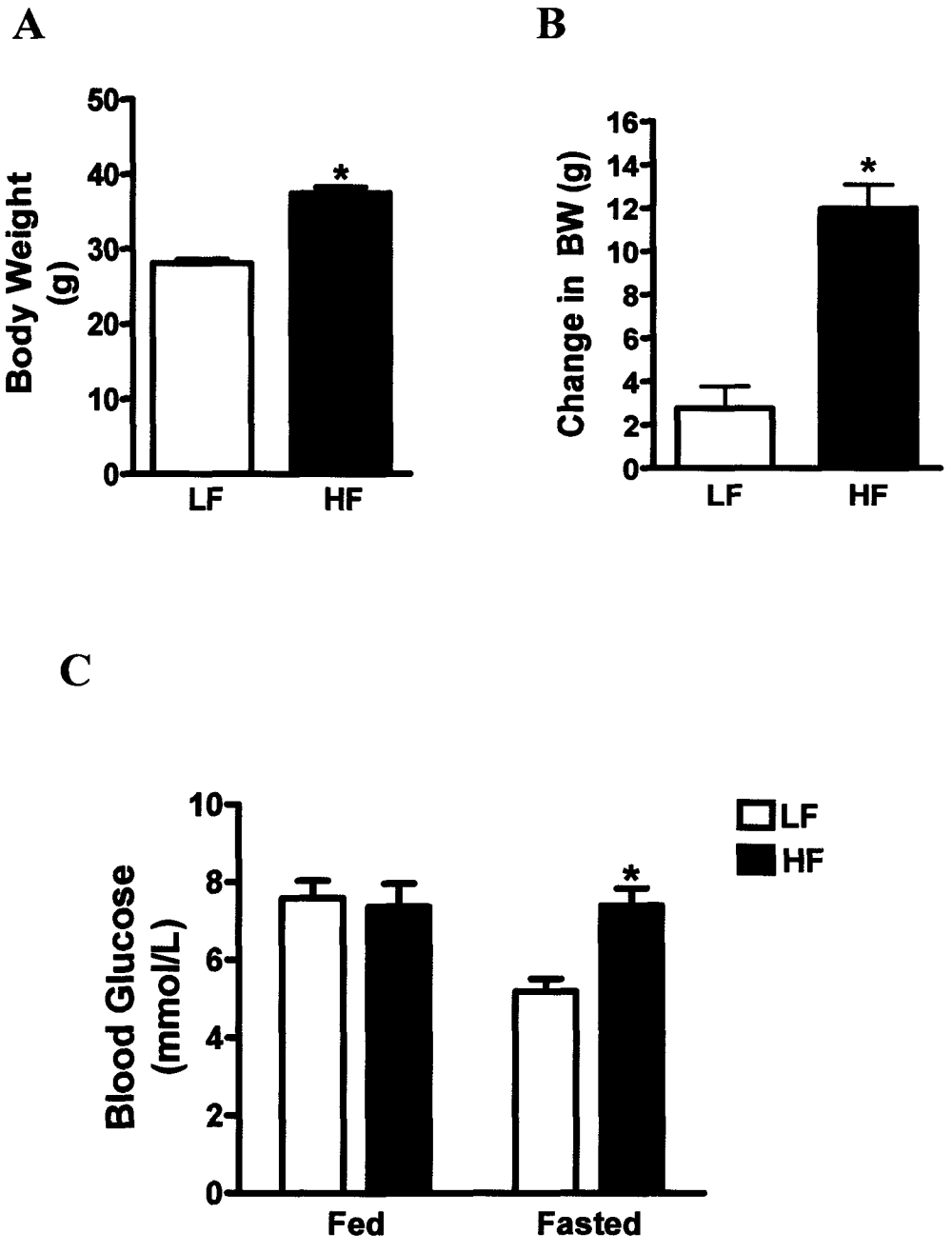

A
B
C
Body Weight (g)
50
40
30
20
10
0
LF
HF
*
Change in BW (g)
16
14
12
10
8
6
4
2
0
Blood Glucose (mmol/L)
Fed
Fasted
LF
HF

Figure 3-10: Impaired glucose clearance and insulin sensitivity in young mice fed a high fat diet

Glucose tolerance tests were performed in young mice following 12 weeks of a LF or HF diet. Blood glucose levels were monitored for 120 min following i.p. bolus dose of glucose (2 g glucose/kg body weight), where readings at time 0 min were baseline glucose measurements following a 6 hr fast just prior to i.p. glucose injection **(A)**. Area under the curve (AUC) calculated for the curves generated by the glucose tolerance test **(B)**. Percent change in blood glucose following i.p. insulin injection (0.3 U insulin/kg body weight) to determine glucose clearance in response to insulin **(C)**. Homogenates were prepared from gastrocnemius muscle collected following an overnight fast from young C57BL/6 mice fed 12 weeks of a LF or HF diet. Homogenates were subjected to SDS-PAGE and immunoblot analysis were performed using anti-phospho-Akt (Ser473) and anti-Akt antibodies **(D)**. $n = 6$ per group, * $p < 0.001$ vs. young LF-fed mice by Student's unpaired t-test **(B)**, * $p < 0.05$ vs. young LF-fed mice by Two-way repeated measures ANOVA with a bonferonni post-hoc test **(A and C)**.

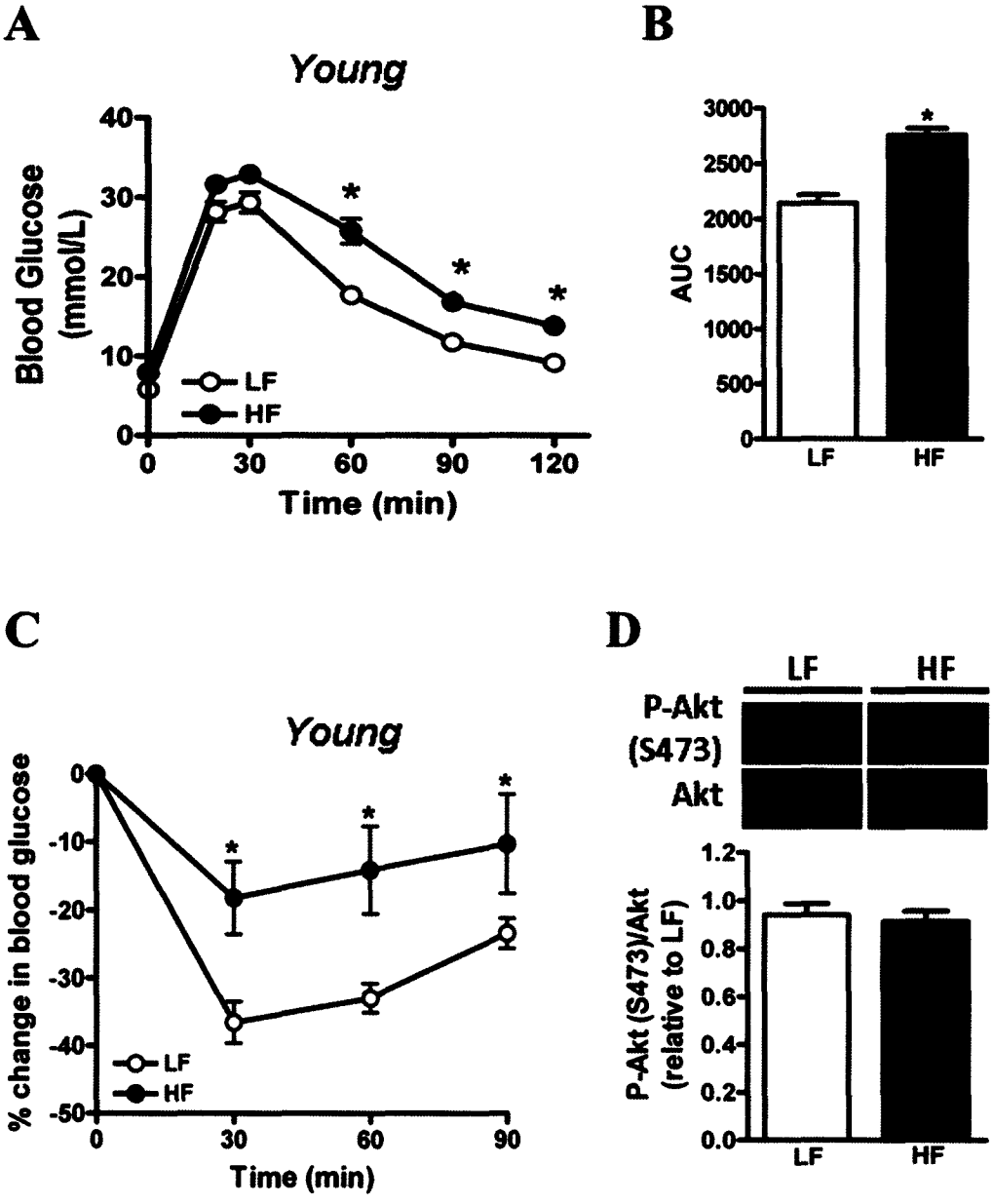

A
Young
Blood Glucose (mmol/L)
40
30
20
10
0
LF
HF
0
30
60
90
120
Time (min)
*
B
AUC
3000
2500
2000
1500
1000
500
0
LF
HF
C
Young
% change in blood glucose
0
-10
-20
-30
-40
-50
LF
HF
0
30
60
90
Time (min)
D
LF
HF
P-Akt (S473)
Akt
P-Akt (S473)/Akt (relative to LF)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF

Figure 3-11: Middle-aged mice gain significant weight following 12 weeks of a high fat diet and develop severe hyperinsulinemia

Absolute body weights in middle-aged mice (40-44 weeks of age) fed a LF (10 kcal% fat) or HF (60 kcal%fat) diet for 12 weeks **(A)**. Plasma insulin levels measured from young (left panel) and middle-aged mice (right panel) on diet during the fed **(B)** and overnight (16 hr) fasted **(C)** states. * $p < 0.05$ vs. aged LF-fed mice by Student's unpaired t-test.

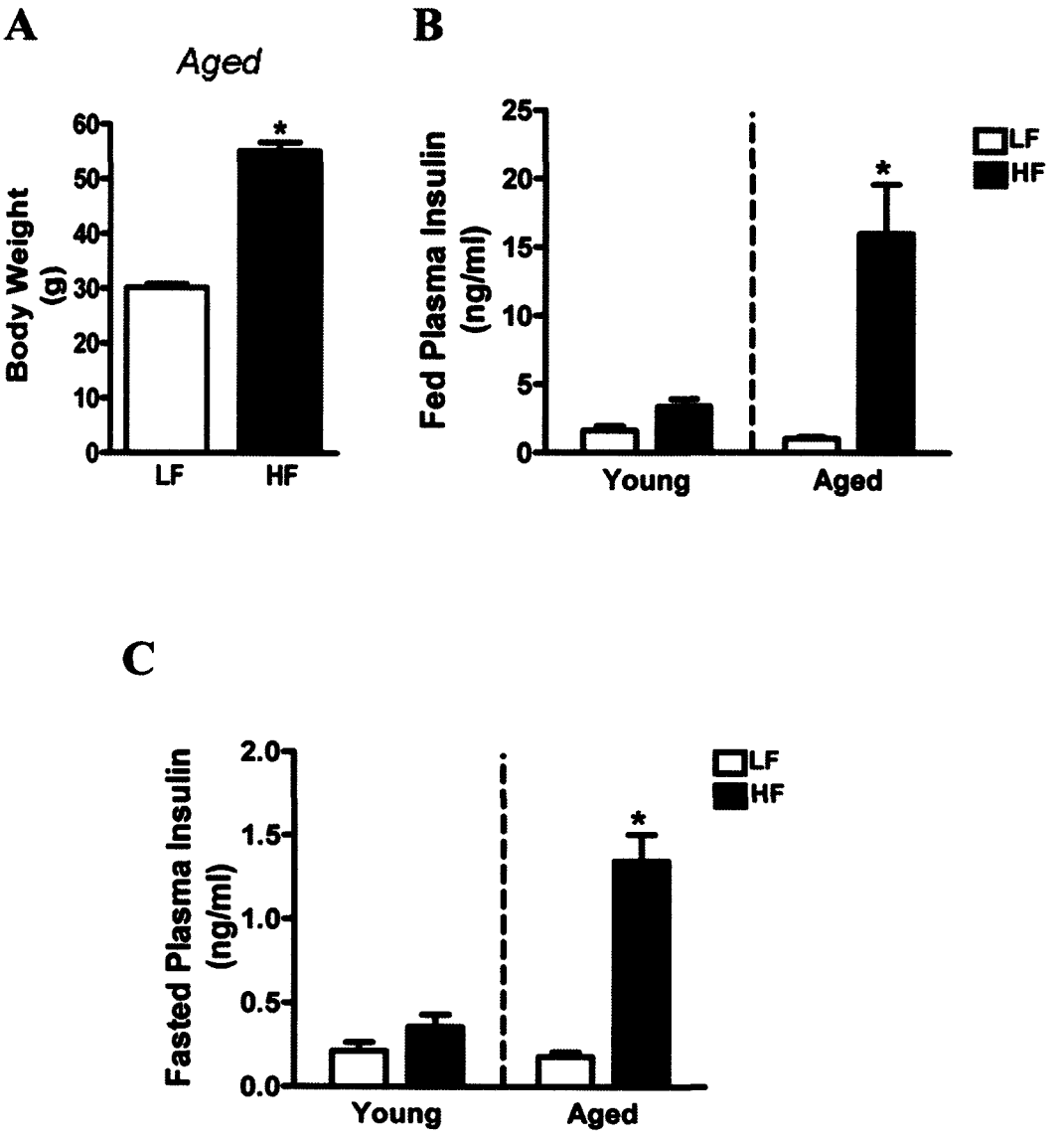

A
Aged
Body Weight (g)
60
50
40
30
20
10
0
*
LF
HF
B
Fed Plasma Insulin (ng/ml)
25
20
15
10
5
0
*
LF
HF
Young
Aged
C
Fasted Plasma Insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
*
LF
HF
Young
Aged

Figure 3-12: Impaired fasting glucose tolerance in middle-aged mice following 12 weeks of high fat diet

Blood glucose concentrations measured in the fed and fasted state from middle-aged mice fed a low fat or high fat diet for 12 weeks, n = 8 per group (**A**). Glucose tolerance tests were performed in young mice following 12 weeks of diet. Blood glucose levels were monitored for 120 min following i.p. bolus dose of glucose (2 g glucose/kg body weight), where readings at time 0 min were baseline glucose measurements following a 6 hr fast just prior to i.p. glucose injection, n = 7-9 per group **(B)**. Area under the curve (AUC) calculated for the curves generated by the glucose tolerance test **(C)**. * $p < 0.05$ vs. aged LF-fed mice in the fasted state **(A),** * $p < 0.01$ vs. aged LF by Two-way ANOVA and Bonferroni post-hoc test **(B),** * $p < 0.05$ vs. aged LF-fed mice by Student's unpaired t-test **(C)**.

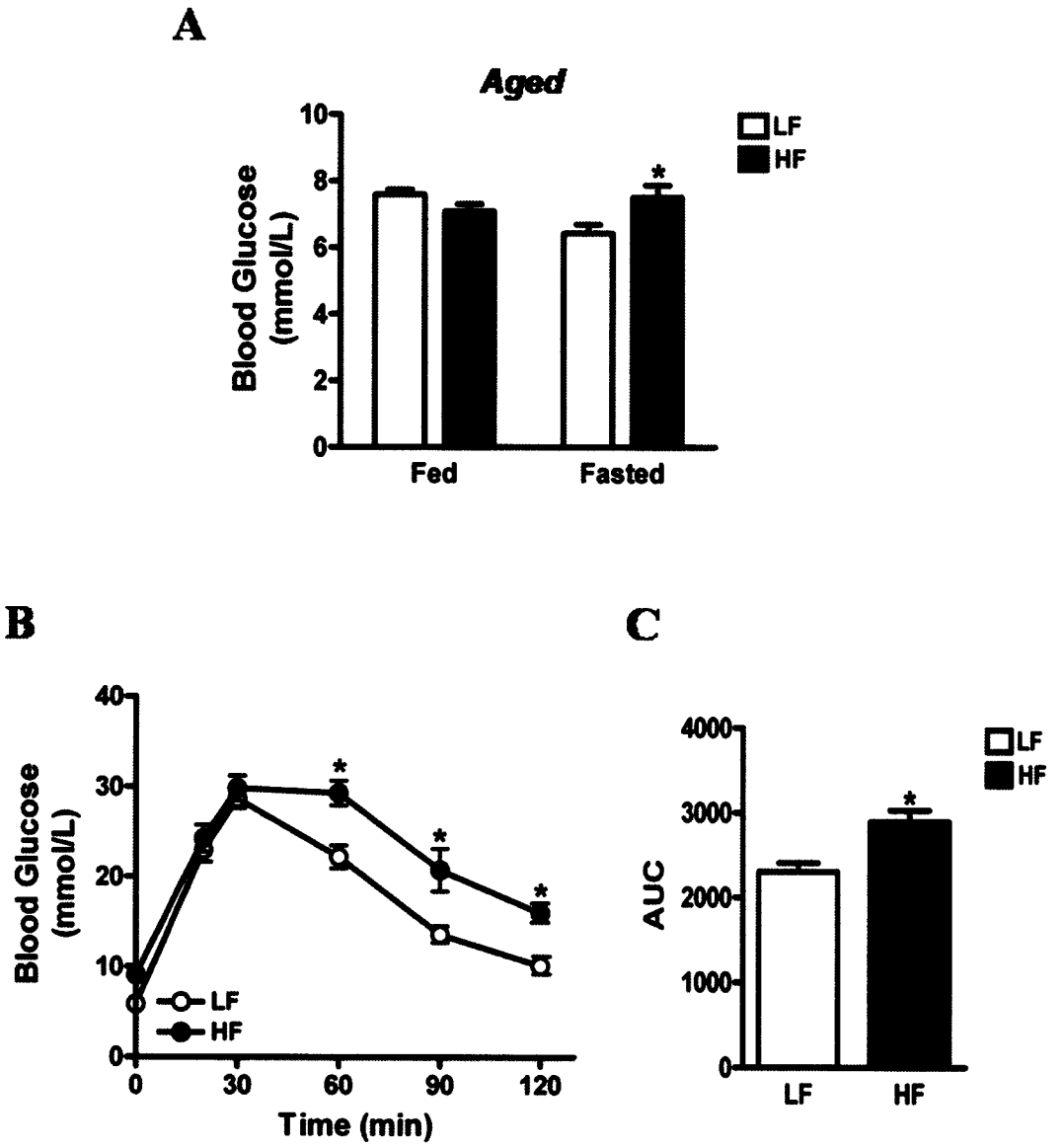
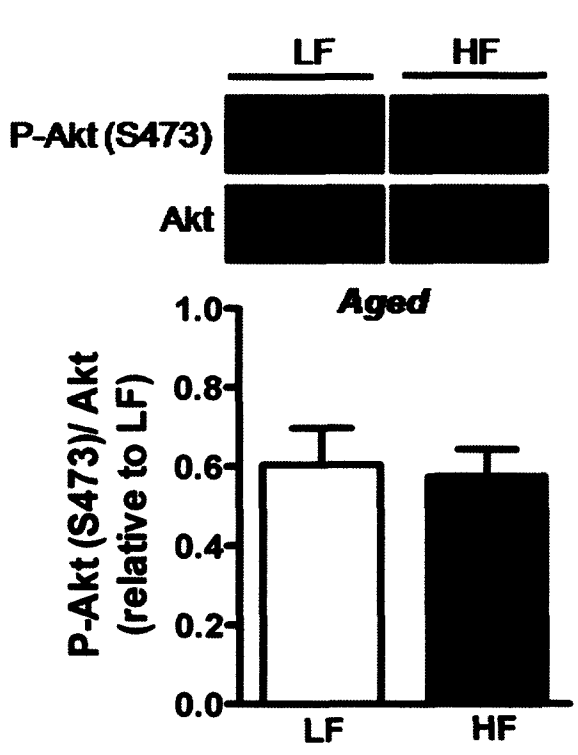

A
Aged
Blood Glucose (mmol/L)
10
8
6
4
2
0
LF
HF
*
Fed
Fasted
B
Blood Glucose (mmol/L)
40
30
20
10
0
LF
HF
*
*
*
0
30
60
90
120
Time (min)
C
AUC
4000
3000
2000
1000
0
LF
HF
*
LF
HF

Figure 3-13: Phosphorylation of Akt is unchanged in skeletal muscle from middle-aged mice after 12 weeks of diet, but HOMA-IR calculations indicate that middle-aged mice are more insulin resistant than young mice

Homogenates were prepared from gastrocnemius muscle collected following an overnight (16 hr) fast from middle-aged WT mice fed 12 weeks of a LF or HF diet. Homogenates were subjected to SDS-PAGE and immunoblot analysis were performed using anti-phospho-Akt (Ser 473) and anti-Akt antibodies (n = 6 per group). Representative immunoblots shown for n = 6 per group and immunoblots of Akt were quantified by densitometry **(A)**. Homeostasis model assessment of insulin resistance (HOMA-IR), used as a surrogate marker of insulin resistance, in young and middle-aged mice fed a HF diet, n = 4-8 per group **(B)**. * $p < 0.01$ vs. aged LF-fed mice as determined by Student's unpaired t-test.

A


LF
HF
P-Akt (S473)
Akt
Aged
P-Akt (S473)/ Akt
(relative to LF)
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF

B


HOMA-IR
14
12
10
8
6
4
2
0
Young
Aged
*
LF
HF

Figure 3-14: Increased CD36 expression in skeletal muscle from high fat fed middle-aged mice is associated with marked accumulation of lipid metabolites

Total CD36 protein levels were determined by immunoblot analysis performed on homogenates prepared from gastrocnemius muscle from middle-aged WT mice fed a LF or HF diet for 12 weeks **(A)**. Triglyceride (TG; **B)**, long chain acyl CoA (LCACoA; **C)** and C18-ceramide **(D)** levels in gastrocnemius muscle collected from overnight (16 hr) fasted middle-aged WT mice fed a LF or HF diet (n = 5-6 per group) and normalized to wet weight (ww) of muscle tissue. * $p < 0.05$ vs. aged LF-fed mice as determined by Student's unpaired t-test.

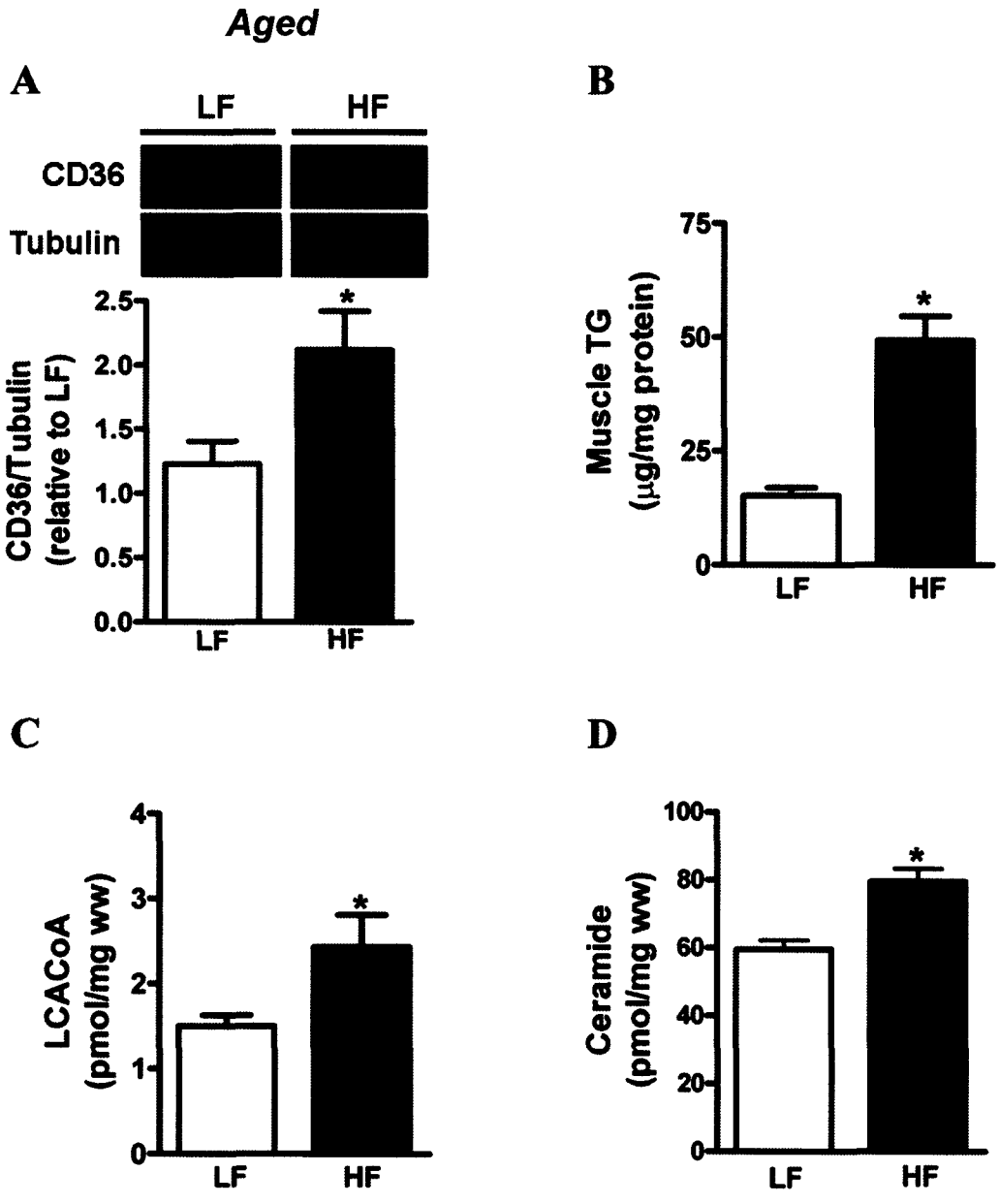
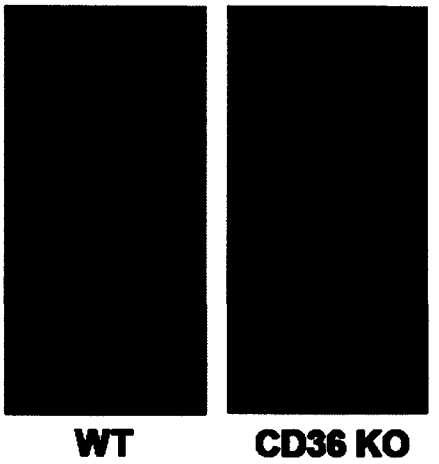

Aged
A
LF
HF
CD36
Tubulin
CD36/Tubulin (relative to LF)
2.5
2.0
1.5
1.0
0.5
0.0
*
LF
HF
B
Muscle TG (μg/mg protein)
75
50
25
0
*
LF
HF
C
LCACoA (pmol/mg ww)
4
3
2
1
0
*
LF
HF
D
Ceramide (pmol/mg ww)
100
80
60
40
20
0
*
LF
HF

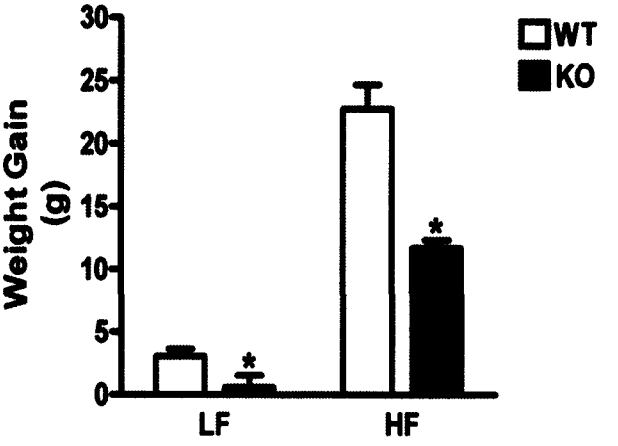
Figure 3-15: Middle-aged CD36 knockout mice are protected from diet-induced obesity despite similar high dietary fat intake

Representative image of middle-aged (48–52 weeks of age) WT and CD36 KO mice fed a HF diet for 12 weeks **(A).** Body weight gain **(B)** and total cumulative 24 hr food intake represented in kcal and adjusted for total body weight **(C)** at the end of 12 weeks of HF diet (n = 6 -11 per group). * $p < 0.05$ vs. WT mice fed either a LF or HF diet as determined by Student's unpaired t-test.

A


WT
CD36 KO

B


Weight Gain (g)
30
25
20
15
10
5
0
*
*
LF
HF
WT
KO

C

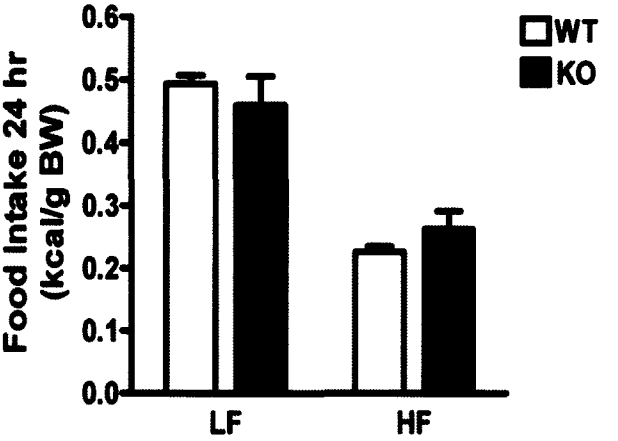

Food Intake 24 hr (kcal/g BW)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
LF
HF
WT
KO

Figure 3-16: CD36 deficiency prevents the decline in metabolic rate in middle-aged mice fed a high fat diet

Indirect calorimetry was performed in middle-aged WT and CD36 KO mice following 12 weeks of a LF or HF diet using the oxymax CLAMS system. Mice were housed in individual metabolic cages and gas exchange was monitored for a full 24 hr cycle consisting of a 12 hr dark phase (0800:2000; active) and 12 hr light phase (2000:0800; inactive). Repsiratory exchange ratio (RER) was calculcated as the ratio of carbon dioxide production and oxygen consumption **(A).** Average rates of oxygen consumption (VO_2; **B)** and carbon dioxide production (VCO_2; **C)** were measured during the dark and light phases (n = 8-11 per group). * $p < 0.01$ vs. HF-fed WT mice in their respective dark or light phase.

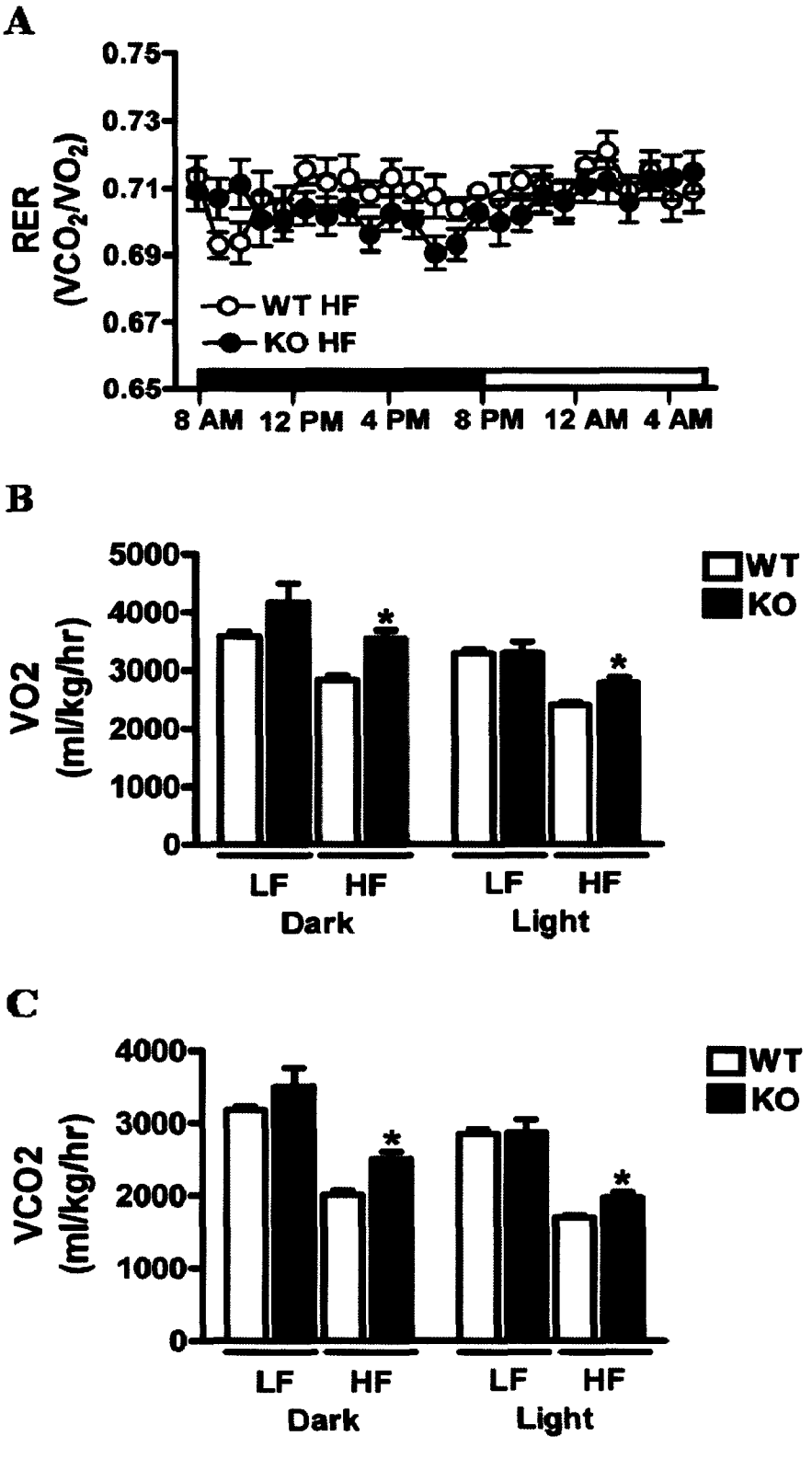

A
0.75
0.73
0.71
0.69
0.67
0.65
RER
(VCO2/VO2)
WT HF
KO HF
8 AM
12 PM
4 PM
8 PM
12 AM
4 AM
B
5000
4000
3000
2000
1000
0
VO2
(ml/kg/hr)
WT
KO
*
LF
HF
Dark
Light
C
4000
3000
2000
1000
0
VCO2
(ml/kg/hr)
WT
KO
*
LF
HF
Dark
Light

Figure 3-17: Increased total activity and heat production in middle-aged CD36 knockout mice as compared to wild-type mice

Indirect calorimetry was performed in middle-aged WT and CD36 KO mice following 12 weeks of a LF or HF diet using the oxymax CLAMS system. Mice were housed in individual metabolic cages and monitored every 13 min for a full 24 hr reverse light cycle consisting of a 12 hr dark phase (0800:2000; active) and 12 hr light phase (2000:0800; inactive). Locomotor activity was monitored by dual axis detection (X, Z axis) using IR photocell technology. Interruption of an IR beam is considered one activity "count." Total activity (counts) represents the sum of all locomotor activities (rearing, jumping, grooming/scratching) performed by the mice while being housed in the metabolic cages **(A)**. Average heat production **(B)** and heat production adjusted for total body weight **(C)** in middle-aged WT and CD36 KO mice fed a LF or HF diet, n = 6-10 per group. * $p < 0.01$ vs. WT mice fed a LF or HF diet, respectively **(A)**, * $p < 0.05$ vs. WT control mice fed an identical diet (LF or HF) during either light or dark phases.

