Glucose Oxidation Control in the Heart

by

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Abstract

Heart failure is associated with major changes in cardiac energy metabolism that decrease cardiac efficiency, which can reduce cardiac function. In severe heart failure there is a shift back toward a fetal heart metabolism, with a decrease in mitochondrial oxidative metabolism and increase in glycolysis, which decreases efficiency. This increased reliance on glycolysis may also contribute to hypertrophy in failing hearts. In response to these observations I became interested in better understanding the changes in energy metabolism that occur on both sides of this continuum of cardiac myocyte maturity and cell growth: cardiac myocyte differentiation and the development of heart failure. Because insulin resistance is believed to decrease cardiac efficiency and is an important contributing factor in the development of heart failure, I also examined the regulation of energy metabolism in cardiac insulin resistance and whether improving cardiac efficiency could improve cardiac function in insulin resistant hearts.

We first focused on the changes in energy metabolism that occur during cardiac myocyte differentiation. To get a better understanding of stem cell energy metabolism I initially characterized energy metabolism of bone marrow mesenchymal stem cells (BMMSC). I showed that BMMSCs derive >97% of ATP production from glycolysis, with the rest coming from glucose and fatty acid oxidation. In the course of these experiments I also discovered that physiological levels of the saturated fatty acid palmitate decrease BMMSC proliferation and cell

survival. Interestingly, the unsaturated fatty acid oleate both protects against detrimental effects of palmitate as well as the decline in palmitate oxidation induced by palmitate treatment. Energy metabolism was then measured in H9C2 cells differentiated toward cardiac myocytes for 7 days. I found that there was a significant increase in glucose oxidation but no significant changes in glycolysis or palmitate oxidation in the differentiated H9C2 cells.

Insulin resistance results in changes in cardiac energy metabolism which are believed to contribute to the development of heart failure. I, therefore, focused on the importance of this decline in glucose oxidation in insulin resistant hearts on the development of cardiac dysfunction. To do this, *db/db* mice were treated for 4 wk with either vehicle or insulin glargine and analysis of *in vivo* cardiac function via echocardiography revealed an improvement in cardiac function. Based on *ex vivo* metabolic and cardiac function measurements using the isolated working heart perfusion I speculate that acute stimulation of glucose oxidation by insulin glargine contributes to the improved cardiac function observed.

We then assessed the changes in cardiac energy metabolism that occur during development of heart failure with preserved ejection fraction (HFpEF). Changes in cardiac energy metabolism were assessed in Dahl salt-sensitive rats fed a high salt diet (HSD), which induces HFpEF, for 0, 3, 6 or 9 wk. Over the course of 9 wk, the HSD increased glycolysis but did not alter glucose oxidation resulting in increased uncoupling of glycolysis and glucose oxidation. After 9 wk

the HSD also decreased palmitate oxidation. These results show that the coupling of glycolysis and glucose oxidation is reduced during the development of HFpEF.

Because of the link between heart failure and insulin resistance I also wanted to better understanding the mechanisms involved in the decreased ability of insulin to stimulate glucose oxidation in insulin resistant hearts. A better understanding of the regulation of cardiac insulin resistance could provide clues to the mechanisms involved in the development of heart failure. We chose to focus on branched chain amino acid oxidation because BCAAs have both been implicated in insulin resistance and there is some indirect evidence to indicate that BCAAs could be involved in heart failure. We tested the proposal that an elevation in BCAA oxidation induces insulin resistance by competing with flux through glucose oxidation and fatty acid oxidation. Our results showed that BCAA oxidation contributes a small amount of the overall ATP production in the heart and is further reduced in response to high fat diet induced insulin resistance. These results suggest that cardiac insulin resistance is not due to BCAA oxidation inhibition of glucose and fatty acid oxidation.

Overall, the results presented in this thesis suggest that stimulating glucose oxidation may be a promising strategy to improve cardiac myocyte differentiation, improve the function of insulin resistant hearts, and treat heart failure.

Preface

The research project, of which this thesis is a part, received approval from the University of Alberta Care and Use Committee, Project Name "Protection of the ischemic myocardium and Breeding Colony", AUP00000288. This project received its annual renewal on August 26, 2015. The ethics committee follows the Canadian Council on Animal Care guidelines.

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I was responsible for all data collection (excluding those indicated below), data analysis, and writing Chapter 4, 5, 6, and 7. CS Wagg performed the isolated working heart perfusions in Chapter 5, 6, and 7. D Beker performed the echocardiography in Chapter 5 and 6. JL Levasseur took care of the rats and performed the echocardiographic analysis L Zhang prepared the HPLC samples and K Strynadka performed the HPLC measurements in Chapter 5. K Milner helped with the western blots in Chapter 5. L Zhang and A Fukushima treated the mice and helped with the western blots in Chapter 7.

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List of Abbreviations

-/-	Knockout
A	Late Diastolic Filling Velocity
A’	Late Mitral Annular Velocity
ACC	Acetyl CoA Carboxylase
ADP	Adenosine Diphosphate
AICAR	5-Aminoimidazole-4-Carboxamide Ribonucleotide
Akt	Protein Kinase B
AMC	7-Amino-4-Methyl Coumarin
AMP	Adenosine Monophosphate
AMPK	5’ AMP-Activated Protein Kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BCAA	Branched Chain Amino Acid
BCATm	Mitochondrial Branched Chain Amino Transferase
BCKA	Branched Chain Ketoacid
BCKD	Branched Chain Ketoacid Dehydrogenase
BCKDKi	Branched Chain Ketoacid Dehydrogenase Kinase Inhibitor
BMMSC	Bone Marrow Mesenchymal Stem Cell
BrDU	Bromodeoxyuridine

BSA	Bovine Serum Albumin
CAL	Coronary Artery Ligation
CAT	Carntine-Acyl Carntine Translocase
CHO	Chinese Hamster Ovary
CO_2	Carbon Dioxide
CoA	Coenzyme A
CPT	Carnitine Palmitoyl Transferase
d	Diastole
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DCA	Dichloroacetate
db/db	Leptin Receptor Deficient
DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amin Acetyl-Asp-Glu-Val-Asp-7-amido-4-Methyl Coumarin
DMEM	Delbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E	Early diastolic filling velocity
E'	Early Mitral Annular Velocity
ECL	Enhanced Chemiluminescence

EDTA	Ethylene Diamine Tetraacetic Acid
EF	Ejection Fraction
EGTA	Ethylene Glycol Tetraacetic Acid
ESC	Embryonic Stem Cell
ETC	Electron Transport Chain
FABP	Fatty Acid Binding Protein
FACS	Fatty Acyl CoA Synthetase
$FADH_2$	Flavin Adenine Dinucleotide, Reduced
FAO	Fatty Acid Oxidation
FAT	Fatty Acid Translocase
FATP	Fatty Acid Transport Protein
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
FS	Fractional Shortening
g	Gram
GLUT	Glucose Transporter
GSK3	Glycogen Synthase Kinase 3
H^+	Proton
HADH	Hydroxyacyl CoA Dehydrogenase
HFD	High Fat Diet
HFI	High Fat Diet Branched Chain Ketoacid Dehydrogenase Kinase

	Inhibitor
HFpEF	Heart Failure With Preserved Ejection Fraction
HFrEF	Heart Failure With Reduced Ejection Fraction
HFV	High Fat Diet Vehicle
HIF	Hypoxia Inducible Factor
HPLC	High Performance Liquid Chromatography
hr	Hour
HSD	High Salt Diet
IDH	Isocitrate Dehydrogenase
iPSC	Induced Pluripotent Stem Cell
IRS	Insulin Receptor Substrate
IVCT	Isovolumetric Contraction Time
IVRT	Isovolumetric Relaxation Time
IVS	Interventricular Septum
kcal	Kilocalorie
kg	Kilogram
LCAD	Long Chain Acyl CoA Dehydrogenase
LDH	Lactate Dehydrogenase
LFD	Low Fat Diet
LV	Left Ventricle
LFI	Low Fat Diet Branched Chain Ketoacid Dehydrogenase Kinase

	Inhibitor
LFV	Low Fat Diet Vehicle
LVID	Left Ventricle Internal Diameter
LVPW	Left Ventricular Posterior Wall Thickness
MCD	Malonyl CoA Decarboxylase
MEM	Modified Eagle Medium
min	Minute
MPC	Mitochondrial Pyruvate Carrier
mTOR	Mechanistic Target of Rapamycin
mTORC	Mechanistic Target of Rapamycin Complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
Na^+	Sodium
NADH	Nicotinamide Adenine Dinucleotide, Reduced
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFDM	Non-Fat Dry Milk
ORIGINAL	Outcome Reduction With Insulin Glargine Intervention
P70S6k	P70S6 kinase
PBS	Phosphate Buffered Saline
PCA	Perchloric Acid
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase

PDK1	Phosphoinositide Dependent Kinase 1
PDP	Pyruvate Dehydrogenase Phosphatase
PFK	Phosphofructokinase
PGAM	Phosphoglycerate Mutase
PI3K	Phosphatidyl Inositol 3 Kinase
PKC	Protein Kinase C
PKM2	Pyruvate Kinase M2 Isoform
PGC1	Peroxisome Proliferator Activated Receptor Coactivator 1
PIP3	Phosphatidyl Inositol (3,4,5) Triphosphate
PP2Cm	Mitochondrial Protein Phosphatase 2C
PPAR	Peroxisome Proliferator Activated Receptor
s	Systole
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
TAC	Transverse Aortic Constriction
TBST	Tris Buffered Saline with Tween
TCA	Tricarboxylic Acid
TMRM	Tetramethylrhodamine Methyl Ester Terminal
TUNEL	Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling

U	Unit
Vol	Volume
w/	With
w/o	Without
wk	Week
wt	Weight

CHAPTER 1

Introduction

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Introduction

1.1 Introduction

Energy metabolism is important in heart function and disease. In particular, a decrease in glucose oxidation can impair cardiac efficiency which can lead to a decrease in cardiac function [33, 152, 159]. Another cause of cardiac inefficiency is insulin resistance. The ability of insulin to stimulate glucose oxidation is decreased in cardiac insulin resistance which also can decrease cardiac function [33, 40, 102, 155, 158, 172, 184, 218, 282, 306, 319]. Research has indicated that cardiac insulin resistance contributes to the cardiac inefficiency observed in heart failure [33, 109, 151, 152, 159, 190, 204, 235, 240, 245, 254, 261, 312, 316]. In severe heart failure another cause of decreased cardiac efficiency is a shift back toward a fetal heart metabolism, with a decrease in mitochondrial oxidative metabolism and an increase in glycolysis [21, 55, 61, 62, 120, 142, 159, 188, 193, 275]. The decreased coupling of glycolysis and glucose oxidation may also contribute to cardiac hypertrophy in heart failure. Part of the studies in this thesis look at the changes in energy metabolism on both ends of the continuum of cardiac myocyte maturity: cardiac myocyte differentiation and the development of heart failure (Figure 1-1). This thesis focuses glucose oxidation control in the hearts, specifically in cardiac myocyte differentiation and the

development of heart failure. This chapter introduces cardiac energy metabolism and what is known about its involvement in cardiac myocyte differentiation and the development of heart failure.

1.2 Glucose metabolism

1.2.1 Glycolysis

Fatty acids and carbohydrates are two of the major substrates used for ATP production in the heart [159]. The glucose transporter (GLUT)1 is primarily responsible for basal glucose uptake in the heart while GLUT4 translocation to the cell membrane and subsequent glucose uptake is regulated by insulin signaling as well as energetic stress and ischemia [41, 82, 310]. Once inside the cell, glucose is phosphorylated by hexokinase, the initial step of glycolysis, and through a series of steps is converted to pyruvate (Figure 1-2 and 1-3) [63]. Alternatively, glucose-6-phosphate can be converted to glycogen, enter the hexosamine biosynthesis pathway, or be diverted to the pentose phosphate pathway. Pyruvate can be transported into mitochondria via the mitochondrial pyruvate carrier (MPC) and undergo glucose oxidation (see below) [31, 103, 159]. Glycolysis produces 2 ATP and 2 NADH. Pyruvate can also be converted to lactate by lactate dehydrogenase (LDH). At the same time, there is an accumulation of protons in the cytosol. This occurs when the flux through glycolysis is greater than flux through glucose oxidation and is referred to as an

uncoupling of glycolysis and glucose oxidation. The subsequent increase in proton production impairs cardiac efficiency (Figure 1-4) [33, 62, 152, 159]. The role of glycolysis in regulating proliferation and the detrimental effects of increased glycolysis in the heart will be discussed later.