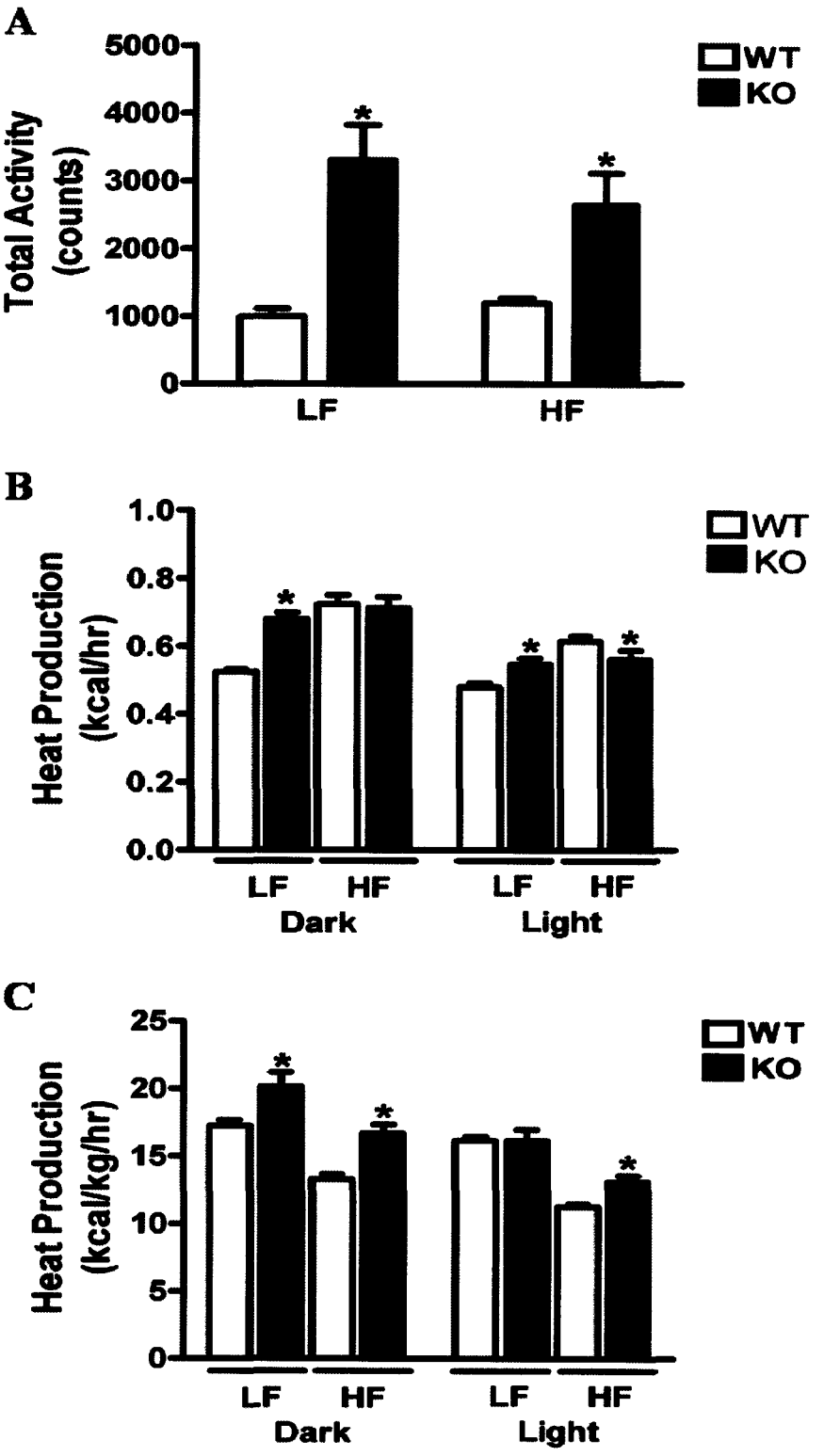

A
Total Activity (counts)
5000
4000
3000
2000
1000
0
LF
HF
WT
KO
B
Heat Production (kcal/hr)
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF
Dark
LF
HF
Light
WT
KO
C
Heat Production (kcal/kg/hr)
25
20
15
10
5
0
LF
HF
Dark
LF
HF
Light
WT
KO

Figure 3-18: Metabolic profiling in skeletal muscle from middle-aged wildtype and CD36 knockout mice following 12 weeks of diet

Short-chain (SC) acylcarnitine levels **(A)**, the sum total of all long-chain (LC) or hydroxylated long chain (LCOH) acylcarnitine species **(B)**, and the ratio of total LCOH–to–long-chain species **(C)** were measured in gastrocnemius muscle collected from overnight fasted (16 hr) middle-aged WT and CD36 KO mice at the end of 12 weeks of LF or HF diet. Values are the mean ± SEM, $n = 6$ mice per group. Main effects of genotype and diet as well as genotype x diet interactions were detected by two-way ANOVA. * $p < 0.05$ indicate metabolites that were affected by genotype, diet or a genotype-diet interaction. Detailed results of the statistical analysis for all metabolite species are presented in Table 3-1.

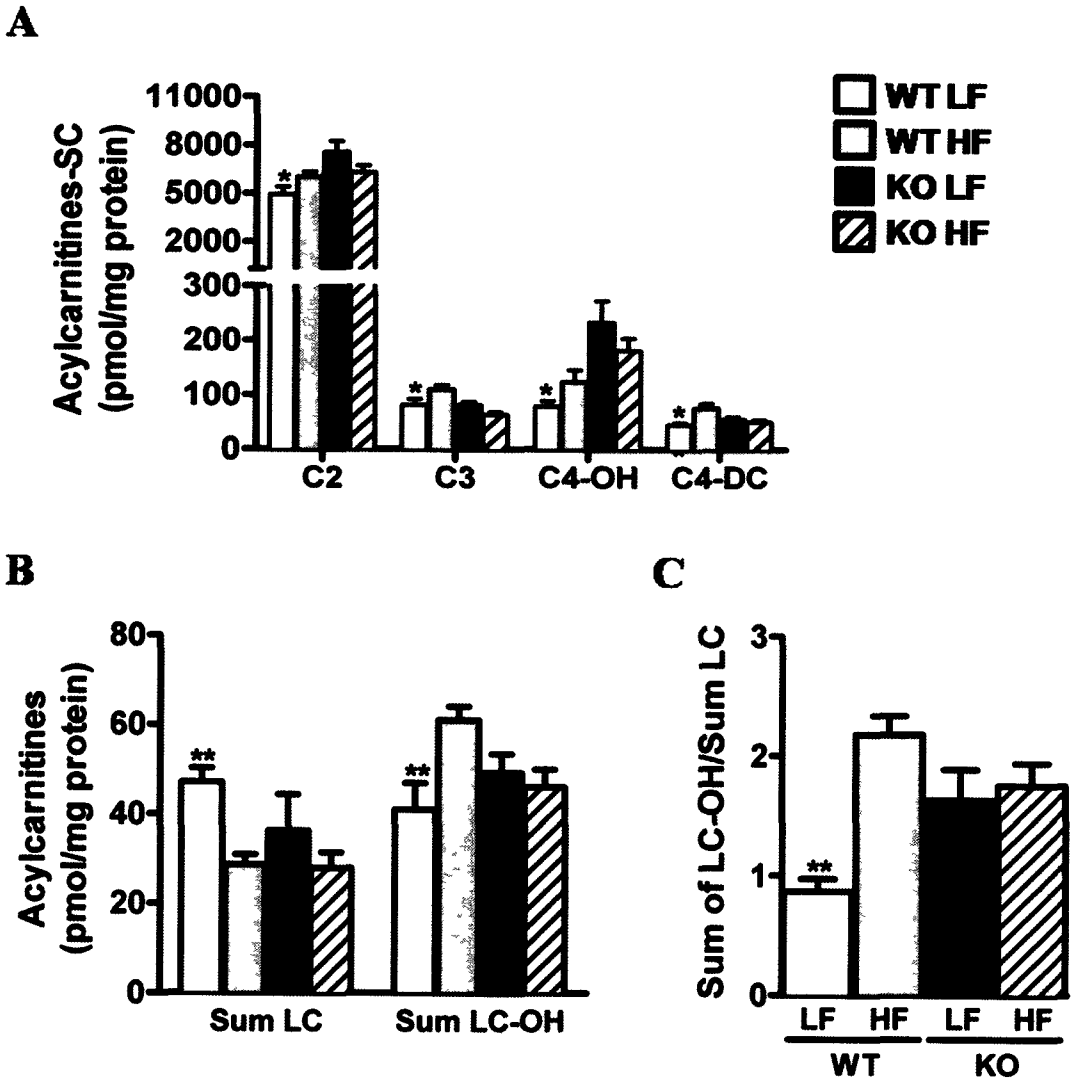

A
Acylcarnitines-SC (pmol/mg protein)
11000
8000
5000
2000
300
200
100
0
C2
C3
C4-OH
C4-DC
WT LF
WT HF
KO LF
KO HF
B
Acylcarnitines (pmol/mg protein)
80
60
40
20
0
Sum LC
Sum LC-OH
C
Sum of LC-OH/Sum LC
3
2
1
0
LF
HF
LF
HF
WT
KO

Figure 3-19: Acylcarnitine levels in gastrocnemius muscle from middle-aged wild-type and CD36 knockout mice following 12 weeks of diet

Acylcarnitine levels were measured by flow-injection tandem mass spectrometry in gastrocnemius muscle collected from overnight-fasted middle-aged (48–52 weeks of age) WT and CD36 KO mice fed a LF or HF diet for 12 weeks. Levels of individual long chain (LC) and medium chain (MC) acylcarnitine species (n = 6 per group). Main effects of genotype, diet, and genotype x diet interactions on acylcarnitine levels were detected by two-way ANOVA. For simplicity, ** indicate metabolites that were affected by genotype, diet, or genotype-diet interaction. Detailed results of the statistical analysis for all acylcarnitine species are presented in Table 3-1.

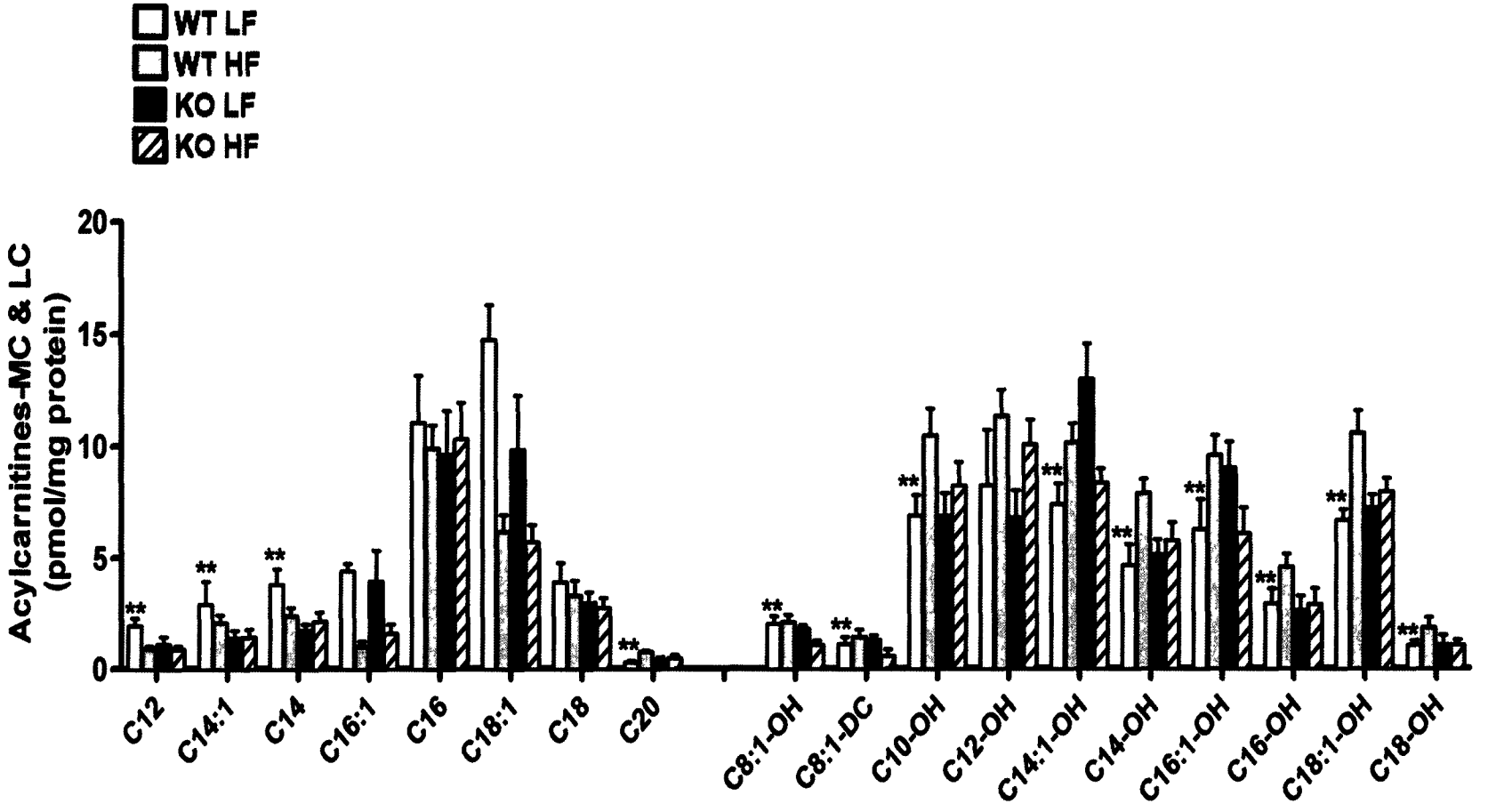

WT LF
WT HF
KO LF
KO HF
Acylcarnitines-MC & LC
(pmol/mg protein)
20
15
10
5
0
**
C12
C14:1
C14
C16:1
C16
C18:1
C18
C20
C8:1-OH
C8:1-DC
C10-OH
C12-OH
C14:1-OH
C14-OH
C16:1-OH
C16-OH
C18:1-OH
C18-OH

Table 3-1: Levels of acylcarnitine metabolites in skeletal muscle from wild-type and CD36 knockout mice following diet

Acylcarnitine levels were measured in gastrocnemius muscle from overnight fasted middle-aged (48-52 weeks of age) WT and CD36 KO mice fed a LF or HF diet for 12 weeks. ANOVA was performed for each metabolite using JMP 8.0 from SAS Institute. Table shows p-values; *$p < 0.05$ indicates metabolites significantly affected by genotype, diet, or a genotype/diet interaction.

Metabolite	Code	Genotype	Diet	Interaction
Free Carnitine	M-CO	0.856	0.751	0.381
Acetyl Carnitine	M-C2	0.006 *	0.858	0.020 *
Propionyl carnitine	M-C3	0.005 *	0.474	0.004 *
Butyryl or Isobutyryl carnitine	C4/Ci4	0.652	0.449	0.479
Tiglyl carnitine	C5:1	0.246	0.090	0.612
Isovaleryl carnitine	C5's	0.904	0.174	0.226
3-Hydroxy-butyryl carnitine	C4-OH	0.000 *	0.883	0.065
Hexanoyl carnitine	C6	0.220	0.123	0.946
3-Hydroxy-isovaleryl or Malonyl carnitine	C5-OH/C3-DC	0.016 *	0.106	0.027 *
Methylmalonyl or Succinyl carnitine	Ci4-DC/C4-DC	0.215	0.037 *	0.005 *
Octenoyl carnitine	M-C8:1	0.159	0.355	0.395
Octanoyl carnitine	M-C8	0.352	0.537	0.691
Glutaryl carnitine	M-C5-DC	0.434	0.685	0.163
3-Hydroxy-octenoyl carnitine	M-C8:1-OH	0.023 *	0.256	0.150
Adipoyl or 3-methylglutaryl carnitine	M-C6-DC	0.938	0.240	0.555
Decatrienoyl carnitine	M-C10:3	0.794	0.625	0.381
Decadienoyl carnitine	M-C10:2	0.913	0.241	0.215
Decenoyl carnitine	M-C10:1	0.745	0.404	0.463
Decanoyl carnitine	M-C10	0.946	0.447	0.110
3-Hydroxy-decanoyl or Suberoyl carnitine	C10-OH/C8-DC	0.304	0.032 *	0.311
Dodecenoyl carnitine	C12:1	0.280	0.756	0.739
Lauroyl carnitine	C12	0.083	0.015 *	0.110
3-Hydroxy-dodecanoyl or Sebacoyl carnitine	C12-OH/C10-DC	0.380	0.047 *	0.955
Tetradecadienoyl carnitine	M-C14:2	0.055 *	0.270	0.215
Tetradecenoyl carnitine	M-C14:1	0.065	0.487	0.452
Myristoyl carnitine	M-C14	0.019 *	0.281	0.062
3-Hydroxy-tetradecenoyl carnitine	M-C14:1-OH	0.072	0.358	0.001 *
3-Hydroxy-tetradecanoyl or Dodecanedioyl	C14-OH/C12-DC	0.298	0.021 *	0.106
Palmitoleoyl carnitine	M-C16:1	0.928	0.000 *	0.466
Palmitoyl carnitine	M-C16	0.770	0.892	0.593
3-Hydroxy-palmitoleoyl or Tetradecanedioyl	C16-OH/C14-DC	0.740	0.871	0.012 *
3-Hydroxy-hexadecanoyl or Tetradecanedioyl	M-C16-OH/C14-DC	0.155	0.170	0.325
Linoleyl carnitine	M-C18:2	0.690	0.145	0.612
Oleyl carnitine	M-C18:1	0.071	0.000 *	0.131
Stearoyl carnitine	M-C18	0.234	0.518	0.765
3-Hydroxy-linoleyl carnitine	M-C18:2-OH	0.922	0.271	0.052 *
3-Hydroxy-octadecenoyl carnitine	M-C18:1-OH	0.159	0.004 *	0.038 *
3-Hydroxy-octadecanoyl or Hexadecanedioyl	C18-OH/C16-DC	0.306	0.281	0.266
Arachidonoyl carnitine	M-C20:4	0.113	0.129	0.136
Arachidoyl carnitine. eicosanoyl carnitine	M-C20	0.403	0.021 *	0.096
Octadecenedioyl carnitine	M-C18:1-DC	0.224	0.137	0.345
3-Hydroxy-eicosanoyl or Octadecanedioyl	M-C20-OH/C18-DC	0.958	0.997	0.868
Total LC 3-hydroxy-acylcarnitines	M-SUM LCOH	0.443	0.063	0.013 *
Total LC acylcarnitines	M-SUM LC	0.209	0.007 *	0.278
Total LCOH/ total LC acylcarnitines	M-LCOH/LC	0.371	0.001 *	0.004 *

Table 3-2: Organic acids in skeletal muscle from wild-type and CD36 knockout mice following diet

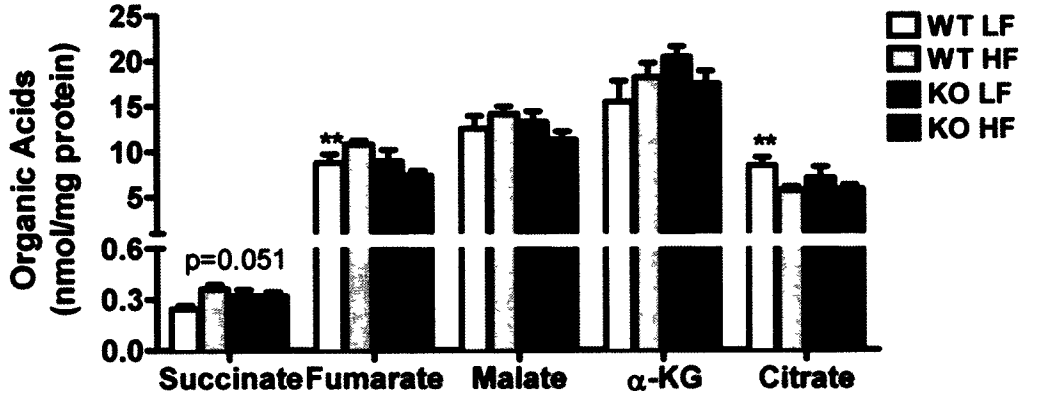
Metabolite	Genotype	Diet	Interaction
M-lactate	0.904	0.782	0.906
M-pyruvate	0.106	0.087	0.163
M-succinate	0.593	0.055	0.051 *
M-fumarate	0.050 *	0.771	0.033 *
M-malate	0.333	0.878	0.107
M-a-ketoglutarate	0.209	0.939	0.100
M-citrate	0.416	0.017 *	0.345

Organic acid levels were measured in gastrocnemius muscle from overnight fasted middle-aged (48-52 weeks of age) WT and CD36 KO mice fed a LF or HF diet for 12 weeks. ANOVA was performed for each metabolite using JMP 8.0 from SAS Institute. Table shows p-values; *$p < 0.05$ indicates metabolites significantly affected by genotype, diet, or a genotype/diet interaction.

Figure 3-20: Similar levels of organic acids of the TCA cycle in skeletal muscle from middle-aged wild-type and CD36 knockout mice

Organic acid levels **(A and B)** were measured in gastrocnemius muscle from overnight fasted middle-aged (48-52 weeks of age) WT and CD36 KO mice fed a LF or HF diet for 12 weeks. Values are the mean ± SEM. of $n = 6$ mice per group. Main effects of genotype and diet as well as genotype x diet interactions were detected by two-way ANOVA. * $p < 0.05$ indicate organic acids that were affected by genotype, diet or a genotype-diet interaction. Detailed results of the statistical analysis for all metabolite species are presented in Table 3-2.

A


Organic Acids
(nmol/mg protein)
25
20
15
10
5
0.6
0.3
0.0
p=0.051
**
**
WT LF
WT HF
KO LF
KO HF
Succinate
Fumarate
Malate
α-KG
Citrate

B

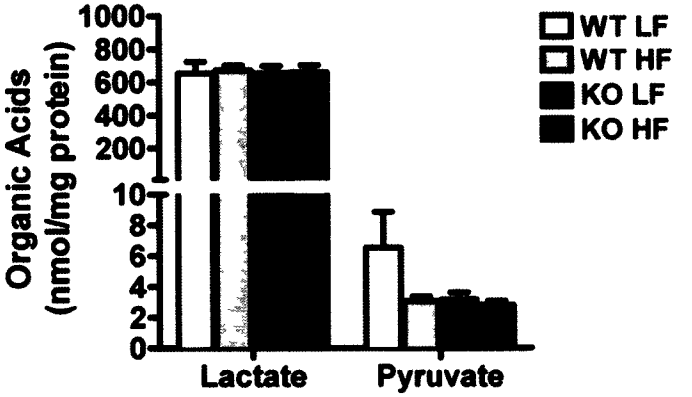

Organic Acids
(nmol/mg protein)
1000
800
600
400
200
10
8
6
4
2
0
WT LF
WT HF
KO LF
KO HF
Lactate
Pyruvate

Figure 3-21: Deletion of CD36 does not restore AMPK-ACC signaling in skeletal muscle from middle-aged mice fed a high fat diet

Immunoblot analysis was performed on homogenates prepared from gastrocnemius muscle collected from overnight fasted WT and CD36 KO mice at the end of 12 weeks of LF or HF diet. Phosphorylation status of AMPK at threonine 172 (T172) and ACC at serine 79 (S79) was detected with the use of anti-phospho-AMPKα and anti-phospho-ACC antibodies. Levels of phosphorylated AMPKα were quantified by densitometry and normalized to total AMPKα levels **(A).** Levels of phosphorylated ACC isoforms were quantified by densitometry and normalized to total ACC levels **(B).** n = 5-7 per group, * $p < 0.01$ vs. LF or HF-fed CD36 KO mice by Student's unpaired t-test.

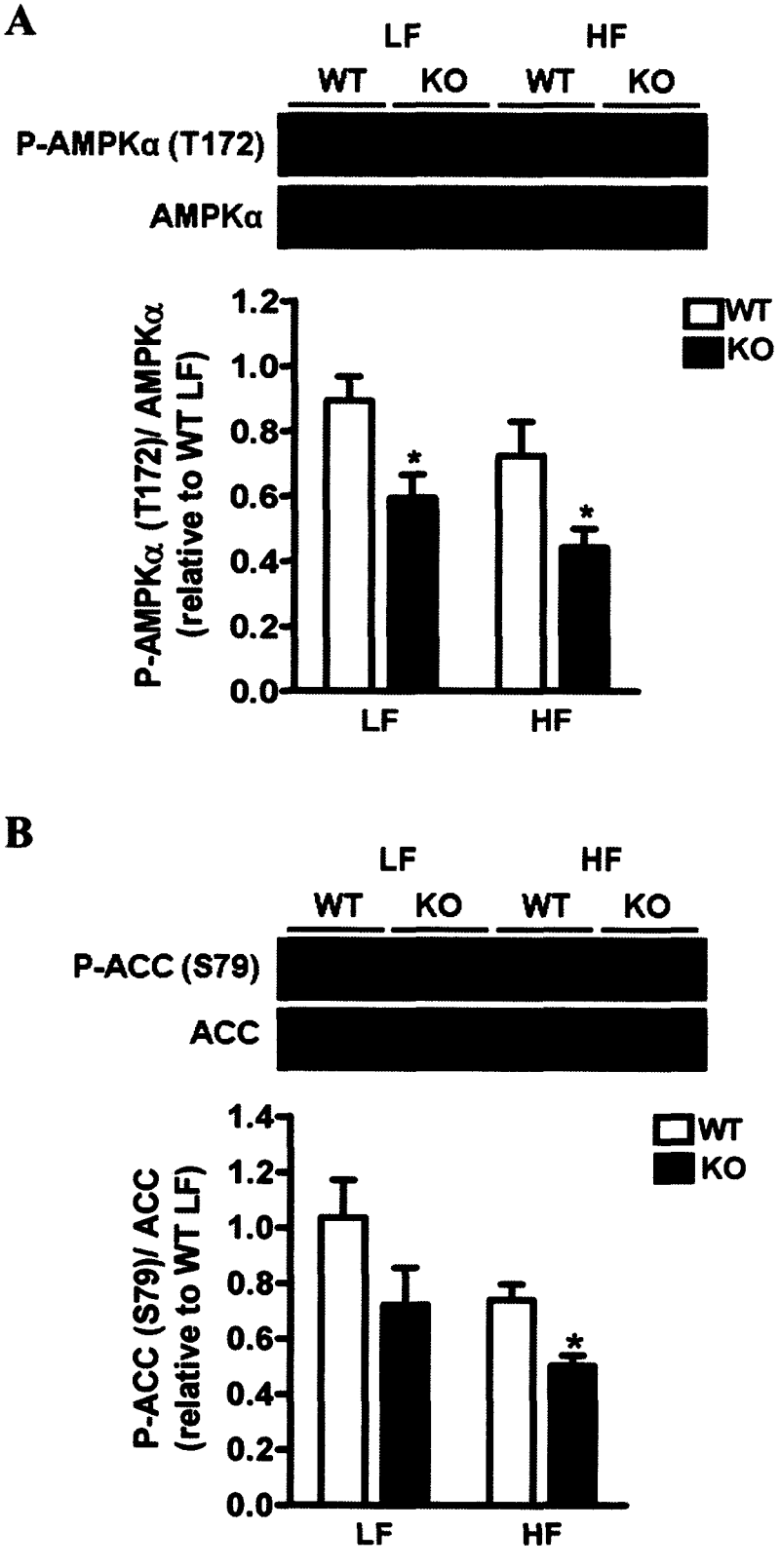

A
LF
HF
WT
KO
WT
KO
P-AMPKα (T172)
AMPKα
P-AMPKα (T172)/ AMPKα
(relative to WT LF)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
*
*
LF
HF
B
LF
HF
WT
KO
WT
KO
P-ACC (S79)
ACC
P-ACC (S79)/ ACC
(relative to WT LF)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
*
LF
HF

Figure 3-22: Diminished intramuscular lipid accumulation in skeletal muscle from CD36 knockout mice, despite high circulating plasma free fatty acid levels

Plasma free fatty acids (FFA) levels in middle-aged WT and CD36 KO mice fed a HF diet in the fasted state, n = 7-11 per group **(A).** Intramuscular triglyceride (TG; **B)**, long chain acyl CoA (LCACoA; **C)** and C-18 ceramide **(D)** levels as determined in gastrocnemius muscle obtained from WT and CD36 KO mice fed a HF diet for 12 weeks, n = 5-6 per group. *$p < 0.01$ vs. WT HF fed mice as determined by Student's unpaired t-test.

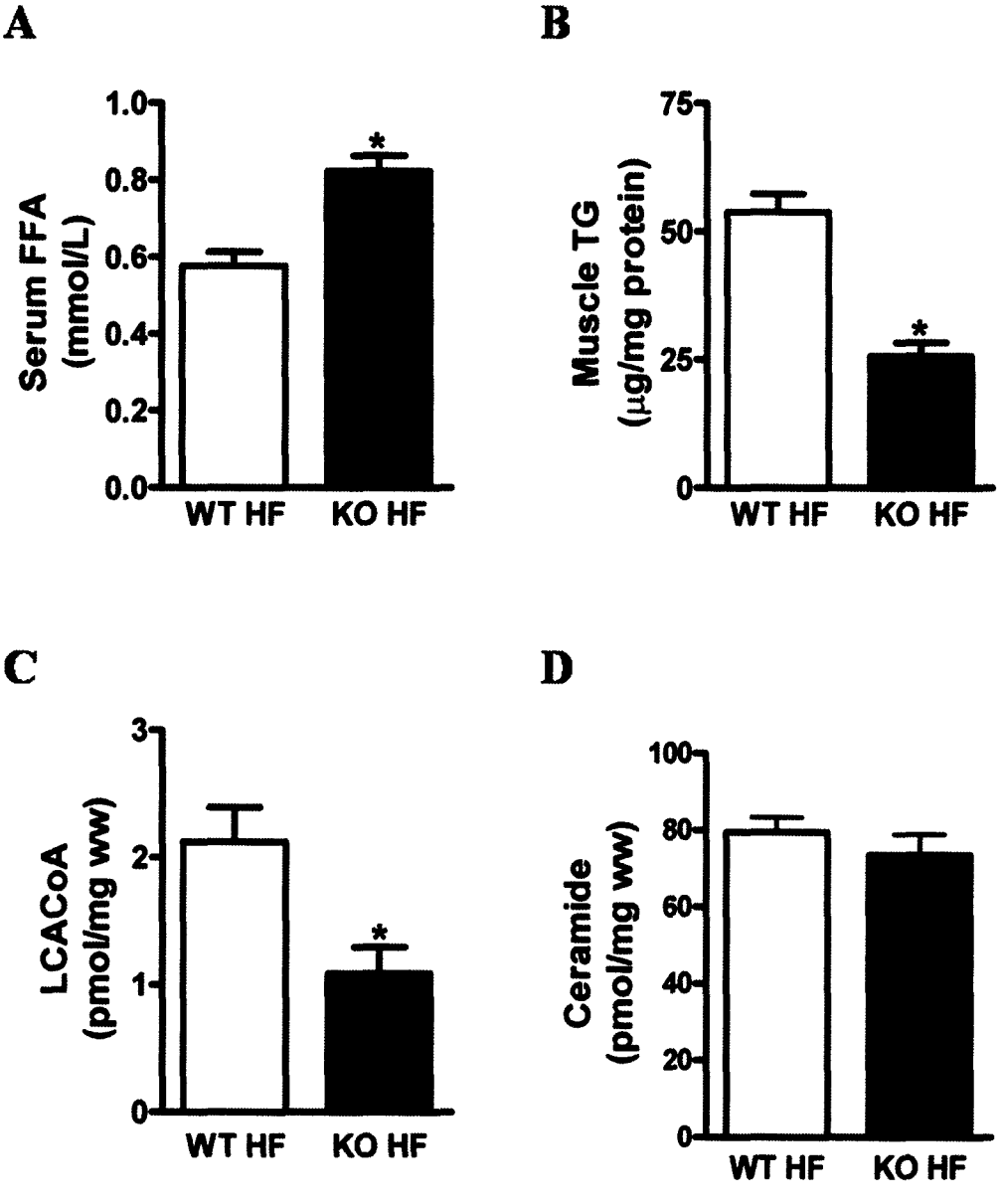

A
Serum FFA (mmol/L)
1.0
0.8
0.6
0.4
0.2
0.0
*
WT HF
KO HF
B
Muscle TG (μg/mg protein)
75
50
25
0
*
WT HF
KO HF
C
LCACoA (pmol/mg ww)
3
2
1
0
*
WT HF
KO HF
D
Ceramide (pmol/mg ww)
100
80
60
40
20
0
WT HF
KO HF

Figure 3-23: Improved glucose tolerance and reduced insulin levels in high fat fed middle-aged CD36 knockout mice as compared to wild-type mice

Fasted levels of blood glucose **(A)** and plasma insulin **(B)** were measured in middle-aged WT and CD36 KO mice after 12 weeks of high fat diet. Glucose tolerance tests were performed in WT and CD36 KO mice following 12 weeks of a HF diet. Blood glucose levels were monitored for 120 min following i.p. bolus dose of glucose (2 g glucose/kg body weight), where readings at time 0 min were baseline glucose measurements following a 6 hr fast just prior to glucose injection **(C).** Area under the curve (AUC) calculated for the curves generated by the glucose tolerance test, n = 7-10 per group **(D).** *$p < 0.05$ vs. WT HF mice **(A, B and D)** by Student's unpaired t-test, *$p < 0.05$ vs. WT HF **(C)** by two-way repeated measures ANOVA with Bonferroni post-hoc test.

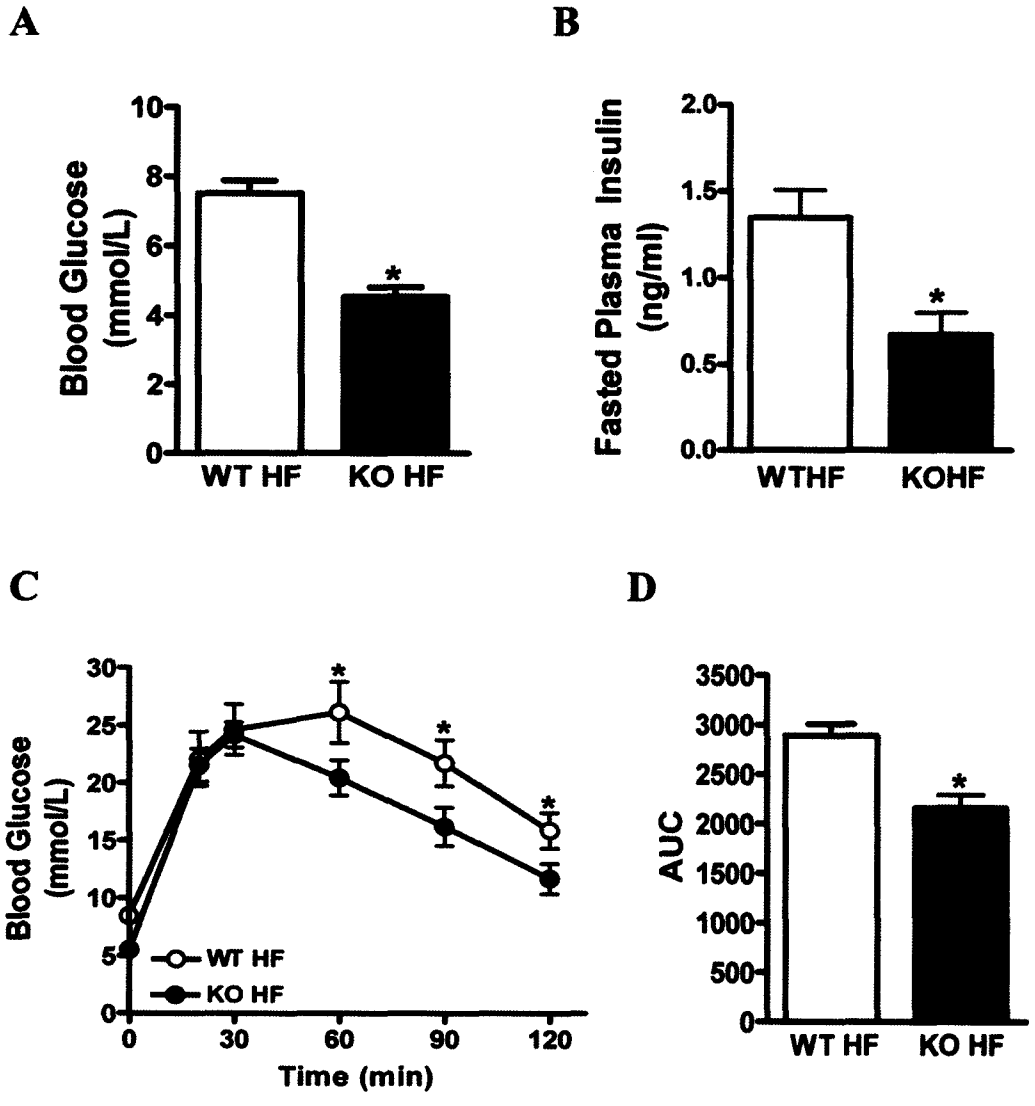
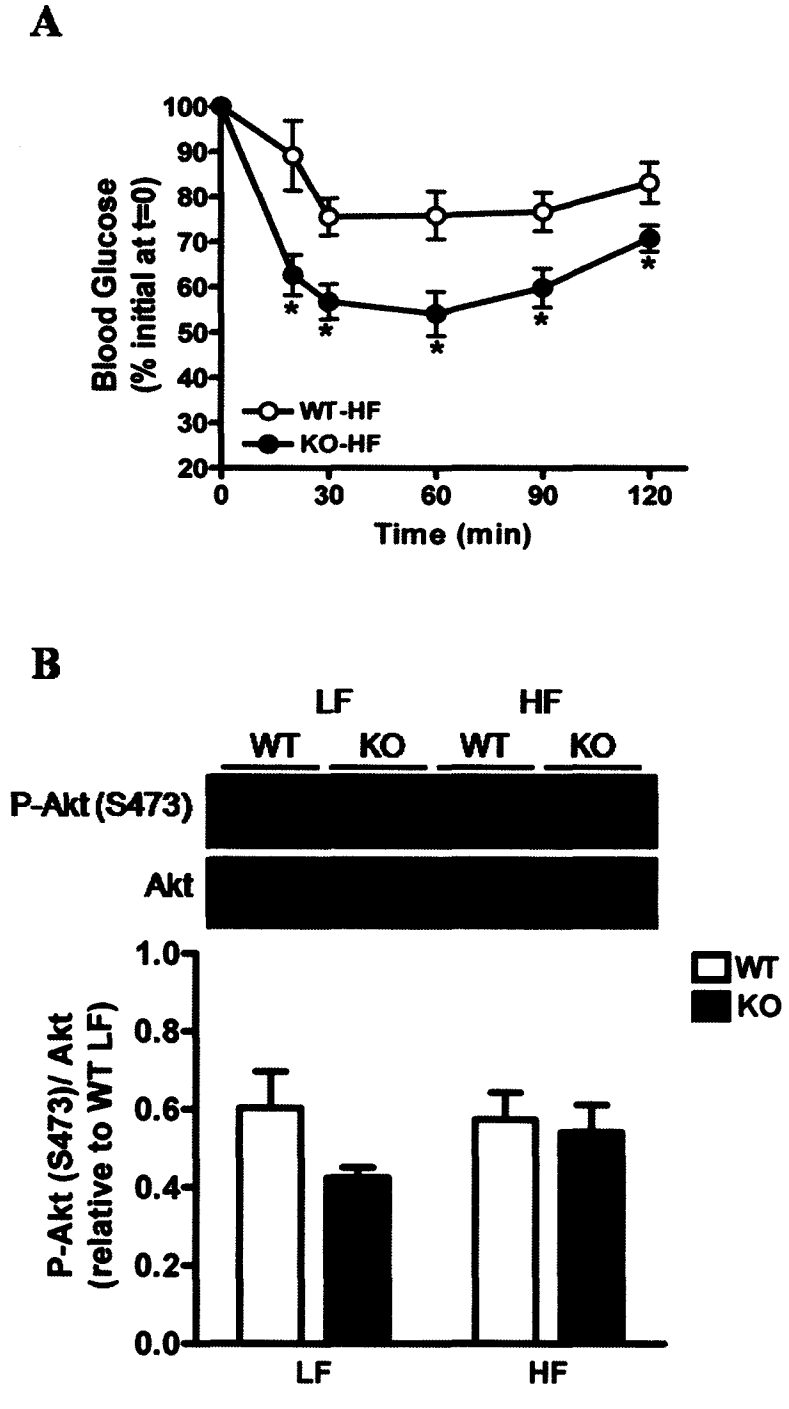

A
B
Blood Glucose (mmol/L)
10
8
6
4
2
0
WT HF
KO HF
*
Fasted Plasma Insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
WTHF
KOHF
C
D
Blood Glucose (mmol/L)
30
25
20
15
10
5
0
WT HF
KO HF
0
30
60
90
120
Time (min)
AUC
3500
3000
2500
2000
1500
1000
500
0

Figure 3-24: Improved insulin sensitivity in middle-aged CD36 knockout mice compared to wild-type mice fed a high fat diet

Insulin tolerance tests were performed in WT and CD36 KO mice following 12 weeks of a HF diet. Blood glucose levels were monitored for 120 min following i.p. bolus dose of insulin (0.7 U insulin/kg body weight), where readings at time 0 min were baseline glucose measurements following a 6 hr fast just prior to insulin injection **(A).** Phosphorylation of Akt at serine 473 (S473) in gastrocnemius muscle collected from aged WT and CD36 KO mice fed a LF or HF diet **(B).** n = 5-6 per group, * $p < 0.05$ vs. WT HF by two-way repeated measures ANOVA and Bonferroni post-hoc test.