1.2.2 Glucose Oxidation

Once inside the mitochondria pyruvate can be decarboxylated to acetyl CoA by pyruvate dehydrogenase (PDH). At the same time a NADH and CO_2 are produced. PDH activity is inhibited by PDH kinase (PDK) phosphorylation [107, 267]. Increases in acetyl CoA and NADH levels stimulate PDK activity while they inhibit PDH activity. The TCA cycle breaks down acetyl CoA to NADH and $FADH_2$ which are utilized by the electron transport chain to produce ATP. Complete oxidation of one glucose molecule yields 31 ATP. LDH can convert lactate to pyruvate, which can be used in the production of ATP in the same manner as pyruvate produced from glycolysis.

1.2.3 Regulation of glucose metabolism by insulin signaling

Insulin increases glucose uptake as well as glycogen production and glucose oxidation [317]. The insulin receptor is composed of 4 subunits: 2 external α subunits and 2 internal β subunits [41]. The binding of insulin to the α subunit causes the β subunits to autophosphorylate each other on tyrosine residues, causing a conformational change and increasing the receptor's tyrosine

kinase activity [41, 317]. The insulin receptor phosphorylates tyrosine residues of substrates including insulin receptor substrate (IRS) proteins. Once phosphorylated, IRS proteins interact with a number of different proteins, including phosphatidyl inositol 3 kinase (PI3K) [41]. PI3K is important in mediating the effects of insulin on glucose uptake and glucose metabolism. PI3K production of phosphatidyl inositol (3,4,5) triphosphate (PIP3) recruits phosphoinositide dependent kinase 1 (PDK1) to the cell membrane. PDK1 increases GLUT4 translocation to the cell membrane and glucose uptake through Akt and protein kinase c (PKC) pathways [41, 82].

1.2.4 Warburg effect

In 1924 Otto Warburg discovered that cancer cells have high rates of glycolysis even under conditions with sufficient oxygen for mitochondrial oxidative metabolism [296, 297]. High rates of glycolysis uncoupled from glucose oxidation under aerobic conditions is now broadly referred to as the Warburg effect (Figure 1-5). The Warburg effect is believed to promote proliferation [285]. This is attributed to NADPH and ribose phosphate production by the pentose phosphate cycle, which diverts glucose away from glycolysis [285]. An increase in glycolysis may also promote proliferation due to an increase in the hexosamine biosynthesis pathway [201]. For example, stimulating glucose oxidation in cancer cells decreases glycolysis which is associated with decreased proliferation and apoptosis [25].

1.3 Fatty acid metabolism

Fatty acid entry into the cell is primarily dependent on proteins including tissue specific fatty acid transport protein (FATP), fatty acid binding protein (FABP), and CD36/fatty acid translocase (FAT) (Figure 1-2) [100, 134, 159, 196, 251, 266].

Once the fatty acid enters the cell, fatty acyl CoA synthetase (FACS) adds a CoA group to the fatty acid. Carnitine palmitoyl transferase 1 (CPT1), an enzyme present on the outer mitochondrial membrane, can then convert this long chain fatty acyl CoA to acyl carnitine allowing it to be transported across the outer mitochondrial membrane. Next, carnitine acylcarnitine translocase transports the long chain fatty acyl carnitine across the inner mitochondrial membrane where it is converted back to a fatty acyl CoA by CPT2 and enters fatty acid β-oxidation [159]. Each cycle of fatty acid β-oxidation yields an acetyl CoA, $FADH_2$, and NADH. For example, complete oxidation of palmitate, a 16 carbon fatty acid, yields 8 acetyl CoA, 7 $FADH_2$, and 7 NADH, producing 105 ATP.

Fatty acid β-oxidation is regulated by a number of factors. Four major factors regulating fatty acid oxidation include malonyl CoA, the Randle Cycle (discussed below), transcriptional regulation, and post-translational regulation. Transcription factors that regulate fatty acid oxidation include the peroxisome proliferator activated receptor (PPAR) family of transcription factors and peroxisome proliferator activated receptor γ coactivator 1 (PGC1)α. PPARα

regulates cardiac fatty acid oxidation. Overexpressing PPARα in the heart stimulates cardiac fatty acid oxidation [81, 211, 243]. Malonyl CoA decreases fatty acid oxidation by inhibiting CPT1 [173, 174, 214]. The synthesis and degradation of malonyl CoA is controlled by acetyl CoA carboxylase (ACC) and malonyl CoA decarboxylase (MCD), respectively. ACC activity is regulated via phosphorylation. For example, 5' AMP-activated protein kinase (AMPK) phosphorylates and inhibits ACC [98, 299]. AMPK is believed to contribute to the increase in fatty acid oxidation in cardiac disease and development by phosphorylating ACC and decreasing malonyl CoA levels [156, 200]. In contrast, MCD is primarily regulated by transcriptional control [38, 244, 311]. An elevation in cardiac MCD activity is associated with a drop in malonyl CoA levels and increase in fatty acid oxidation [38, 70, 159, 160, 200, 283]. Cardiac MCD expression and overall activity is increased under multiple settings associated with elevated cardiac fatty acid oxidation, including diabetes, obesity, and postnatal development [160, 200, 311]. Another post translational modification known to regulate fatty acid oxidation enzyme activity is acetylation. There is not yet a consensus on the effect of acetylation on fatty acid oxidation and fatty acid oxidation enzymes [5, 6, 24, 105, 106]. However, several studies have reported a positive correlation between fatty acid oxidation and acetylation of long chain acyl CoA dehydrogenase (LCAD) and 3-hydroxy acyl CoA dehydrogenase (HADH), indicating that acetylation activates these fatty acid oxidation enzymes [13, 245, 290].

1.4 Randle cycle

Fatty acid oxidation and glucose metabolism (glucose oxidation and glycolysis) inter-regulate each other [229]. For example, acetyl CoA produced by PDH inhibits the fatty acid oxidation enzyme 3-ketoacyl CoA thiolase [202]. Further, the NADH produced during glucose oxidation also inhibits fatty acid oxidation by decreasing HADH activity [73]. Fatty acid oxidation also inhibits glucose oxidation and glycolysis through multiple mechanisms. For example, an increase NADH/NAD+ levels and acetyl CoA in mitochondria inhibit glucose oxidation by activating PDK and subsequently increasing PDH phosphorylation [117, 125, 267]. In addition, citrate, which increases when fatty acid oxidation is elevated, can inhibit the glycolytic enzyme phosphofructokinase 1 (PFK1) and can also indirectly inhibit hexokinase by increasing glucose-6-phosphate levels [159, 228].

1.5 Energy metabolism and heart failure

In heart failure there is as an insufficient supply of blood to the body due to a decreased ability of the heart to fill with and/or eject blood. Common causes of heart failure include hypertension and myocardial infarction as well as idiopathic dilated cardiomyopathy, and alcoholic cardiomyopathy [175]. Progression of heart failure with diastolic dysfunction is not well defined but it is frequently associated with diabetes and is accompanied by the development of

cardiac hypertrophy [17, 42]. The resulting increased pressure in the left ventricle leads to elevated atrial pressure and pulmonary congestion [17, 132]. In some cases as cardiac dysfunction progresses systolic dysfunction can also develop in these hearts. In heart failure with systolic dysfunction the decline in ejection fraction results from a greater elevation in end systolic volume than end diastolic volume [42]. The ventricle becomes spherical which can cause misalignment of the papillary muscles and mitral valve and subsequent mitral regurgitation [42]. Mitral regurgitation can then cause further ventricle enlargement and remodeling [42, 237]. In addition to ventricle dilatation, failing hearts with decreased systolic function can also have increased left ventricle hypertrophy [17, 42]. In both systolic and diastolic dysfunction, an increase in catecholamines, an elevation in the sympathetic nervous system, and subsequent activation of the renin-angiotensin-aldosterone system contribute to cardiac remodeling (which includes increased myocardial fibrosis and collagen volume), increases systemic vascular resistance, causes inflammation, and has prothrombotic effects [17, 42, 237].

The two main types of heart failure are heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). HFrEF is characterized by heart failure with an ejection fraction ≤40% [307]. Heart failure is defined as HFpEF when ejection faction is ≥50% [307]. HFrEF and HFpEF each occur in about half of heart failure patients [175, 307]. However, heart failure research, including research on energy metabolism, has primarily focused on HFrEF.

Alterations in cardiac energy metabolism are believed to contribute to the impaired cardiac function present in heart failure. In heart failure there is an overall decrease in mitochondrial oxidative metabolism and increase in glycolysis [21, 34, 55, 61, 69, 120, 142, 159, 188, 193, 275]. The degree of this drop in cardiac mitochondrial oxidative metabolism depends on the cause and stage of heart failure [159]. In general, cardiac fatty acid oxidation is decreased in animal models of heart failure [34, 69, 142, 208, 225, 316] and in heart failure patients [59, 191, 210, 309], contributing to this decline in overall mitochondrial oxidative metabolism. These changes in energy metabolism also result in an increase in the relative contribution of glycolysis to energy production in the heart and increase in the uncoupling of glycolysis and glucose oxidation, either due to an increase in glycolysis or decrease in glucose oxidation [61, 69, 120, 170, 315]. Increased uncoupling of glycolysis and glucose oxidation results in increased H^+ and lactate production, decreasing cardiac efficiency [62, 159]. At the same time the Na^+/H^+ exchanger transports H^+ out of the cell and Na^+ inside [159]. When Na^+ is transported back out of the cell via the Na^+/Ca^{2+} exchanger, calcium is transported inside which must then be transported back across the cell membrane or into vesicles by Ca^{2+} ATPases to maintain intracellular calcium levels [159] (Figure 1-3). The utilization of ATP in maintaining ionic homeostasis reduces cardiac efficiency (Figure 1-6). Interestingly, these changes in metabolism that accompany heart failure are consistent with a shift back toward a fetal or less differentiated metabolism [21, 61, 120, 142, 159]. In failing human hearts the

expression of many of the metabolic proteins decrease toward that of the fetal heart including a decrease in GLUT1 and 4, medium chain acyl CoA dehydrogenase, and CPT1β [231, 272]. Expression of other proteins including contractile protein expression also shifts back toward the expression profile of the fetal heart. Work conducted in failing human hearts showed an increase in atrial natriuretic factor but both myosin heavy chain α and β decrease [231, 250, 272].

The metabolic changes observed in heart failure are attributed to changes in the expression and activity of enzymes involved in these pathways. Briefly, several post translational modifications have been found to alter these enzymes' activities and flux through their respective pathways including phosphorylation, acetylation, and malonylation. The specific roles of each of these post translational modifications in heart failure are still being elucidated. However, recent work indicates that acetylation and succinylation of mitochondrial enzymes may impair cardiac function [29, 141]. Changes in the expression and activity of transcription factors including PGC1α (mitochondrial oxidative metabolism), hypoxia inducible factor (HIF)1α (glycolysis), and PPARα (fatty acid oxidation) are believed to contribute to the metabolic changes observed during heart failure. For example, in pressure overload induced hypertrophy HIF-1α is increased and PPARα and PGC1α are decreased [12, 74, 123, 159, 186]. Further, overexpressing PPARα in the heart stimulates cardiac fatty acid oxidation and decreases cardiac function [81, 211, 243].