A
Blood Glucose (% initial at t=0)
100
90
80
70
60
50
40
30
20
WT-HF
KO-HF
0
30
60
90
120
Time (min)
B
LF
HF
WT
KO
WT
KO
P-Akt (S473)
Akt
P-Akt (S473)/ Akt (relative to WT LF)
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
LF
HF

Discussion

It is widely accepted that aging is an important risk factor for the development of insulin resistance and T2D [30, 31]. Indeed, mitochondrial dysfunction has been observed in healthy elderly humans [32, 33, 35] and it has been proposed that this may contribute to intramuscular lipid accumulation and subsequently the development of insulin resistance. Taken together, these changes could predispose middle-aged individuals to the onset of T2D and may explain the high prevalence of metabolic disease and T2D in the elderly population. In the present study we investigated how middle-age impacts skeletal muscle metabolism and whole-body glucose utilization, as well as the susceptibility of healthy middle-aged mice to the development of HF diet-induced insulin resistance.

In agreement with previous studies in humans showing a decrease in metabolic rate and energy expenditure with age [32], our data also show a significant decline in metabolic rate in middle-aged mice (52-58 weeks) compared to young mice (12-14 weeks) as indicated by reductions in VO_2, VCO_2 and heat production. The prevailing theory is that skeletal muscle oxidative metabolism is impaired with age, and that is a direct consequence of age-related mitochondrial dysfunction. Both mitochondrial oxidative enzyme activity [32, 34] and mitochondrial content/ number [21, 35, 38-40] have been shown to decline with age, which may contribute to impaired mitochondrial metabolism. Based on this, we examined the levels of oxidative phosphorylation complexes in muscle from

young and aged mice in our study by immunoblot; however, contrary to previous findings we found no difference in mitochondrial protein content between age groups. Although mitochondrial function was not directly measured in this study, the decline in overall metabolic rate as measured by indirect calorimetry did not appear to stem from compromised mitochondrial function in skeletal muscle of middle-aged mice compared to young mice, suggesting other factors may influence substrate oxidation in muscle, as well as overall metabolic rate. Indeed, whole body RER was modestly decreased in middle-aged mice and skeletal muscle activity of β-HAD increased, suggesting there is a shift in whole-body substrate utilization from carbohydrates towards a greater reliance on FAs with aging. Although elevated β-HAD activity suggests that skeletal muscle FA oxidation is increased, it should be noted that these are measurements of maximal enzyme activities in the presence of an excess of substrate, therefore, *in vivo* substrate flux into the mitochondria maybe a limiting factor.

An important site of regulation for FA oxidation is found at the level of FA translocation across the mitochondrial membrane. Malonyl CoA regulates FA oxidation by allosteric inhibition of CPT1, the rate-limiting enzyme involved in mitochondrial FA uptake [13]. Malonyl CoA is synthesized primarily by ACC, and AMPK is known regulate lipid metabolism by phosphorylation and inhibition of ACC [41]. Recent studies show an age-associated reduction in skeletal muscle AMPK activation and mitochondrial biogenesis [43, 44]. Given the impact that reduced AMPK activity may have on mitochondrial function and lipid metabolism, we sought to investigate whether AMPK and ACC signaling is

impaired with aging in our study. Indeed, we observed a significant reduction in phosphorylation of both AMPK and its downstream target ACC in skeletal muscle from middle-aged mice. While we did not directly measure malonyl CoA levels, increased phosphorylation of ACC suggests that FA uptake into the mitochondria are reduced and subsequently FA oxidation rates may also be diminished. In addition, free carnitine levels were significantly reduced in skeletal muscle from middle-aged mice as compared to young mice. Indeed, carnitine insufficiency has been observed in rodent models of aging and diabetes, and is associated with marked perturbations in mitochondrial FA metabolism [45]. As such, due to the essential role of carnitine in permitting mitochondrial translocation and oxidation of long chain FAs, age-related decline in skeletal muscle carnitine levels in our model may contribute to alterations in FA metabolism that are independent of changes in the AMPK-ACC signaling pathway, however, the potential significance of this finding requires further study.

Consistent with whole-body glucose utilization being impaired with aging, basal and insulin-stimulated Akt phosphorylation were also markedly reduced in skeletal muscle from middle-aged as compared to young mice. In agreement with the intramuscular lipid accumulation theory of insulin resistance, we observed a significant elevation in intramuscular TG levels in skeletal muscle from middle-aged mice. It is not yet clear whether intramuscular TG accumulation is a causative factor in the development of age-related insulin resistance or maybe an adaptive mechanism to sequester FAs in the TG pool and prevent the cytosolic accumulation of potentially toxic bioactive lipid intermediates secondary to

increased FA supply [57]. Therefore, further studies are required to determine the potential contribution of altered TG levels and metabolism to skeletal muscle insulin resistance observed with aging. Taken together, we hypothesize that impaired activation of skeletal muscle insulin-signaling parameters and elevations in intramuscular TG levels may increase the susceptibility of aged mice to developing diet-induced insulin resistance. Potentially, elevated intramuscular TG levels may reduce the buffering capacity of the muscle to handle a chronic oversupply of FAs as one would observe with the consumption of a HF diet. If elevated intramuscular TG levels are directly or indirectly involved in the development of skeletal muscle insulin resistance, then the large TG accumulation itself may be detrimental or when the TG stores are filled to capacity there may be spill over of FA into other potentially toxic lipid intermediates, such as LCACoAs or ceramides.

To determine whether middle-aged mice are indeed more susceptible to developing insulin resistance in response to a HF diet as compared to their younger counterparts, we fed young and middle-aged mice 12 weeks of a LF or HF diet. As expected, middle-aged mice gained significantly more weight than young mice when fed a HF diet for the same period of time. Although impairment of glucose tolerance in response to HF diet was similar between age groups, severe HF diet-induced hyperinsulinemia was only observed in middle-aged mice, indicating that increases in β-cell insulin secretion were sufficient to compensate for reduced peripheral insulin sensitivity in middle-aged mice and to offset the appearance of overt hyperglycemia. Moreover, the HOMA-IR index, which is an

accepted surrogate marker of insulin resistance [48], was significantly elevated in HF-fed aged mice, suggesting that 12 weeks of HF feeding induces a more dramatic insulin resistance in middle-aged mice as compared to young mice. Given that prolonged hypersecretion of insulin by the pancreatic β-cells to compensate for peripheral insulin resistance can ultimately lead to β-cell failure [58] and subsequently the onset of T2D [59], our data strongly suggest that advanced age heightens the susceptibility of middle-aged mice to the development of diet-induced insulin resistance. However, a caveat to this conclusion is that many of the age-related changes observed were correlated with increased body weight in these mice, which we have found is primarily due to increased adiposity (unpublished data, Sung M and Dyck J). Therefore, the increased susceptibility to diet-induced insulin resistance may not be due completely to aging *per se*, but also increased adiposity. As increased adiposity often accompanies aging it is difficult to discriminate between the effects of each factor on skeletal muscle metabolism or the development of insulin resistance in our current study. Future studies are required to determine the effects of 'aging' independent of increases in adiposity, potentially by caloric restriction or exercise to limit weight gain. Nevertheless, as aging and increased adiposity co-associate our findings likely reflect the majority of middle-aged humans in the western world who are at risk of developing insulin resistance.

Interestingly, the phosphorylation status of Akt was similar between diet groups in both the young and middle-aged mice. Although we cannot be certain why this is the case, it may be related to the increase in circulating insulin levels

in the HF fed compared to the LF fed mice, which could maintain Akt phosphorylation at similar levels in both groups of mice. We suspect that this is also the case in the HF fed aged mice where phosphorylation of Akt is not reduced in skeletal muscle despite obvious impairments in whole-body insulin sensitivity. Regardless, obvious changes were observed in both glucose and insulin tolerance tests between LF and HF-fed mice.

Although age-related intramuscular lipid accumulation is often attributed to mitochondrial dysfunction [32, 34], the build-up of intramuscular lipids can also occur independent of marked changes in mitochondrial function, for instance when rates of FA transport into muscle exceed the capacity for their oxidation [8, 10, 60-62]. Therefore, next we assessed whether accumulation of lipids and lipid-derived intermediates in skeletal muscle of middle-aged mice fed a HF diet was accompanied by an increase in expression of FA transport proteins. We focused on the FA transport protein CD36 because it is known to be a major contributor to increased FA uptake into skeletal muscle in human obesity and T2D [8], as well as in aged rodents [61, 62]. Consistent with our hypothesis, we observed a 2-fold increase in CD36 protein expression in skeletal muscle of HF fed middle-aged mice compared to LF fed middle-aged mice. In accordance with increased CD36 protein expression, skeletal muscle TG levels were significantly elevated in HF fed middle-aged mice compared to LF fed mice, as were levels of LCACoAs and ceramides. Since concentrations of sarcolemmal-associated CD36 were not measured in muscle from HF-fed middle aged mice, it is not known whether levels of CD36 translocation to the plasma membrane or FA transport are elevated

in these mice. However, marked intramuscular lipid accumulation suggests that increased CD36 protein expression does reflect elevated skeletal muscle FA uptake. Taken together, these data suggest that increased CD36-mediated FA uptake may contribute to lipid accumulation, and impaired insulin sensitivity in skeletal muscle of middle-aged mice fed a HF diet.

Although aging-associated intramuscular lipid accumulation did not appear to result from overt mitochondrial dysfunction, the 2-fold increase in CD36 protein expression in skeletal muscle of HF-fed middle-aged mice compared to LF-fed middle-aged mice suggests there is an imbalance between FA uptake and subsequent FA utilization. While it is not known what mediates the increase in skeletal muscle CD36 content in our study, similar to many proteins involved in lipid handling the expression of CD36 is thought to be at least indirectly under the transcriptional control of PPARs, therefore chronic FA oversupply may result in PPAR-mediated upregulation of CD36 expression [63]. Moreover, it has been shown in humans and young rodents that high levels of glucose or insulin may directly or indirectly regulate CD36 expression through both transcriptional and/or translational mechanisms [64, 65]. Consistent with this, approaches that effectively blunt HF diet-induced hyperglycemia also reduce CD36 expression in skeletal muscle [50]. Since obesity is associated with an increase in circulating levels of glucose, insulin and FAs, one of these factors may likely contribute to the increase in CD36 levels in skeletal muscle from aged mice. We predicted that limiting FA transport and metabolism could rescue the HF-diet induced phenotype, and therefore, we utilized the CD36 KO mouse, which has skeletal

muscle FA uptake rates ~40-70% of those in WT mice [51, 52]. Consistent with our hypothesis, CD36 deficiency was associated with preserved metabolic rate and energy expenditure in middle-aged mice fed a HF diet as compared to middle-aged WT mice fed a HF diet. Since food intake was similar between groups, it is possible that increased energy expenditure in HF fed middle-aged CD36 KO mice contributes to their protection against diet-induced obesity. Furthermore, skeletal muscle levels of TGs and LCACoA esters were reduced in middle-aged CD36 KO mice. Surprisingly, ceramides which have been strongly implicated in the pathogenesis of insulin resistance due to effects on insulin signaling and glucose uptake [56] were not decreased in middle-aged CD36 KO mice, although insulin sensitivity was dramatically improved in these mice. Another lipid species that may also contribute to impaired insulin signaling is DAG, however, DAG levels were not investigated in this current study and it is not clear what role DAG may play in our aging- HF diet model.

The blunted decline in metabolic rate observed in middle-aged CD36 KO mice fed a HF diet was accompanied by alterations in muscle concentrations of several metabolic intermediates, but interestingly this did not include an improvement in AMPK and ACC phosphorylation. The impact of the diet on muscle metabolites in this study differed to some extent as compared to a previous report [29] in which tissue specimens were harvested from younger animals in the fed state. In the current study, tissues were collected after an overnight fast as we sought to evaluate a state of heightened FA oxidation. Under these conditions, HF feeding lowered several LC acylcarnitines but increased LCOH acylcarnitines.

The decline in LC acylcarnitines could reflect decreased FA availability, lower CPT1 activity or increased LCCoA flux through LCAD. Several lines of evidence support the third possibility. First, the HF diet increases rather than decreases lipid delivery to muscle. Second, of the two major products of CPT1 (palmitoylcarnitine (C16) and oleylcarnitine (C18:1)), only the unsaturated species was reduced by the diet, suggesting upregulation of the isomerase enzyme that catalyzes conversion of the double bond [66]. Third, the generalized rise in LCOH species along with the high LCOH/LC ratio in response to chronic lipid exposure suggest a shift in flux limitation from the earlier to later steps in β-oxidation. Lastly, as expected a HF diet resulted in a robust drop in whole body RER even in middle-aged mice, indicative of a systemic increase in FA oxidation in response to a HF-diet. Notably, CD36 deficiency alone in middle-aged mice altered baseline levels of several muscle metabolites, and in general, tended to mitigate diet-induced changes in several acylcarnitine species. This apparent resistance to diet-induced metabolic perturbations in the middle-aged CD36 KO mice might be directly related to a reduction in tissue FA uptake and metabolism and/or secondary to enhanced energy expenditure and insulin sensitivity. Although further work is necessary to fully understand the implications of these results, it is evident that loss of CD36-mediated FA uptake has a global impact on muscle fuel metabolism. Although this study focused primarily on skeletal muscle, the coordinated control of energy metabolism requires communication between and involvement of multiple organs and so it is likely that other organs, such as adipose tissue, liver and brain [67, 68], are also involved in maintaining a

high level of whole body energy expenditure in the middle-aged CD36 KO mice. Indeed, the phenotype observed in the HF fed middle-aged CD36 KO mouse is strikingly similar to that observed in HF-fed mice with acute hepatic expression of PPAR-γ2, where hepatic steatosis initiates a neuronal signaling pathway originating from the liver resulting in enhanced energy expenditure and improved systemic insulin sensitivity in these mice [68]. Whether or not this pathway is involved in producing the phenotype observed in CD36 KO mice is currently unknown, and requires further investigation.

Overall, ablation of CD36 was associated with an improvement in whole-body glucose utilization and insulin sensitivity in mice fed a HF diet. Although the precise mechanisms responsible for this are not fully understood, excessive intramuscular lipid accumulation induced by HF feeding was prevented by CD36 deficiency. Whereas elevated intramuscular TG levels were not associated with insulin resistance in young mice as evidenced by low HOMA-IR values, preventing the more dramatic age- and diet-induced accumulation of intramuscular TG and LCACoA levels in middle-aged CD36 KO mice correlated with improved whole-body insulin sensitivity. Taken together, our data demonstrate that lowering FA uptake in the presence of a chronic oversupply of FA due to over-nutrition guards against whole-body and skeletal muscle insulin resistance in middle-aged mice fed a HF diet. This finding infers a potential therapeutic strategy for combating age-related metabolic disease by targeting FA uptake and preventing potential downstream consequences including intramuscular lipid accumulation and mitochondrial stress.

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CHAPTER 4.

Increased CD36 Expression in Middle-Aged Mice Contributes to Obesity-related Cardiac Hypertrophy in the Absence of Cardiac Dysfunction

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My role in this work involved performing all the experiments (except those noted below), as well as analyzing the data and writing the manuscript. The high fat feeding experiments in aged wild-type and CD36 knockout mice were performed in direct collaboration with post-doctoral fellow Debby Koonen, while I performed biochemical analyses of the hearts following the 12 weeks of diet. René Jacobs performed the triglyceride assays, and Carrie-Lynn Soltys performed some of the immunoblots. Echocardiography was performed by technical staff in the Cardiovascular Research Centre (CVRC) *in vivo* imaging core (University of Alberta, Edmonton, AB).

CHAPTER 4.

Increased CD36 Expression in Middle-Aged Mice Contributes to Obesity-related Cardiac Hypertrophy in the Absence of Cardiac Dysfunction

Abstract

As aging is a significant risk factor for the development of left ventricular hypertrophy and cardiovascular disease, we hypothesized that hearts from middle-aged mice may be more sensitive to the effects of a high fat (HF) diet than hearts from young mice. To investigate this, young (10-12 week old) and middle-aged (40-44 week old) male C57BL/6 mice were fed a low fat (LF) or high fat (HF) diet (10 or 60% kcalfat, respectively) for 12 weeks. Following this 12 week period, we show that CD36 protein expression was not changed in hearts from young mice yet was increased 1.5-fold in the middle-aged HF group compared to LF-fed age-matched counterparts. Correlated with increased CD36 expression, middle-aged mice displayed a greater degree of cardiac hypertrophy compared to the young mice when fed a HF diet. Furthermore, middle-aged CD36 knockout mice were protected against HF diet-induced cardiac hypertrophy, supporting a link between CD36 and cardiac hypertrophy. To further explore potential mechanisms that may explain why middle-aged mice are more susceptible to HF

diet-induced cardiac hypertrophy, we investigated mediators of cardiac growth. We show that myocardial ceramide levels were significantly increased in middle-aged mice fed a HF diet compared to LF controls, which was also correlated with inhibition of AMP-activated protein kinase (AMPK). Consistent with AMPK being a negative regulator of cardiac hypertrophy, decreased AMPK activity also resulted in activation of the mTOR-p70S6K pathway, which is known to enhance protein synthesis associated with cardiac hypertrophy. Together, these data suggest that increased myocardial CD36 expression in hearts from middle-aged mice may contribute to HF diet-induced cardiac hypertrophy and that this may be mediated by elevated ceramide levels signaling through AMPK. Overall, we suggest that inhibition of CD36-mediated fatty acid uptake may prevent obesity-related cardiomyopathies in the middle-aged population.

Introduction

Lipotoxic cardiomyopathy is a cardiac condition that arises due to excessive accumulation of damaging fatty acid intermediates within the cardiomyocyte that results in impaired cardiac performance [1-3]. While the pathogenesis of lipotoxic cardiomyopathy is multifactorial, in most instances it is precipitated by increased lipid storage in the cardiac myocyte (cardiac steatosis) resulting from excessive fatty acid (FA) uptake [4, 5] and/or impaired FA utilization [6]. Conditions associated with cardiac steatosis and eventual lipotoxic cardiomyopathy include; obesity, insulin resistance, and diabetes [1]. These conditions are associated with elevated circulating levels of FAs and it is generally accepted that excessive FA uptake into the cardiac myocyte is primarily responsible for initiating the observed cardiac steatosis [7]. Indeed, expression of FA transport proteins such as CD36, which are involved in mediating FA uptake into the cardiac myocyte have been shown to increase in response to diet-induced obesity [7-10]. Interestingly, we have also shown that myocardial CD36 protein expression increases with age and is associated with excessive cardiac steatosis and contractile dysfunction [11]. As a result of these two findings, we propose that cardiac steatosis may be more pronounced with advanced age [11, 12] and/or that the aged myocardium may be more susceptible to enhanced accumulation of lipids during increased dietary fat intake.

In addition to the positive correlation between increased CD36 protein expression and cardiac dysfunction, our previous work has also shown that

increased levels of CD36 protein in hearts of middle-aged wild-type (WT) mice is correlated with cardiac hypertrophy [11]. However, due to the degree of cardiac dysfunction observed in those middle-aged mice, it was not possible to assess if increased CD36 expression could contribute to cardiac hypertrophy independent from causing contractile dysfunction. To date, the precise role of CD36 in mediating cardiac hypertrophy remains unclear. Interestingly, the middle-aged mice evaluated in our previous study were maintained on a 10% fat diet for a full year, which contains a higher dietary fat content than standard rodent chow (4%kcal fat). As a result, we speculated that the middle-aged WT mice in that study displayed an exaggerated detrimental cardiac phenotype due to a prolonged exposure to elevated FAs and elevated body weight. Therefore, in the present study we assessed whether a shorter duration of increased dietary fat intake (12 *vs.* 52 weeks) would also increase CD36 expression and induce cardiac steatosis, cardiac dysfunction as well as cardiac hypertrophy in middle-aged mice. Furthermore, we aimed to better understand the downstream signaling pathways by which upregulation of CD36 may contribute to cardiac hypertrophy and/or contractile dysfunction. We hypothesized that advanced age would increase the susceptibility for developing high fat (HF) diet-induced cardiomyopathy and that this would be associated with increased myocardial CD36 expression. Given that the elderly population is rapidly expanding and the fact that there is an increasing health burden of obesity and its co-morbidities, understanding the consequences of obesity on the heart particularly in the context of aging as well as the role that CD36 plays in this process is of significant clinical importance.

Materials and Methods

Animals

The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with Canadian Council of Animal Care guidelines. Young (10-12 week old) and aged (40-44 week old) male C57BL/6J mice were purchased from The Jackson Laboratory. Mice were housed in a temperature controlled environment with a 12:12 hr reversed light/dark cycle. Mice were fed either a low fat (LF; 10 kcal% fat) or high fat (HF; 60 kcal% fat) diet (D12450B and D12492, Research Diets, Inc., respectively) for 12 weeks during which time they were allowed free access to food and water unless otherwise stated.

CD36 Deficient Mice

CD36 knockout (KO) mice were generated in the laboratory of Dr. Maria Febbraio as described in Chapter 2. In brief, CD36 KO and wild-type (WT; C57BL/6) male mice were derived from littermates and were 6X backcrossed to a C57BL/6 background strain [13]. Mice were left relatively undisturbed until 32-34 weeks of age and given free access to water and standard rodent diet (5001; LabDiet). At 32-34 weeks of age CD36 KO and WT mice were randomly divided into two groups and fed either a LF or HF diet for a period of 12 weeks.

In Vivo Assessment of Cardiac Function by Echocardiography

Transthoracic echocardiography was performed on mildly anesthetised mice using a Vevo 770 High-Resolution Imaging System equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Ontario) and cardiac function and ventricular dimensions were determined as described in Chapter 2.

Determination of Myocardial Triglyceride, Long-chain Acyl CoA (LCACoA) and Ceramide Levels

Triglycerides (TGs), LCACoAs and C-18 ceramides were extracted from frozen powdered ventricular tissue of fasted (16 hr) mice and assayed for lipids as described in Chapter 2.

Immunoblot Analysis

Tissue was homogenized, SDS-PAGE gels run and membranes probed with antibodies as described in Chapter 2. Signals were visualized using chemiluminescence and densitometry performed using Image J software.

Statistical Analysis

All data are presented as means ± SEM. Comparisons between groups were performed using an unpaired Student's two-tailed t-test or one way ANOVA with a Bonferroni post-hoc test where appropriate. A value of $p < 0.05$ is considered significant.

Results

Increased dietary fat intake in middle-aged mice does not alter systolic function after 12 weeks

In order to characterize differential effects of dietary fat intake in young and aged mice, we fed young (10-12 weeks old) and middle-aged (40-44 weeks old) C57BL/6 mice a LF (10 kcal% fat) diet or a HF (60 kcal% fat) diet for 12 weeks. At the end of this period, as expected young mice fed a HF diet had a significant increase in body weight compared to mice fed a LF diet (Fig. 4-1A) and displayed systemic glucose intolerance as determined by glucose and insulin tolerance tests (data not shown). Despite that middle-aged mice had a similar daily food intake as young mice fed a HF diet (Fig. 4-1B), at the end of the 12 week period middle-aged mice were found to have gained considerably more weight than young mice on the same HF diet (Fig. 4-1A). Interestingly, even with 12 weeks of high dietary fat intake and the resulting obesity, transthoracic M-mode echocardiography revealed that *in vivo* systolic function, as determined by %EF and %FS, were not impaired in either young or middle-aged mice (Fig. 4-2A-C). As diastolic dysfunction can often precede the onset of systolic dysfunction in many disease processes [14], we examined isovolumic relaxation time (IVRT), as an index of diastolic function, but found similar diastolic function between groups regardless of diet and/or age (Fig. 4-2D).

Interestingly, while there were no changes cardiac performance in both young and middle-aged mice, HF diet significantly increased the heart weight

(HW) to tibia length (TL) ratio in both groups of mice (Fig. 4-3A). However, while the increase in the HW/TL ratio was modest in the young mice, the HF-mediated effect was more pronounced in the middle-aged mice (Fig. 4-3B) and walls appeared thicker in M-mode images (Fig. 4-2A), suggesting an increased sensitivity of middle-aged mice to HF-mediated stimulation of cardiac growth.

High fat feeding in middle-aged mice induces CD36 expression without altering myocardial triglyceride levels

Previous work from our lab has shown that increased CD36 protein expression in the heart is associated with diastolic and systolic dysfunction, as well as cardiac hypertrophy in aged WT mice [11]. Although our previous findings support the concept that increased CD36 protein expression and FA accumulation may cause cardiac hypertrophy, but since cardiac dysfunction was also present in these hearts it is difficult to distinguish whether CD36 plays a direct role in mediating cardiac hypertrophy. Therefore, next we examined if myocardial CD36 expression was altered in response to a HF diet in young and middle-aged mice and ascertained whether these differences were correlated with the increased HW/TL ratio observed in Fig. 4-3A. Heart homogenates from LF- and HF-fed mice were subjected to immunoblot analysis with anti-CD36 antibody. CD36 protein expression in the heart was not different between young mice on LF and HF diet (Fig. 4-4A). However, a significant 1.5-fold increase in CD36 protein levels was detected in hearts from middle-aged mice after 12 weeks of HF diet (Fig. 4-4B). Interestingly, despite increased CD36 protein expression in hearts from middle-aged mice on a HF diet, there was no measureable

difference in myocardial TG levels in the HF-fed compared to the LF-fed middle-aged mice (Fig. 4-4C), suggesting that myocardial TG levels *per se* are not responsible for the increased HW/TL ratio observed in Figure 4-3A. In addition, since systolic and diastolic function was normal in aged WT mice fed either a LF or HF diet (Fig. 4-2B-D), our data suggest that increased CD36 protein levels in the aged heart precede the development of and may contribute to diet-induced cardiac dysfunction.

Increased CD36 expression in response to dietary fat intake in middle-aged mice contributes to cardiac hypertrophy

As CD36 expression is increased in middle-aged WT mice following 12 weeks of HF feeding, we utilized this age of mice and this HF feeding protocol to examine the relationship between increased CD36 expression and cardiac hypertrophy in the absence of cardiac dysfunction. To determine whether CD36 expression is responsible for any observed changes in cardiac growth in response to a diet high in dietary fat, we fed middle-aged (32-34 weeks of age) CD36 WT and CD36 KO mice either a LF or HF diet for 12 weeks. Consistent with our previous findings (Fig. 4-2B), at the end of 12 weeks of HF feeding, neither CD36 WT nor CD36 KO mice demonstrated any evidence of overt systolic or diastolic dysfunction (Fig. 4-5A and 4-B, respectively). However, even in the absence of systolic dysfunction, M-mode echocardiographic analysis revealed significant thickening of both the interventricular septum (IVS) and left ventricular (LV) posterior wall in hearts from CD36 WT mice fed a HF diet compared to CD36 WT mice fed a LF diet (Fig. 6A and C, respectively). While a modest thickening

of the septal wall was evident in hearts from HF-fed CD36 KO mice (Fig. 4-6A), LV posterior wall thickness remained unchanged (Fig. 6C). In addition, the absolute changes in wall thickening due to 12 weeks of HF diet was found to be significantly greater in CD36 WT hearts than CD36 KO hearts (Fig. 4-6B and D). Consistent with these *in vivo* measurements of cardiac hypertrophy, at the end of 12 weeks of HF feeding, CD36 WT mice had a significant increase in HW/TL ratio compared to LF-fed CD36 WT mice (Fig. 4-6E). Moreover, in agreement with a previous report [5], our data show that hearts from LF-fed CD36 KO mice trended towards being larger than their WT counterparts at baseline (Fig. 4-6E). Despite this, whereas 12 weeks of HF diet resulted in a 1.5-fold increase in HW/TL in CD36 WT mice, HF feeding did not increase HW/TL of CD36 KO mice (Fig. 4-6E and F), suggesting that CD36 KO mice were resistant to HF-mediated cardiac hypertrophy. Taken together, these data suggest that CD36 contributes to the development of HF diet-induced cardiac hypertrophy, and that ablation of CD36 may prevent HF-mediated cardiac hypertrophy.

Increased ceramide levels in middle-aged wild-type mice fed a high fat diet is associated with cardiac hypertrophy

To investigate the potential mechanisms that may be involved in CD36 ablation preventing cardiac hypertrophy, we analyzed hearts from middle-aged CD36 WT and CD36 KO mice fed either a LF or HF diet for 12 weeks. Interestingly, hearts from middle-aged CD36 WT mice fed a HF diet had similar intramyocardial TG levels as LF-fed CD36 WT mice (Fig. 4-7A), suggesting, as in Figure 4-4C, that TG accumulation *per se* is not responsible for the increase in

cardiac hypertrophy observed in the HF-fed CD36 WT mice. In agreement with CD36 deletion preventing FA uptake and eventual TG storage, hearts from CD36 KO mice had significantly lower intramyocardial TG levels as compared to CD36 WT mice on either LF or HF diet (Fig. 4-7A).

Since there are confounding variables associated with whole body CD36 ablation that may influence cardiac function or growth [15, 16], as well as the fact that only the middle-aged CD36 WT mice exhibited HF-mediated cardiac hypertrophy, we focused the remainder of our studies on the WT mice to delineate the underlying mechanisms. Firstly, since TG levels were not increased in middle-aged CD36 WT mice fed a HF diet, we hypothesized that lipid-derived intermediates may contribute to promoting cardiac hypertrophy in these mice. Although TG levels were not different, total LCACoA levels trended to increase in hearts from middle-aged WT CD36 mice fed a HF diet compared to the LF group (Fig. 4-7B). In order to provide more detailed analysis of this increase in LCACoA levels, we also isolated the major LCACoA species in these hearts. Interestingly, despite oleoyl-CoA and stearyl-CoA levels trending to be higher in hearts from HF-fed middle-aged CD36 WT mice ($p=0.06$ and 0.08, respectively), palmitoyl-CoA levels were not significantly different between groups (Fig. 4-7C). To determine if this lower than expected level of palmitoyl-CoA in the CD36 WT HF mice could be explained by increased *de novo* ceramide synthesis in that group, we also measure ceramides levels. As expected, ceramide levels were almost 2-fold higher in hearts from middle-aged CD36 WT mice fed a HF diet compared to hearts from middle-aged CD36 WT mice fed a LF diet (Fig. 4-7D).

As HF diet-induced increases in cardiac hypertrophy were more pronounced in middle-aged CD36 WT mice fed a HF diet compared to age matched mice fed a LF diet (Fig. 4-6A), we postulated that the 2-fold increase in ceramides levels in the WT hearts may alter signaling pathways that govern myocardial growth. Indeed, elevated levels of ceramides have previously been shown to inhibit the phosphorylation and activation of a major negative regulator of cardiac myocyte cell growth [17-19] and cardiac hypertrophy [17, 20], namely AMP-activated protein kinase (AMPK) via PP2A activation [21], suggesting that inhibition of AMPK may be involved in regulating the cardiac growth in these mice.

Impaired AMPK signaling in middle-aged wild-type mice fed a high fat diet is associated with cardiac hypertrophy

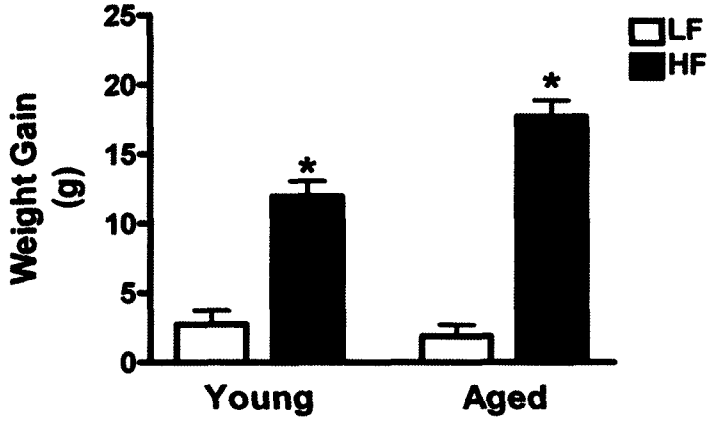
To determine if decreased AMPK activity could contribute to the development of cardiac hypertrophy in the middle-aged CD36 WT mice fed a HF diet, we first examined ceramides levels and P-AMPK in hearts from young mice and found both parameters to be unchanged (Fig. 4-8A and B). To determine if decreased AMPK activity could contribute to the development of cardiac hypertrophy in the middle-aged CD36 WT mice fed a HF diet, we performed immunoblot analysis for P-AMPK (Fig. 4-9A). In agreement with our hypothesis that AMPK activity is down regulated in the presence of elevated ceramides levels, AMPK phosphorylation was significantly decreased in hearts from middle-aged CD36 WT mice fed a HF diet as compared to hearts from LF-fed middle-aged CD36 WT mice (Fig. 4-9A). This decrease in AMPK activity was not due to a decrease in total AMPK levels (Fig. 4-9B) or a general decrease in kinase

activity as we observed no changes in Akt phosphorylation between groups (Fig. 4- 9C). This is particularly important as Akt activity has also been shown to be inhibited by ceramides [22-24] and can regulate cardiac growth [25-27]. Since increased AMPK activity reduces cardiac myocyte cell growth and inhibits protein synthesis by decreasing p70S6K phosphorylation [17-19], we speculated that depressed cardiac AMPK activity observed in the HF-fed middle-aged CD36 WT mice may create a permissive environment allowing for cardiac hypertrophic growth. In agreement with this, the known downstream effector molecules that regulate protein synthesis such as the mammalian target of rapamycin (mTOR), p70s6K and its downstream target, s6 ribosomal protein were activated in hearts from middle-aged CD36 WT mice fed a HF diet compared to LF-fed middle-aged CD36 WT mice (Fig. 4-9D and F-H). Interestingly, phosphorylation of eukaryotic elongation factor (eEF)-2 was unchanged in hearts from middle-aged CD36 WT mice (Fig. 4-9E), suggesting that activation of the s6 pathway is sufficient to promote protein synthesis.