1.6 Cardiac Insulin Resistance

1.6.1 Metabolic changes in insulin resistance and diabetes.

Insulin resistance alters cardiac energy metabolism. Normally, insulin stimulates cardiac glucose oxidation and inhibits fatty acid oxidation [317]. The ability of insulin to stimulate glucose oxidation and inhibit fatty acid oxidation is impaired in insulin resistance and diabetes (Figure 1-7) [33, 40, 102, 155, 158, 172, 184, 218, 282, 306, 319]. In addition, in insulin resistant states, such as diet-induced obesity and diabetes, overall fatty oxidation increases [28, 33, 39, 78, 80, 126, 158, 282, 318]. This increase in fatty acid oxidation and decrease in glucose oxidation reduces cardiac efficiency, which contributes to the decreased cardiac function seen in diabetes and heart failure [33, 109, 151, 152, 159, 178, 190, 204, 235, 240, 245, 254, 261, 312, 316]. Fatty acid oxidation is a less efficient source of energy than glucose oxidation because fatty acid oxidation produces less ATP per O_2 molecules consumed. Fatty acid oxidation can also reduce cardiac efficiency by inhibiting glucose oxidation, resulting in less coupling of glycolysis and glucose oxidation [88, 152]. In fact, insulin resistance either at the whole body or heart level increases the chance of developing heart failure and the severity of heart failure [32, 114, 117, 119, 124, 159, 170, 182, 183, 190, 240, 245, 261, 269, 315, 316]. It was also recently reported that improving cardiac insulin sensitivity, due to a diet intervention, in the setting of heart failure or diabetes improves cardiac function [168, 245]. An increased incidence of vascular disease and atherosclerosis (which increases the risk of heart failure) in diabetes

has been attributed to hyperglycemia and hyperlipidemia [27, 58, 176, 234, 264, 274]. Elevated levels of blood glucose also result in increased protein glycosylation which is associated with cardiac hypertrophy and heart failure and may contribute to the development of heart failure [163, 199]. For example, O-linked glycosylation, which increases in the heart under conditions of hyperglycemia, modulates the activity of ion channels and increases Ca^{2+}/calmodulin-dependent protein kinase II which alter electric signaling and cause arrythmias [75, 181]. In fact, a recent clinical trial showed that a sodium glucose cotransporter inhibitor both lowers circulating glucose levels, mortality, and hospitalizations due to heart failure in Type 2 diabetic patients [84, 322].

An accumulation of lipid metabolites is believed to contribute to insulin resistance. Diacylglycerol (DAG) is believed to be the major lipid intermediate contributing to cardiac insulin resistance [319]. While triacylglycerol also accumulates, it is likely not involved in cardiac insulin resistance [16, 282, 319]. It has been proposed that this accumulation of lipid metabolites could be due to two factors, decreased fatty acid β-oxidation and a surplus of fatty acids. However, cardiac fatty acid oxidation is elevated in obesity and diabetes [18, 109, 204, 218, 219, 235, 254]. An increased supply of fatty acids is more likely to be responsible for this accumulation of fatty acid intermediates. Circulating fatty acids are elevated in diabetes and other insulin resistant conditions such as diet induced obesity [135, 172, 232, 233, 268]. Insulin can reduce free fatty acid (FFA) levels by inhibiting lipolysis in adipose tissue and stimulating triacylglycerol

synthesis. Therefore, in insulin resistance an increase in adipose tissue lipolysis and triacylglycerol hydrolysis may contribute to the elevated plasma FFA levels.

1.6.2 Role of BCAA oxidation in cardiac insulin resistance.

Branched chain amino acids (BCAAs) (which include leucine, isoleucine, and valine) have been implicated in insulin resistance. During the initial step of BCAA oxidation, BCAAs are reversibly transaminated to branched chain ketoacids (BCKA) (Figure 1-8). In muscle, the main branched chain amino transferase is the mitochondrial branched chain amino transferase (BCATm) [189, 270]. Branched chain ketoacid dehydrogenase (BCKD), located on the inner mitochondrial membrane, catalyzes the next step committing the BCKAs to oxidation. The final products of BCAAs are acetyl CoA or succinyl CoA. One of the key ways BCKD is regulated is via phosphorylation. BCKD is phosphorylated, and inhibited, by BCKD kinase and dephosphorylated by protein phosphatase 2Cm (PP2Cm) [161].

Several studies have shown that BCAAs accumulate in skeletal muscle in obesity and insulin resistance [111, 195]. BCAAs are strongly associated with whole body insulin resistance in both obesity and diabetes [9, 76, 164, 195] as well as in heart failure, another condition characterized by insulin resistance [120, 246]. Work that directly modulated the supply of BCAAs also implicates BCAAs in insulin resistance. For example, increasing BCAAs in the diet exacerbates insulin resistance by impairing insulin signaling in liver and skeletal muscle [195,

277]. In contrast, in a mouse model of diabetes and obesity, the *db/db* mouse, whole body insulin sensitivity is improved by reducing the amount of BCAAs in their diet [302].

Multiple mechanisms have been proposed for how BCAAs induce insulin resistance. One of these mechanisms suggests that increased BCAA levels results in elevated BCAA oxidation which inhibits fatty acid oxidation and glucose oxidation [194]. Increased BCAA levels may increase the level of BCAA oxidation intermediates (suggesting elevated BCAA oxidation) and incomplete fatty acid oxidation in skeletal muscle [194]. However, there is evidence, especially in the heart, that insulin resistance in the setting of obesity is actually associated with an elevation in fatty acid oxidation [102, 155, 184, 282, 306, 319]. Alternatively, BCAAs themselves may inhibit insulin signaling through activation of mechanistic target of rapamycin (mTOR) signaling (Figure 1-9). BCAAs can stimulate mTOR signaling, and thereby reduce insulin signaling, by activating mTOR complex (mTORC)1 [9, 68, 97, 145, 165, 242, 301]. Increased BCAA levels are associated with activation of the mTOR pathway and insulin resistance in skeletal muscle [164, 165, 195]. Importantly, insulin resistance is also associated with increased mTOR signaling in the heart [319]. It has also been proposed that the accumulation of mitotoxic products of BCAAs inhibits insulin signaling [164]. An example of these products are BCKAs, which can accumulate when there are defects in BCAA oxidation [8, 203]. However, there is also evidence to suggest that these negative effects of BCKAs are not due to

direct actions of BCKAs themselves but are actually mediated by BCAAs. BCKAs can not exert their effects at the level of the pancreas in BCATm knockout mice, likely because they need to be converted back to BCAAs [321].

1.7 Stem cell energy metabolism

1.7.1 Energy metabolism and stem cell proliferation

The Warburg effect is believed to be important in proliferation [285]. While much of this was investigated in cancer cells, it also may provide clues to the importance of energy metabolism in stem cell proliferation. In fact, while not identical, embryonic stem cells (ESC) and embryonal carcinoma cells have similar concentrations of metabolites, particularly those involved in glycolysis [7]. Frequently, the survival and proliferation of these cells is reduced in response to a decrease in glycolysis [187, 285, 287]. Stimulating glucose oxidation in cancer cells decreases glycolysis which is associated with apoptosis and decreased proliferation [25].

One of the explanations suggested for the benefit which glycolysis exerts in cancer cells is that glycolysis increases the production of substrates required for cell growth [285]. For example, the NADPH produced by the pentose phosphate cycle, which shunts glucose away from glycolysis, is required for lipogenesis [37, 305]. Certain isoforms of glycolytic proteins, such pyruvate kinase M2 (PKM2) and LDHA, become preferentially expressed in cancer and are important to

maintain high rates of glycolysis and proliferation [48, 140, 286]. Inhibition of LDH-A impairs tumor growth [140].

There is also evidence that fatty acid oxidation promotes proliferation and survival. During glucose deprivation stimulation of fatty acid oxidation protects glioblastoma cells normally dependent on Akt for glycolysis and survival [36]. In addition, perhexiline, an inhibitor of mitochondrial fatty acid uptake, reduces the proliferation of several cancer cell lines [236]. This ability of fatty acid oxidation to promote cancer cell proliferation and survival is not true for all cancer cells and may be unique to cancer cells. These findings do suggest that oxidative metabolism, and specifically fatty acid oxidation, is not necessarily at odds with the ability of a cell to proliferate.

Fatty acids may also regulate proliferation and cell survival via regulation of the Warburg effect. The effect of fatty acids on survival is dependent on the type of fatty acid. In general, saturated fatty acids, such as palmitate, induce cell death while unsaturated fatty acids, such as oleate, do not. In fact, oleate can prevent saturated fatty acid induced cell death [99, 180]. Several cell types including Chinese hamster ovary (CHO) cells and bone marrow mesenchymal stem cells (BMMSC) are believed to undergo palmitate induced cell death via apoptosis [133, 150, 162]. This deleterious effect of fatty acids may be due to an increase fatty acid oxidation, which has been described by the Randle cycle to decrease overall glucose metabolism. In breast cancer cells palmitate-induced death was associated with reduced PI3K activity which should reduce glucose

uptake [99]. In fact, coupling of glycolysis to glucose oxidation in cancer cells results in decreased glycolysis accompanied by apoptosis [25]. Further, inhibiting glycolysis in BMMSCs with 2-deoxyglucose also enhances hypoxia-induced BMSC death and increases caspase 3 activity [187]. This suggests that the deleterious effects of saturated fatty acids on proliferative cells may be partially due to inhibition of the Warburg effect. Interestingly, there is also evidence that increasing fatty acid oxidation actually protects against palmitate induced cell death [101, 162].

1.7.2 Energy metabolism and cardiac myocyte differentiation

Energy metabolism has been implicated in stem cell-to-cardiac myocyte differentiation and heart development [50, 51, 156, 160]. Metabolic studies conducted during postnatal heart maturation and in stem cells have increased our understanding of the changes that occur in energy metabolism during cardiac muscle maturation. The metabolism of the fetal heart is still highly glycolytic, likely partially due to the fact that its environment has low oxygen levels and that the heart cells are still proliferative [11, 156, 259]. Immediately after birth, the newborn heart is still highly glycolytic which has been suggested to be due to hypertrophy [11, 156, 259]. However, following birth there is a very dramatic and fast shift in metabolism to that of the adult heart. For instance, just 7 days after birth glycolysis only provides 10% of the ATP produced in the rabbit heart [116, 156]. The increase in fatty acid oxidation that occurs after birth is believed

to be largely due to a drop in malonyl CoA levels, which would relieve inhibition of fatty acid oxidation [116, 156, 157, 160]. The drop in malonyl CoA has been attributed to increased degradation of malonyl CoA via MCD and inhibition of ACC caused by phosphorylation by AMPK [156, 160, 167]. AMPK is believed to be important in differentiation and the maturation of energy metabolism in the heart [72, 89, 156, 160, 167]. These changes in energy metabolism that occur during heart maturation provide important information on some of the changes that will need to occur during complete differentiation of a stem cell into a mature cardiac myocyte.

Methods used to study the changes in energy metabolism during cardiac myocyte differentiation have been much less direct. Thus far, indirect measurements of energy metabolism have been used, such as lactate production, oxygen consumption, and mitochondrial amount and morphology, to study energy metabolism during stem cell-to-cardiac myocyte differentiation. Actual rates of the energy metabolic pathways have rarely been measured directly in stem cells. Some of the cell types that have been used to study cardiac myocyte differentiation both in basic science and in clinical studies include ESCs, BMMSCs, H9C2 cells, and induced pluripotent stem cells (iPSC) [50, 51, 54, 93, 177, 253, 280]. Much of the initial work looking at energy metabolism in cardiac myocyte differentiation was conducted in ESCs which require a cocktail of factors to differentiate [50, 51, 131]. The use of iPSCs in metabolism studies is increasing due to the progress that has been made recently in differentiating iPSCs into

cardiac myocytes efficiently [93, 253, 280]. A major limitation of the cell types being used in stem cell heart therapy is the degree and success rate of differentiation. Even the most promising BMMSC-to-cardiac myocyte differentiation protocols only result in a small proportion and degree of differentiation while requiring a cocktail of factors [22]. In fact, transplanted stem cells such as BMMSCs or cardiac stem cells have negligible rates of differentiation [10, 19, 22, 93, 265, 284]. Cell lines such as H9C2 cells are also being studied *in vitro* to improve our understanding of cardiac myocyte differentiation since they can be much more readily differentiated [30, 54, 177, 217]. I hypothesize that an improved understanding of energy metabolism in cardiac myocyte differentiation will provide novel strategies for inducing efficient cardiac myocyte differentiation.

Evidence of increased mitochondrial oxidative metabolism is consistently observed during cardiac myocyte differentiation (Figure 1-5). Mitochondria maturation is observed during stem cell-to-cardiac myocyte differentiation [22, 51, 54, 207]. In addition, AMPK is involved in cardiac myocyte differentiation [72, 89]. AMPK may be contributing to an increase in mitochondrial oxidative metabolism by inducing mitochondrial biogenesis [89]. There is evidence indicating that inhibition of the electron transport chain with antimycin A in ESCs promotes pluripotency [288]. Inhibition of the electron transport chain also partially inhibits ESC-to-cardiac myocyte differentiation [51]. A more recent study using cardiac stem cells with mitochondrial mutations also suggested that

cardiac myocyte differentiation is reliant on mitochondrial oxidative metabolism [207].

The increase in mitochondrial oxidative metabolism during cardiac myocyte differentiation is also accompanied by a decrease in glycolysis. ESC-to-cardiac myocyte differentiation is associated with a restructuring of the glycolytic pathway indicating a decrease in glycolysis and glycolytic capacity and lower lactate production [50, 51]. The concept that a shift away from glycolysis is important in maturation is further supported by the finding that iPSC generation requires a shift to a more glycolytic and lower oxidative metabolism which is similar to ESC metabolism [87, 224, 289]. This apparent shift toward a glycolytic phenotype appears to be at least partially caused by an increase in hexokinase II expression, a decrease in PDH expression, and an increase in PDH phosphorylation [289]. It is therefore not surprising that high glucose levels suppress ESC cardiogenesis [72]. These changes in glycolysis may be due to the decline in proliferation that occurs during cardiac myocyte differentiation. This suggests that a reduction in glycolysis might promote differentiation through regulation of proliferation.

The transcription factors that regulate energy metabolism would also be expected to contribute to the increase in mitochondria and oxidative metabolism that occurs during cardiac myocyte differentiation. While this has not been investigated during stem cell-to-cardiac myocyte differentiation, it was recently reported that increased PPARδ activity and fatty acid oxidation appears to be

essential for hematopoietic stem cell differentiation [115]. In addition, pretreatment of BMMSCs with a PPARγ agonist increases cardiac myocyte differentiation *in vitro* [256]. However, this drug did not increase differentiation when administered during the differentiation protocol [256]. Treatment of ESCs with agonists of PPARα, a transcription factor that regulates fatty acid oxidation and mitochondrial biogenesis, enhances cardiac myocyte differentiation [65, 66].

1.8 Modulation of energy metabolism as a potential strategy to improve cardiac myocyte differentiation and cardiac function in diabetes and heart failure

1.8.1 Modulation of energy metabolism as a potential treatment for diabetic cardiomyopathy and heart failure

Heart failure is commonly treated with angiotensin converting enzyme inhibitors, aldosterone antagonists, angiotensin receptor blockers, beta blockers, and diuretics [307]. Angiotensin converting enzyme inhibitors target the renin angiotensin system and have been shown to reduce mortality and morbidity of patients with HFrEF [1, 4, 92]. The angiotensin receptor blockers are given to patients with HFrEF who are intolerant to the angiotensin converting enzyme inhibitors or who are on this drug and beta blockers but need further therapy [53, 166, 220]. Beta blockers improve cardiac function by inhibiting the sympathetic

nervous system and allowing the ventricles to fill more completely with blood [2, 3, 23, 223]. Diuretics are used for both HFpEF and HFrEF to deal with fluid retention [307]. As of yet, there are not effective therapies on the market for HFpEF. Drugs recommended for treatment of HFpEF focus on treatment of edema and pressure overload with diuretics and treatment of hypertension with beta blockers, angiotensin converting enzyme inhibitors, and/or angiotensin receptor blockers [47, 144, 307]. Therefore, it will be especially important to develop therapies for HFpEF. Cardiac energy metabolism represents a promising target for the treatment and prevention of HFpEF and HFrEF.

Stimulating glucose oxidation, either directly or by inhibiting fatty acid oxidation, is a promising strategy for the treatment of diabetes and heart failure [120, 126, 151, 152, 159, 182, 240, 282]. Since cardiac efficiency is believed to contribute to both diabetes and heart failure, similar strategies may be promising for the treatment of both diseases. Further, since insulin resistance is a major risk factor for heart failure, improving insulin resistance can also be beneficial in heart failure [114, 117, 119, 124, 159, 170, 182, 183, 190, 240, 245, 261, 269, 315, 316]. Some of the drugs used in basic and clinical studies to modulate glucose and fatty acid oxidation include dichloroacetate (DCA; PDK inhibitor), perhexeline and MCD inhibitors (mitochondrial fatty acid uptake inhibitors), PPARα agonists, and trimetazidine (fatty acid oxidation inhibitor) [44, 70, 90, 91, 121, 159, 238, 239, 241, 249, 262, 313].

Fatty acid oxidation is a promising target for the treatment of heart disease. For example, in heart failure trimetazidine improves cardiac function and lowers proton levels by improving the coupling of glycolysis and glucose oxidation [90, 91, 241]. Trimetazidine also improves cardiac function of diabetic mice [147]. Interestingly, some studies have also reported beneficial effects of PPAR agonists on cardiac function. Beneficial effects of PPAR agonists on cardiac function have been attributed to stimulation of peripheral tissue fatty acid oxidation which lowers circulating fatty acids and cardiac fatty acid oxidation [35, 56, 121, 159, 238, 239, 249, 313]. Another way to inhibit fatty acid oxidation is by reducing mitochondrial fatty acid uptake. While MCD inhibitors have not yet been tested in heart failure, MCD inhibition both improves insulin sensitivity and protects against ischemia [44, 70, 262, 282]. It is important to note, however, that directly inhibiting fatty acid oxidation may not be desirable in heart failure because it may decrease ATP levels in a condition where ATP levels are already reduced. Therefore, strategies to treat heart failure that target energy metabolism need to be carefully considered in order to not further exacerbate a condition with a low ATP supply.

Directly stimulating glucose oxidation is also a promising strategy for improving cardiac function. For example, it has been reported that DCA improves cardiac function in both right ventricle hypertrophy and during ischemia/reperfusion [120, 152, 221, 222, 273]. DCA increases PDH activity and glucose oxidation by inhibiting PDK activity. Further, treatment of Dahl salt-

sensitive rats on a high salt diet, a model of heart failure, with DCA was also beneficial. DCA was effective at activating cardiac PDH, decreasing plasma lactate levels, and improving diastolic function and overall survival [120]. It will be important to further examine whether direct stimulation of glucose oxidation can improve and prevent cardiac dysfunction in diabetes and heart failure.

BCAA oxidation is another promising target for improving cardiac function. As discussed previously, BCAAs regulate insulin resistance indicating that they contribute to the changes in cardiac energy metabolism in diabetes and heart failure. A positive correlation has been reported between BCAA related metabolic clusters and heart disease [110, 252]. Further, BCAAs are elevated in both human and rodent failing hearts [120, 246]. Patients with propionic aciduria and methylmalonic acidemia, diseases caused by defects in BCAA oxidation enzymes, frequently develop cardiomyopathy [110]. This suggests that strategies that reduce BCAA levels, such as stimulating BCAA oxidation, could be beneficial in heart failure. Further work will be needed to determine the importance of BCAA oxidation in the development of heart failure and if promoting BCAA oxidation can be beneficial in heart failure.

1.8.2 Modulation of energy metabolism as a potential strategy to promote cardiac myocyte differentiation

One of the major factors that hinder the success of heart stem cell therapy is negligible rates of stem cell-to-cardiac myocyte differentiation. Most studies

report low rates of differentiation *in vitro* (≤30%) and negligible cardiomyocyte differentiation *in vivo* [19, 22, 93, 303, 304]. I believe that the development of strategies to fix these issues will require an understanding of stem cell energy metabolism.

The evidence points to three possible strategies for modulating energy metabolism and promoting cardiac myocyte differentiation. The first potential strategy for promoting cardiac myocyte differentiation is stimulating overall mitochondrial oxidative metabolism. In addition to mitochondrial biogenesis and elevated mitochondrial oxidative metabolism occurring during cardiac myocyte differentiation, inhibition of the electron transport chain inhibits this differentiation [22, 51, 54, 217]. It may be necessary to stimulate either glucose oxidation or fatty acid oxidation specifically to promote stem cell-to-cardiac myocyte differentiation. Evidence to suggest that stimulating fatty acid oxidation can promote cardiac myocyte differentiation comes from work looking at the importance of PPARs [65, 66, 256]. Cardiac myocyte differentiation is enhanced by PPARs, which can promote fatty acid oxidation [65, 66, 256]. Improving the coupling of glycolysis by decreasing glycolysis and/or stimulating glucose oxidation is another potential strategy to promote stem cell-to cardiac myocyte differentiation and heart regeneration. During cardiac myocyte differentiation there is a decrease in lactate production which may be due to a restructuring of the glycolytic pathway that would be expected to result in increased coupling of glycolysis and glucose oxidation [50, 51]. Further, proliferation ceases upon

terminal cardiac myocyte differentiation [11, 156, 259]. This suggests that inhibiting glycolysis or improving the coupling of glycolysis and glucose oxidation could enhance differentiation, potentially by promoting this reduction in proliferation. It may also be that at different stages of cardiac myocyte differentiation different energy metabolism pathways need to be regulated to promote differentiation. The effect of stimulating glucose oxidation and fatty acid oxidation on cardiac myocyte differentiation will need to be determined.

Lessening the Warburg effect by stimulating glucose oxidation can actually reduce cancer and endothelial cell proliferation and may be beneficial in the treatment of cancer [128, 291]. *In vitro* experiments have indicated that stimulating glucose oxidation with DCA or by re-expressing MPC both stimulates glucose oxidation and decreases the proliferation and survival of cancer cells *in vitro* [25, 127, 179, 247]. In addition, there is evidence that DCA also reduces the proliferation and survival of endothelial cells and angiogenesis *in vitro* [127, 179]. Further, DCA reduces glioblastoma growth in human patients [179]. This was also accompanied by a decrease in angiogenesis, indicating an effect of DCA on endothelial cell proliferation in addition to effects directly on cancer cells [179]. Last year a study was published showing that treatment of animals with DCA both decreases the growth of tumors from human kidney cancer cells and angiogenesis [127]. However, further research is needed to follow up on recent research indicating that DCA may need to be combined with other drugs to be an effective therapy in the clinic [49, 128].

1.9 Hypothesis

General Hypothesis

In heart failure changes in cardiac energy metabolism occur that decrease cardiac efficiency, which is believed to reduce cardiac function. A decrease in oxidative metabolism and increase in glycolysis is observed in severe heart failure, which resembles a shift back toward a fetal heart metabolism. This increase in glycolysis relative to glucose oxidation decreases cardiac efficiency. I hypothesize that the changes in energy metabolism that occur during cardiac myocyte differentiation and heart failure are on opposite ends of a continuum. While there is a decrease in glycolysis and increase in mitochondrial oxidative metabolism during cardiac myocyte differentiation, there is an increase in glycolysis and decrease in mitochondrial oxidative metabolism in heart failure. I also hypothesize that a reliance on an efficient energy source is important in maintaining cardiac function. Optimizing the heart's use of glucose and fatty acid oxidation can improve cardiac function.

Chapter 3 Specific Hypothesis

I hypothesize that stem cell energy metabolism is primarily glycolytic and has low rates of mitochondrial oxidative metabolism and that this metabolism is important in regulating stem cell survival and proliferation. This will be tested by measuring glucose oxidation, fatty acid oxidation, and glycolysis in BMMSCs. I

will then assess the effect of fatty acids on the proliferation and survival of BMMSCs and at the same time determine what changes in these metabolic pathways occur in response to fatty acids. This will add to our understanding of the energy metabolism that characterizes a cell prior to cardiac myocyte differentiation and the role of energy metabolism in proliferation, a form of cell growth which decreases during cardiac myocyte differentiation.