Figure 4-1: Middle-aged mice gain more weight following 12 weeks of a high fat diet as compared to young mice, despite similar food consumption

Young (10-12 weeks) and middle-aged (40-44 weeks) C57BL/6 mice were fed a LF (10 kcal%fat) or HF (60 kcal%fat) diet for 12 weeks. Body weight gain at the end of 12 weeks of feeding, $n = 6$ per group and $n = 4$-5 per group of young mice and middle-aged mice, respectively **(A)**. Total food intake (kcal) measured over 24 hr cycle with 12 hr dark/active and 12 hr light/inactive phase at the end of 12 weeks of diet and adjusted to body weight (BW) in grams **(B)**. * $p < 0.05$ vs. age-matched LF-fed control mice as determined by Two-way ANOVA and Bonferroni post-hoc test where appropriate.

A


Weight Gain (g)
25
20
15
10
5
0
LF
HF
*
*
Young
Aged

B

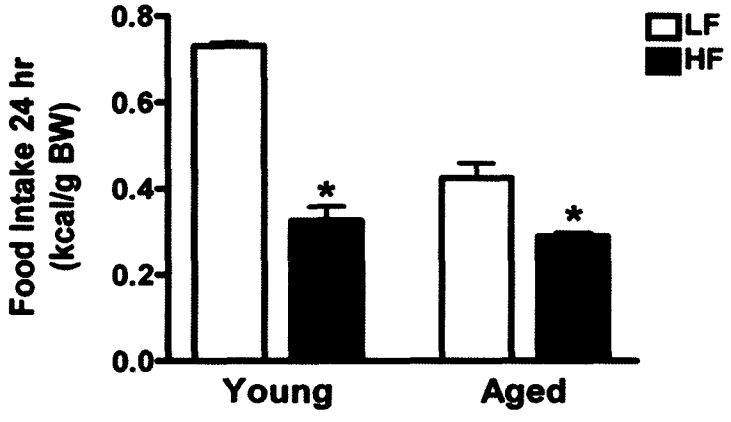

Food Intake 24 hr (kcal/g BW)
0.8
0.6
0.4
0.2
0.0
LF
HF
*
*
Young
Aged

Figure 4-2: Systolic and diastolic function is unchaged in hearts from young and middle-aged mice following 12 weeks of a high fat diet

Represntative M-mode echocardiographic recordings of cardiac performance taken from mildly anesthetised young and middle-aged mice at rest after 12 weeks of a LF or HF diet **(A)**. Echocardiographic assessment of percent ejection fraction (%EF) as an indice of left ventricular systolic performance in LF- and HF-fed young (n = 6 per group) and aged (n = 4-5 per group) mice **(B)**. Echocardiographic assessment of percent fractional shortening (%FS) as an indice of left ventricular systolic performance, which is calculated by taking into account changes in left ventricular diameter as opposed to volume that is used %EF **(C)**. Isovolumic relaxation time (IVRT) as an indice of diastolic function in the heart **(D)**. Data are expressed as means ± SEM. No significant differnece between age-matched LF- and HF-fed groups as determined by Student's unpaired t-test.

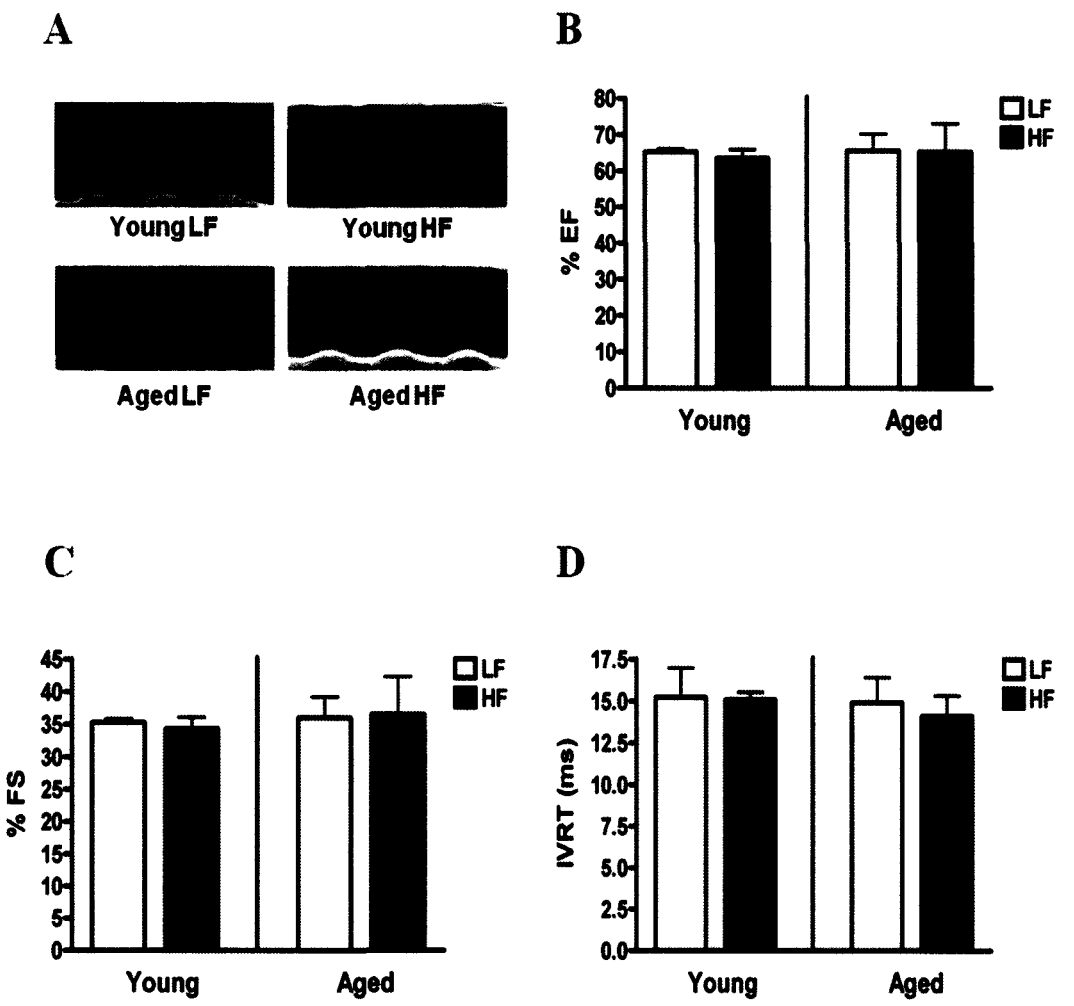
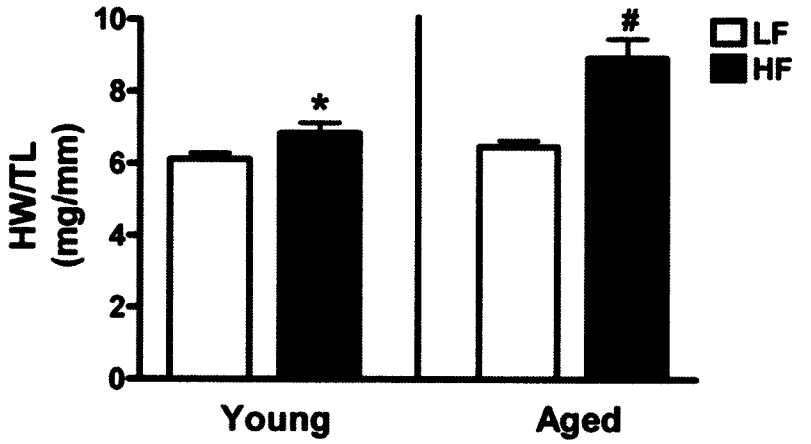

A
Young LF
Young HF
Aged LF
Aged HF
B
% EF
80
70
60
50
40
30
20
10
0
LF
HF
Young
Aged
C
% FS
45
40
35
30
25
20
15
10
5
0
LF
HF
Young
Aged
D
IVRT (ms)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
LF
HF
Young
Aged

Figure 4.3 – Middle-aged mice develop more dramatic cardiac hypertrophy in reponse to a high fat diet than do young mice

Hearts from young and middle-aged mice were extracted following 12 weeks of a LF or HF diet. Absolute heart weights (HW; mg) were normalized to tibia length (TL; mm), n = 6/ group for young mice and n = 5-8/ group for middle-aged mice (**A**). The fold change in HW/TL ratio in response to HF feeding as compared to average HW/TL of age-matched LF fed controls (**B**). *$p < 0.05$ vs. young LF-fed mice, $^{\#}$ $p < 0.01$ vs. aged LF-fed mice (**A**). * $p < 0.05$ vs. young mice in (**B**) as determined by Student's unpaired t-test.

A


10
8
6
4
2
0
HW/TL (mg/mm)
*
#
LF
HF
Young
Aged

B

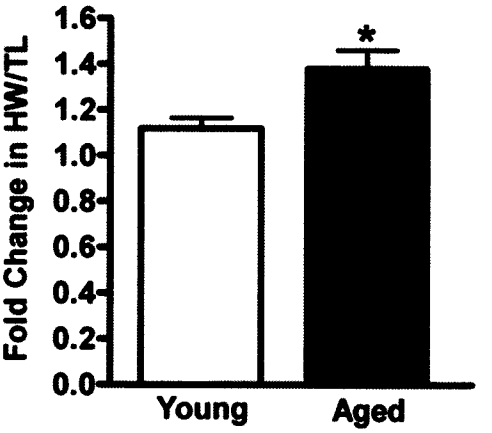

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fold Change in HW/TL
*
Young
Aged

Figure 4-4: Increased expression of CD36 in hearts from middle-aged mice fed a high fat diet, but intramyocardial triglyceride levels remain unchanged

Whole heart homogenates were prepared from young (10-12 week old) and middle-aged (40-44 week old) C57Bl6 mice fed 12 weeks of a LF or HF diet. Homogenates were subjected to SDS-PAGE and immunoblot analysis were performed using anti-CD36 polyclonal and anti-tubulin antibodies. Levels of CD36 in hearts from young mice **(A)** and aged mice **(B)** were quanitifed by densitometry and normalized against tubulin as a control for protein loading. Representative immunblots are shown above each graph, n = 6 per group for young mice and aged mice. Triglyceride levels measured from frozen powdered ventricular tissue in aged-mice fed a LF or HF diet **(C)**. $^{\#}$ $p < 0.01$ vs middle-aged LF-fed mice as determined by Student's unpaired t-test.

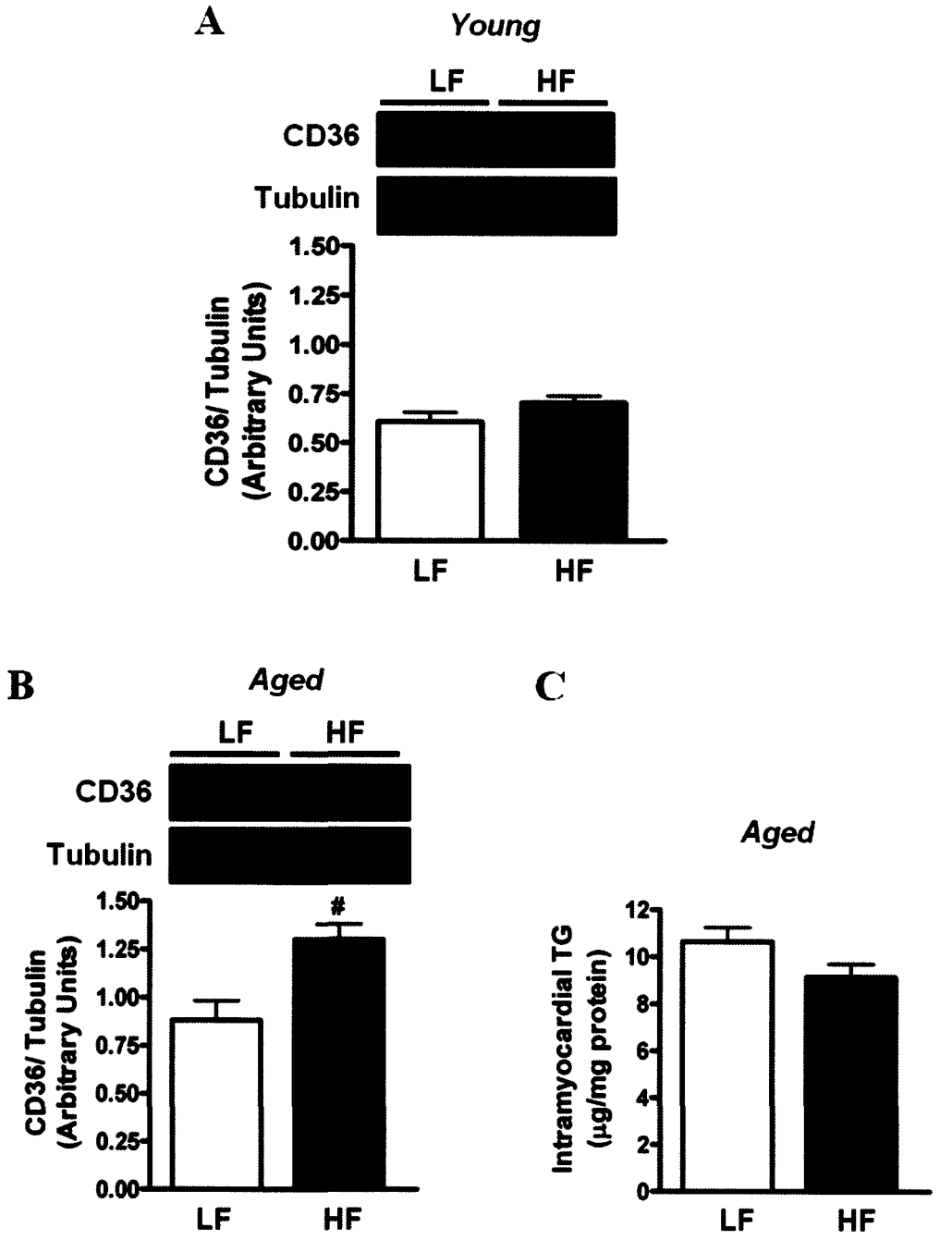
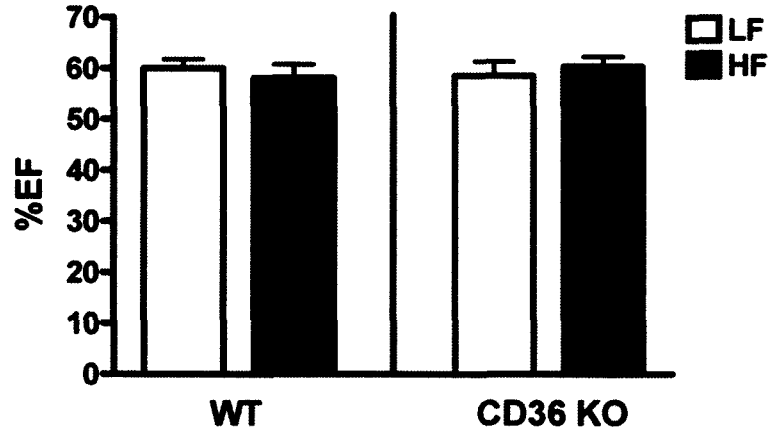

A
Young
LF
HF
CD36
Tubulin
CD36/ Tubulin (Arbitrary Units)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
LF
HF
B
Aged
LF
HF
CD36
Tubulin
#
CD36/ Tubulin (Arbitrary Units)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
LF
HF
C
Aged
Intramyocardial TG (µg/mg protein)
12
10
8
6
4
2
0
LF
HF

Figure 4-5: Systolic and diastolic performance in middle-aged wild-type and CD36 knockout mice

Middle-aged (32-34 weeks old) WT and CD36 knockout (KO) C57Bl mice were fed 12 weeks of a LF (10 kcal% fat) or HF (60 kcal% fat) diet. Echocardiographic assessment was performed on mildly anaestetized mice and percent ejection fraction (%EF) was determined as an indice of systolic ventricular function, n = 8-11 per group (**A**). Isovoloumic relaxation time (IVRT) determined as an indice of diastolic function (**B**). Comparisions were made between LF and HF fed WT or CD36 KO mice by Student's unpaired t-test.

A


70
60
50
40
30
20
10
0
%EF
LF
HF
WT
CD36 KO

B

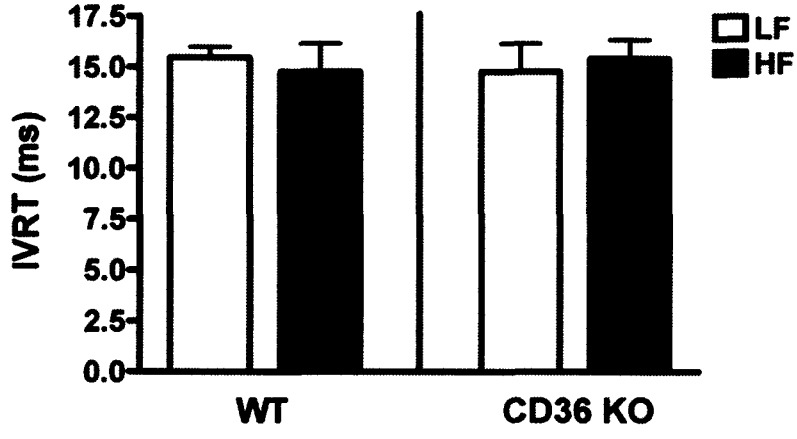

17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
IVRT (ms)
LF
HF
WT
CD36 KO

Figure 4-6: High fat diet-induced cardiac hypertrophy in middle-aged CD36 wild-type mice is prevented in middle-aged CD36 knockout mice

Middle-aged (32-34 weeks old) WT and CD36 knockout (KO) C57BL/6 mice were fed a LF (10 kcal% fat) or HF (60 kcal% fat) diet and echocardiography was performed at the end of 12 weeks of diet. Absolute thickness of the interventricular septal (IVS) wall and left ventricular (LV) posterior wall were measured by M-mode echocardiography (**A** and **C**, respectively). Change in IVS and LV psoterior wall thickness of HF-fed WT and CD36 KO mice as compared to their LF-fed WT and/or CD36 KO counterparts (**B** and **D**, respectively). Mice were fasted overnight prior to heart extraction. Heart weight (HW) to tibia length (TL) ratio in WT and CD36 KO mice (**E**). Fold change in HW/TL ratio in middle-aged WT vs. CD36 KO mice fed a HF diet as compared to their respective age-matched LF fed WT or CD36 KO mice, n = 5-11 per group (**F**). *$p < 0.05$ vs. WT LF or WT HF (**B, D, F**), $^{\#}p < 0.05$ vs. CD36 WT LF as determined by Student's unpaired t-test.

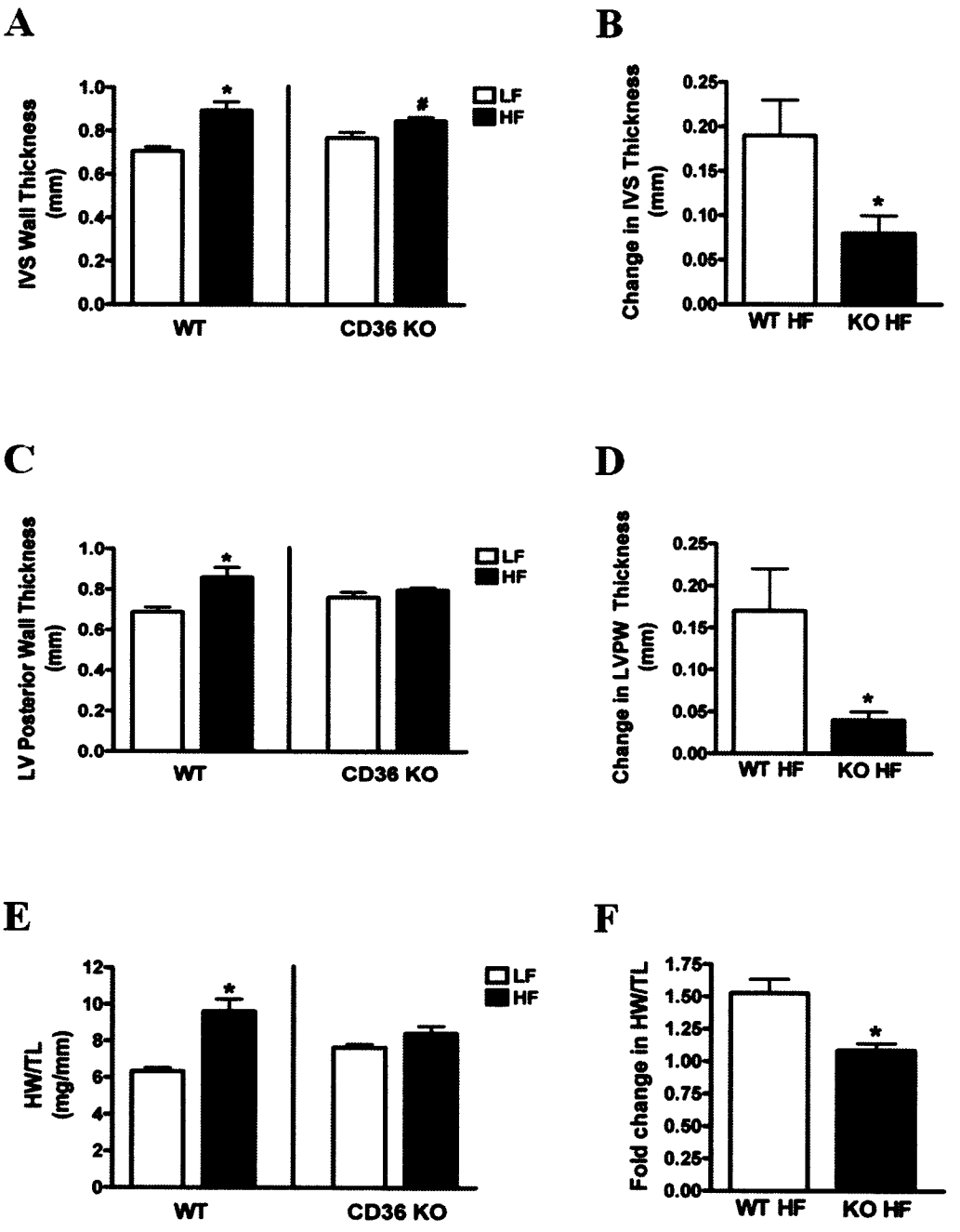

A
IVS Wall Thickness (mm)
1.0
0.8
0.6
0.4
0.2
0.0
*
#
LF
HF
WT
CD36 KO
B
Change in IVS Thickness (mm)
0.25
0.20
0.15
0.10
0.05
0.00
*
WT HF
KO HF
C
LV Posterior Wall Thickness (mm)
1.0
0.8
0.6
0.4
0.2
0.0
*
LF
HF
WT
CD36 KO
D
Change in LVPW Thickness (mm)
0.25
0.20
0.15
0.10
0.05
0.00
*
WT HF
KO HF
E
HW/TL (mg/mm)
12
10
8
6
4
2
0
*
LF
HF
WT
CD36 KO
F
Fold change in HW/TL
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
WT HF
KO HF

Figure 4-7: Triglycerides are similar in hearts from middle-aged wild-type mice fed a low fat or high fat diet, but intramyocardial ceramide levels are increased in high fat-fed wild-type mice

Triglyceride (TG) levels were measured by gas chromatography in frozen powdered ventricular tissue from middle-aged WT and CD36 KO mice following 12 weeks of a LF or HF diet and normalized to mg of protein, n = 5-6 in each group (**A**). Total long chain acyl CoA (LCACoA) levels and major long chain species (C16:0, C18:1 and C18:0) were measured in hearts from WT mice fed a LF or HF diet, n = 5 per group (**B** and **C**, respectively). Myocardial C:18 ceramide levels were determined by high performance liquid chromatography (HPLC), n = 7-8 in each group (**D**). * $p < 0.05$ vs. WT LF-fed mice as determined by Student's unpaired t-test.

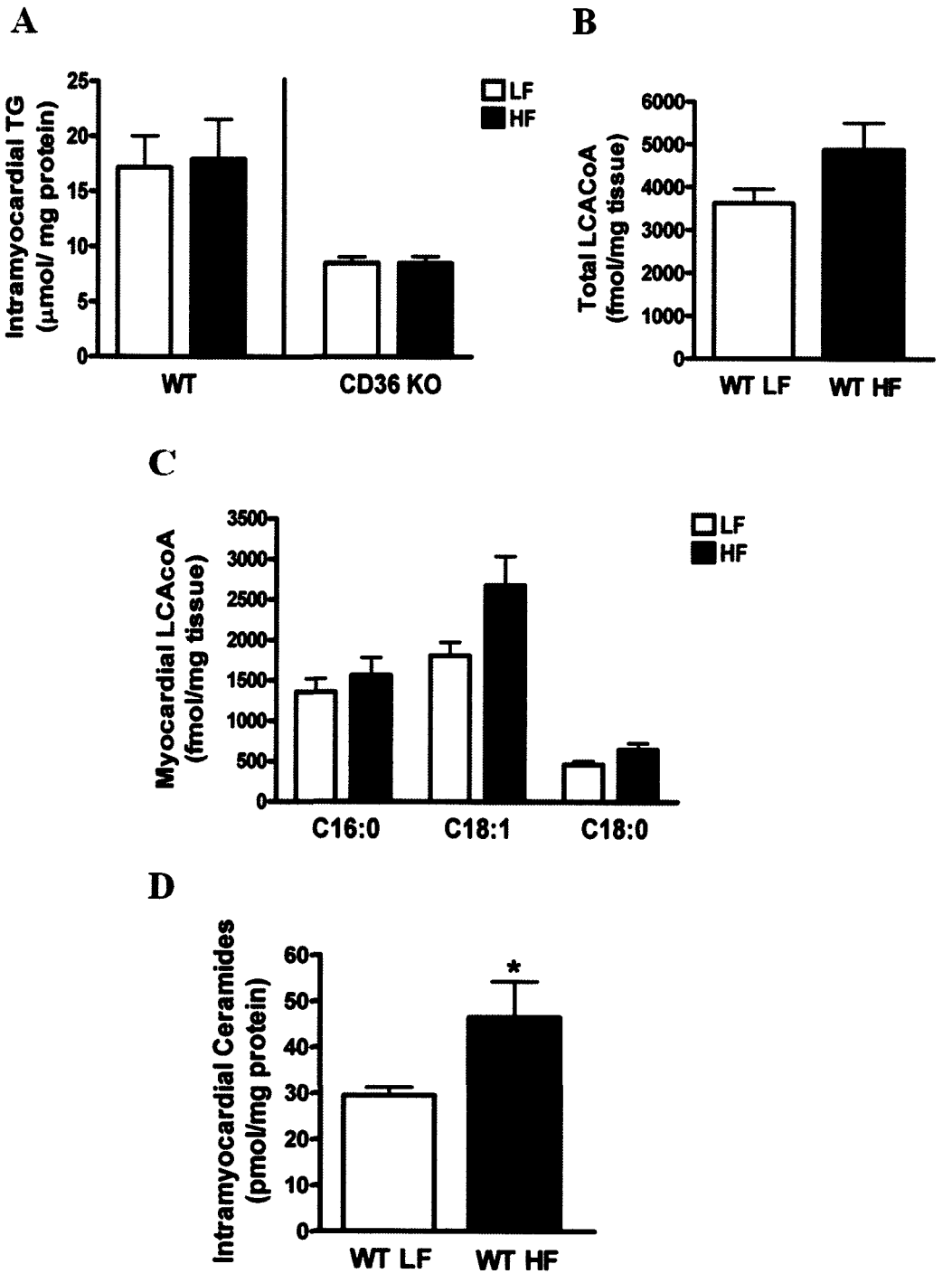

A
Intramyocardial TG (µmol/ mg protein)
25
20
15
10
5
0
LF
HF
WT
CD36 KO
B
Total LCACoA (fmol/mg tissue)
6000
5000
4000
3000
2000
1000
0
WT LF
WT HF
C
Myocardial LCAcoA (fmol/mg tissue)
3500
3000
2500
2000
1500
1000
500
0
LF
HF
C16:0
C18:1
C18:0
D
Intramyocardial Ceramides (pmol/mg protein)
60
50
40
30
20
10
0
*
WT LF
WT HF

Figure 4-8: Levels of intramyocardial ceramides and AMPK activation in hearts from young mice fed a low fat or high fat diet

Myocardial C18-ceramides content from young mice following 12 weeks of LF or HF diet **(A)**. Immunoblot analysis was performed on ventricular homogenates from young mice on diet. Levels of phosphorylated AMPKα at threonine 172 (P-AMPKα, T172) were quantified by densitometry and normalized to total AMPKα levels **(B)**, n = 6 hearts in each group. Differences were determined using Student's unpaired t-test.

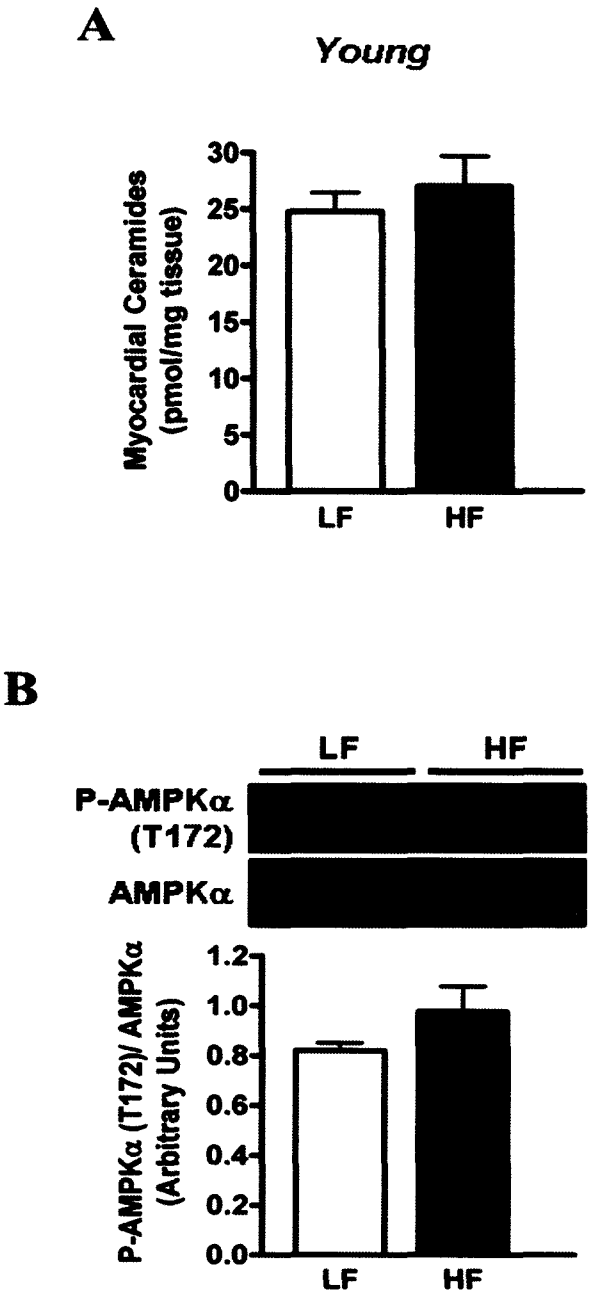
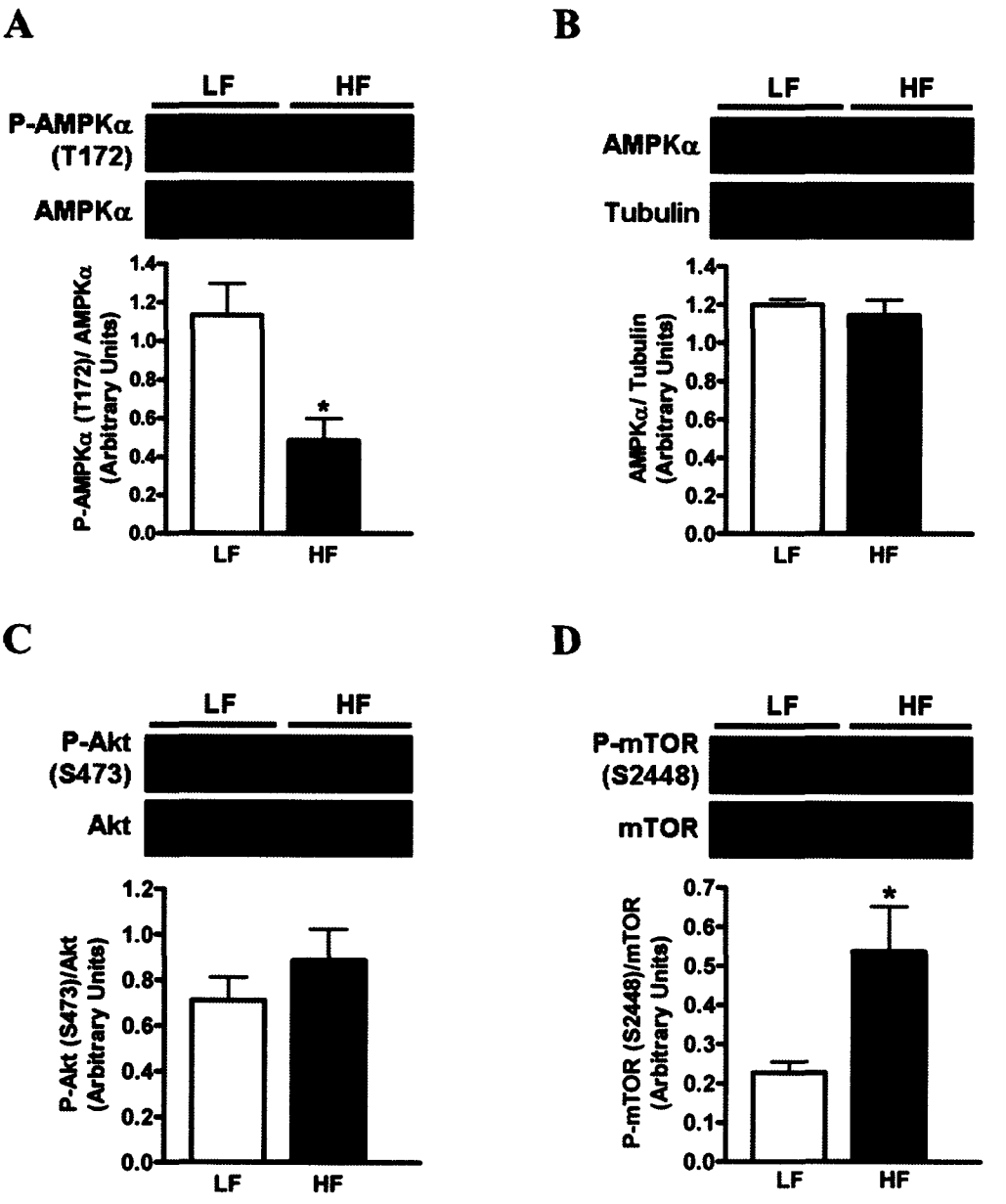
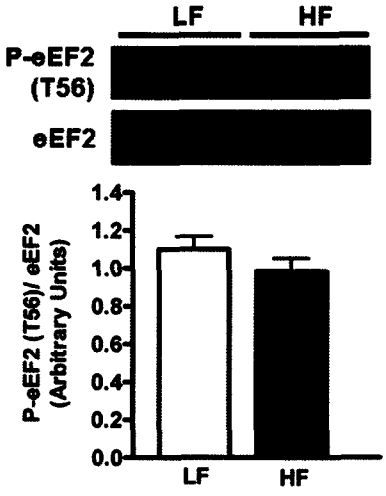

A
Young
Myocardial Ceramides (pmol/mg tissue)
30
25
20
15
10
5
0
LF
HF
B
LF
HF
P-AMPKα (T172)
AMPKα
P-AMPKα (T172)/ AMPKα (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF

Figure 4-9: Phosphorylation of AMPK is reduced in hearts from wild-type mice fed a high fat diet, and this is associated with the activation of pro-hypertrophic pathways involved in protein synthesis

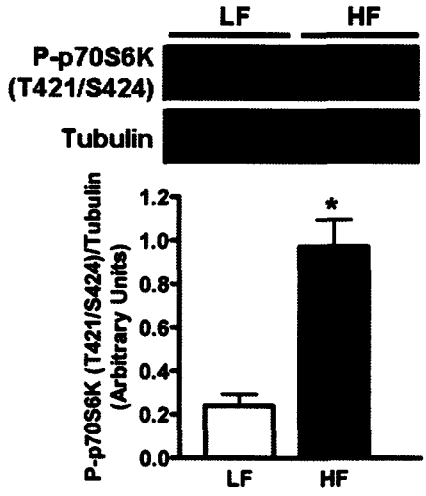
Representative immunoblots and densitometry of ventricular homogenates prepared from middle-aged WT and CD36 KO C57Bl6 mice (32-34 week old) following 12 weeks of a LF or HF diet (n = 5-6 in each group). Levels of phosphorylated AMPKα (P-AMPKα, T172) were quantified and normalized to total AMPKα **(A)**. Total AMPKα levels as normalized to tubulin as a loading control **(B)**. Levels of phosphorylated Akt (P-Akt, S473) were quantified and normalized to total Akt **(C)**. Levels of phosphorylated mTOR (P-mTOR, S2448) were quantified and normalized to total mTOR **(D)**. Levels of phosphorylated eEF2 (T56) were normalized to total levels of eEF2 **(E)**. Phosphorylated levels of p-70S6 kinase (p-70S6K, T421/S424 and T389; **F and G**, respectively) and S6 ribosomal protein (S240/244; **H)** were quantified and normalized to tubulin. * $p < 0.05$ vs. LF as determined by Student's unpaired t-test.