Chapter 4 Specific Hypothesis

I hypothesize that during cardiac myocyte differentiation overall mitochondrial oxidative metabolism, including glucose and fatty acid oxidation, increase and glycolysis decreases. This will be determined by examining the change in glucose oxidation, fatty acid oxidation, and glycolysis that occur in response to 7 days of H9C2 cell differentiation toward a cardiac myocyte. This will increase our understanding of the changes in energy metabolism that occur during early cardiac myocyte differentiation.

Chapter 5 Specific Hypothesis

I hypothesize that decreased cardiac efficiency contributes to the development of heart failure and that stimulating glucose oxidation can prevent the development of heart failure. This will be tested in this chapter by determining if insulin glargine, which stimulates glucose oxidation and inhibits fatty acid oxidation, can improve the function of *db/db* mouse hearts. I will focus on cardiac

function in this model of insulin resistance due to the importance of insulin resistance in the development of heart failure. 18 wk old *db/db* mice will be treated with insulin glargine (a long acting insulin) for 4 wk. *In vivo* cardiac function will be determined using ultrasound echocardiography and *ex vivo* cardiac function will be assessed in the isolated working heart. I will also assess the long term effect of insulin glargine on cardiac energy metabolism using the isolated working heart. Finally, I will measure levels of short chain CoAs and other parameters of cardiac insulin signaling in these hearts.

Chapter 6 Specific Hypothesis

I hypothesize that heart failure is broadly characterized by a decline in mitochondrial oxidative metabolism and increase in glycolysis. This will be examined by determining what changes in cardiac energy metabolism occur during the development of heart failure with preserved ejection fraction (HFpEF). Energy metabolism will be assessed using the isolated working heart perfusion in hearts from Dahl salt-sensitive rats fed a high salt diet (which induces HFpEF) for 0, 3, 6, or 9 wk. *In vivo* cardiac function will be determined using ultrasound echocardiography and *ex vivo* cardiac function will be assessed in the isolated working heart.

Chapter 7 Specific Hypothesis

I hypothesize that BCAA oxidation is involved in the impairment of insulin stimulated glucose oxidation observed in cardiac insulin resistance. It has been suggested that BCAA oxidation may contribute to insulin resistance by competing with flux through glucose oxidation. I will begin testing this hypothesis by determining the contribution of BCAA oxidation to ATP production in high fat diet-induced obese mice, which have cardiac insulin resistance. BCAA oxidation, glycolysis, glucose oxidation, and fatty acid oxidation will be measured and used to determine the contribution of BCAA oxidation to overall ATP production in the heart. I will then determine the effect that stimulating BCAA oxidation *in vivo* and *ex vivo* has on cardiac insulin sensitivity using the insolated working heart. This research will increase our understanding of how insulin stimulated glucose oxidation becomes impaired in cardiac insulin resistance. Because insulin resistance is an important factor in the development of heart failure this may also provide further insight into the development of heart failure.

Figure 1-1. Diagram of hypothesis of the changes in energy metabolism that occur during cardiac myocyte differentiation and development of heart failure. I hypothesized that the changes in energy metabolism that occur during cardiac myocyte differentiation and heart failure are on opposite ends of a continuum. During cardiac myocyte differentiation there is a decrease in glycolysis and increase in mitochondrial oxidative metabolism while there is an increase in glycolysis and decrease in mitochondrial oxidative metabolism in heart failure.

Figure 1-1

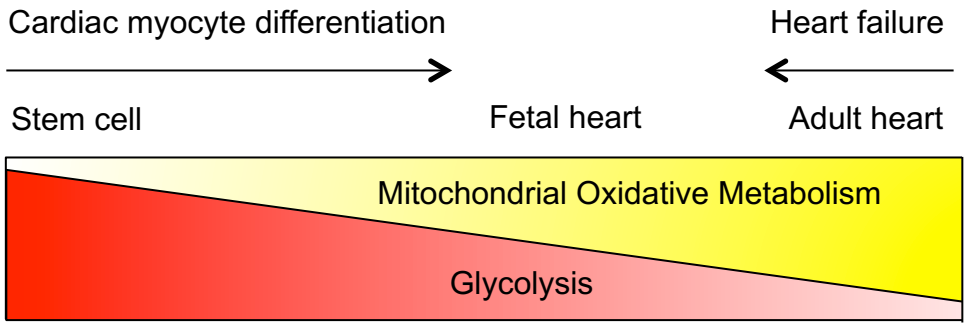

Cardiac myocyte differentiation
Heart failure
Stem cell
Fetal heart
Adult heart
Mitochondrial Oxidative Metabolism
Glycolysis

Figure 1-2. Overview of cardiac glucose and fatty acid energy metabolism. Two of the major substrates the heart uses for energy production are fatty acids and glucose. In the cardiac myocyte the dominant glucose transporter is GLUT4. Once the intracellular glucose is phosphorylated by hexokinase it can enter glycolysis which will produce pyruvate. Pyruvate can be transported into the mitochondria via MPC and undergo glucose oxidation (be converted to acetyl CoA by PDH) which can be used by the TCA cycle and electron transport chain to produce ATP. The pyruvate can also be converted to lactate by LDH. This also results in an increase in cytoplasmic proton levels. Fatty acids primarily enter the cell through fatty acid transporters such as CD36. Once it is converted to fatty acyl CoA it can be transported into the mitochondria via a multi protein process. Fatty acid oxidation then breaks down fatty acids to acetyl CoAs that can be used, again, for ATP production.

Figure 1-2

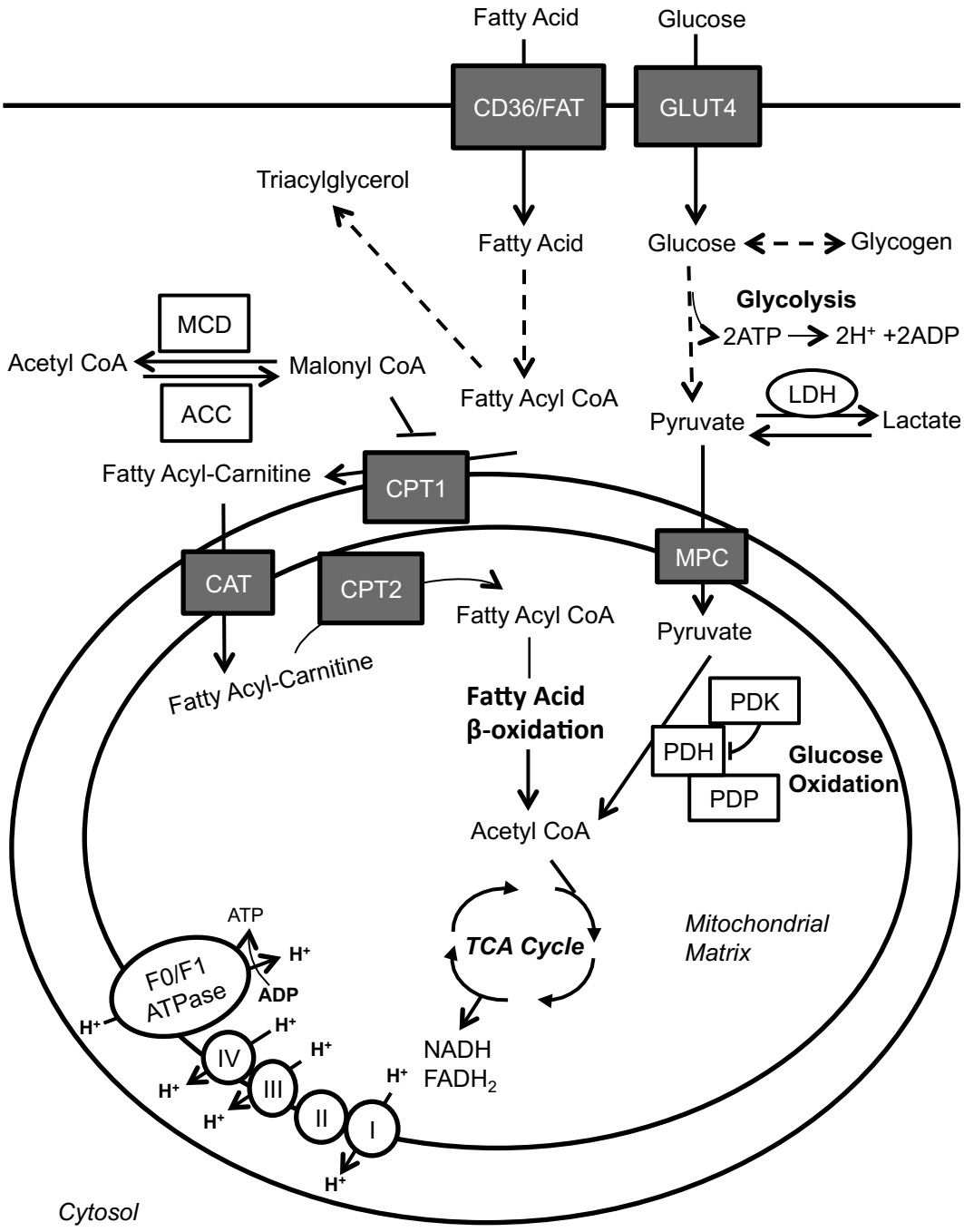

Fatty Acid
Glucose
CD36/FAT
GLUT4
Triacylglycerol
Fatty Acid
Glucose
Glycogen
Glycolysis
2ATP
2H+ +2ADP
MCD
Acetyl CoA
Malonyl CoA
ACC
Fatty Acyl CoA
LDH
Pyruvate
Lactate
Fatty Acyl-Carnitine
CPT1
MPC
CAT
CPT2
Fatty Acyl CoA
Pyruvate
Fatty Acyl-Carnitine
Fatty Acid
β-oxidation
PDK
PDH
PDP
Glucose
Oxidation
Acetyl CoA
ATP
F0/F1
ATPase
ADP
H+
TCA Cycle
Mitochondrial
Matrix
NADH
FADH2
IV
III
II
I
Cytosol

Figure 1-3. Glycolysis.

Figure 1-3

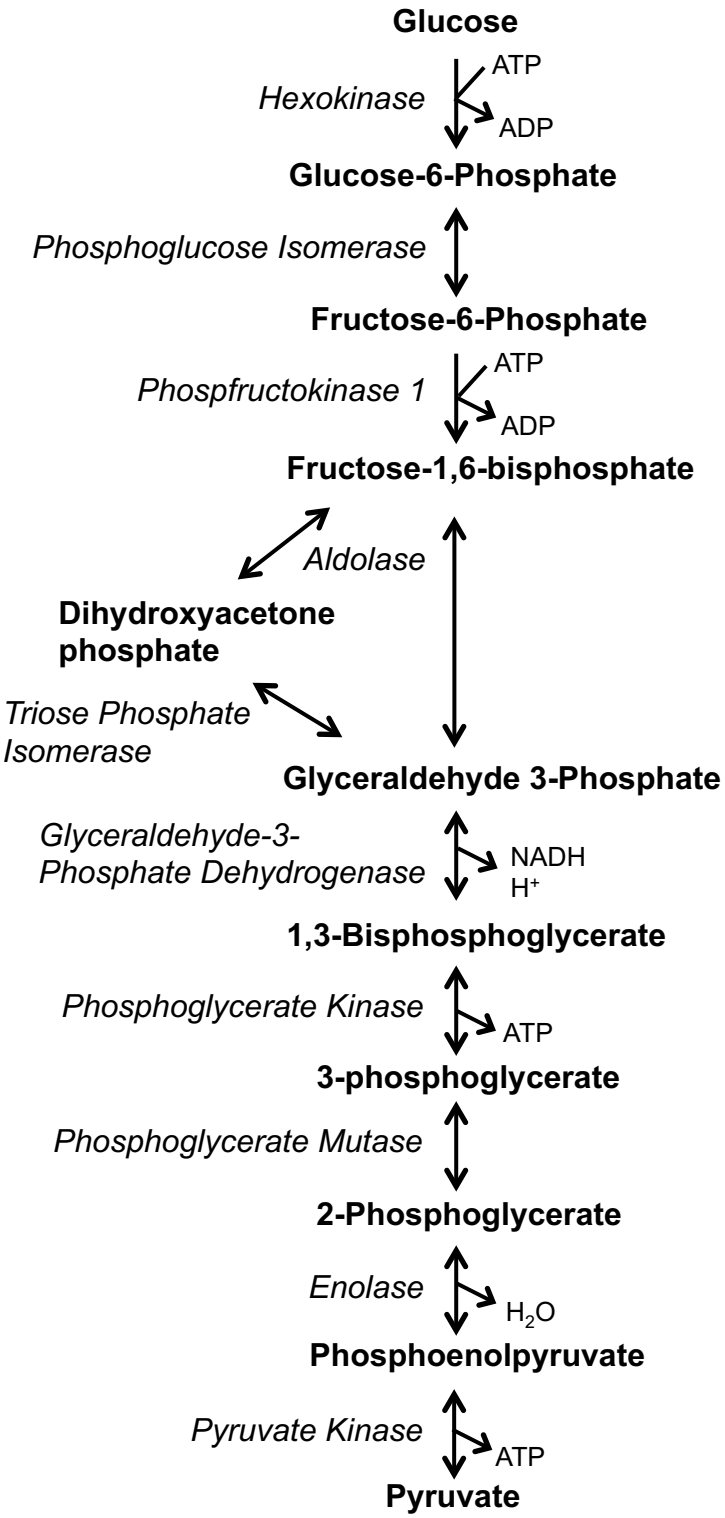

Glucose
ATP
Hexokinase
ADP
Glucose-6-Phosphate
Phosphoglucose Isomerase
Fructose-6-Phosphate
ATP
Phospfructokinase 1
ADP
Fructose-1,6-bisphosphate
Aldolase
Dihydroxyacetone phosphate
Triose Phosphate Isomerase
Glyceraldehyde 3-Phosphate
Glyceraldehyde-3-Phosphate Dehydrogenase
NADH
H+
1,3-Bisphosphoglycerate
Phosphoglycerate Kinase
ATP
3-phosphoglycerate
Phosphoglycerate Mutase
2-Phosphoglycerate
Enolase
H2O
Phosphoenolpyruvate
Pyruvate Kinase
ATP
Pyruvate

Figure 1-4. Uncoupling of glycolysis and glucose oxidation decreases cardiac efficiency. A decrease in the coupling of glycolysis and glucose oxidation results in increased proton levels. Removal of these protons results in a net increase in calcium transport into the cell. ATP is used to remove this calcium and maintain calcium homeostasis.

Figure 1-4

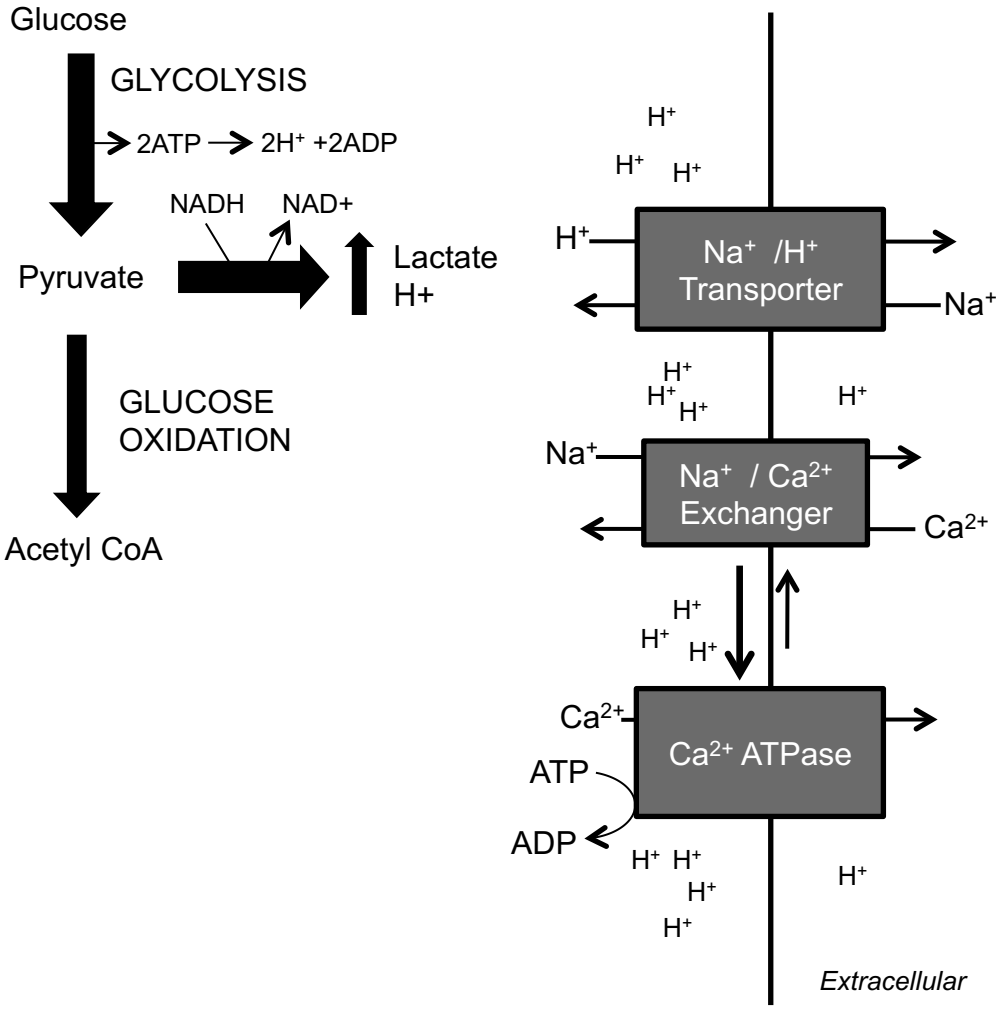

Glucose
GLYCOLYSIS
2ATP → 2H+ +2ADP
NADH
NAD+
Pyruvate
Lactate
H+
GLUCOSE
OXIDATION
Acetyl CoA
H+
H+ H+
H+
Na+ /H+
Transporter
Na+
H+
H+ H+
H+
Na+
Na+ / Ca2+
Exchanger
Ca2+
H+
H+ H+
Ca2+
Ca2+ ATPase
ATP
ADP
H+ H+
H+
H+
H+
Extracellular

Figure 1-5. Warburg effect and differentiation. The Warburg effect refers to a high uncoupling of glycolysis and glucose oxidation under aerobic conditions. Proliferative cells are frequently characterized by a high uncoupling of glycolysis and glucose oxidation and a low mitochondrial oxidative metabolism. Non-proliferative cells, such as differentiated cells, have better coupling of glycolysis and glucose oxidation and higher mitochondrial oxidative metabolism.

Figure 1-5

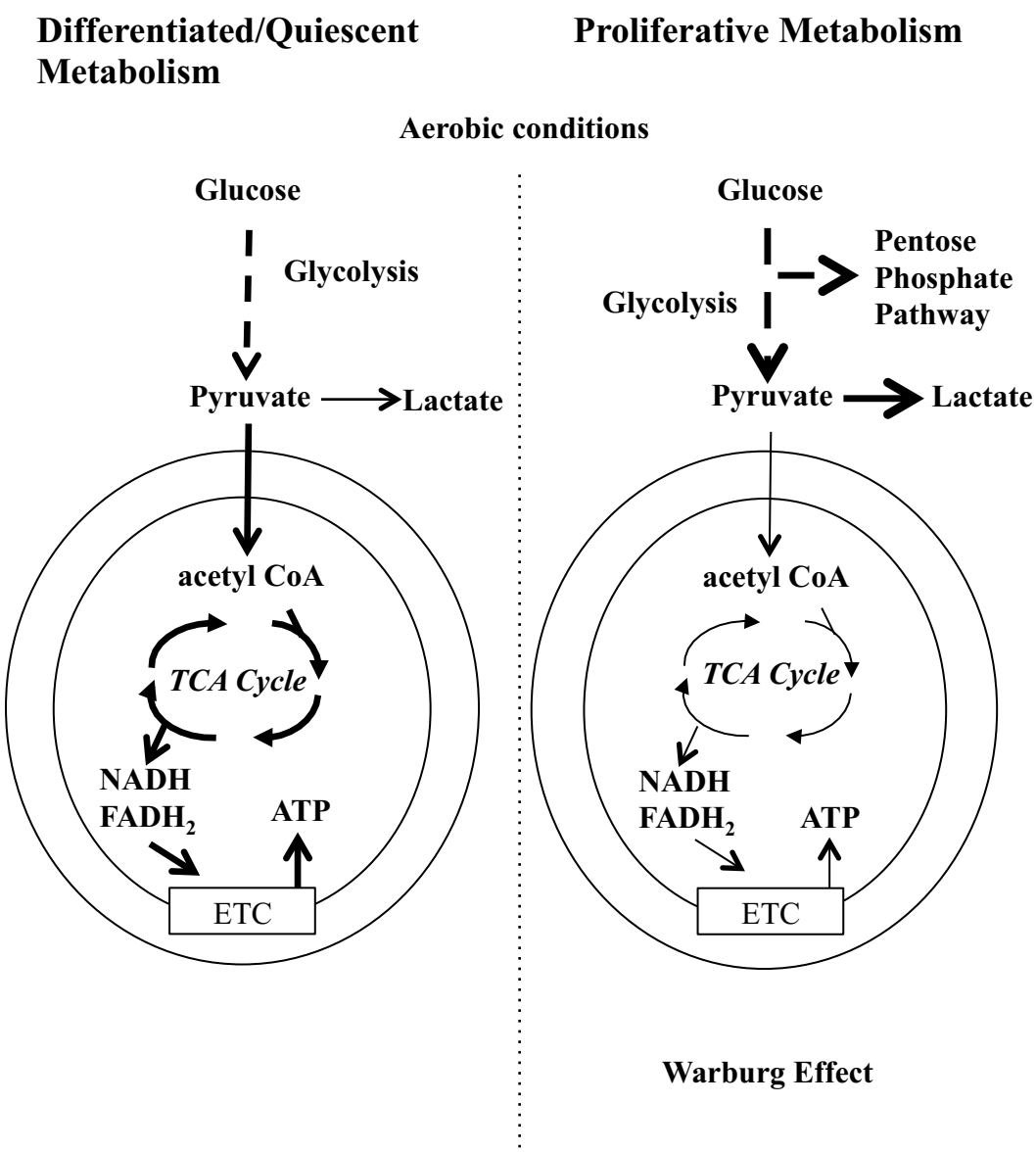

Differentiated/Quiescent Metabolism
Proliferative Metabolism
Aerobic conditions
Glucose
Glycolysis
Pyruvate
Lactate
acetyl CoA
TCA Cycle
NADH
FADH2
ATP
ETC
Glucose
Pentose Phosphate Pathway
Glycolysis
Pyruvate
Lactate
acetyl CoA
TCA Cycle
NADH
FADH2
ATP
ETC
Warburg Effect

Figure 1-6. Uncoupling of glycolysis and glucose oxidation and heart failure. Heart failure is generally associated with a decrease in overall mitochondrial oxidative metabolism and increase in glycolysis. This increase in uncoupling of glycolysis and glucose oxidation results in increased proton levels. ATP is utilized to both remove these protons and maintain sodium and calcium homeostasis. These changes in energy metabolism, therefore, reduce cardiac efficiency and are believed to contribute to heart failure.