A
LF
HF
P-AMPKα (T172)
AMPKα
P-AMPKα (T172)/ AMPKα (Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
LF
HF
B
LF
HF
AMPKα
Tubulin
AMPKα/ Tubulin (Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF
C
LF
HF
P-Akt (S473)
Akt
P-Akt (S473)/Akt (Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF
D
LF
HF
P-mTOR (S2448)
mTOR
P-mTOR (S2448)/mTOR (Arbitrary Units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
LF
HF

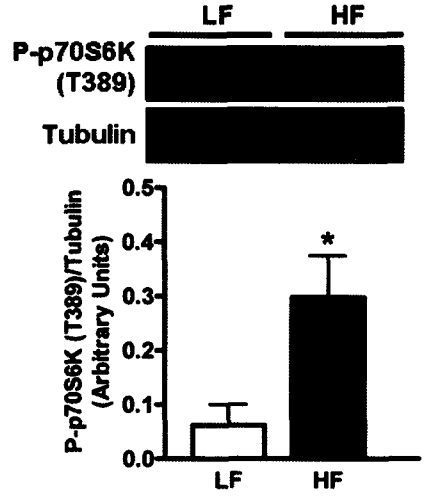
E


LF
HF
P-eEF2
(T56)
eEF2
P-eEF2 (T56)/ eEF2
(Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LF
HF

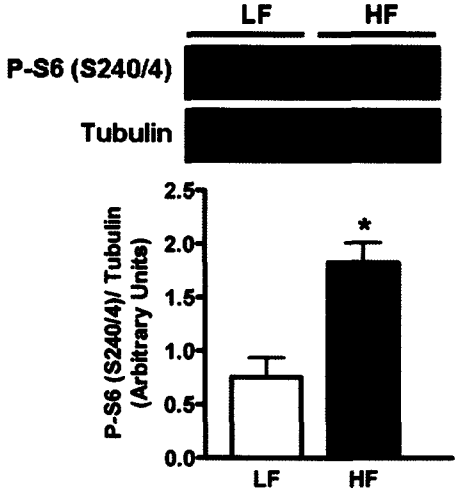
F


LF
HF
P-p70S6K
(T421/S424)
Tubulin
P-p70S6K (T421/S424)/Tubulin
(Arbitrary Units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
LF
HF

G


LF
HF
P-p70S6K
(T389)
Tubulin
P-p70S6K (T389)/Tubulin
(Arbitrary Units)
0.5
0.4
0.3
0.2
0.1
0.0
*
LF
HF

H


LF
HF
P-S6 (S240/4)
Tubulin
P-S6 (S240/4)/ Tubulin
(Arbitrary Units)
2.5
2.0
1.5
1.0
0.5
0.0
*
LF
HF

Discussion

Our previous data have indicated that upregulation of CD36 protein in hearts of middle-aged mice contributes to intramyocardial lipid accumulation, cardiac dysfunction and cardiac hypertrophy [11]. However, this dramatic age-mediated cardiac phenotype could not be reproduced in the absence of higher than normal levels of dietary fatty acids. As such, we speculated that aging and elevated levels of dietary fatty acids interacted to produce the cardiac phenotype that we reported previously [11]. Based on this, our current study examined the link between CD36, lipid accumulation and cardiac hypertrophic growth in a physiological model of obesity within the context of aging. Specifically, the present study investigated whether 12 weeks of HF diet could induce cardiac hypertrophy and/or cardiac dysfunction in middle-aged mice and whether the aged heart would be more susceptible than the young heart to the detrimental effects of a diet high in dietary fat.

As described in chapter 3, middle-aged mice gained more weight on a HF diet as compared to young mice on the same diet (Fig. 4-1A). Despite this difference in weight gain and greater degree of glucose intolerance and insulin resistance in the middle-aged mice fed a HF diet [28], these effects did not alter either systolic (Fig. 4-2B and C) or diastolic (Fig. 4-2D) function in any of the groups. Contrary to our hypothesis, these data provide evidence that the middle-aged heart is not more susceptible to diet-induced cardiac dysfunction compared to the young heart. However, a caveat to this conclusion is that we may not have fed the mice for a long enough period of time and differences may have appeared

between young and middle-aged HF-fed mice with greater than 12 weeks of diet. Although cardiac insulin resistance has been found to develop by 1.5 weeks of a HF diet in young C57BL/6 mice, systolic function was not significantly impaired until 20 weeks of feeding [29]. As aging is associated with inherent metabolic and structural alterations of the heart [30, 31], it remains possible that advanced aging increases the susceptibility of the heart to obesity-related cardiomyopathy and should be further investigated. Interestingly, in the absence of impaired contractile performance, middle-aged mice were more sensitive to developing HF diet-induced cardiac hypertrophy than young mice (Fig. 4-3A and 4-B). Although many changes are occurring in hearts from middle-aged mice fed a HF diet that could contribute to the development of cardiac hypertrophy, we show that this is associated with increased CD36 protein expression (Fig. 4-4B). Moreover, CD36 ablation prevents HF diet-induced cardiac hypertrophy in middle-aged mice (Fig. 4-6) providing further evidence that CD36 is at least partially responsible for HF diet-induced cardiac hypertrophy. In most of the animal studies suggesting a link between CD36 and cardiac hypertrophy, these animals also have impaired cardiac performance [5, 10, 11], therefore making it difficult to establish whether CD36 directly plays a role in mediating hypertrophy and/or cardiac dysfunction. Indeed, from our previous study in aged mice it was unclear whether upregulation of myocardial CD36 preceded the development of age-associated cardiac hypertrophy and it was possible that hypertrophy developed secondary to cardiac lipotoxicity and diminished cardiac function in these mice [11]. Therefore, our

current study allowed us to investigate the early events and effects of obesity on the heart while function is preserved.

The translocation of CD36 from intracellular stores to the plasma membrane is an important site of post-translational regulation for CD36. Although total myocardial CD36 levels were found to be increased in HF-fed middle-aged mice, as we did not measure sarcolemmal-associated levels of CD36 we cannot know for certain if CD36 is localized at the plasma membrane to increase FA transport. Although the precise mechanism leading to increased myocardial CD36 expression in our study is unknown, it may have resulted from high levels of plasma glucose and/or insulin that have been shown to regulate CD36 expression through both transcriptional and/or translational modifications in rodents and humans [32, 33]. The factors that regulate CD36 expression and subcellular localization in the obese heart will require further investigation.

Although we have shown that increased CD36 expression contributes to HF diet-induced cardiac hypertrophy in middle-aged mice, interestingly there was no measureable difference in myocardial TG levels in the HF-fed middle-aged mice compared to the LF-fed middle-aged mice (Fig. 4-7A). While we do not provide evidence to explain this, it is possible that accelerated rates of FA oxidation observed in the early stages of obesity but prior to the onset of overt diabetes may contribute to the prevention of increased TG accumulation [34-36]. Indeed, several studies have shown that cardiac FA oxidation rates are accelerated in the setting of obesity and diabetes [37-41], and a similar scenario likely occurs early on in hearts from middle-aged mice when challenged with HF. However, studies

in obese and diabetic animals have shown that long-term chronic exposure of the heart to FA overload and prolonged over-reliance on FA can lead to myocardial inefficiency, generation of ROS and mitochondrial dysfunction [38, 42-45]. Thus, it has been proposed that through these mechanisms that increased FA utilization can ultimately contribute to contractile dysfunction. This concept would argue against the use of agents to promote FA oxidation in the obese and diabetic heart that while it may initially reduce cardiac steatosis this may actually be counter-productive by impairing cardiac efficiency and potentially accelerating the development of mitochondrial dysfunction. In the context of aging, this issue becomes more complex as evidence suggests that aging is associated with impaired mitochondrial function. Therefore, future studies will be required to ascertain whether FA oxidation is in fact impaired in the aged heart and what impact this has in obesity and diabetes. This suggests though that clinical treatment may need to be more tailored to the patient based on their age. Regardless, these data demonstrate that increased myocardial TG levels *per se* do not contribute the cardiac hypertrophy observed in middle-aged HF-fed mice. This supports the concept that myocardial TG itself may not be harmful to the cell but by virtue of their highly dynamic nature and rapid hydrolysis rates can act as an intracellular source for other potentially toxic lipid intermediates, such as LCACoAs and ceramides [46]. Although myocardial TG levels were unchanged, we do show that there is a trend towards increased LCACoA levels in hearts from middle-aged mice fed a HF diet compared to the same mice fed a LF diet (Fig. 4-7B and C), which are lipid-derived intermediates that may indirectly alter

signaling pathways regulating cardiac hypertrophy. More importantly, we also show that ceramide levels are increased in hearts from middle-aged mice fed a HF diet (Fig. 4-7D) and that these ceramides are likely produced from palmitoyl-CoA by *de novo* synthesis catalyzed by serine palmitoyl transferase (SPT)-1 (Fig. 4-7C), however it is possible that sphingomyelin hydrolysis may also contribute to elevated ceramide levels in these hearts. Among the different lipid species potentially implicated in lipotoxic cardiomyopathy, ceramides are considered one of the more toxic species and studies have shown that the addition of cell-permeable ceramide analogs or sphingomyelinase to promote ceramide accumulation induces apoptosis in various cell types [47]. Indeed, pharmacological inhibition of *de novo* ceramide synthesis with myriocin or the genetic deletion of a subunit of SPT in models of lipotoxic cardiomyopathy prevents cardiac hypertrophy, as well as improves glucose metabolism and systolic function [2]. Furthermore, accumulation of ceramides is also thought to contribute to impaired insulin signaling via the activation of downstream effectors, including serine/threonine protein phosphatases (i.e. PP2A) [48, 49], as well as multiple kinases that can phosphorylate and block tyrosine phosphorylation of IRS to impair insulin signaling [50].

Since ceramides levels were increased in hearts from middle-aged mice fed a HF diet as compared to age-matched mice fed a LF diet, we investigated the activity of AMPK and Akt; two signaling kinases that are known to be regulated by ceramides, as well as contribute to cardiac hypertrophy. Interestingly, despite that ceramides have been shown to impair Akt activity by a) preventing Akt

translocation to the membrane [51, 52] and b) promoting the dephosphorylation of Akt via activation of protein phosphatases [23, 53], we observed that Akt phosphorylation status was not altered in hearts from middle-aged mice fed a HF diet as compared to age-matched LF-fed controls (Fig. 4-9C). However, phosphorylation of AMPK was significantly reduced in the hearts from middle-aged mice fed a HF diet compared to the LF-fed group (Fig. 4-9A). This finding of reduced AMPK activation is consistent with a previous study showing that elevated levels of ceramides inhibit the phosphorylation and activation of AMPK via PP2A activation [21]. Furthermore, the study by Wu *et al.* [21] also found that inhibition of PP2A using siRNA could reverse the HF diet-induced inhibition of AMPK in the aorta. Since PP2C is also known to dephosphorylate AMPK at the threonine 172 residue on the α subunit [54], it may also be activated by ceramides in the heart to inhibit AMPK activity in our model. Alternatively, our lab has recently shown that oxidative stress can inhibit the LKB1-AMPK signaling pathway via 4-hydroxy-2-nonenal (HNE) adduct formation with LKB1 to reduce its activity [20]. Although we have not measured LKB1 activity and/or markers of oxidative stress in hearts from aged mice fed a HF diet, we have shown that short-term adenoviral-mediated increases in CD36 expression in isolated neonatal cardiac myocytes results in marked lipid accumulation, as well as significant superoxide generation, as determined by confocal imaging with fluorescent dyes (Sung M and Dyck J, unpublished data). Moreover, ceramide analogs can directly impair respiration of isolated mitochondria by inhibiting electron transport chain activity, specifically at complex III, and increase the generation of ROS [55, 56],

suggesting that the mitochondria may be a potential lipotoxic target for ceramides. As such, the increased myocardial ceramide levels observed in our study may lead to enhanced oxidative stress and subsequent inhibition of LKB1, and this may also be responsible for the reduction in AMPK observed in our animal model of aging and obesity.

In addition to the physiologic effects of AMPK in control of energy metabolism, AMPK also regulates several key modulators of protein synthesis [57, 58]. Protein synthesis is a key component that contributes to increased myocyte size associated with cardiac hypertrophy, and is controlled in part by the process of peptide chain elongation. As protein synthesis is a highly energy-consuming process [59], it is not surprising that AMPK, which responds to the energy demand of the cell to turn off anabolic pathways, plays an important role in regulating this process. In agreement with our previous findings that AMPK is a major negative regulator of cardiac myocyte cell growth [17-19] and cardiac hypertrophy [17, 20], we show that the activating phosphorylation status of mTOR, p70s6K and s6 are all increased in hearts from middle-aged mice fed a HF diet as compared to the LF fed group (Fig. 4-9). This is especially important given that activation of the mTOR-p70S6K-s6 pathway increases protein synthesis and cardiac hypertrophy [18, 20]. Activated mTOR is able to phosphorylate and activate p70S6K [60] which then phosphorylates the 40S ribosomal protein S6 [61] to activate mRNA translation and ultimately increase protein synthesis. Evidence has shown that AMPK is able to regulate mTOR signaling through the phosphorylation and regulation of tuberous sclerosis

complex (TSC)-2, an upstream regulator of mTOR. Phosphorylation of TSC2 by AMPK promotes the activity of TSC2 to suppress mTOR signaling and p70S6K [62]. Studies from our lab in neonatal cardiac myocytes have shown that pharmacological activation of AMPK with AICAR or resveratrol attenuates phenylephrine and/or Akt-induced cardiac hypertrophy and that this is associated with inhibition of p70S6K [17, 19]. Furthermore, AMPK has been shown to be able to directly phosphorylate mTOR at a novel site (threonine 2446) which correlates with reduced activation of p70S6K [63]. In addition, AMPK is known to be able to phosphorylate (serine 398) and activate eEF2 kinase. Active eEF2 kinase can in turn phosphorylate and inhibit eEF2 at threonine 56 to inhibit protein synthesis, specifically peptide chain elongation [64-66]. Interestingly, phosphorylation status of eEF2 (threonine 56) is unchanged in hearts from middle-aged mice fed a HF diet compared to LF-fed mice (Fig. 4-9E), suggesting that robust activation of the mTOR-p70S6K-S6 pathway is sufficient to promote cardiac hypertrophy in these hearts. In summary, there are multiple mechanisms by which AMPK can negatively regulate protein synthesis. Taken together, our data suggest that decreased AMPK signaling in hearts from aged WT mice fed a HF diet allows for increased activation of pro-hypertrophic pathways that may contribute to the cardiac hypertrophy observed in this group of mice. Indeed, we have observed similar results in the spontaneously hypertensive rat (SHR), a widely-used genetic model of hypertension and cardiac hypertrophy, where cardiac hypertrophy in the SHR is associated with dramatic reductions in AMPK activity with subsequent activation of the mTOR-p70S6K pathway [20].

Moreover, activation of AMPK by administration of resveratrol to the SHR blunted hypertrophic growth of the heart and downregulated activation of the mTOR-p70S6K pathway. As mentioned above, we predict that this reduction in AMPK may occur due to ceramide-induced activation of PP2A or in a similar manner as we have previously reported in the SHR involving oxidative stress-induced modifications of the upstream AMPK kinase, LKB1.

Overall, our findings show that aging increases the sensitivity to developing HF diet-induced cardiac hypertrophy and that increased CD36 expression may play an important role in the early pathogenesis of obesity-related cardiomyopathy. Moreover, we show that inhibition of CD36 via genetic ablation in middle-aged mice blunts the hypertrophic response of the heart to 12 weeks of a HF diet. Although we did not observe impaired cardiac function in middle-aged mice after 12 weeks of HF diet, diet-induced cardiac hypertrophy may ultimately lead to cardiac dysfunction. As the size of the elderly population is rapidly expanding and the prevalence of obesity increases, our findings provide important insight to understanding the differential consequences of obesity on the heart in the aging population as compared to their young counterparts. Indeed, inhibition of myocardial CD36 and subsequent lipid accumulation may be a strategy for the treatment of obesity-related ventricular hypertrophy and also have the potential to reduce the incidence of CVD in the aging population and may slow the progression of age-related cardiac dysfunction.

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CHAPTER 5.

Improved Cardiac Metabolism and Activation of the RISK Pathway Contribute to Improved Post-ischemic Recovery in Calorie Restricted Mice

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My role in this work involved performing all the experiments (except those noted below), as well as analyzing the data and writing the manuscript. Grant Masson performed the *ex vivo* heart perfusions to determine function and metabolism of these hearts. Carrie-Lynn Soltys and Jamie Boisvenue performed some of the immunoblots.

CHAPTER 5.

Improved Cardiac Metabolism and Activation of the RISK Pathway Contribute to Improved Post-ischemic Recovery in Calorie Restricted Mice

Abstract

Recent evidence has suggested that activation of AMP-activated protein kinase (AMPK) induced by short-term caloric restriction (CR) protects against myocardial ischemia-reperfusion (I/R) injury. Because AMPK plays a central role in regulating energy metabolism we investigated whether alterations in cardiac energy metabolism contribute to the cardioprotective effects induced by CR. Hearts from control or short-term CR mice were subjected to *ex vivo* I/R and energy substrate metabolism, as well as post-ischemic functional recovery, was measured. Even in the presence of elevated levels of fatty acids, CR significantly improved recovery of cardiac function following ischemia. While rates of fatty acid oxidation or glycolysis from exogenous glucose were similar between groups, improved functional recovery post-ischemia in CR hearts was associated with high rates of glucose oxidation during reperfusion compared to controls. Consistent with CR improving energy supply, hearts from CR mice had increased ATP levels, as well as lower AMPK activity at the end of reperfusion compared to

controls. Furthermore, in agreement with the emerging concept that CR is a non-conventional form of pre-conditioning, we observed a significant increase in phosphorylation of Akt and Erk1/2 at the end of reperfusion. These data suggest that activation of the reperfusion salvage kinase (RISK) pathway also contributes to the beneficial effects of CR in reducing post-ischemia contractile dysfunction. These findings suggest that short-term CR improves post-ischemic recovery by promoting glucose oxidation, and activating the RISK pathway. As such, pre-operative CR may be a clinically relevant strategy for increasing ischemic tolerance of the heart.

Introduction

Myocardial ischemia/reperfusion (I/R) injury is a common complication of open heart surgery resulting in varying degrees of contractile dysfunction [1, 2]. Several pre- and intra-operative strategies have been employed to protect against I/R injury post-cardiac surgery, including ischemic preconditioning, where brief, repeated non-lethal ischemic episodes confer profound protection against a subsequent prolonged index ischemic period [3]. While conventional forms of preconditioning have been extensively studied, the novel strategy of short-term calorie restriction (CR) has been shown in rodents to lessen myocardial damage and preserve post-ischemic contractile function [4-6]. While the underlying mechanisms responsible for short-term CR improving ischemic tolerance are currently being elucidated, one component still to be investigated is alterations in cardiac energy metabolism.

In both human studies [7] and animal models [8] there is clear evidence that a component of I/R injury is dependent upon the types of substrates metabolized by the heart during and following the ischemic period. Circulating plasma levels of fatty acids (Fas) are significantly increased during ischemia [9], resulting in accelerated rates of FA oxidation during reperfusion which dramatically inhibits glucose oxidation via the Randle cycle [10]. This imbalance between glycolysis and glucose oxidation results in alterations in myocardial ionic homeostasis and impaired post-ischemic function [8, 11, 12]. Therefore, it has been proposed that promoting glucose oxidation following ischemia will improve cardiac function

and may be a beneficial approach to lessening ischemic damage [10, 12-14]. Whether alterations in glucose metabolism contribute to improved function in hearts from short-term CR mice is currently unknown.

Recent reports have indicated that activation of the energy sensing kinase, AMP-activated protein kinase (AMPK) by short-term CR is centrally involved in the improvement in myocardial ischemic tolerance in mice [6]. Since AMPK is a major regulator of cardiac energy metabolism [10], this further supports the likelihood that alterations in cardiac energy metabolism play a key role in improved contractile function post-ischemia. Indeed, Shinmura *et al.* [6] suggest that activation of myocardial AMPK by short-term CR prior to ischemia increases glucose uptake and promotes glycolysis, thus maintaining energy reserves (in the form of high-energy phosphates and glycogen) necessary to sustain an ischemic episode. However, as controversy still exists as to the beneficial or detrimental effects of AMPK activation during ischemia, it is imperative that the impact of CR on myocardial AMPK activity during I/R be fully explored. This is particularly relevant given that AMPK activation may increase glycolysis and FA oxidation [10]. Thus, in the setting of elevated circulating levels of FAs observed in patients (i.e >1 mmol/L), activation of AMPK has the potential to reduce cardiac efficiency and impair functional recovery post-ischemia by uncoupling glycolysis from glucose oxidation [12].

Based on the aforementioned rationale, the purpose of this study was to investigate the role of AMPK and myocardial energy metabolism in meditating the cardioprotective effects of short-term CR. Our studies were also designed to

explore the temporal relationship of kinase signaling, glycogen levels and cardiac energetics prior to, during, and following ischemia in hearts from control and CR mice. We also investigated the involvement of prosurvival kinases of the reperfusion injury salvage kinase (RISK) pathway, namely Akt and Erk1/2, both of which have been strongly implicated as mediators in the cardioprotection afforded by ischemic preconditioning [15]. Together, the data provided herein provide insight into the mechanisms involved in CR-induced cardioprotection. These findings have significant clinical importance given that pharmacological mimetics of short-term CR have been proposed as a cardioprotective strategy to improve ischemic tolerance [6].

Materials and Methods

Animals

This investigation conforms with the guidelines of the Canadian Council on Animal Care, and the University of Alberta Animal Policy and Welfare Committee. C57BL/6 mice (8 weeks of age) were obtained from The Jackson Laboratory and maintained on a 12:12 hr dark-light cycle (0800:2000 dark) with free access to food and water for a 2-week acclimatization period. Mice were randomly assigned into groups and fed *ad libitum* an AIN93M standard chow diet (Research Diets, New Brunswick, New Jersey) for 5 weeks (control) or a CR diet.

Calorie Restriction Feeding Protocol

CR mice received 90% of the average baseline caloric intake of the control mice for three weeks, followed by 40% of the average caloric intake for the final two weeks of the experiment. CR diets (Research Diets) were enriched in vitamins, minerals and salts such that restricted animals were not deficient compared to the control animals. The day prior to *ex vivo* heart perfusions, both control and CR mice were fasted overnight (starting at 1600), and experiments performed the following day (0800).

Isolated Working Heart Perfusions

Overnight fasted control and CR mice were anaesthetized with sodium pentobarbital and mouse hearts were perfused in the working ejecting mode with modified Krebs–Henseleit solution containing 5 mmol/L glucose and 1.2 mmol/L palmitate pre-bound to 3% FA–free albumin in the presence of 50 μU/mL insulin

as previously described in Chapter 2. Rates of glycolysis, glucose oxidation and FA oxidation were measured in the aerobic periods by quantitative collection of the $^{3}H_2O$ and $^{14}CO_2$, respectively, as derived from [5-^{3}H/U-^{14}C]glucose and [9, 10-^{3}H]palmitate as described in detail in Chapter 2.

Analysis of Plasma Parameters

Blood was collected from fed control mice and overnight (20 h) fasted CR mice via the tail vein and plasma free FAs and blood glucose levels were assayed as described in the Methods chapter (Chapter 2).

Measurement of Adenine Nucleotide Content

Adenine nucleotides were extracted from frozen ventricular tissue (20 mg) of fasted mice by homogenization in ice-cold 6% (v/v) perchloric acid solution. The tissue-perchloric acid mixture was neutralized with 0.5 M K_2CO_3 (pH 5-7) and centrifuged, and the resulting supernatant collected and assayed as described in Chapter 2.

Measurement of Glycogen Content

Glycogen was extracted from frozen ventricular tissue (5-10 mg), and hydrolyzed to glucose by reaction with 2N H_2SO_4 and quantified using a glucose kit (Sigma-Aldrich, St. Louis, Missouri). Glycogen was measured as μmol glucosyl units/ gram wet weight of tissue.

Immunoblot Analysis

Frozen heart tissue was homogenized, SDS-PAGE gels run and membranes probed with antibodies as described in Chapter 2. Signals were visualized using chemiluminescence and densitometry performed using Image J software.

Statistical Analysis

Data are expressed as means ± SEM. Comparisons between control and CR groups were performed at respective timepoints using an unpaired Student's two-tailed t-test, or repeated-measures Two-way analysis of variance (ANOVA) where appropriate, with a Bonferroni post-hoc test. A value of $p < 0.05$ is considered significant.

Results

Improved post-ischemic recovery in hearts from calorie restricted mice

Control mice were *ad libitum* fed for 5 weeks with control diet while CR mice were fed for 3 weeks at 90% of their average caloric intake (10% CR) followed by 2 weeks at 60% of the average caloric intake (40% CR) (Fig. 5-1A). As expected, body weight significantly decreased in CR mice over the course of 5 weeks of food restriction as compared to *ad libitum* fed control mice (Table 5-1). To assess whether CR improved post-ischemic functional recovery in the presence of high levels of FAs normally observed during ischemia [9], hearts were subjected to *ex vivo* I/R using a Krebs–Henseleit solution containing 1.2 mmol/L palmitate and 5 mmol/L glucose. These buffer concentrations were also chosen to mimic circulating glucose and free fatty acid (FFA) levels observed *in vivo* in control and CR mice following an overnight fast (glucose: control - 5.5 ± 0.6 vs. CR - 5.9 ± 0.5 mmol/L; FFA: control - 1.04 ± 0.05 vs. CR - 0.94 ± 0.08 mmol/L). During the aerobic period prior to ischemia, cardiac function was similar between control and CR groups (Fig. 5-1B and 5-1D; Table 5-2). However, post-ischemic contractile function was dramatically improved in hearts from CR mice compared to control mice (Fig. 5-1B and 5-1D; Table 5-2). Indeed, HR x PSP and cardiac power were significantly increased in hearts from CR mice, with a > 22% improvement in recovery in both parameters (Fig. 5-1C and 5-1E).

Myocardial metabolism is altered at baseline in hearts from calorie restricted mice

To determine if alterations in energy metabolism contribute to improved functional recovery observed in hearts from CR mice, we measured glucose and FA metabolism in these hearts. During the aerobic period prior to ischemia, there were no differences in rates of palmitate oxidation and glycolysis between groups (Fig. 5-2A and 5-2B, respectively). However, glucose oxidation was increased nearly 2-fold in hearts from CR mice compared to control mice (Fig. 5-2C). Consistent with this, hearts from CR mice had significantly elevated glucose-derived TCA cycle acetyl CoA production (Fig. 5-2D).

Calorie restriction is not associated with pre-ischemic increases in myocardial P-AMPK status, glycogen levels or ATP content

Previous reports have shown that reducing myocardial glycogen levels is associated with the loss of CR-induced cardioprotection, and that AMPK activation is essential for maintenance of glycogen levels [6]. To investigate this potential CR-mediated AMPK/glycogen axis, we examined AMPK phosphorylation at its activating phosphorylation site (Thr 172; P-AMPK) to determine whether AMPK was activated in response to CR. Since there are significant alterations in feeding behaviour associated with CR, we fasted both groups of mice prior to heart extraction so as to account for differential nutritional status between *ad libitum* fed and CR mice (i.e. both groups of mice were without food for the same duration). Interestingly, P-AMPK levels were similar in hearts

from CR mice compared to control mice prior to ischemia (Fig. 5-3A). Previous reports have shown that P-AMPK levels are elevated in the heart during fasting [16], therefore, fasting likely resulted in myocardial P-AMPK levels being similar between groups. In order to confirm the level of activation of AMPK in these hearts, the phosphorylation status of both the α and β isoforms of acetyl CoA carboxylase (ACC), a downstream target of AMPK, were determined. Similar to levels of P-AMPK, the phosphorylation status of ACC was also not different between groups (Fig. 5-3B-D). However, our findings are inconsistent with a previous report showing increased P-AMPK following an almost identical feeding protocol [6], which may not have controlled for changes in cardiac AMPK activity from fasted vs. fed mice. Regardless of the effect of CR on cardiac P-AMPK levels, we observed no differences in baseline myocardial glycogen levels (Fig. 5-3E) or AMP/ATP ratio (Fig. 5-3F) between groups, suggesting altered pre-ischemic glycogen or increased P-AMPK levels do not contribute to improved functional recovery post-ischemia observed in hearts from CR mice.

Calorie restriction is not associated with increased myocardial P-AMPK, increased glycogen levels or improved energetics at the end of ischemia

Although we did not observe changes in P-AMPK or myocardial glycogen at baseline, previous studies have shown that depleted myocardial glycogen stores during ischemia may impair functional recovery during reperfusion [17]. Therefore, to investigate whether CR improves energetic status of hearts during

ischemia, we measured P-AMPK, glycogen levels and ATP levels in hearts collected at the end of 18 min ischemia. Interestingly, P-AMPK and P-ACC were not different at the end of ischemia in hearts from either control or CR groups (Fig. 5-3A-D). Furthermore, myocardial glycogen content (Fig. 5-3E) and AMP/ATP ratio (Fig. 5-3F; Table 5-3) were not different in hearts from control or CR mice. Together, these data suggest that glycogen energy reserve or energetic status were not improved in hearts from CR mice compared to controls prior to reperfusion.

Improved post-ischemic recovery in hearts from calorie restricted mice is associated with improved myocardial energetics during reperfusion

Since we did not observe any significant changes in P-AMPK, glycogen content, or ATP levels between CR and control hearts at the end of ischemia, we investigated whether alterations in myocardial energy metabolism at reperfusion may contribute to the improved functional recovery observed in hearts from CR mice following ischemia. Consistent with the metabolic profile of hearts prior to ischemia, rates of palmitate oxidation and glycolysis (Fig. 5-4A and 5-4B, respectively) were not altered between groups yet glucose oxidation was significantly increased in hearts from CR mice compared to control mice (Fig. 5-4C). When TCA cycle acetyl CoA production was calculated there was a 2-fold increase in total acetyl CoA produced, with a greater proportion coming from glucose oxidation in hearts from CR mice compared to control (Fig. 5-4D). Increased glucose oxidation rates during reperfusion were associated with an almost 5-fold increase in phosphorylation of Akt substrate of 160 kDa (AS160) in

hearts from CR mice (Fig. 5-4E). AS160 is thought to regulate GLUT4 translocation to the plasma membrane and phosphorylation of AS160 is commonly used as a surrogate marker of increased GLUT4-mediated glucose uptake [18-20]. In addition, hearts from CR mice had significantly increased ATP and reduced AMP levels compared to control hearts at the end of reperfusion, resulting in a significant decrease in AMP to ATP ratio (Fig. 5-4F; Table 5-3). Together, these data suggest improved energy supply in hearts from CR mice compared to controls (Fig. 5-4F), and may explain the enhanced functional recovery in these hearts as compared to control hearts (Fig. 5-1B and 5-1D). AMPK is activated by increases in AMP to ATP ratio, an indicator of cellular energy status, in order to restore or maintain ATP levels in times of energetic and/or metabolic stress, so as such we determined phosphorylation of AMPK at threonine 172 [21, 22]. Consistent with elevated ATP levels, P-AMPK was significantly reduced in hearts from CR mice compared to controls (Fig. 5-4G), indicating improved energy supply in these hearts.

Elevated myocardial RISK signaling is associated with improved post-ischemic recovery in hearts from calorie restricted mice

Although improved myocardial energy metabolism may contribute to the beneficial effects of CR during reperfusion following ischemia, multiple mechanisms are likely involved including activation of specific signaling pathways. Since activation of the Akt signal<ling pathway has been proposed to be essential in preconditioning-induced cardioprotection [15, 23-25], and previous reports have shown that brief periods of CR increase phosphorylation of Akt in

skeletal muscle [26-28] and heart [29], we examined P-Akt levels in hearts from control and CR mice. Interestingly, at baseline P-Akt levels were similar between groups (Fig. 5-5A) however, at the end of reperfusion P-Akt was significantly increased in hearts from CR mice compared to control mice (Fig. 5-5B).

In addition to Akt phosphorylation, we also examined an additional member of the RISK pathway namely Erk1/2 [15]. At baseline, phosphorylation of Erk1/2 was similar between hearts from control and CR mice (Fig. 5-5C). Consistent with the time course of activation of the RISK pathway in classical ischemic preconditioning, we observed a dramatic increase in phosphorylation of Erk1/2 in hearts from CR mice at the end of reperfusion (Fig. 5-5D), which has previously been shown to be necessary for preconditioning-induced cardioprotection [15]. Inactivation of GSK3β has been proposed as a key point of convergence for both Akt and Erk 1/2 signaling pathways in mediating cardioprotection [30-32]. As phosphorylation of GSK3β at serine 9 is inversely correlated with its activity and indicative of its inhibition, we measured phosphorylation status by immunoblot in hearts from control and CR mice. Although GSK3β phosphorylation was increased at baseline (Fig. 5-5E), phosphorylation status was not altered at the end of reperfusion (Fig. 5-5F). Evidence has shown that inhibition of GSK3β prior to ischemia, as well as during reperfusion, is cardioprotective and improves recovery of post-ischemic contractile function [31, 33, 34]. However, contrary to this idea a recent study using inactivation-resistant GSK3α/β knock-in mice, demonstrated that the protective effects of ischemic pre- or post-conditioning were preserved GSK3α/β knock-in mice and that pharmacological inhibition of GSK3 failed to

recapitulate conditioning-induced cardioprotection [35]. Although this previous study suggests that inhibition of GSK3 is not required for cardioprotection afforded by either pre- or post-conditioning, several studies have proposed that inhibition of GSK3 signaling is the mechanism responsible for cardioprotection in several other models. Therefore, increased phosphorylation of GSK3β observed in hearts from CR mice at baseline may confer some protection against ischemia by mechanisms that are still to be elucidated. Indeed, future studies are required to confirm the role of activation of members of the RISK pathway in cardioprotection mediated by short-term CR.

Table 5-1: Physical and plasma parameters of control and calorie restricted mice after 5 weeks of diet.

	Control	CR	
Final body weight (g)	28.7±0.5	21.1±0.3	*
Change in body weight (%)	8.7±1.5	-23.2±0.8	*
Blood glucose (mmol/L)	7.19±0.26	6.03±0.17	*
Plasma FFA (mmol/L)	0.51±0.07	0.94±0.08	*

Values are means ± SEM. Body weights of n = 27-28 control and CR mice following 5 weeks of *ad libitum* fed or calorie restricted diet, respectively. Glucose and free fatty acids (FFA) were measured in blood and/or plasma collected from fed control mice and CR mice at the same time of day (1200-1400), at which time CR mice were under fasting conditions. * $p < 0.05$ for CR vs. control by Student's unpaired t-test.

Table 5-2: Pre- and post-ischemia measures of cardiac function in *ex vivo* perfused hearts from control and calorie restricted mice.

	Aerobic		Reperfusion		
	Control	CR	Control	CR	
Heart rate (beats/min)	279±10	300±10	191±14	236±13	*
Peak systolic pressure (mmHg)	67±1	67±1	28±4	49±4	*
Developed pressure (mmHg)	22±1	21±1	6±2	12±2	*
Cardiac output (mL/min)	8.5±0.6	9.0±0.4	1.8±0.5	3.9±0.5	*
Cardiac Work (mL/mmHg/min x10^{-2})	5.7±0.4	6.0±0.3	0.9±0.3	2.4±0.4	*
Coronary flow (mL/min)	2.4±0.1	2.3±0.1	1.0±0.2	2.1±0.2	*
Aortic outflow (mL/min)	6.1±0.6	6.6±0.4	0.8±0.4	1.8±0.4	

Values are means ± SEM of hearts from control (n = 21) and CR (n = 24) mice perfused *ex vivo* following 5 weeks of diet. Data shown represent averages for the 0-to-30-minute (aerobic) and 48-to-88-minute (reperfusion) perfusion period. * $p < 0.05$ for CR vs. control during reperfusion as determined by Student's unpaired t-test.

Table 5-3: Adenine nucleotide content in cardiac tissue from control and calorie restricted mice.

	End Ischemia		Reperfusion	
	Control [3]	CR [5]	Control [13]	CR [17]
ATP (μmol/g wet wt)	1.37 ± 0.27	1.60 ± 0.20	1.00 ± 0.13	1.35 ± 0.11 *
AMP (μmol/g wet wt)	0.75 ± 0.07	0.55 ± 0.07	0.29 ± 0.06	0.16 ± 0.03 *
ADP (μmol/g wet wt)	1.18 ± 0.15	1.12 ± 0.14	0.51 ± 0.04	0.45 ± 0.02
AMP/ATP	0.62 ± 0.18	0.37 ± 0.07	0.37 ± 0.07	0.17 ± 0.04 *

Differences were determined using Student's unpaired t-test. * represents a significant difference ($P<0.05$) between CR group and the corresponding control group at the respective timepoints at end of 18 min ischemia or end of 40 min aerobic reperfusion post-ischemia. Values are means ± SEM of hearts from control and CR mice.