Figure 1-6

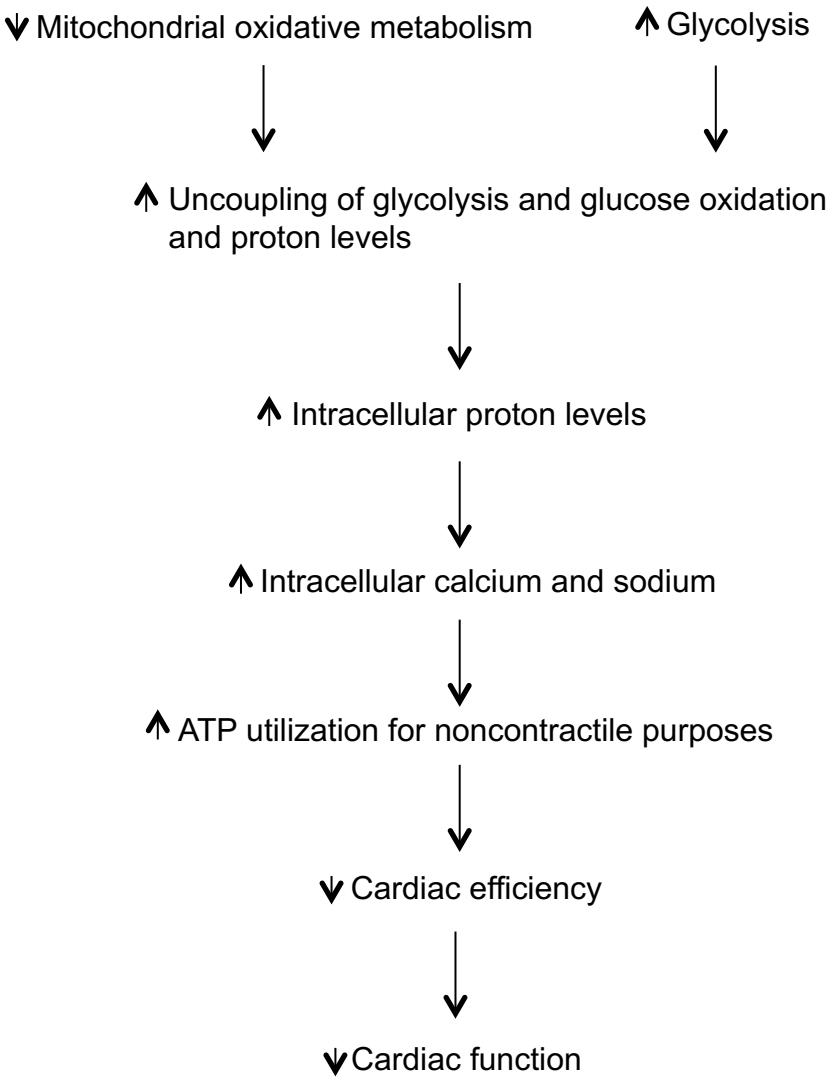

↓ Mitochondrial oxidative metabolism
↑ Glycolysis
↑ Uncoupling of glycolysis and glucose oxidation and proton levels
↑ Intracellular proton levels
↑ Intracellular calcium and sodium
↑ ATP utilization for noncontractile purposes
↓ Cardiac efficiency
↓ Cardiac function

Figure 1-7. Energy metabolism and diabetic cardiomyopathy. Diagram of how alterations in energy metabolism and insulin resistance can lead to cardiac dysfunction.

Figure 1-7

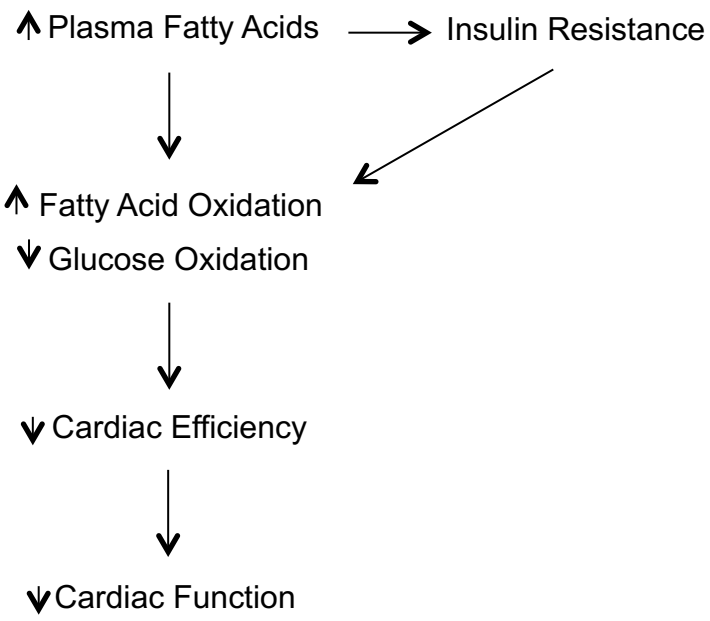

↑ Plasma Fatty Acids
Insulin Resistance
↑ Fatty Acid Oxidation
↓ Glucose Oxidation
↓ Cardiac Efficiency
↓ Cardiac Function

Figure 1-8. BCAA oxidation.

Figure 1-8

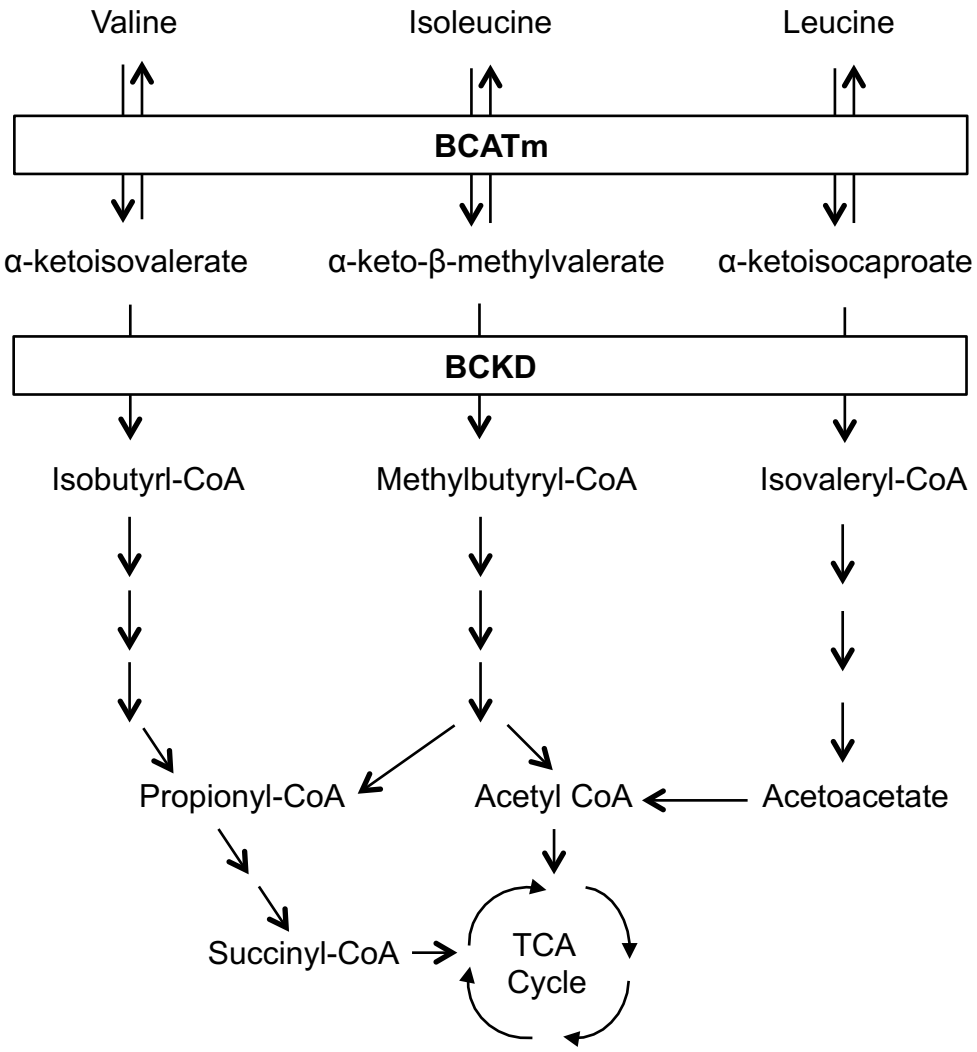
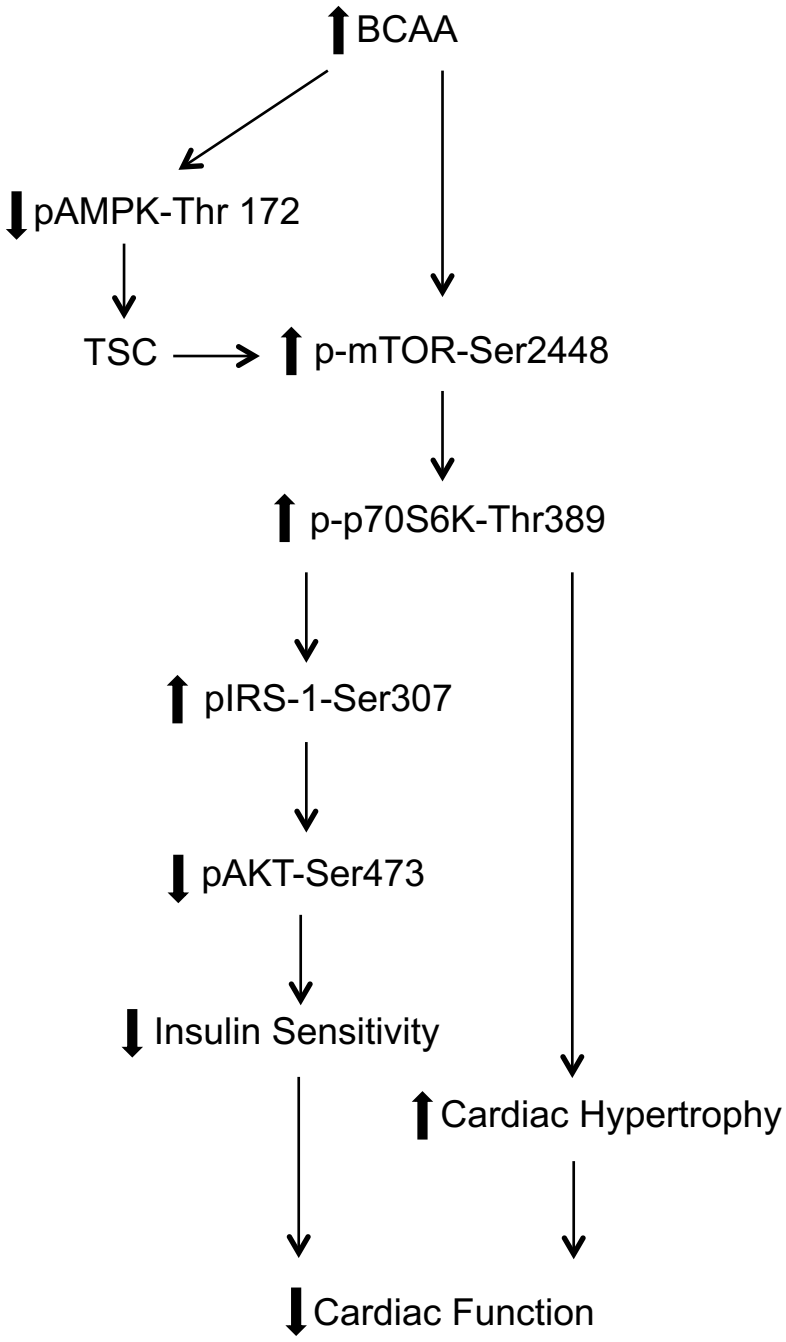

Valine
Isoleucine
Leucine
BCATm
α-ketoisovalerate
α-keto-β-methylvalerate
α-ketoisocaproate
BCKD
Isobutyrl-CoA
Methylbutyryl-CoA
Isovaleryl-CoA
Propionyl-CoA
Acetyl CoA
Acetoacetate
Succinyl-CoA
TCA
Cycle

Figure 1-9. Proposed pathway for BCAA induced insulin resistance through regulation of the mTOR pathway. BCAAs have been suggested to stimulate the mTOR pathway through inhibition of AMPK and activation of mTOR. Increased mTOR signaling can impair insulin signaling. mTOR can induce cardiac hypertrophy which may also contribute to the cardiac dysfunction frequently observed in diseases associated with insulin resistance including diabetic cardiomyopathy and heart failure.