Figure 5-1: Caloric restriction improves recovery of post-ischemic cardiac function of *ex vivo* perfused hearts

Following two weeks of acclimitization to AIN93M rodent diet, C57BL/6 mice (10 weeks) were allocated into control or calorie restriction (CR) groups. CR mice received 90% of the average baseline caloric intake of the control mice for three weeks, followed by 40% of the average caloric intake for the final two weeks of the experiment **(A)**. Isolated perfused working mouse hearts were perfused in the presence of 5 mmol/L glucose, 1.2 mmol/L palmitate and 50 μU/mL insulin. Heart rate x peak systolic pressure (HR x PSP; **B)** and cardiac power **(C)** during I/R, where cardiac power (mWatts) = (cardiac output x peak systolic pressure x 2.22)/ 1000). Average percent recovery of HR x PSP and cardiac power, respectively, during 40 min reperfusion following 18 min ischemia. Values are means ± SEM of $n = 21$ and $n = 24$ control and CR hearts, respectively. *$p < 0.05$ vs. control by two-way repeated measures ANOVA with a Bonferroni post-hoc test in **(B)** and **(D)**. * $p < 0.05$ vs. control as determined by Student's unpaired t-test in **(C)** and **(E)**.

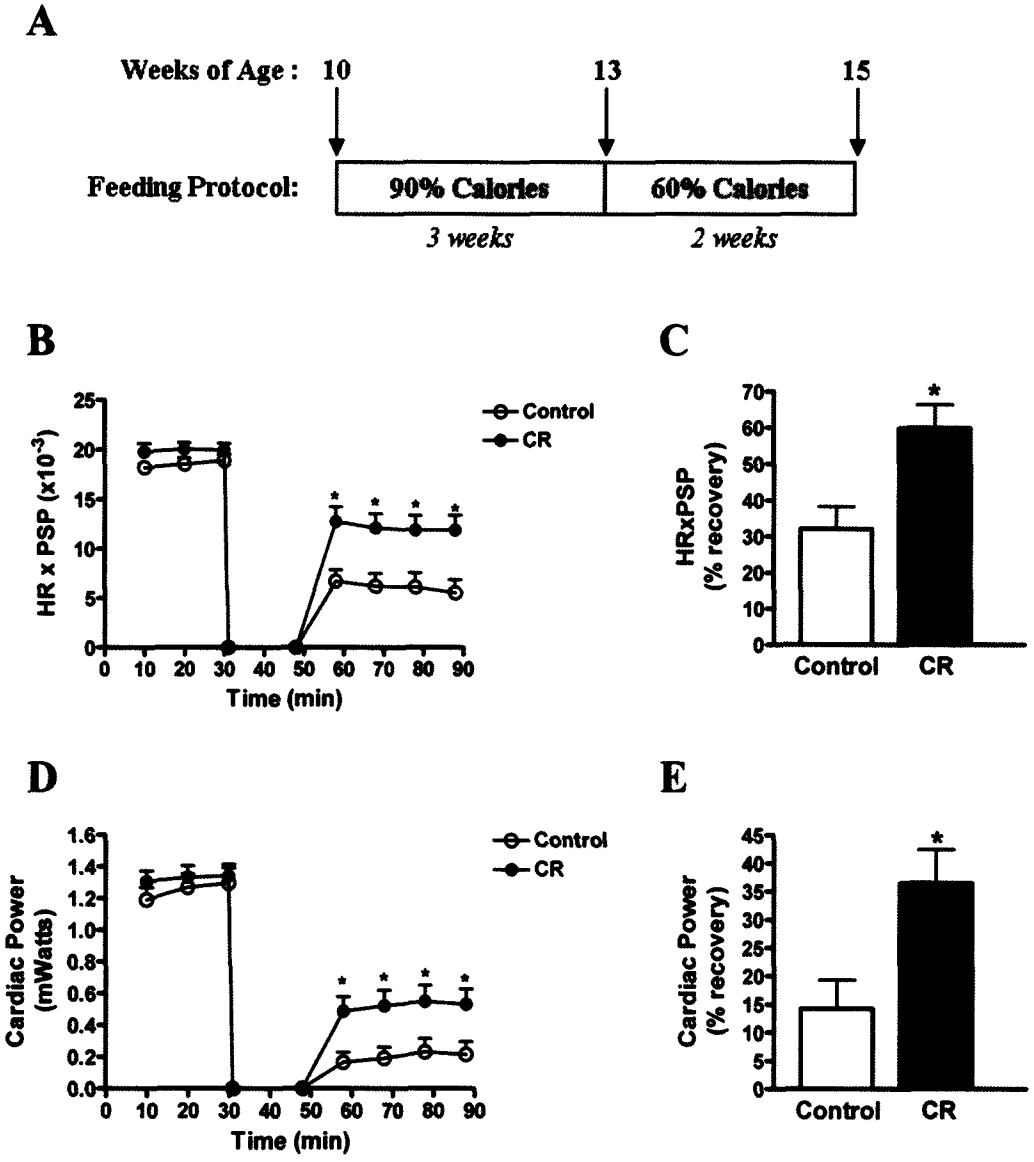

A
Weeks of Age : 10
13
15
Feeding Protocol:
90% Calories
60% Calories
3 weeks
2 weeks
B
HR x PSP (x10-3)
Time (min)
Control
CR
C
HRxPSP (% recovery)
Control
CR
D
Cardiac Power (mWatts)
Time (min)
Control
CR
E
Cardiac Power (% recovery)
Control
CR

Figure 5-2: Cardiac energy substrate metabolism in hearts from control and calorie restricted mice during aerobic perfusion

Average rates of palmitate oxidation (n = 13 per group, **A)**, glycolysis from exogenous glucose (n = 15-17 per group, **B)**, glucose oxidation (n = 9-11 per group, **C)**, and the contribution of glucose and palmitate to acetyl CoA entering the TCA cycle **(D)** during 30 min aerobic perfusion in the presence of 5 mmol/L glucose, 1.2 mmol/L palmitate and 50 μU/mL insulin. * $p < 0.05$ vs. control as determined by Student's unpaired t-test.

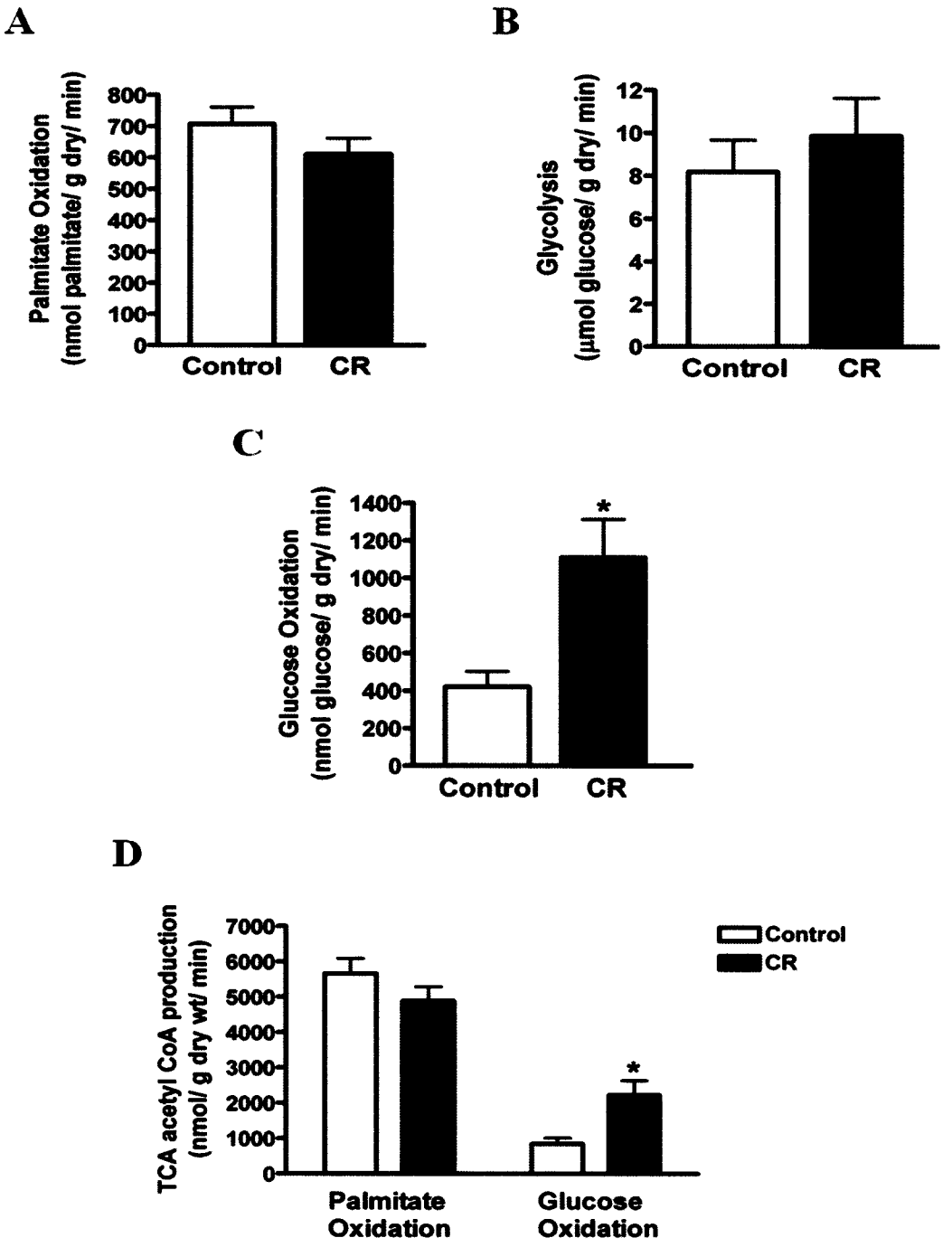
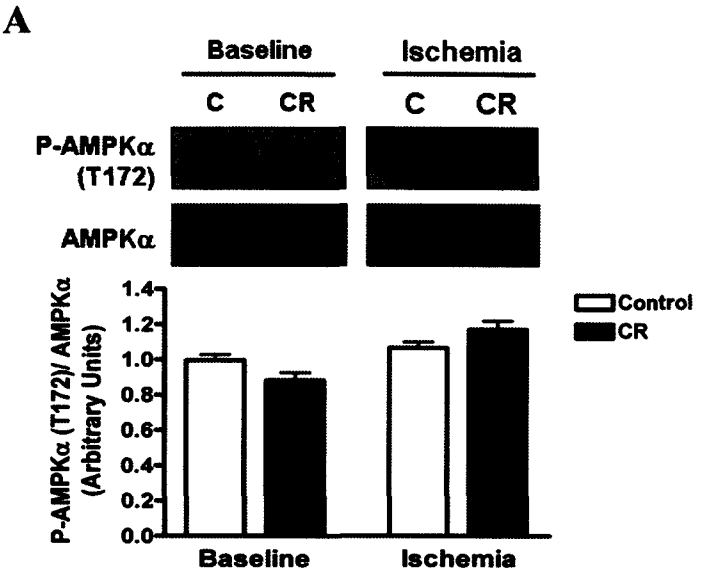
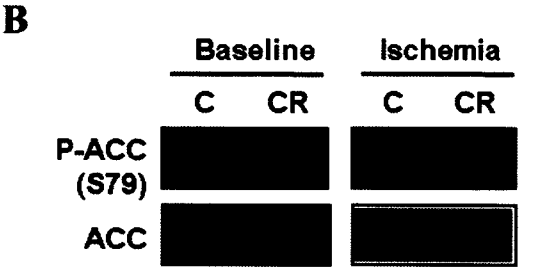
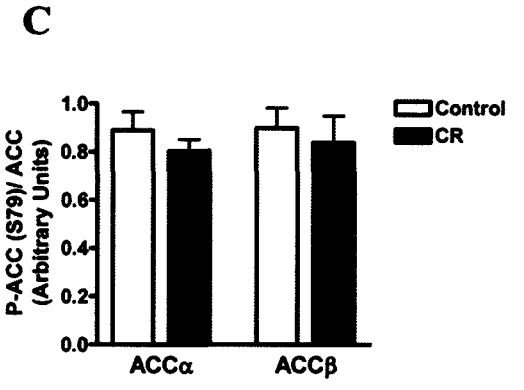

A
Palmitate Oxidation
(nmol palmitate/ g dry/ min)
800
700
600
500
400
300
200
100
0
Control
CR
B
Glycolysis
(µmol glucose/ g dry/ min)
12
10
8
6
4
2
0
Control
CR
C
Glucose Oxidation
(nmol glucose/ g dry/ min)
1400
1200
1000
800
600
400
200
0
*
Control
CR
D
TCA acetyl CoA production
(nmol/ g dry wt/ min)
7000
6000
5000
4000
3000
2000
1000
0
*
Control
CR
Palmitate
Oxidation
Glucose
Oxidation

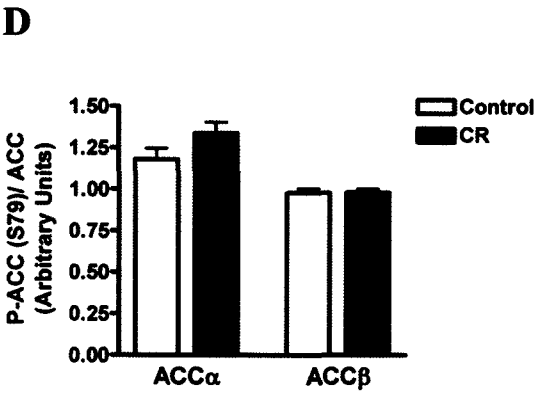
Figure 5-3: Energetic status of hearts from control and calorie restricted mice

Immunoblot analysis was performed on homogenates prepared from hearts isolated from overnight fasted control and CR mice after 5 weeks of diet (baseline) and from *ex vivo* perfused mouse hearts collected at the end of 18 min ischemia. Levels of phosphorylated AMPK α (Thr 172) were quantified by densitometry and normalized against total AMPK α **(A)**. Representative immunoblots **(B)** of phosphorylated ACC (Ser 79) were quantified by densitometry and normalized against respective total ACC α (bottom band, **C)** and β (top band, **D)**. Myocardial glycogen levels were measured from frozen powdered ventricular tissue collected from control and CR mice and expressed as μmol glucose/g wet weight of tissue **(E)**. Myocardial AMP and ATP levels were measured by HPLC and expressed as a ratio of AMP to ATP **(F)**. $n = 5$ hearts per group at baseline and $n = 3 - 5$ hearts per group at end of ischemia. * $p < 0.05$ vs. control at their respective time points as determined by Student's unpaired t-test.

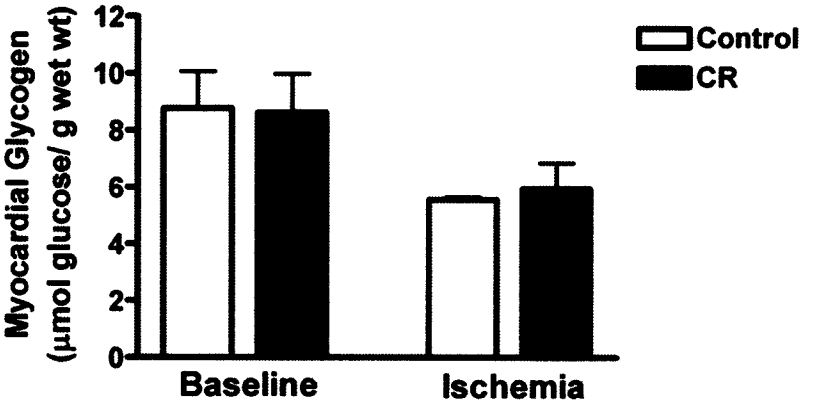

A
Baseline
Ischemia
C
CR
C
CR
P-AMPKα
(T172)
AMPKα
P-AMPKα (T172)/ AMPKα
(Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CR
Baseline
Ischemia


B
Baseline
Ischemia
C
CR
C
CR
P-ACC
(S79)
ACC


C
P-ACC (S79)/ ACC
(Arbitrary Units)
1.0
0.8
0.6
0.4
0.2
0.0
Control
CR
ACCα
ACCβ


D
P-ACC (S79)/ ACC
(Arbitrary Units)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Control
CR
ACCα
ACCβ

E


Myocardial Glycogen
(µmol glucose/ g wet wt)
12
10
8
6
4
2
0
Baseline
Ischemia
Control
CR

F

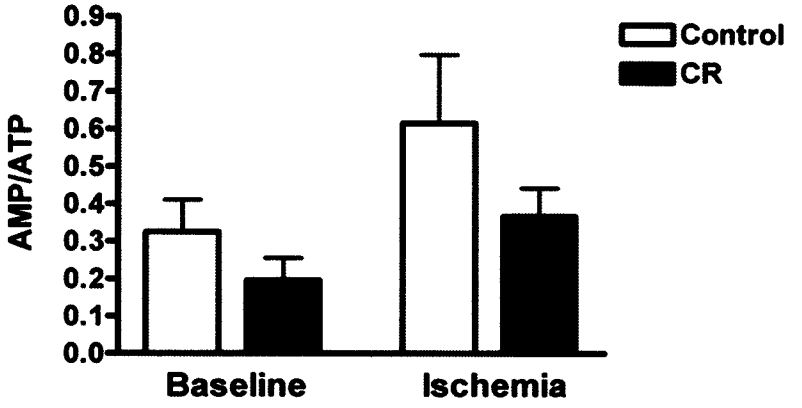
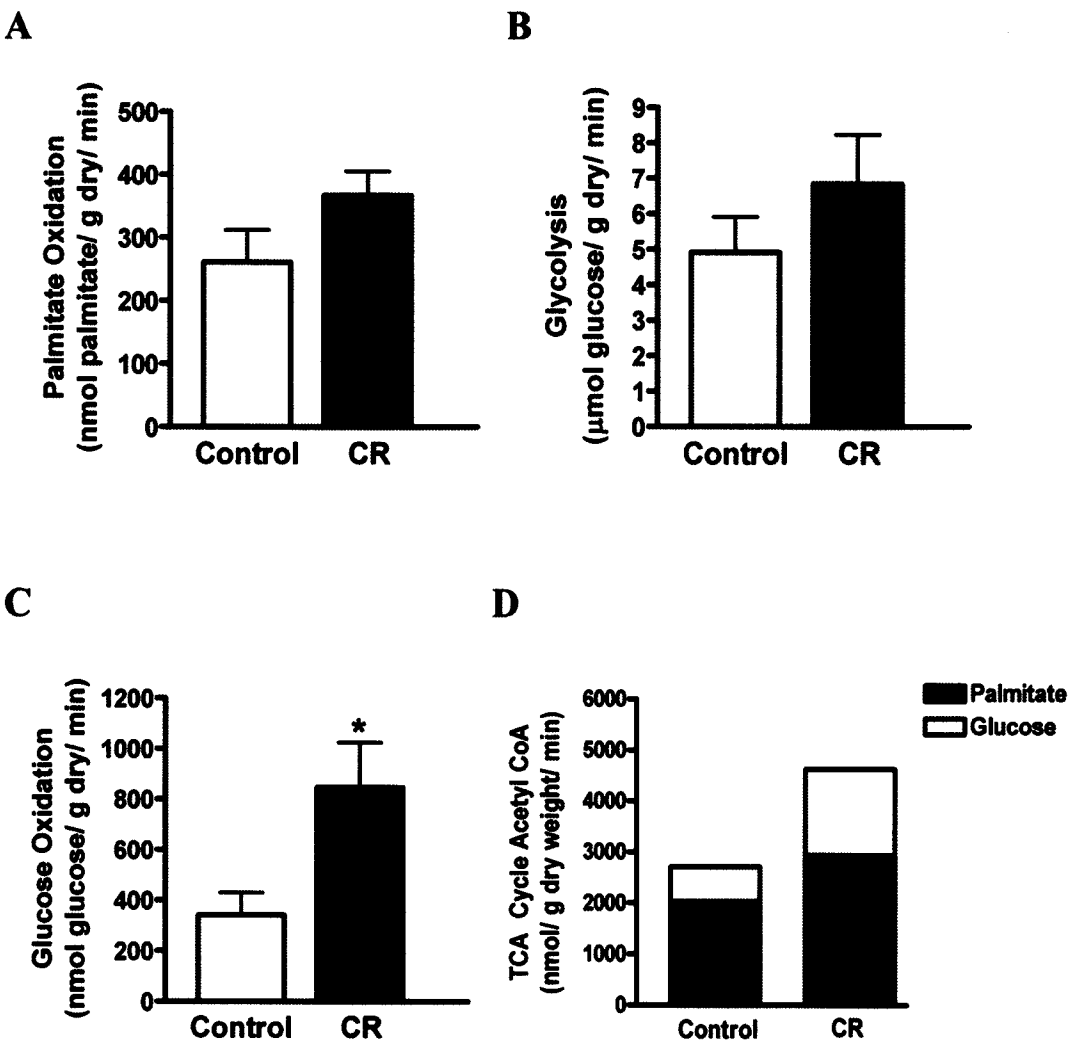

AMP/ATP
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Baseline
Ischemia
Control
CR

Figure 5-4: Cardiac energy metabolism and energetic status of hearts from control and calorie restricted mice during aerobic reperfusion following ischemia

Rates of palmitate oxidation (n = 13 per group, **A)**, glycolysis from exogenous glucose (n = 15-17 per group, **B)** and glucose oxidation (n = 9-11 per group, **C)** in isolated ejecting mouse hearts during reperfusion. Values represent the average rates over 40 min of reperfusion following 18 min of global, no-flow ischemia. Contribution of glucose and palmitate to acetyl CoA entering the TCA cycle in isolated perfused hearts from control and CR mice **(D)**. Levels of phosphorylated AS160 (Thr 462) as determined by immunoblot analysis performed on control and CR hearts (n = 7 per group) at the end of 40 min reperfusion **(E)**. Ratio of AMP:ATP in control and CR hearts at the end of reperfusion, n = 13-18 hearts per group **(F)**. Immunoblot analysis was performed on control and CR hearts (n = 7 per group) at the end of reperfusion with anti-phosphorylated AMPKα (Thr 172) and anti-AMPKα **(G)**. Representative immunoblots are shown above the graph of densitometry. * $p < 0.05$ vs. control as determined by Student's unpaired t-test.


A
Palmitate Oxidation
(nmol palmitate/ g dry/ min)
500
400
300
200
100
0
Control
CR
B
Glycolysis
(µmol glucose/ g dry/ min)
9
8
7
6
5
4
3
2
1
0
Control
CR
C
Glucose Oxidation
(nmol glucose/ g dry/ min)
1200
1000
800
600
400
200
0
*
Control
CR
D
TCA Cycle Acetyl CoA
(nmol/ g dry weight/ min)
6000
5000
4000
3000
2000
1000
0
Palmitate
Glucose
Control
CR

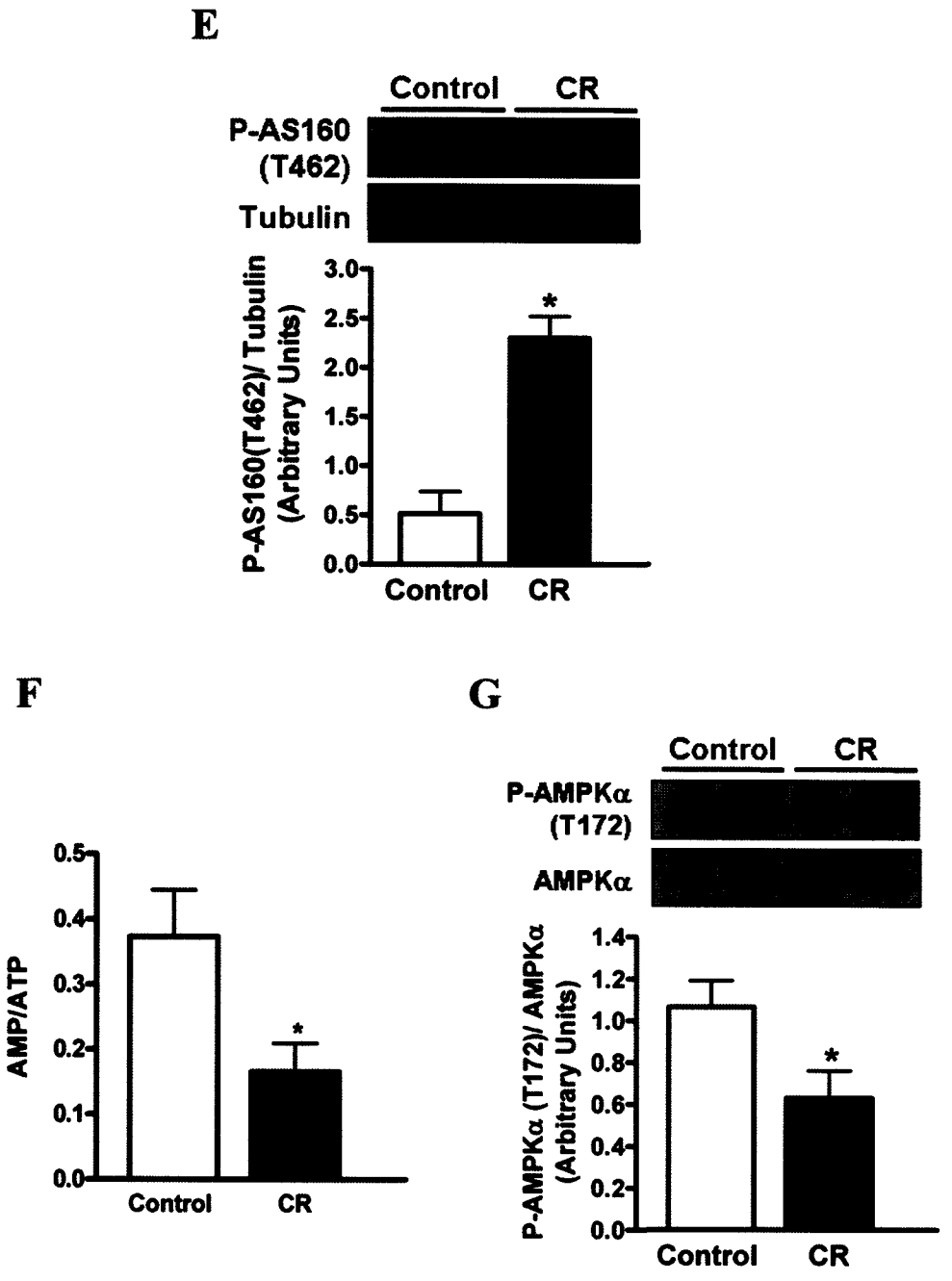
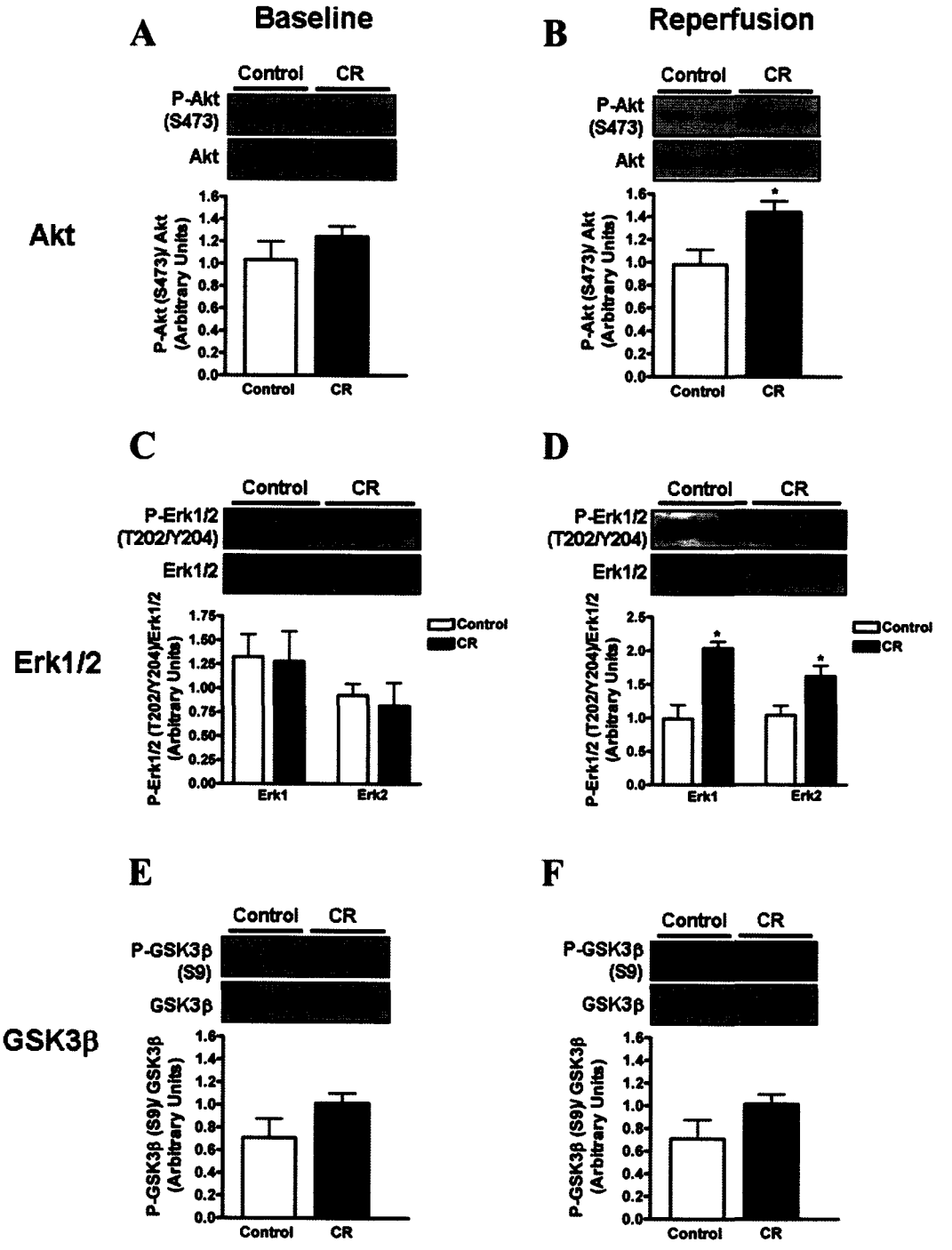

E
Control
CR
P-AS160
(T462)
Tubulin
P-AS160(T462)/ Tubulin
(Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
CR
F
AMP/ATP
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
CR
G
Control
CR
P-AMPKα
(T172)
AMPKα
P-AMPKα (T172)/ AMPKα
(Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
CR

Figure 5-5: Caloric restriction increases phosphorylation of Akt, GSK3β and Erk1/2 in the heart

Immunoblot analysis was performed on heart homogenates from overnight fasted control and CR mice at baseline and at the end of reperfusion following 18 min ischemia **(A,C,E)** Levels of phosphorylated Akt at serine 473 (S473), GSK3β at serine 9 (S9) and Erk1/2 at threonine 202/tyrosine 204 (T202/Y204) were quantified by densitometry and normalized against total protein levels in hearts collected at baseline (n = 5 per group), and **(B,D,F)** at the end of 40 min reperfusion following 18 min ischemia (n = 7 per group). Representative immunoblots are shown above each graph. * $p < 0.05$ vs. control at their respective time points as determined by Student's unpaired t-test.


A
Baseline
B
Reperfusion
Control
CR
P-Akt (S473)
Akt
Akt
P-Akt (S473)/ Akt (Arbitrary Units)
C
D
P-Erk1/2 (T202/Y204)
Erk1/2
Erk1/2
P-Erk1/2 (T202/Y204)/Erk1/2 (Arbitrary Units)
Erk1
Erk2
E
F
P-GSK3β (S9)
GSK3β
GSK3β
P-GSK3β (S9)/ GSK3β (Arbitrary Units)

Discussion

CR has emerged as an effective strategy for extending lifespan and while the precise mechanisms responsible remain to be fully elucidated, the current theory suggests that CR is a mildly stressful condition that provokes a survival response helping the organism to endure a more severe stress [36]. This theory of CR is analogous to the phenomenon of myocardial ischemic preconditioning, where periods of brief sub-lethal ischemia given prior to the prolonged ischemic event confer significant cardioprotection [3]. Consistent with previous studies [5, 6], we show that short-term CR is cardioprotective in isolated perfused mouse hearts subjected to I/R resulting in a >22% improvement in recovery of cardiac contractile function (Fig. 5-1C and 5-1E). Importantly, we demonstrate that this beneficial effect is maintained even in the presence of high concentrations of FAs that would normally be observed in the plasma of patients following ischemia (i.e. >1 mmol/L) [9]. This finding is of particular significance given that FA concentration in the perfusate of isolated perfused hearts can dramatically affect the recovery of hearts following ischemia [10] and it was not known what affect CR would have in this more clinically relevant setting. As such, these findings add strength to the argument that pre-operative short-term CR may be a novel strategy for increasing ischemic tolerance and improving cardiovascular outcomes post-surgery.

Several lines of evidence indicate that the beneficial cardiovascular effects induced by CR are mediated, in part, by increasing adiponectin levels and

subsequent activation of AMPK [6, 37-39]. However, in the setting of myocardial I/R, it remains controversial whether AMPK activation is beneficial or harmful to the heart during reperfusion [10]. Despite this, it has been shown that short-term CR induces adiponectin-mediated AMPK activation which contributes to improved cardiac function post-ischemia [6]. Indeed, that study found that the cardioprotective effects of CR were abrogated in adiponectin antisense transgenic mice, which was associated with blunted CR-induced AMPK activation. Furthermore, that study showed that pharmacological inhibition of AMPK with adenine 9-D arabinofuranoside (AraA) given just prior to perfusion abrogated CR-induced cardioprotection, suggesting that AMPK plays an integral role in mediating the protective effects of CR against I/R injury. However, these previous findings are inconsistent with the data presented herein which suggest that increased AMPK activity may not be fully responsible for reducing I/R injury (Fig. 5-3A). While we cannot fully explain why the findings from the previous study are different from the data presented herein, it may be due to the fact that our mice were fasted for 14-16 hours prior to the I/R protocol. As the control mice have free access to food they consume food throughout the day (12hr-dark/ 12hr-light cycle) as per normal, with greater food intake during the dark phase (active) then in the light phase (inactive). Since CR mice rapidly consume all of their food when fed, they are fasting by the time the I/R protocol is performed. We attempted to account for this difference in feeding behaviour by fasting the control mice so that both groups were without food for a similar duration of time. As myocardial P-AMPK levels have been shown to increase significantly in

fasted rodents [16], we controlled for this potential cofounding variable by fasting both control and CR mice for the same period of time prior to experimentation. Therefore, fasting likely acutely increased myocardial P-AMPK levels, which resulted in hearts from both control and CR mice having similar levels of P-AMPK prior to undergoing our *ex vivo* I/R protocol. Interestingly, although P-AMPK levels were similar between groups at baseline (Fig. 5-3A), post-ischemic functional recovery was still significantly increased in hearts from CR mice compared to control (Fig. 5-1B and 5-1D). While previous work has demonstrated that the cardioprotective effects of short-term CR are mediated by an adiponectin/AMPK signaling axis and that these beneficial effects could be abrogated by inhibiting AMPK [6], our data suggest that AMPK may not be a central mediator of this effect. Moreover, a recent study demonstrated that adiponectin-mediated cardioprotection was retained in dominant-negative AMPKα2 transgenic mice during *in vivo* I/R [27]. Those findings, together with the data presented herein argue against an adiponectin-AMPK signaling axis being the central mediator of CR-induced cardioprotection. However, as AMPK is known to regulate the expression and activity of multiple downstream metabolic targets [21] it is possible that AMPK is activated early on in CR to alter gene expression but its activity is normalized by 5 weeks of feeding or during the reperfusion period. Since we still observed significant cardioprotection in our study, this suggests that acute activation of AMPK prior to perfusion is not obligatory for CR-induced cardioprotection.

While our study focused primarily on investigating the contribution of AMPK activation to short-term CR induced protection against myocardial I/R, we have yet to examine the role of adiponectin in mediating these beneficial effects. Adiponectin is an adipocyte-derived hormone found in high concentrations in plasma (range, 3-30 μg/mL) and circulating adiponectin levels are negatively correlated with increased BMI [40, 41]. The downregulation of adiponectin levels in obesity-related conditions, such as obesity and T2D [42], as well as other CVDs [43-46], suggest that the reduction in plasma adiponectin contributes to disease development. Evidence has implicated adiponectin as a key signaling molecule responsible for mediating the cardioprotective effects of short-term CR on isolated mouse hearts subjected to I/R [6]. As mentioned above, many of the protective effects of adiponectin in the heart have been ascribed to the activation of AMPK. However, in addition to its proposed effects on AMPK, adiponectin has also been shown to confer protection against myocardial I/R through other molecular mechanisms including a) inhibition of inducible nitric oxide synthase (iNOS) and reduction of oxidative/nitrative stress [47], b) activation of cyclooxygenase (COX)-2 to suppress inflammatory pathways [38] and c) activation of endothelial nitric oxide synthase (eNOS) and production of nitric oxide [37, 48]. Indeed, several studies have demonstrated that administration of exogenous adiponectin to be cardioprotective during myocardial I/R [38, 48] and conversely adiponectin deficient mice are more susceptible to I/R injury [38, 47]. Therefore, it is possible that CR may increase levels of adiponectin observed *in vivo* prior to perfusion which may exert protective actions throughout reperfusion

following ischemia through one of the mechanisms described above or through a yet to be elucidated mechanism.

Despite earlier work suggesting an involvement of myocardial glycogen in contributing to improved ischemic recovery in short-term CR mice [6], total myocardial glycogen and ATP levels were comparable between groups both at baseline and end of ischemia (Fig. 5-3E and 5-3F). As such, these data suggest that CR does not enhance post-ischemic recovery by increasing energy stores to better equip the heart to withstand ischemia. However, a limitation of our findings is that baseline glycogen levels are potentially on the low side and may be the combined result of fasting the mice, as well as mild hypoxia/ischemia that is known to occur during heart extraction. Our present findings do not preclude that glycogen turnover may be accelerated in CR hearts. As a result, increases in glucose uptake and glycogen synthesis may be masked by concomitant increases in glycogen degradation which could account for no change in glycogen in the CR heart.

While glycogen accumulation in CR hearts did not appear to contribute to improved functional recovery post-ischemia, our findings do not rule out the possibility that CR hearts have improved cardiac energetics. Indeed, increased cardiac glucose oxidation has been shown to lessen I/R injury during both the ischemic period and subsequent reperfusion of the myocardium [14]. Based on this, we investigated whether alterations in cardiac metabolism may contribute to the cardioprotective effects of CR in our model of reversible I/R injury. Indeed, while FA oxidation and glycolytic rates were similar, we observed a >2-fold

increase in glucose oxidation rates during aerobic perfusion both prior to and following ischemia in hearts from CR mice (Fig. 5-2C and 5-4C). As pharmacological agents that promote glucose oxidation have been shown to be beneficial to post-ischemic recovery [7, 14, 49], our findings suggest that high rates of glucose oxidation in hearts from CR mice likely contribute to improved cardiac energy supply and enhanced post-ischemic cardiac function. Importantly, elevated glucose oxidation was also observed in hearts from CR mice during the aerobic period when cardiac function was similar between groups. As such, this suggests that elevated glucose oxidation is induced by CR and is not simply a consequence of improved function following ischemia. Although the precise mechanism involved in elevated glucose oxidation rates is unknown, it is possible that the activity of pyruvate dehydrogenase (PDH), the rate limiting step in glucose oxidation, is increased or that substrate supply to PDH is increased in hearts from CR mice. Consistent with studies showing that CR increases insulin-stimulated GLUT4 translocation and glucose uptake in skeletal muscle [26, 50], we observed a marked increase in P-AS160 in CR hearts (Fig. 5-4E). Insulin- and/or contraction-stimulated phosphorylation of AS160 is associated with enhanced GLUT4 trafficking to the plasma membrane and glucose uptake [18, 51], therefore it is commonly used a surrogate marker of GLUT4-mediated glucose uptake. The increased P-AS160 in hearts from CR mice suggests that similar to effects observed in skeletal muscle that CR also improves cardiac insulin sensitivity and glucose uptake and may contribute to the increased glucose oxidation rates in these hearts. As it is well-established that insulin is

cardioprotective in experimental models of I/R injury [52-55], it is possible that CR may act as an insulin-sensitizing factor, and the protective effects that we observed may be mediated by insulin. As a subset of hearts in this CR study were not perfused in the absence of insulin, we cannot rule out this possibility. Interestingly most of these insulin studies were performed in hearts perfused in the absence of FAs and recent work has showed that the cardioprotective effect of insulin in the isolated ejecting mouse heart is highly dependent upon the concentration of FAs in the perfusate [54]. In fact the cardioprotective effect of insulin is lost in the presence of 1.2 mM palmitate; therefore potentially CR may restore the cardioprotective actions of insulin in the presence of high concentrations of FAs.

Interestingly, despite that glucose oxidation rates were elevated, there were no changes in palmitate oxidation rates (Fig. 5-2A and 5-4A), as might be predicted by the Randle cycle. While it is unclear why palmitate oxidation rates are unchanged in hearts from CR mice, it is possible since fasting increases levels of lipoprotein lipase that TG-derived FA delivery to the heart is increased [16] and as such, FA oxidation rates may be increased in both groups under these conditions. Another possible explanation involves AMPK, as mentioned above fasting activates AMPK [16] which can regulate FA oxidation by phosphorylation and inhibition of ACC. ACC produces malonyl CoA, which is a potent inhibitor of CPT-1 mediated FA uptake into the mitochondria [56-58]. Therefore, as a consequence of AMPK activation, levels of malonyl CoA are reduced and this relieves inhibition on CPT-1 allowing for accelerated entry of FAs into the

mitochondria and enhanced β-oxidation [56, 57, 59]. Indeed, studies have shown that fasting increases rates of FA oxidation in the rat heart [60, 61].

Previous studies have shown that short-term CR improves insulin-stimulated glucose uptake in skeletal muscle, which is mediated in part by activation of the PI3K-Akt pathway [27, 50, 62]. Interestingly, Akt phosphorylation was significantly increased in hearts from CR mice at the end of reperfusion (Fig. 5-5B), which may indirectly act to accelerate the recovery of glucose oxidation rates during reperfusion and improve myocardial efficiency. In addition to modulating cardiac energy metabolism, Akt is a key mediator of cardioprotection as conferred by ischemic preconditioning and together with Erk1/2 make up the RISK pathway [15]. As hypothesized, in addition to Akt activation we also observed a significant increase in Erk1/2 phosphorylation at the end of reperfusion (Fig. 5-5D). Together, this suggests that activation of pro-survival kinase cascades may play a role in the cardioprotective effects of short-term CR. Activation of these kinases during reperfusion following ischemia has been proposed to protect the heart by preventing cell death and limiting infarct size in more severe models of I/R and pharmacological inhibition of these kinases completely abrogates the protective effects of ischemic preconditioning [15, 25]. Although our model of I/R does not involve a long enough period of reperfusion to induce significant apoptosis or necrosis, there may still be beneficial effects associated with activating the RISK pathway that contributes to improved recovery post-ischemia in our model. As such, the data presented herein suggest that short-term CR can precondition the heart against more severe ischemic insults

by activating the RISK pathway. To elucidate the role of elevated activation of the Akt pathway in the cardioprotective effects of calorie restriction, future studies should be performed in the presence of PI3K inhibitors, such as wortmannin, or in PI3Kα dominant-negative [63]and PI3Kγ KO mice [64].

Our data from *ex vivo* isolated heart perfusions suggest that alterations in glucose metabolism may contribute to the beneficial effects of CR to improve ischemic tolerance of the heart. Although there are obvious limitations in our *ex vivo* heart perfusion protocol, the improved recovery of post-ischemic function we observed in this study clearly necessitates further *in vivo* studies of CR as a potential novel therapeutic approach and/or the use of CR-mimetics to reduce damage caused by myocardial ischemia and subsequent reperfusion. Hearts in this study were perfused with a crystalloid buffer, which has a low oxygen carrying capacity and requires high coronary flow rates in order to deliver adequate amounts of oxygen to the heart as compared to using oxygenated whole-blood or red-blood cell enriched perfusate. However, this is overcome by gassing the perfusate with carbogen (95% oxygen and 5% carbon dioxide) that results in a high oxygen partial pressure in the perfusate and allows for sufficient delivery of oxygen to the heart, as well as helps maintain perfusate pH [65]. Indeed, studies have shown that gassing the perfusate with 70% oxygen is adequate to maintain basal cardiac function and when challenged with inotropic stimuli hearts are able to maintain increased workload for sustained periods of time without inducing injury to the heart [65]. A second limitation of this model is the substrate composition of the crystalloid perfusate. The bicarbonate buffer utilized is

formulated to attempt to match the primary ionic content and pH (7.4 at 37 °C) found in blood/plasma, and contains insulin, as well as both glucose and FAs as energy substrates. However, the perfusate does not fully reproduce physiological conditions with respect to other substrates including lactate, ketone bodies, pyruvate, lipoproteins and other FA species, as well as hormones, neutrophils, platelets and adipokines. In particular, CR is known to increase circulating levels of adiponectin [6], but adiponectin was absent in our perfusion buffer and this may have influenced our results. Previous studies have shown that administration of exogenous adiponectin during I/R limits infarct size and improves post-ischemic functional recovery in both isolated perfused hearts [48] and *in vivo* models of myocardial infarction [38, 47]. Therefore, we may have underestimated the protective effect of CR under our perfusion conditions, and it is possible that high circulating levels of adiponectin found *in vivo* may also exert a direct beneficial effect against I/R. Although the monounsaturated FA oleate is the most abundant fatty acid in blood [66], perfusions in this study were performed with only palmitate as a source of fat. Although it has been shown that FA oxidation and glucose oxidation rates are comparable in the isolated working mouse heart perfused with either palmitate or oleate [67], the absence of oleate in the buffer may have influenced rates of FA and glucose oxidation. In addition, the absence of alternative substrates, such as ketone bodies, means that we may be overestimating the contribution of FAs [68]. Furthermore, we measured rates of glycolysis and glucose oxidation of exogenous labelled glucose, however we did not measure the rate of conversion of glucose to glycogen and rates of glycogen

synthesis, or endogenous substrate utilization and thus we may be underestimating total glucose metabolism.

In summary, our data suggest that alterations in cardiac energy metabolism contribute to the improved post-ischemic recovery of mechanical function of hearts from CR mice. We demonstrate that short-term CR promotes the recovery of glucose oxidation and that this may improve cardiac ATP production during reperfusion in the presence of clinically relevant concentrations of FAs. Furthermore, our study indicates that the cardioprotective effects of short-term CR in I/R injury are mediated by an AMPK-independent mechanism and may involve the post-ischemic activation of Akt and Erk1/2. These findings suggest that CR can precondition the heart to withstand more severe ischemic insults by activating the RISK pathway. Together, the present study provides insight into the mechanisms involved in short-term CR-induced cardioprotection and highlights the potential of this intervention for increasing myocardial ischemic tolerance.

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CHAPTER 6.

Discussion and Conclusions

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Cardiovascular disease (CVD) accounts for the deaths of over 69,000 Canadians every year and is the single leading cause of death in Canada [1]. Therefore it is imperative to understand the underlying mechanisms responsible for the development and progression of CVD with the goal of improving treatment strategies to reduce morbidity and mortality associated with these diseases. CVD is often co-associated with a myriad of other chronic diseases, most notably is type 2 diabetes (T2D). Indeed, the chronic metabolic changes that occur in T2D greatly increase the risk of developing CVD by 2-4 fold, as well patients with CVD who also have T2D sustain a worse prognosis for survival. It is becoming increasingly evident that perturbations in energy metabolism are an integral component to the etiology of a number of cardiovascular conditions, including cardiac hypertrophy, diabetic cardiomyopathy, ischemia-reperfusion (I/R) injury, ischemic heart disease, myocardial infarction, as well as chronic metabolic disorders such as insulin resistance and T2D [2-8]. Several of these disease states are characterized by increased fatty acid availability and excessive fatty acid uptake into cells [9], this is most evident in conditions such as obesity and T2D. When the uptake of fatty acids into the cell is far in excess of the capacity for its oxidation, this can result in the excessive intracellular accumulation of detrimental fatty acid metabolites, which has been strongly linked to impaired insulin signaling in the heart and

skeletal muscle [5, 10-13]. This phenomenon is collectively known as 'lipotoxicity' and is especially damaging to the cardiomyocyte, potentially leading to myocardial contractile dysfunction and cardiomyocyte apoptosis [14-17]. Since aging is a significant risk factor for the development of insulin resistance and CVD, we sought to determine the physiological changes that occur during the onset of middle-age and the influence age may have on the development and pathogenesis of obesity-related insulin resistance and cardiomyopathy.

In general, the aims of this thesis were to examine how alterations in energy metabolism contribute to the pathogenesis of skeletal muscle insulin resistance and diabetic cardiomyopathy, as well as how optimization of energy metabolism may have the potential to treat and/or prevent the onset these diseases. This thesis has investigated several aspects of skeletal muscle and cardiac energy metabolism under the conditions of calorie excess and calorie restriction, particularly in the context of aging, with an emphasis on: 1) determining the changes in whole-body and skeletal muscle metabolism that occur in the process of aging and how these age-related alterations may be a predisposing risk factor for developing high fat (HF) diet-induced insulin resistance; 2) identifying the mechanisms by which the aged heart may be more susceptible to the development of obesity-related cardiomyopathy and; 3) investigating how the novel dietary strategy of calorie restriction (CR) may confer protection against myocardial I/R injury by optimizing cardiac energy metabolism.

A growing body of evidence has shown that skeletal muscle mitochondrial oxidative capacity and mitochondrial function decline with advancing age, and this

has been proposed to be a key contributor to the development of insulin resistance. It is generally believed that this mitochondrial dysfunction can give rise to excessive accumulation of lipid intermediates, such as TG, DAG, LCACoA and ceramides, that can interfere with insulin signaling and result in impaired glucose tolerance and insulin resistance in the elderly population [11, 18]. A similar scenario is thought to also be responsible for the development of insulin resistance in the setting of obesity [12, 18-21]. In this thesis, we show that whole-body metabolic rate is markedly reduced and intramuscular TG levels are elevated in healthy middle-aged mice as compared to young mice. Furthermore, middle-aged mice become more obese and insulin resistant when challenged with a HF diet than young mice. Therefore, an overall reduction in substrate metabolism/utilization in healthy middle-aged mice may be an early event in the process of aging, which could progress into mitochondrial dysfunction and predispose middle-aged mice to diet-induced obesity and insulin resistance. We further show that excessive fatty acid uptake into skeletal muscle is a significant contributing factor to the onset of insulin resistance in middle-aged mice fed a HF diet. Indeed, limiting fatty acid uptake in mice by genetic deletion of the fatty acid transport protein, CD36, attenuated intramuscular lipid accumulation and effectively protected against the development of diet-induced obesity and insulin resistance.

Similarly, the middle-aged heart is also more susceptible to developing obesity-induced cardiac hypertrophy, which is associated with the upregulation of myocardial CD36 expression and lipid accumulation. Our data support a potential link between increased CD36 levels and cardiac hypertrophy. Interestingly,

although myocardial TG were unchanged, ceramide levels were dramatically elevated in these hearts, thus implicating ceramides as one of the major lipid intermediates involved in mediating obesity-related cardiomyopathy. Taken together, our data show that the aged skeletal muscle and heart are more susceptibility to the effects of obesity and that increased CD36-mediated fatty acid uptake are important contributing factors to the development of insulin resistance and cardiomyopathy.

Moving away from the condition of excess caloric intake and obesity we next investigated the dietary strategy of CR. Interestingly, moderate CR has been shown to have significant beneficial effects to slow and/or reverse the progression of several metabolic and CVDs. In the latter part of this thesis, we show that 5 weeks of moderate CR resulted in improved recovery of contractile function following ischemia. In contrast to the perturbations in fatty acid handling and the concomitant impairment in whole-body glucose utilization in the setting of obesity and insulin resistance, we show that CR induces marked stimulation of myocardial glucose oxidation rates and that this cardioprotective effect appears to be independent of acute AMPK activation in these hearts.

Overall, the findings in this thesis have clearly demonstrated that limiting excessive fatty acid uptake and reducing the accumulation of lipid metabolites can protect against the effects of obesity on skeletal and cardiac muscle in middle-aged mice. Indeed, we have shown that alterations in fatty acid handling are major contributors to disease etiology, which strongly suggest that strategies aimed at optimizing energy metabolism may have significant potential as treatments for the

aforementioned diseases. Next we moved beyond the study of obesity and excess caloric intake towards the other end of the dietary spectrum, namely CR. We clearly demonstrate that the novel dietary strategy of CR exhibits profound cardioprotective effects against I/R injury and that this may be mediated in part by promoting glucose metabolism. The remainder of this chapter will discuss in more detail the implications of the findings in this thesis, as well as justification of methodology, limitations of the current studies and lastly future directions.

General Discussion

Age-related alterations in whole-body and skeletal muscle energy metabolism

Advanced age is considered a significant risk factor for the development of T2D [22]. Given the obesity epidemic and the growing size of the aging population, the prevalence of T2D in the western world is expected to rise dramatically over the next 10-20 years [23, 24]. Based on this rationale, we investigated the metabolic and molecular signaling changes that occur during the normal process of aging in C57Bl mice, with a particular emphasis on skeletal muscle as it is one of the predominant tissues involved in whole-body glucose disposal. Secondly, we determined if middle-aged mice were more susceptible to the effects of obesity by feeding young and middle-aged mice a HF diet for a period of 12 weeks.

In comparison to young mice (12-14 weeks), middle-aged mice (52-58 weeks) had a significant decline in resting metabolic rate, suggesting that overall energy production from fat and carbohydrates is reduced in healthy middle-aged mice. Our data is consistent with previous reports showing that muscle mitochondrial oxidative capacity and mitochondrial ATP synthesis rates decline with advanced age and may be a normal consequence of aging in humans [11, 25, 26]. Interestingly, the reduced metabolic rate and energy expenditure observed in middle-aged mice did not appear to be the result of altered mitochondrial content and/or maximal β-oxidation or TCA cycle enzyme activity in skeletal muscle.

However, activation of the AMPK-ACC signaling pathway was markedly reduced in skeletal muscle from middle-aged mice, suggesting that malonyl CoA-mediated inhibition of CPT1 is increased which would limit fatty acid entry into the mitochondria and ultimately result in decreased rates of mitochondrial β-oxidation. The data presented herein are consistent with a study by Reznick *et al.* [27] showing that AMPK activation is impaired in aged muscle and may be an important contributing factor to reduced mitochondrial function and perturbations in lipid metabolism commonly associated with aging. This idea is further supported by findings in our current study showing that free carnitine levels are diminished in muscle from middle-aged mice. Carnitine plays an essential role in the mitochondrial transport and oxidation of long chain fatty acids and as such, carnitine insufficiency is associated with marked perturbations in mitochondrial metabolism [28]. Taken together, these data suggest that fatty acid import into the mitochondria may be a limiting factor leading to a reduction in β-oxidation and may contribute to the overall reduction in resting metabolic rate observed in middle-aged mice. As middle-aged mice developed more severe insulin resistance in response to HF feeding, this suggests that alterations in metabolic rate and fatty acid handling can predispose middle-aged mice to developing obesity and diet-induced insulin resistance and may help explain the high incidence of insulin resistance and T2D in the aging population.

Despite that several lines of evidence suggest that skeletal muscle mitochondrial function declines with advanced age in both rodents and humans [11, 29-32], some studies have failed to show age-associated changes in muscle

metabolism and mitochondrial function [33-36]. The discrepancy between aging studies may be explained by differences in how aging impacts different muscle groups (glycolytic versus oxidative), variation between studies in regards to age range of both young and aged groups, methodological considerations particularly in regards to mitochondrial isolation, and/or the degree of adiposity and physical activity of subjects studied. The latter explanation has received a great deal of attention as levels of habitual physical activity were not adequately controlled for in several earlier studies and/or were often assessed based on self-reported activity questionnaires. Indeed, physical activity is known to strongly influence mitochondrial function where skeletal muscle from young healthy subjects is capable of increasing mitochondrial oxidative capacity and metabolism in response to chronic exercise [37-40]. In general, although often to a lesser degree than in younger subjects older adults still maintain the capacity to adapt to exercise training by increasing oxidative capacity in muscle [41-44]. Moreover, exercise training has been shown to partially reverse some of the age-related reductions in mitochondrial function and improve insulin sensitivity [42, 45]. Therefore, the extent to which aging directly impacts mitochondrial function continues to be a topic of vigorous debate, and it is proposed that rather lifestyle factors, such as obesity and physical inactivity, are the primary determinants of mitochondrial function rather than aging *per se* [30, 41]. However, data in this thesis demonstrate that metabolic rate and energy expenditure are dramatically reduced with aging despite near identical levels of physical activity between age groups of mice. Therefore, it seems that independent of physical activity at least a component of

altered mitochondrial function and oxidative capacity may be the result of aging *per se*. An important caveat to this conclusion is that healthy middle-aged mice were heavier in body weight than young mice; therefore this increased susceptibility to metabolic disease may not be solely due to aging *per se*, but also increased adiposity. Nevertheless, as aging and increased adiposity co-associate our findings likely reflect the majority of middle-aged humans in the western world who are at risk of developing insulin resistance.

Consistent with reduced energy expenditure, increased adiposity and reductions in AMPK and ACC activity in skeletal muscle from middle-aged mice, there was a greater than 1.5-fold increase in intramuscular TG levels in skeletal muscle from middle-aged as compared to young mice. As will be discussed in more detail below, a popular theory states that mitochondrial dysfunction can lead to intramuscular lipid accumulation and the development of insulin resistance. A similar increase in intramuscular TG content has been observed in healthy elderly human subjects [11], supporting that the study of aging in rodents may still provide some important insight into the etiology of age-related diseases in humans. Interestingly, although basal and insulin-stimulated Akt phosphorylation was significantly impaired in skeletal muscle of middle-aged mice compared to their younger counterparts, this correlated with only a modest impairment in whole-body glucose tolerance. Importantly, these data suggest that age-induced alterations in skeletal muscle fatty acid metabolism, insulin signaling and TG accumulation may precede the development of overt systemic insulin resistance.

Role of fatty acid uptake and intramuscular lipid accumulation in the pathogenesis of skeletal muscle insulin resistance

It is widely accepted that impaired skeletal muscle insulin sensitivity is one of the earliest observable changes and precedes the onset of T2D [46, 47]. Although the precise underlying mechanisms responsible for obesity-related insulin resistance have not been fully elucidated and are likely multifactorial, it is generally believed that impaired insulin signaling in skeletal muscle results from the aberrant and excessive accumulation of lipids arising from a chronic imbalance between fatty acid uptake and fatty acid utilization [4, 18, 19]. While rates of fatty acid uptake are sensitive to fatty acid availability to the muscle [48], membrane transport proteins also play an essential role in regulating efficiency of muscle fatty acid transport. Among the fatty acid transport proteins identified so far, CD36 is one of the most well-characterized and is responsible for facilitating 40-70% of fatty acid transport in skeletal muscle [49, 50]. The increase in rates of muscle fatty acid transport in models of moderate insulin resistance, such as the obese Zucker rat, is often attributed to increased abundance of CD36 at the plasma membrane, and not related to elevated protein expression of fatty acid transporters [51-56]. However, in this thesis we clearly show that CD36 protein levels are increased in skeletal muscle from middle-aged mice in response to 12 weeks of a HF diet, and this was associated with marked increases in levels of several intramuscular lipid metabolites, including TG, LCACoA and ceramides. Similarly, in the Zucker diabetic fatty rat, an animal model of severe insulin resistance that rapidly progresses to T2D, there is also an increase in CD36 expression as well as

plasmalemmal content in muscle [57], suggesting that elevated CD36 protein levels may be indicative of a more severe insulin resistant state. A major finding of the study presented in this thesis was that inhibition of CD36-mediated uptake of fatty acids via genetic ablation protected middle-aged mice from developing HF diet-induced obesity and insulin resistance. Consistent with the essential role of CD36 in facilitating fatty acid uptake, CD36 deficiency prevented the accumulation of TG and LCACoA in skeletal muscle observed in middle-aged WT mice following HF feeding. Taken together, these data further support that increased CD36-mediated fatty acid uptake is an early predisposing factor contributing to intracellular lipid overload and the development of impaired insulin sensitivity in skeletal muscle of middle-aged mice following a HF diet. However, the underlying mechanism responsible for the upregulation of CD36 protein levels in aged muscle is still unknown, and may involve changes in PPAR-related transcriptional and/or post-transcriptional mechanisms potentially resulting in increased translational efficiency or reduced protein turnover [58, 59].

Although there is a close association between intramuscular TG accumulation and the severity of insulin resistance in obesity, aging and T2D [60], it is not entirely clear whether elevated TGs play a causative role or are simply a biomarker of insulin resistance. The modest elevation in TG levels detected in skeletal muscle from middle-aged mice was only associated with a small decrement in glucose tolerance as compared to young mice. Interestingly, the fact that glucose tolerance remained relatively normal despite elevated intramuscular TG levels would seem to argue against the concept that higher TG

levels *per se* contribute to skeletal muscle insulin resistance. That said, the elevated TG levels observed in skeletal muscle of middle-aged mice is relatively modest and it is possible that they may not have reached high enough levels and/or been present for a long enough period of time to initiate the signaling events that eventually lead to skeletal muscle insulin resistance. Indeed, we observed a more dramatic 3-fold increase in intramuscular TG in middle-aged mice fed a HF diet and this correlated with severe insulin resistance, suggesting that elevated intramuscular TG levels may potentially directly or indirectly contribute to the development of skeletal muscle insulin resistance. As middle-aged mice have higher baseline intramuscular TG levels at the onset of HF feeding, we suspect that these mice may potentially have a reduced capacity for handling high fatty acid levels as compared to young mice, thus making them more susceptible to developing insulin resistance. Further investigation will be required to determine the threshold levels of TG in skeletal muscle that must be reached before buffering capacity is exceeded and whether excessive TG storage may become a source for other potentially toxic lipid intermediates. Although our data show a correlation between excessive TG accumulation and insulin resistance, a true cause and effect relationship still remains to be established.

However, recent evidence has challenged this idea that TG play a causative role in insulin resistance, suggesting a third alternative that TG may act as a protective buffer for intramyocellular lipids [61]. This concept first emerged when highly insulin sensitive endurance trained athletes were shown to have high intramuscular lipid levels, in a phenomenon known as the "athlete's paradox" [62,

63]. Indeed, moderate exercise training results in intramuscular TG accumulation and a reduction in muscle levels of DAG and ceramides [64], which implies that the partitioning of lipids in the TG pool may actually protect against the accumulation other potentially toxic lipid intermediates. Similarly, transgenic mice with a muscle-specific overexpression of diacylglycerol acyltransferase (DGAT1), a key enzyme in TG synthesis, is able to recapitulate the "athlete's paradox" observed in humans. Increased TG content in muscle from these mice is associated with increased muscle insulin sensitivity, as well as decreased levels of DAG and ceramide [65, 66]. Taken together, this suggests that other lipid intermediates than TG contribute to the development of insulin resistance and that limiting their accumulation should be the target of potential therapies. Interestingly in our current study, improved insulin sensitivity in the middle-aged CD36 KO mice was not associated with a decrease in ceramide levels, but LCACoA levels were reduced. Since DAG levels were not measured in this study, it is unclear whether DAG may be a mediator of lipid-induced skeletal muscle insulin resistance in middle-aged mice. As DAG has been shown to activate novel PKC isoforms and other stress-kinases which interfere with the insulin signaling pathway, it makes an attractive candidate for mediating lipid-induced insulin resistance [13]. Future experiments will be required to examine the contribution of DAG to diet-induced skeletal muscle insulin resistance in the context of aging, as well as delineation of the downstream molecular signaling pathways that are involved in this lipid-induced impairment of insulin action.

Role of altered mitochondrial substrate oxidation in the pathogenesis of insulin resistance

There continues to be significant debate as to whether or not insulin resistance develops secondary to impaired mitochondrial fatty acid oxidation. Many studies have proposed that accelerating fatty acid oxidation in skeletal muscle may be a potential therapeutic approach for the treatment of obesity-induced insulin resistance [10, 12, 18, 27, 67-69]. However, some studies now indicate that skeletal muscle insulin resistance may not arise from impaired fatty acid oxidation, but rather results from excessive fatty acid oxidation [70, 71]. In contrast to the popular theory that mitochondrial fatty acid oxidation is reduced in insulin resistance, Koves *et al.* [70] propose that fatty acid oxidation rates are actually increased but exceeds flux through the TCA cycle and electron transport chain resulting in incomplete fatty acid oxidation, accumulation of lipid metabolites and subsequently mitochondrial stress. Furthermore, they show that limiting fatty acid oxidation secondary to an inhibition in mitochondrial fatty acid uptake can mitigate obesity-related insulin resistance [70]. Therefore, this remains a topic of vigorous debate and it is imperative that we fully understand how fatty acid oxidation rates are altered and more importantly whether these alterations play a causative role in the development of insulin resistance. Within the context of aging, this adds another layer of complexity to the issue as several studies have shown that mitochondrial function and oxidative capacity decline with advanced age [11, 25, 26, 31, 72]. As well, data from this thesis provides important data showing that HF-diet induced obesity produces differential effects according to age

and highlight the need for more studies examining the etiology of insulin resistance in the aging population.

In this thesis, we show an impairment in the AMPK-ACC signaling pathway in muscle from healthy middle-aged mice as compared to young mice, which suggest that there may be a potential decline in mitochondrial fatty acid oxidation with aging. Moreover, this interpretation is consistent with the observation that overall metabolic rate is reduced in middle-aged mice. However, as we did not directly measure rates of fatty acid oxidation nor levels of acylcarnitines in muscle from young and middle-aged mice, future studies will be required to fully characterize the potential alterations in mitochondrial fatty acid oxidation that occur with advanced age and whether or not this plays a role in the pathogenesis of diet-induced insulin resistance in these mice. Interestingly, using mass spectrometry-based metabolic profiling we showed that several long-chain acylcarnitine species were reduced while at the same time levels of hydroxylated long-chain acylcarnitines were increased in muscle of aged mice fed a HF diet as compared to age-matched LF-fed controls. This pattern of acylcarnitines is suggestive of a flux limitation at β-HAD, an enzyme of the β-oxidation pathway. By comparison, many of these metabolite changes were mitigated in middle-aged CD36 KO mice.

Although this thesis focused largely on alterations in fatty acid handling in the context of aging and if/how this contributes to the development of obesity-related insulin resistance, our data suggest that aging may also be associated with intrinsic perturbations in skeletal muscle glucose handling. It will be important to

determine whether glucose uptake, glycolysis and glucose oxidation are altered with aging. Indeed, inherent deficits in glucose metabolism with advanced age would likely set the stage for development of more severe insulin resistance in skeletal muscle following a HF diet. As discussed in Chapter 1, AMPK is a cellular fuel gauge and a major regulator of both fatty acid and glucose metabolism. Therefore, the age-related reduction in AMPK activity in skeletal muscle may act to decrease levels of glucose uptake and glycolysis via the known effects of AMPK on GLUT4 and PFK-2, respectively. This further highlights the need for further study to fully characterize the metabolic alterations that occur in skeletal muscle with advanced age.

Link between increased myocardial CD36, lipid accumulation and obesity-related cardiac hypertrophy

Obesity is a rapidly growing health problem that is reaching epidemic proportions worldwide and is known to dramatically increase the risk of developing CVD, including coronary artery disease, myocardial infarction, heart failure and premature death [73-76]. Excess body weight is also associated with multiple co-morbidities, such as hypertension, dyslipidemia and T2D, which are further risk factors for development of CVD and likely contribute to the increased incidence of CVD in obese patients [23, 77, 78]. A growing body of evidence has linked obesity to the development of left ventricular hypertrophy and cardiac dysfunction [75, 79, 80]. While the pathophysiological mechanisms responsible for obesity-related cardiomyopathy are certainly complex and multifactorial, there is strong evidence showing that derangements in cardiac fatty acid metabolism play a

fundamental role in the development of cardiac dysfunction [3, 16, 81-84]. An increased supply of fatty acids to the heart [9] and elevated myocardial fatty acid uptake [14, 85-88] are thought to play essential roles in the accumulation of potentially deleterious lipid derivatives in the cardiac myocyte, which can lead to cardiac myocyte apoptosis and contractile dysfunction in a phenomenon known as 'lipotoxic cardiomyopathy' [82, 89]. Indeed, expression of fatty acid transport proteins such as CD36, which are involved in mediating fatty acid uptake into the cardiac myocyte have been shown to be increased in models of obesity and T2D [86, 87, 90]. Aging is known to be a significant risk factor for the development of CVD and based on this rationale; we sought to investigate if the aged myocardium may be more susceptible to enhanced accumulation of lipids and development of cardiac dysfunction in response to increased dietary fat intake.

Interestingly, systolic and diastolic function was similar in mice fed a LF or HF diet for 12 weeks regardless of age, suggesting that middle-age mice were not more susceptible to diet-induced cardiac dysfunction than young mice. However, while we did not observe any alterations in cardiac function with 12 weeks of diet, it is possible that either a longer duration of HF feeding and/or the use of mice older than 12 months of age may have revealed age-dependent differences in response to a HF diet. Previous studies have shown that cardiac function progressively declines during the course of HF feeding in young mice, with significant impairments in systolic function only evident by echocardiography following 20 weeks of diet [16]. In support of the latter idea, we have preliminary data showing that older mice (> 12 months of age) subjected to an identical 12

week HF feeding protocol develop significant diastolic dysfunction (unpublished data, Sung M and Dyck J). Therefore, timecourse studies will be required to serially assess *in vivo* cardiac function in mice of various ages at different time points of HF feeding to determine whether increased dietary fat intake leads to cardiac dysfunction and if middle-aged mice are in fact more susceptible to developing cardiac contractile dysfunction in response to a HF diet.

While there were no changes in cardiac performance, diet-induced obesity did result in cardiac hypertrophy that was more pronounced in middle-aged mice as compared to young mice, and this was associated with a 1.5-fold increase in myocardial CD36 expression in the middle-aged heart. Furthermore, we provide evidence that middle-aged CD36 KO mice are protected against the development of cardiac hypertrophy as compared to middle-aged WT mice, suggesting that increased CD36 expression in response to high dietary fat intake is an important contributing factor to the pathogenesis of cardiac hypertrophy. These findings are consistent with previous work from our laboratory demonstrating that CD36 expression is increased in the aged WT murine heart and that this was associated with marked cardiac hypertrophy and contractile dysfunction [91]. However, since cardiac hypertrophy was present together with cardiac dysfunction in these aged WT mice, it was difficult to determine if increased CD36 expression directly contributed to the development of cardiac hypertrophy. The absence of overt cardiac dysfunction in this current study allowed us to investigate the early signaling changes that occur in the aged heart in response to a HF diet, as well as establish a potential role for CD36 in mediating diet-induced cardiac hypertrophy.

Although we have shown that increased CD36 expression contributes to HF diet-induced cardiac hypertrophy in middle-aged mice, interestingly myocardial TG levels were not altered in the HF-fed as compared to LF-fed middle-aged mice, suggesting that TG accumulation *per se* is not responsible for these obesity-related effects on the heart. At this point we can only speculate as to why TG levels were not altered in the aged heart after HF diet. As a large body of evidence proposes that cardiac fatty acid oxidation rates are increased in the setting of obesity and diabetes [3, 92, 93], it is possible that fatty acid oxidation rates are accelerated in the middle-aged heart in response to HF diet and may contribute to TG levels being unchanged in these hearts. Furthermore, similar to skeletal muscle it has also been proposed that sequestration of lipids in the TG pool may be protective in times of lipid overload by reducing intracellular levels of potentially lipotoxic fatty acid metabolites. Indeed, cardiac overexpression of DGAT1 prevents cardiac lipotoxicity and improves cardiac function in a murine model of lipotoxic cardiomyopathy, and this is associated with reductions in levels of myocardial DAG and ceramide [94]. Consistent with this concept, we show there is a strong trend towards increased LCACoA levels and significantly increased ceramide content in hearts from aged, obese mice. It has been proposed that in the obese and diabetic state that the excessive accumulation of lipid metabolites, such as LCACoA, DAG and ceramide, results in cardiac dysfunction and the development of cardiomyopathy (as reviewed in [82, 95-99]). In particular, ceramides are thought to be one of the primary mediators of lipotoxic cardiomyopathy. High levels of ceramides have been shown under *in vitro* and *in vivo* conditions to

induce cardiomyocyte apoptosis and lead to the development of cardiac dysfunction [20, 84, 100-102]. Moreover, the pharmacological inhibition of ceramide formation with myriocin has been shown to improve glucose oxidation rates and restore normal cardiac function in mice with a cardiac-specific overexpression of glycosylphosphatidylinositol membrane-anchored form of LPL, a well-characterized model of dilated cardiomyopathy [17]. Based on these findings, it is tempting to speculate that prolonged exposure to elevated ceramide levels may have resulted in impaired cardiac function in middle-aged WT mice had the HF feeding protocol been extended. Future experiments should be performed whereby myriocin is administered to middle-aged mice to see if inhibition of ceramide formation can prevent the onset of diet-induced cardiac hypertrophy.

Despite that we did not measure cardiac fatty acid oxidation rates in middle-aged mice in our current study, the increase in CD36 expression and myocardial lipid accumulation support the idea that increased fatty acid supply and transport into the heart is a major contributor to obesity-related cardiomyopathy. Even in the possible presence of accelerated fatty acid oxidation rates in the middle-aged heart, there appears to be an imbalance whereby fatty acid uptake exceeds the capacity for its oxidation in the setting of obesity. As many of the studies examining alterations in cardiac energy substrate metabolism have been performed in young animals, similar studies need to be done in aged mice. Our findings show that advanced age increases the sensitivity to developing HF diet-induced cardiac hypertrophy and suggest that aging may be associated with inherent metabolic alterations in the heart that may predispose the middle-aged population to obesity-

related cardiomyopathy.

It is becoming increasingly apparent that fatty acid transport and oxidation may not be the only factors contributing to cardiac lipotoxicity, and that TG metabolism plays an important and underappreciated role [96]. As mentioned above, DGAT1 overexpression and increased TG synthesis in the heart protects against lipotoxic cardiomyopathy and improves cardiac function [94]. Interestingly, together with reduced levels of cardiac ceramide, DAG and fatty acids this protection was associated with enhanced fatty acid oxidation, which suggests that TG metabolism through still yet to be defined mechanisms can influence mitochondrial fatty acid oxidation. Therefore, it will be important to investigate alterations in TG synthesis and turnover that may occur in obesity, diabetes and aging, and how this interacts with both fatty acid uptake and oxidation to contribute to the pathogenesis of lipotoxic cardiomyopathy.

AMPK as a negative regulator of cardiac hypertrophy in the aged, obese heart

In this thesis we show that AMPK activation is markedly reduced in the presence of a 2-fold increase in ceramide levels in hearts from HF-fed middle-aged WT mice as compared to LF-fed age-matched control mice. Some debate exists as to the exact role of AMPK in regulating cardiac myocyte hypertrophy. Increased AMPK activity has been shown to be correlated with the development of pressure-overload-induced cardiac hypertrophy [103]. However, it is unclear if AMPK activation in this setting is a compensatory mechanism for impaired energetic status of the heart secondary to pressure overload or whether it plays a primary

pathogenic role in the development of hypertrophy. Contrary to this, a growing body of evidence shows that AMPK is a major negative regulator of cardiac myocyte cell growth and cardiac hypertrophy [104-108]. Indicative of a pro-hypertrophic environment we observed marked activation of the mTOR-p70S6K-s6 pathway in hearts from middle-aged mice. Interestingly, this occurred in the absence of changes in Akt signaling, which was important for two reasons as it is known that 1) Akt activation promotes protein synthesis and cardiac hypertrophy [109] and 2) Akt can negatively regulate AMPK activity in the heart [110]. Therefore, these data strongly suggest that independent of changes in Akt activation that impaired AMPK signaling may create a permissive environment allowing for the stimulation of pro-hypertrophic pathways and protein synthesis that contribute to HF diet-induced cardiac hypertrophy in the middle-aged heart. Findings in this thesis are supported by a recent report from our laboratory showing that myocardial AMPK activation is impaired in the spontaneously hypertensive rat (SHR), a well-established model of hypertension and cardiac hypertrophy. In a similar fashion, impaired AMPK activity was associated with robust activation of the mTOR-p70S6K pathway in the hypertrophic SHR heart. Moreover, administration of resveratrol to SHRs led to an increase in AMPK activation and a reduction in cardiac hypertrophy [107]. Taken together, these studies lend support to the idea that AMPK inactivation may contribute to the pathogenesis of cardiac hypertrophy, and suggest that therapies targeted at increasing AMPK activity may be beneficial to attenuate hypertrophic growth of the heart under pathological conditions, including obesity and hypertension.

Although the precise mechanism responsible for the inhibition of AMPK in these hearts remains unclear, a previous study by Wu *et al.* [111] demonstrated that elevated ceramide levels result in the robust activation of PP2A and subsequent dephosphorylation and thus inhibition of AMPK activity. These authors also confirmed this finding in young mice fed a HF diet for 12 weeks and show that selectively inhibiting PP2A using small interfering RNA could reverse HF diet-induced AMPK inhibition in aortas from these mice [111]. Therefore, based on this we propose that myocardial AMPK inactivation observed in our present study may be the result of increased ceramide-induced PP2A activation. Interestingly, our laboratory has recently demonstrated that oxidative stress can covalently modify LKB1, an important upstream kinase of AMPK in the heart, resulting in the inhibition of AMPK signaling. Moreover, cardiac-specific deletion of LKB1 leads to cardiac hypertrophy associated with reduced AMPK signaling and activation of mTOR and p70S6K [112]. Although levels of oxidative stress were not measured in middle-aged hearts in this current study, reports in the literature show evidence of increased levels of reactive oxygen species (ROS) and lipid peroxidation in other rodent models of lipotoxic cardiomyopathy [113-115]. Indeed, ceramides and ROS appear to be intricately linked with ceramides able to induce ROS formation and vice versa [116, 117]. Therefore, this may be a potential alternative mechanism to explain depressed AMPK activity in hearts from HF-fed middle-aged mice. Indeed, future studies will be required to explore the potential mechanisms that lead to impaired AMPK activation in the aged heart and the potential contributions of altered PP2A and LKB1 activity.

Contribution of glucose metabolism to the cardioprotective effects of short-term calorie restriction

Next we shifted our attention towards I/R injury, which is a common cardiac condition often observed following open-heart surgery, as well as acute myocardial infarction. While several different strategies have been explored in attempts to limit the severity of I/R injury and salvage the myocardium, many of these approaches have proven to have limited clinical success, largely due to methodological considerations, as well age-dependence, potential interference by other pharmacological agents and refractoriness of the patient to preconditioning stimuli, such as ischemia [118-120]. Therefore, the search continues for potential preventative therapies to restore normal cardiac function and protect the heart against I/R injury. One such therapy that has emerged is the novel dietary strategy of short-term CR which has been shown to reduce CV risk and delay the onset of age-associated CVD in humans [121-123]. CR is best known for its actions in lifespan extension in several different species [124], however, recent studies have shown that CR also dramatically improves ischemic tolerance of the heart [125, 126]. It is well-established that the types of substrates metabolized by the heart during reperfusion following the ischemic period contribute to the degree of cardiac injury [6, 127]. During and following ischemia, circulating plasma free fatty acid levels are dramatically elevated (i.e. >1 mmol/L) in patients [128] and this can result in excessive fatty acid oxidation rates during reperfusion, which can dramatically inhibit glucose oxidation and lead to impaired post-ischemic recovery of cardiac function [127, 129-135]. Importantly, in this thesis we show

that even when perfused in the presence of high levels of fatty acids that 5 weeks of moderate CR markedly improved recovery of post-ischemic contractile function. This finding is of particular significance given that fatty acid concentration in the perfusate of isolated perfused hearts can dramatically affect recovery of hearts following ischemia [127, 136] and it was not known what effect CR would have in this more clinically relevant setting. As such, these findings support the idea that pre-operative short-term CR may be an effective new strategy for increasing ischemic tolerance and improving cardiovascular outcomes post-surgery. In linking the findings in this CR study to the previous aging studies in this thesis, it is of great interest that the aged heart is more susceptible to ischemia [137, 138] and furthermore that the degree of cardioprotection associated with more conventional pre- and post-conditioning interventions is severely blunted with advanced age [120, 125, 138, 139]. This age-dependent loss of cardioprotection has likely hampered the success of such interventions in the clinic where the majority of patients being treated for ischemic heart disease are in the elderly population. However, CR restores the protective effects of ischemic pre-conditioning in the aged heart [125, 140] and provides further rationale for the clinical application of CR in humans since the prevalence of ischemic heart disease is highest in the aging population. As well, understanding the mechanisms of how CR improves myocardial ischemic tolerance and reverses cardiovascular aging is of paramount importance and may lead the way to the discovery of novel pharmacological targets to recapitulate the powerful cardioprotective effects of CR.

Strategies that either directly or indirectly promote glucose oxidation during reperfusion following ischemia have been shown to improve cardiac efficiency and recovery of cardiac function and may be a beneficial approach to lessening ischemic damage [131, 133, 141-143]. Based on this, we investigated whether alterations in cardiac energy substrate metabolism may contribute to CR-induced cardioprotection in our isolated heart perfusion model of reversible I/R injury. Indeed, improved myocardial ischemic tolerance in hearts from CR mice was associated with a greater than 2-fold increase in glucose oxidation rates during aerobic periods prior to and following ischemia. Interestingly, this increase in glucose oxidation was not associated with alterations in glycolysis and fatty acid oxidation rates. Consistent with previous studies showing that short-term CR increases insulin-stimulated glucose uptake in skeletal muscle [144-148], we show that phosphorylation of AS160 and Akt are markedly increased in hearts from CR mice, suggesting that glucose uptake and insulin sensitivity are improved in hearts following CR and this may indirectly contribute to the high rates of glucose oxidation observed in these hearts. Although the precise mechanism involved in mediating increased glucose oxidation rates in hearts from CR mice is still unclear, we speculate that activity of pyruvate dehydrogenase (PDH) and/or the substrate supply to PDH is increased. It will be important to determine how CR impacts myocardial glucose handling and insulin signaling, as well as the molecular mechanisms by which glucose oxidation is elevated in these hearts. As well, future studies will be required to determine the downstream mechanisms by which improved glucose oxidation is linked to improved cardiac function. As glucose is

known to be a more efficient substrate for ATP synthesis, this may increase cardiac efficiency of hearts from CR mice [143]. Alternatively, increased glucose oxidation may improve coupling with glycolysis and reduce proton production and/or prevent disturbances in ionic homeostasis [131, 135]. Overall, these findings suggest that promoting glucose oxidation may be an important mechanism by which CR improves post-ischemic recovery of cardiac function. This adds further validity to the approach of optimizing energy metabolism, in particular targeting glucose oxidation, as a therapeutic strategy in the prevention and/or management of I/R injury.

Role of AMP-activated protein kinase and alternative kinase signaling pathways in the cardioprotective effects of calorie restriction

Recent reports have proposed that the cardioprotective effects of short-term CR are the result of adiponectin-mediated activation of AMPK [126]. However, data presented herein suggest that AMPK activation may not be the primary mediator of increased myocardial ischemic tolerance in CR mice. Indeed, we show that phosphorylation status of AMPK is unchanged in hearts from CR mice as compared to control mice at baseline. As there are significant differences in feeding behaviour in CR mice, we fasted both groups of mice prior to perfusion to attempt to account for differential nutritional status. Since previous studies have shown that fasting increases phosphorylation of AMPK [149], we suspect that fasting the mice prior to heart extraction may have resulted in myocardial AMPK activation being similar between control and CR mice. However, despite that AMPK activity was not increased in hearts from CR mice prior to ischemia these

hearts still exhibited dramatic improvement in post-ischemic recovery of contractile function, which suggests that activation of AMPK may not be obligatory for CR-induced cardioprotection. In addition to high rates of glucose oxidation, hearts from CR mice had increased ATP and reduced AMP levels, as well as reduced AMPK phosphorylation at the end of 40 min reperfusion following ischemia, which suggests improved energy supply in these hearts and may partly explain the increased recovery of function. Although our data implicate that alternative mechanisms aside from acute AMPK activation and signaling are involved in CR-induced cardioprotection, because AMPK is known to regulate the expression and activity of several downstream targets, it is possible that AMPK is activated early on during the course of CR to alter expression of genes involved in regulating metabolism, but its activity is normalized by the end of 5 weeks of CR or as a result of fasting and/or perfusion protocol. Indeed, further investigation will be necessary to ascertain if more chronic changes in the AMPK signaling pathway play a role in mediating CR-induced cardioprotection against I/R injury.

In addition to its role in insulin signaling pathways regulating cardiac energy metabolism, Akt is a also necessary mediator of the powerful cardioprotection conferred by ischemic preconditioning (IPC) and together with Erk1/2 make up the reperfusion injury salvage kinase (RISK) pathway [150, 151]. Similar to the time course of activation of pro-survival kinases in IPC, we show a robust activation of Akt and Erk 1/2 in hearts from CR mice at the end of reperfusion. Future studies will be required to determine if activation of Akt and Erk 1/2 directly contribute to the cardioprotective effects elicited by short-term CR, including performing I/R on

hearts from CR mice in the presence of pharmacological PI3K inhibitors, such as wortmannin, and Erk inhibitors, such as PD-98059 [151] or in transgenic mice with a genetic deletion of the these kinases. Activation of the RISK pathway at the time of reperfusion is thought to protect the heart against apoptotic and necrotic cell death and limit infarct size, in part by inhibiting the opening of the mitochondrial permeability transition pore (mPTP) [152, 153]. Although our model of I/R does not involve a long enough period of reperfusion to induce significant cardiac cell death, there are likely still beneficial effects from activating the RISK pathway that contributes to cardioprotection in our model. Therefore, the data presented herein suggest that short-term CR may precondition the heart to better withstand more severe ischemic insults by activating the RISK pathway and further highlights the numerous cardioprotective effects of short-term dietary restriction.

Future Directions

Age-related Alterations in Skeletal Muscle Fatty Acid Handling Predispose Middle-aged Mice to Development of Diet-induced Insulin Resistance

The data presented in this thesis suggest that aging is associated with an overall reduction in metabolic rate that may be a predisposing risk factor for the development of obesity-related insulin resistance. However, the mechanisms contributing to this decline in basal metabolic rate are not fully understood. The

potential significance of impaired skeletal muscle mitochondrial function in producing this aging-related phenotype remains to be fully investigated. Based on protein levels of electron transport chain complexes, mitochondrial content appeared to be similar in skeletal muscle from young and middle-aged mice, however this should be confirmed by measuring mitochondrial DNA abundance and expression and activity of other mitochondrial enzymes in the TCA cycle and β-oxidation pathway. Changes in muscle oxidative capacity should be assessed in young and aged mice *in vivo* using ^{31}P magnetic resonance spectroscopy and correlated with the changes we observed in whole-body metabolism by calorimetry. Other possible explanations for the reduced metabolic rate that would also be of interest of investigate include a reduction in lean fat-free mass and muscle fiber type switching to less oxidative type II fibers .

Since phosphorylation of AMPK and ACC were markedly reduced in muscle from middle-aged mice as compared to young mice, this suggests that malonyl CoA-mediated inhibition of CPT1 is increased thus, leading to suppression of mitochondrial fatty acid import and fatty acid oxidation. Tissue malonyl CoA levels should be measured from gastrocnemius tissue by high performance liquid chromatography, as well as CPT1 activity, to see if reduced ACC phosphorylation in aged muscle is associated with increased malonyl CoA synthesis. Furthermore, fatty acid oxidation rates should be measured from whole homogenates of gastrocnemius muscle, as well as from isolated mitochondria, from healthy young and middle-aged mice. A growing number of studies have proposed that obesity-related skeletal muscle insulin resistance is the result of mitochondrial overload

and elevated levels of incomplete fatty acid oxidation [70]. Therefore, it would be important to determine if a similar scenario occurs in the process of aging to contribute to the development of age-related insulin resistance. Measurement of muscle acylcarnitine levels by mass spectrometry-based metabolic profiling should be performed in young and aged mice in both the fed and fasted states, as well, rates of incomplete oxidation in skeletal muscle should be measured [70, 71]. Although the age-related decline in AMPK activation was not improved in CD36 KO mice, given the critical role that AMPK plays in the regulation of fatty acid and glucose metabolism it would be interesting to see if restoring AMPK activity would also prevent the development of obesity-induced insulin resistance in middle-aged mice. Indeed, we have already begun preliminary studies feeding middle-aged mice a HF-diet supplemented with resveratrol, which has been shown to be a potent activator of AMPK [154], and have found that resveratrol improves insulin sensitivity in these mice.

The literature continues to be divided on whether skeletal muscle fatty acid oxidation is increased or decreased in response to obesity and how this contributes to the etiology of insulin resistance and T2D [155]. Therefore, measurements of fatty acid oxidation rates and mitochondrial oxidative capacity should be performed in muscle from young and middle-aged mice following 12 weeks of a HF diet. A clear understanding of this is of vital significance since promoting fatty acid oxidation is being promoted as a novel therapeutic strategy for the treatment of insulin resistance and T2D, whereby accelerating lipid metabolism would limit intramuscular lipid accumulation [4, 10, 18, 67, 68, 156]. Since aging may be

associated with inherent perturbations in mitochondrial substrate metabolism, the pathogenesis of obesity-related insulin resistance may be different and/or accelerated in aged mice as compared to young mice. Indeed, further studies are needed to determine how diet-induced obesity impacts rates of fatty acid oxidation in skeletal muscle from aged mice, and potentially if accelerating fat oxidation in the presence of a pre-existing reduction in mitochondrial substrate utilization may be beneficial to increase overall energy production. An important concern with this strategy is if in doing so whether the Randle cycle will lead to a concomitant inhibition of glucose oxidation, which would be an undesirable effect that could further exacerbate insulin resistance.

Based on the findings in this thesis that intramuscular TG levels were elevated in aged mice compared to young mice, it would be interesting to determine lipase activity and expression of levels of DGAT and other enzymes involved in TG synthesis and degradation to elucidate if alterations in TG metabolism also contribute to lipid accumulation and insulin resistance. As well, similar characterization of TG metabolism should be performed in young and aged mice following HF feeding.

As the CD36 deficient mouse model used in the current study was a whole-body KO, the beneficial effects of inhibiting CD36-mediated fatty acid uptake on diet-induced obesity and insulin resistance may also be mediated by other tissues, such as adipose tissue. Future studies should be performed in muscle-specific CD36 KO mice which would allow us to determine if inhibiting CD36-mediated fatty acid uptake and lipid accumulation in muscle is sufficient for protection

against the development of HF diet-induced insulin resistance in the middle-aged mouse and eliminate the potential confounding effects of inhibiting CD36 in other tissues. Indeed, we are currently in the process of developing tissue-specific CD36 deficient mice (skeletal muscle, heart and liver) which will give us a better understanding of how inhibiting CD36 protects against diet-induced obesity and insulin resistance. Furthermore, as multiple organs and tissues are affected by obesity and contribute to systemic insulin resistance, it would be important to study the alterations in fatty acid handling that occur in other tissues as a result of aging and obesity. In particular, adipose tissue is becoming increasingly recognized for its role as a major secretory and endocrine organ involved in the regulation of several metabolic functions and not simply being a site for lipid storage [157, 158]. Adipose tissue is the site of production for multiple peptide hormones classified as 'adipokines' including adiponectin, leptin, and resistin, as well as proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, which can regulate peripheral energy homeostasis. Therefore, it would be interesting to further examine the role of 'adipokines' and inflammation in the development of age- and obesity-related insulin resistance [159-163].

Increased CD36 Expression in Middle-Aged Mice Contributes to Obesity-related Cardiac Hypertrophy in the Absence of Cardiac Dysfunction

Although cardiac dysfunction was not observed in middle-aged mice following 12 weeks of HF diet, it is possible that a longer duration of HF feeding

may have revealed age-related differences in cardiac function. Therefore, long-term HF feeding study ($\geq$ 20 weeks) should be performed in young mice in parallel with middle-aged mice *in vivo* cardiac systolic and diastolic function be serially assessed by echocardiography in order to establish a timecourse for the onset of dysfunction and identify any differential effects that may arise as a function of age. As well, hearts should be analyzed from cohorts of young and aged mice on LF or HF diet sacrificed at various timepoints during this long-term HF feeding study and myocardial levels of TG, ceramides, LCACoA and DAG measured in order to correlate magnitude of intramyocardial lipid accumulation and cardiac dysfunction. Furthermore, in order to fully explore the relationship between increased CD36 expression and the development of obesity-related cardiac hypertrophy, shorter duration HF feeding studies should be performed in middle-aged mice to determine at what timepoint of HF feeding that levels of CD36 and ceramide are upregulated in these hearts in relation to the development of hypertrophy.

As data presented herein proposes that ceramide accumulation may result in impaired AMPK activity and activation of pro-hypertrophic pathways, middle-aged mice subjected to diet-induced obesity should be treated with pharmacological agents such as myriocin or L-cycloserine to see if inhibiting *de novo* synthesis of ceramides offers protection against the development of cardiac lipotoxicity and diet-induced cardiac hypertrophy. More specifically, it would be interesting to determine whether reducing myocardial ceramide accumulation and/or content restores AMPK activation in hearts from middle-aged mice, thus

permitting AMPK to resume its actions as a negative regulator of cardiac hypertrophy and preventing activation of the mTOR-p70S6K-S6 pro-hypertrophic signaling pathway. Since studies have shown that pharmacological activation of AMPK *in vitro* [104-106, 164] and *in vivo* [165] can attenuate cardiac hypertrophic growth and protein synthesis, it would be interesting to determine if administration of AMPK activators, such as AICAR, can reverse the cardiac hypertrophy observed in middle-aged mice. As well, PP2A expression levels and activity should be measured in hearts from HF-fed middle-aged mice to see if activity is indeed increased in these hearts to mediate the reduction in AMPK phosphorylation observed following HF feeding.

To confirm the connection between ceramides and impaired AMPK activity, studies could be performed in cell culture of neonatal rat cardiac myocytes, or if possible adult cardiac myocytes from rat or mouse which would allow us to directly investigate molecular signaling pathways. Cardiac myocytes could be treated with cell permeable ceramide analogs and assayed for PP2A and AMPK activity. As well, in the next step PP2A could be inhibited using small interfering RNA to see if ceramide-induced impairment in AMPK activity can be rescued.

We have recently shown in the spontaneously hypertensive rat that the electrophilic aldehyde lipid peroxidation by-product 4-hydroxy-2-nonenal (HNE) is able to modify LKB1, an important AMPK kinase in the heart, resulting in inhibition of AMPK and subsequent activation of the mTOR-p70S6K pathway [107]. Thus, levels of HNE-protein adduct formation and LKB1 phosphorylation and activity should be measured in hearts from middle-aged mice following 12

weeks of HF diet. This may represent a potential alternative mechanism by which AMPK activity is reduced in these hearts creating a permissive environment for hypertrophic growth.

In addition to inhibition of protein synthesis, we and others have shown that the anti-hypertrophic effects of AMPK are also mediated by suppression of the NFAT-calcineurin pathway [104, 165]. While activation of the NFAT-calcineurin has been found to be involved in several models of pathological cardiac hypertrophy, such as pressure overload hypertrophy [166, 167], it is less clear whether this hypertrophic pathway contributes to HF-diet induced cardiac hypertrophy and diabetic cardiomyopathy. Therefore, it would be interesting to perform assays of calcineurin activity in hearts from middle-aged mice fed a LF or HF diet to determine if activation of the calcineurin-NFAT pathway also contributes to diet-induced cardiac hypertrophy in the middle-aged heart.

Since CD36 is expressed in several different cell types, whole-body deletion of CD36 leads to multiple effects in different tissues aside from the heart, it is important to repeat these HF feeding studies in inducible and heart-specific CD36 KO mice. This transgenic mouse model would limit potential compensatory changes that occur due to lifelong CD36 deficiency and allow for time-dependent control of CD36 gene disruption. The CD36 gene could be disrupted prior to initiation of the HF feeding protocol, which would allow us to determine if cardiac-specific inhibition of CD36-mediated fatty acid is able to recapitulate the protection against diet-induced cardiac hypertrophy that we observe in the whole-body CD36 KO mouse.

In regards to cardiac metabolism, future studies should measure rates of palmitate and glucose oxidation in *ex vivo* perfused mouse hearts to identify alterations that occur as a result of aging, as well as in conjunction with HF feeding. It would be interesting to measure *in vivo* myocardial substrate uptake using combined positron emission tomography and computerized tomography (PET/CT), which would confirm that increased CD36 expression was associated with increased myocardial fatty acid transport in hearts from middle-aged mice fed a HF diet.

Improved Cardiac Metabolism and Activation of the RISK Pathway Contribute to Improved Post-ischemic Recovery in Calorie Restricted Mice

As we observed increases in phosphorylation of AS160 in hearts from CR mice, this suggests that glucose uptake is increased and thus, it would be interesting to measure levels of cardiac glucose uptake *in vivo* using F18-fluorodeoxyglucose (FDG) and PET/CT as an imaging tool. Another option is to calculate glucose uptake in *ex vivo* perfused hearts using the sum of the rates of glycolysis and rate of incorporation of radiolabelled glucose into glycogen. Given that several studies show that skeletal muscle insulin sensitivity is improved following CR [144-148], we should also examine insulin sensitivity in hearts from CR mice. Tissues should be collected from control and CR mice following injection with insulin or saline and immunoblots performed on frozen ventricular tissue to determine the levels of insulin-induced activation of members of the

insulin-signaling pathway, including insulin receptor, IRS-1 and Akt. As well, it would be interesting to determine gene expression of proteins involved in regulating glucose metabolism including Pdk4, GLUT-1 and GLUT-4. Furthermore, since translocation of GLUT-4 to the plasma membrane is necessary to facilitate glucose entry, subcellular membrane fractionation could be performed to measure GLUT-4 protein content at the plasma membrane following injection with insulin or saline. Although total myocardial glycogen content was unchanged between control and CR groups, it is possible that glycogen turnover is accelerated in these hearts. Therefore, the degree of incorporation of radiolabelled glucose into glycogen should be determined which will correspond to the rate of glycogen synthesis. All the aforementioned experiments will provide a clearer understanding of the impact of CR on glucose handling in the heart and may help explain the high glucose oxidation rates observed in these hearts.

The contribution of Akt and Erk 1/2, which together comprise the RISK pathway, to the cardioprotective effects of CR against I/R injury require further investigation. *Ex vivo* perfusion studies of hearts from control and CR mice should be performed in the presence of pharmacological inhibitors of PI3K, such as wortmannin or LY 294002, or MEK 1/2 inhibitors, such as PD-98059, administered during reperfusion to see if inhibiting either of these kinases can abrogate CR-induced cardioprotection against I/R injury. A more sophisticated approach to address this question would be to repeat CR feeding studies in PI3Kα dominant-negative [168] and PI3Kγ KO mice [169]. Together these experiments will help determine if Akt plays a role in mediating the improved myocardial

ischemic tolerance observed in CR mice. Since Erk1/2 is essential for embryonic development, similar CR experiments in Erk1/2 transgenic mice may not be a viable experimental option. Indeed, targeted disruption of Erk2 has been shown to be embryonically lethal due to abnormal placental development [170, 171]. Erk1 deficient mice are viable, fertile and of normal size, however, this seems to be the result of greater activation of Erk2 to compensate for the loss of Erk1 [172]. Therefore, as described above pharmacological inhibition of Erk1/2 may be the best approach for identifying the contribution of Erk1/2 to CR-induced cardioprotection. Since activation of the RISK pathway is thought to protect the heart against ischemic insults by preventing cell death and limiting infarct size, our results suggest that short-term CR may act to precondition the heart to withstand a more severe ischemic insult. Therefore, it would be interesting to perform *in vivo* infarct by LAD ligation in CR mice to see if protection is also afforded by CR in this more severe model of myocardial injury and if this is mediated by activation of the RISK pathway. This would further extend our knowledge about the beneficial effects of CR on the cardiovascular system and provides further rationale that nutritional approaches to limit caloric intake may have clinical applications in patients with coronary artery disease.

Adiponectin has been shown to have numerous protective effects on the cardiovascular system (as reviewed in [173] and [174]) and is proposed to be a key mediator in protecting the heart against I/R injury [175, 176]. CR is known to result in an increase in circulating adiponectin levels [126] and therefore, it would be important to investigate whether adiponectin contributes to improved ischemic

tolerance in our model of short-term CR. Ideally, CR should be performed in adiponectin KO mice to see if protection against I/R injury can be abrogated as a result of the absence of adiponectin. The protective effects of adiponectin are complex and involve multiple signaling pathways that would also be of interest to investigate in the setting of CR, including activation of COX-2 signaling pathways [177] and reduction in oxidative stress [176, 178].

As the goal is to ultimately translate this research into a clinical setting, it would be important to perform timecourse studies to determine the shortest duration of CR that effectively elicits an improvement in ischemic tolerance, as well as the minimal restriction of calories needed (ie. 10% or 40% CR). In this thesis we classify 5 weeks as being short-term, however, in terms of applying this strategy to clinical practice this is still a considerable amount of time that will require significant patient discipline. Ideally, if an even shorter period of mild dietary restriction could reproduce some of the cardioprotection as observed in our 5 week protocol, this would likely be a more viable therapeutic approach that could be recommended by physicians prior to elective open-heart surgery in order to improve patient outcomes post-surgery.

Justification of Methodology and Experimental Limitations

High Fat Diet-Induced Model of Insulin Resistance

Over the past several decades, a variety of rodent models of obesity have been engineered to attempt to mimic the human condition both metabolically and

pathophysiologically, including the Zucker diabetic (fa/fa) rat [179], JCR:LA-cp rat [180], as well as mouse models including db/db (truncated leptin receptor) [181] and ob/ob (defective leptin) mouse [181, 182]. However, the most relevant model to the obese, insulin resistant state in humans is the model of diet-induced obesity where rodents are fed a diet high in fat content, and develop weight gain, elevated fat mass, dyslipidemia and insulin resistance [183]. This is an appropriate model for study as a majority of individuals in the population with insulin resistance and/or T2D are also obese, which is due in part to consumption of foods high in dietary fat [23, 184]. Recently, it has been argued that the diet used in our current studies which contain 60 kcal% fat from lard is not representative of the diet consumed in the western world. There has been a growing trend in the literature towards using a 45 kcal% fat diet, or so-called 'Western diet' which contains a greater amount of sucrose [185]. Indeed, studies have shown that diets high in simple sugars (ie. sucrose, fructose) can lead to impaired insulin sensitivity [186, 187]. We acknowledge that it will be of importance to repeat some of the experiments to compare the effects of a 60 and 45 kcal% fat diet to determine the effect of high fat/high sucrose on the development of obesity and insulin resistance, as they likely both play roles in altering glucose metabolism albeit through distinct mechanisms [188]. However, the HF diet model utilized in this study is still valid and allowed us to study more specifically the effects of high levels of fatty acids in the etiology of insulin resistance and T2D.

Indirect Calorimetry to Assess In Vivo Whole-Body Metabolic Rates

A limitation of the measurements of whole-body VO_2 consumption we obtained using the oxymax CLAMS system was that these values are largely reflective of the mouse at rest, and therefore, VO_2 rates are considerably lower as compared to rates during exercise [189]. Ideally, measurements should have also been performed under exercised conditions using metabolic cages equipped with an animal treadmill and shockgrid where mice would be subjected to involuntary treadmill running, in order to monitor metabolism during exercise and under varying workloads. Calorimetric measurements obtained during exercise can reveal alterations in metabolism that are not evident at rest. However, it should be noted that mice display high locomotor activity during the dark phase, this includes jumping, rearing, and ambulatory behaviours (grooming, scratching), as compared to the light phase. As we show in chapter 3, there were still significant differences observed in VO_2, VCO_2 and RER between young and aged mice over the course of 24 hr cycle therefore, our data still provide important insight into the whole-body metabolic alterations that occur in aging.

Another potential limitation of our calorimetric measurements was that VO_2 and VCO_2 values were not normalized lean body mass. As we did not possess the equipment to measure lean and fat mass at the time these experiments were performed, data obtained from the oxymax CLAMS system was normalized to total body mass. As energy expenditure varies with body composition and body mass, it is necessary to normalize VO_2 consumption to body mass particularly when comparing groups of animals that differ in body mass. However, correcting

to total body mass makes the assumption that all tissues have equivalent metabolic demand. Indeed, adipose tissue (fat mass) has been suggested to have a lower metabolic activity as compared to lean mass. Therefore, previous studies have proposed that normalizing to lean body mass is a more accurate approach that can help eliminate the confounding factor of increased fat mass between lean and obese phenotypes [190]. However, normalization of lean mass can lead to drastic differences in results and it continues to be an area of contention which approach is more accurate in interpreting energy expenditure data [191]. As measurements of metabolic rate and energy expenditure are vital to obesity research, future studies will be required to determine the best approach to account for changes in both fat mass and lean mass.

Isolated Working Mouse Heart Perfusions

The isolated perfused working mouse heart is a widely used model with a high rate of reproducibility that can be used to measure physiological, biochemical and metabolic parameters. Despite its relative simplicity, it is a very powerful tool for the study of cardiac energy metabolism and its relation to cardiac function, as work performed by the heart is a primary determinant cardiac metabolism and ATP production. Cardiac energy metabolism can be measured under physiologically relevant preload and afterload conditions in the presence of varying concentrations of energy substrates, such as glucose and fatty acids [192]. *Ex vivo* heart perfusion removes the potential confounding influence of circulating hormones and signaling from the nervous system.

A limitation with our isolated working heart perfusion model is the use of crystalloid buffer in lieu of blood to perfuse the hearts, while crystalloid buffer is prepared to try to mimic certain physiological aspects of blood, such as pH and ionic constituents, it lacks the haemoglobin, hormones and white-blood cell components of blood. It is recognized that there is an inflammatory component to myocardial I/R injury, in particular neutrophils are known to be key contributors of reperfusion injury following ischemia [193, 194]. Therefore, studies should also be performed in *in vivo* models of I/R injury in order to confirm results obtained from the isolated working heart model. Indeed, *ex vivo* heart perfusion with whole-blood is a possibility and has its advantages, including more efficient oxygen delivery to the myocardium allowing for lower coronary flow rates. However, there is an added cost to obtaining the donor blood and this will likely add more variability to the results as there are multiple energy substrates available that are not controlled for like in crystalloid buffer. In addition, erythrocytes and other circulating cells such as leukocytes and neutrophils also metabolize glucose, which may add a degree of error to estimates of cardiac glucose metabolism.

However, despite some obvious limitations to this experimental model as described above and in chapter 5, there are several advantages to the use of isolated perfused hearts over either isolated cardiac myocyte or isolated muscle preparations. While isolated cardiac myocytes are useful for the dissection of intracellular signaling mechanisms, after isolation they are largely quiescent and do not perform the same degree of work as we would observe in the isolated working heart. Therefore, overall oxidative rates are lower in isolated cardiac

myocytes as compared to the isolated beating heart, likely resulting in increased lactate and proton accumulation [195]. Superfused isolated muscle preparations, such as papillary muscle, are useful to measure mechanical contractile properties of the isolated tissue. However, there are concerns regarding adequacy of oxygenation and nutrient supply across the entire isolated muscle, as well as the accumulation of metabolites [196].

Metabolic Profiling

Mass spectrometry-based metabolic profiling is a sophisticated, state of the art technique used for the quantitative analysis of metabolite levels in biological samples, including tissue and plasma. Since diabetes is a complex disorder involving multiple organ systems and the interaction of several factors, including diet, lifestyle and genetics, this presents a significant challenge in understanding the multitude of molecular and metabolic pathways that contribute to the onset and progression of this disease. Targeted metabolic profiling is a powerful tool that allows for the high-throughput, comprehensive assessment of metabolites, including acylcarnitines, organic acids and amino acids, which can provide significant insight into disease pathogenesis and mechanisms of action and toxicity of drug therapies. However, this field is still in its infancy and more studies are required to establish patterns of metabolite changes in disease states, such as insulin resistance and T2D, and interpretations of these large data sets can be a challenge [197]. Alterations in metabolite concentrations give insight into substrate flux through mitochondrial oxidative pathways and identify potential limitations at particular enzymes; however, concentrations of metabolites

measured represent only one specific point in time. Therefore, metabolic profiling data should be combined with more direct measurements of flux through oxidative pathways. For a greater understanding of the metabolic phenotype exhibited in observed in our aged mice fed a HF diet, it is necessary to examine these changes in light of data measuring mitochondrial and muscle fatty acid oxidation rates, levels and activities of oxidative enzymes and rates of ATP synthesis.

Conclusions

Overall, this thesis has provided important insights in regards to how alterations in energy metabolism contribute to the development of metabolic disorders and CVD, as well as how optimizing substrate metabolism is a potential therapeutic strategy in the prevention and/or treatment of these skeletal muscle and cardiac conditions. The findings presented in this thesis demonstrate that aging increases the susceptibility for developing obesity-induced skeletal muscle insulin resistance and cardiac hypertrophy, in part due to alterations in fatty acid handling. As well, the novel dietary strategy of short-term CR markedly improves ischemic tolerance of the heart and this is associated with an improvement in myocardial glucose metabolism. Individually, these studies demonstrate that:

1) A reduction in overall whole-body metabolic rate is an early event in the aging process and is associated with alterations in skeletal muscle fatty acid handling and TG accumulation. These age-related changes in energy metabolism may predispose middle-aged mice to the development of obesity-induced insulin resistance. However, limiting CD36-mediated fatty acid uptake via CD36 ablation protects against the development of HF diet-induced obesity and insulin resistance potentially by mitigating intramuscular lipid accumulation and improving metabolic rate.
2) Aging increases the sensitivity of the heart to the development of obesity-related cardiac hypertrophy, which is associated with increased CD36 expression, myocardial lipid accumulation and activation of pro-hypertrophic pathways. Inhibition of CD36 by genetic deletion protects the

heart against diet-induced cardiac hypertrophy, suggesting that CD36 is an important contributor to the early pathogenesis of obesity-related cardiomyopathy in the aged heart.

3) Alterations in cardiac glucose metabolism may contribute to the improved post-ischemic recovery of mechanical function of hearts from CR mice. Indeed, the cardioprotective effects of short-term CR in I/R injury may be mediated by an AMPK-independent mechanism and may involve activation of the RISK pathway.

Taken together, these studies show that aging is an important risk factor in the development of obesity-related metabolic disorders and CVD and may act to accelerate the onset and/or progression of disease. Alterations in substrate energy metabolism play an important role in the pathogenesis of both skeletal muscle insulin resistance and cardiac hypertrophy, and represent potential therapeutic targets in the treatment of these diseases. Along those lines, optimizing cardiac energy metabolism may contribute to the improved ischemic tolerance of the heart produced by short-term CR and is a novel therapeutic approach to lessen myocardial ischemic damage.

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