Figure 1-9


BCAA
pAMPK-Thr 172
TSC
p-mTOR-Ser2448
p-p70S6K-Thr389
pIRS-1-Ser307
pAKT-Ser473
Insulin Sensitivity
Cardiac Hypertrophy
Cardiac Function

CHAPTER 2

Materials and Methods

CHAPTER 2

Materials and methods

2.1 Introduction

In this thesis I focused on the regulation of glucose oxidation in cardiac myocyte differentiation, heart failure, and cardiac insulin resistance. *In vitro* experiments were conducted in bone marrow mesenchymal stem cells (BMMSC) and H9C2 cells to characterize stem cell energy metabolism and the changes in energy metabolism that accompany cardiac myocyte differentiation. The methods that were used to assess energy metabolism and other parameters in cell culture are provided below. Details that are specific to a given set of experiments are provided in the "Materials and methods" sections of Chapters 3 and 4.

To assess energy metabolism in insulin resistance and heart failure a series of *in vivo* experiments were also conducted. An initial set of *ex vivo* experiments in the isolated working heart perfusion were conducted in C57bl6 mice and *db/db* mice to characterize the effect of insulin glargine on cardiac energy metabolism. *Db/db* mice were then treated for 4 wk with insulin glargine and energy metabolism and cardiac function were assessed. To determine the changes in energy metabolism that occur in the development of heart failure with preserved ejection fraction (HFpEF) I induced diastolic dysfunction in Dahl salt-sensitive rats by feeding them a high salt diet (HSD). Energy metabolism and cardiac function were assessed in Dahl salt-sensitive rats after 0, 3, 6, or 9 wk on the

HSD. Finally, I conducted a series of experiments to examine whether branched chain amino acids (BCAA) oxidation contributes to cardiac insulin resistance by impairing insulin stimulated glucose oxidation. Initially, the contribution of BCAA oxidation to overall energy metabolism was measured in mice fed a high fat diet (HFD) for 10 wk to induce cardiac insulin resistance. In the second experiment, mice were fed a HFD for 5 wk. During the 5th wk BCAA oxidation was stimulated with a branched chain ketoacid dehydrogenase (BCKD) kinase inhibitor and the effect on cardiac insulin resistance was determined. Finally, the direct effect of inhibiting BCKD kinase on cardiac BCAA oxidation and insulin sensitivity was assessed *ex vivo* in the isolated working heart perfusion. Cardiac metabolic rates were measured using the isolated working heart perfusion. The methods used to assess energy metabolism, including the isolated working heart perfusion, high performance liquid chromatography (HPLC) to measure changes in short chain CoA levels, and assessment of enzyme expression by western blot, are described below. Cardiac function was examined both *ex vivo* using the isolated working heart and *in vivo* using ultrasound echocardiography. Details that are specific to a given study will be provided in the "Materials and methods" sections of Chapters 5, 6, and 7.

2.2 Materials

[5-^{3}H] glucose, [U-^{14}C] glucose, [1-^{14}C] palmitate, [1-^{14}C]oleate, [9,10-^{3}H] palmitate, [U-^{14}C] lactate, [U-^{14}C] valine/leucine/isoleucine and Western Lightning Plus- enhanced chemiluminescence (ECL) substrate were obtained from Perkin Elmer (Waltham, Massachusetts). ScintiSafe aqueous counting scintillation fluid was obtained from Fisher Scientific (Fair Lawn, New Jersey). Hyamine hydroxide was obtained from Curtis Laboratories (Bensalem, Pennsylvania). Fatty acid free bovine serum albumin (BSA) was obtained from Equitech-Bio Inc (Kerrville, Texas). Free fatty acid (FFA) assay kit was obtained from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Insulin (Novolin ge Toronto) was obtained from Novo Nordisk (Mississauga, Ontario) through the University of Alberta hospital stores. Insulin glargine was obtained from Sanofi (Laval, Quebec). Mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Dahl salt sensitive rats were purchased from Charles River Laboratories (Wilmington, Massachusetts). Nova-Pak Supelcosil, 4 μm particle size, C18 pre-column cartridge was purchased from Waters Company (Milford, Massachusetts) and Adsorbosphere C18, 3μm particle size, 150 x 4.6 mm column was obtained from Mandel Scientific (Guelph, Ontario) for HPLC measurement of short chain CoAs. Cell culture supplies were purchased from Sigma Aldrich (St Louis, Missouri) and Life Technologies (Carlsbad, California). Tetramethylrhodamine methyl ester (TMRM), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit, and 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) was obtained from Life Technologies (Carlsbad, California). Ki67 and bromodeoxyuridine (BrdU) antibody was obtained from Dako (Markham, Ontario). Hypoxia inducible factor (HIF)1α primary antibody was obtained from Novus Biologics (Littleton, Colorado). phospho BCKD (pBCKD) Ser293 primary antibody was purchased from Bethyl Laboratories (Montgomery, Texas). Mitochondrial branched chain amino transferase (BCATm) primary antibody was purchased from Thermo Fisher Scientific (Waltham, Massachusetts). Lactate dehydrogenase (LDH)A, glucose transporter (GLUT)1, GLUT4, and cytochrome c primary antibodies and goat anti rabbit, goat anti mouse, and donkey anti goat antibodies were obtained from Santa Cruz (Santa Cruz, California). Isocitrate dehydrogenase (IDH), hydroxyacyl CoA dehydrogenase (HADH), long chain acyl CoA dehydrogenase (LCAD), BCKD, BCKD kinase, mitochondrial protein phosphatase 2C (PP2Cm), and peroxisome proliferator activated receptor (PPAR)α primary antibodies were obtained from Abcam (Toronto, Ontario). Phosphoglycerate mutase 1 (PGAM1), pyruvate dehydrogenase (PDH), 5' AMP-activated protein kinase (AMPK), pAMPK Threonine 172, p70S6 kinase (p70S6K), p-p70S6K Threonine 389, pAkt Serine 473, Akt, mechanistic target of rapamycin (mTOR), p-mTOR Serine 2448, glycogen synthase kinase (GSK)3β, pGSK3α/β Serine 21/9 primary antibodies were obtained from Cell Signaling (Danvers, Massachusetts). pACC Serine 79, insulin receptor substrate (IRS)1, and pIRS1 Serine 307 primary antibody was obtained from Millipore (Darmstadt, Germany). Peroxidase labeled streptavidin

was obtained from Jackson Immunoresearch (West Grove, Pennsylvania). pPDH Serine 293 primary antibody was obtained from Calbiochem (San Diego, California). Human BMMSCs were obtained from Texas A&M Health Science Center. Rodent diets were obtained from Research Diets (New Brunswick, New Jersey). Vevo 770 was obtained from VisualSonics (Toronto, Ontario). Zeiss LSM 510 software and Zeiss LSM 510 NLO microscope were obtained from Carl Zeiss Microscopy GmbH (Jena, Germany). Nitrocellulose membrane was obtained from BioRad Laboratories (Munich, Germany). Medical x-ray film was obtained from FUJIFILM Corporation (Tokyo, Japan). All other chemicals used in these studies were obtained from Sigma Aldrich (St Louis, Missouri).

2.3 Methods

2.3.1 Cell culture

Human BMMSCs and H9C2 cells were used in the cell culture experiments described in this thesis. Cells were cultured at 37°C and 5% CO_2 and standard cell culture procedures were followed [177]. More detail for each cell line and the experiments conducted with each cell line are provided below.

Already characterized human BMMSCs were purchased from the Texas A&M Health Science Center. Human BMMSCs were cultured in low glucose α-modified eagle medium (MEM) supplemented with 16.5% fetal bovine serum (FBS), 1% glutamine, and 1% streptomycin/penicillin. A series of experiments

were conducted to assess the chronic and acute effects of palmitate and oleate on BMMSCs. BMMSCs were treated with cell culture media supplemented with either 4% fatty acid free BSA (Equitech-Bio Inc BAH66) or 4% BSA bound to fatty acid (the type and concentration of fatty acid is provided for each experiment in Chapter 3) to assess the chronic effect of fatty acids. BMMSCs were cultured under normal conditions described above before assessing the acute effect of fatty acids during assays measuring glucose oxidation, palmitate oxidation, oleate oxidation or glycolysis. More details on this assay and the media supplemented with BSA and fatty acids are provided in the "Measurement of energy metabolism in cells" section.

H9C2 cells were cultured in media containing high glucose delbecco's modified eagle medium (DMEM), 10% FBS, 1% penicillin/streptomycin, and 0.25 mM carnitine. To induce cardiac myocyte differentiation H9C2 cells were allowed to reach 90% confluency and then were differentiated for 7 days in differentiation media (high glucose DMEM, 1% fetal bovine serum, 1% penicillin/streptomycin, 0.25 mM carnitine, 10 nM retinoic acid). Media was changed every 48-72 hr during the 7 day differentiation period.

2.3.2 Insulin glargine treatment

10 wk old C57bl6 and *db/db* mouse hearts were treated acutely with vehicle or insulin glargine during the working heart perfusion. Vehicle or increasing amounts of insulin glargine (C57bl6: 0, 25, 50, 100 μU/ml; db/db: 0,

50, 200, 500 μU/ml) were added to the perfusate every 18 min. A set of 18 wk old *db/db* mice were subcutaneously injected with either vehicle (saline) or insulin glargine (150 U/kg BW) daily for 4 wk. This set of experiments was conducted in male mice. Mice were fasted for 12 hours prior to measurement of whole body glucose tolerance. Mice were in the fed state when euthanized.

2.3.3 BCKD kinase inhibitor treatment

This set of experiments was conducted in male mice. Mice were in the fed stated when euthanized. Mice received intraperitoneal injections with vehicle (5% dimethyl sulfoxide (DMSO), 10% cremophor EL, and 85% 0.1 M sodium bicarbonate, pH 9.0) or BCKD kinase inhibitor (20mg/kg/day; Sigma, L499110) daily for 1 wk. The vehicle or BCKD kinase inhibitor was administered to mice on the standard chow diet or during their 5^{th} wk on the HFD.

Vehicle or BCKD kinase inhibitor was added to hearts from another set of mice at the beginning of the isolated working heart perfusion. The BCKD kinase inhibitor (200 μM) was either dissolved in DMSO or a chremophore solution (5% DMSO, 10% chremophore, 85% 0.1 M sodium bicarbonate, pH=9.0).

2.3.4 High fat diet feeding protocol

This set of experiments was conducted in male mice. Mice were in the fed state when euthanized. Mice were either fed a standard laboratory chow diet or a HFD (Research Diets, D12492) *ad libitum*. The standard chow diet contains 4%

kcal from fat while 60% kcal are from in the HFD. Lard makes up approximately 90% of the kcal from fat and soybean contributes about 10% of the kcal in the HFD. Depending on the experiment, mice were fed the HFD for 5 or 10 wk.

2.3.5 High salt diet feeding protocol

8 wk old Dahl salt-sensitive malerats were either fed a low salt diet (0.3% NaCl) or a HSD (8% NaCl) *ad libitum*. Rats were fed the HSD for either 0, 3, 6, or 9 wk. Rats were in the fed stated when euthanized.

2.3.6 Isolated working heart perfusions

Rodents were anesthetized with sodium pentobarbital. Once fully anesthetized, the hearts were quickly removed, the aorta was cannulated and hearts were equilibrated in the Langendorff mode in Krebs-Henseleit buffer (25 mM $NaHCO_3$, 4.7 mM KCl, 118.5 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$). Once the heart were trimmed of excess tissue and the left atrium was cannulated the heart was switched to working mode by opening up the left aortic inflow and blocking flow into the heart through the aorta inflow line. Hearts were perfused with oxygenated Krebs-Henseleit buffer supplemented with 5 mM glucose, 0.8 mM palmitate bound to 3% fatty acid-free BSA, and appropriate radioactive labeled substrate. Either 0.5 mM lactate or BCAAs (0.15 mM leucine, 0.15 mM isoleucine, and 0.2 mM valine) were also added to the Krebs-Henseleit buffer depending on the study. [U-^{14}C] glucose, [5-^{3}H] glucose,

[9,10-^{3}H] palmitate, [U-^{14}C] lactate, or [U-^{14}C] valine/leucine/isoleucine were added to the Krebs-Henseleit buffer in order to measure glucose oxidation, glycolysis, palmitate oxidation, lactate oxidation, and BCAA oxidation, respectively. Glycolysis and palmitate oxidation were calculated based on the amount of $^{3}H_2O$ produced. Glucose oxidation, lactate oxidation, and BCAA oxidation were calculated based on $^{14}CO_2$ production [20, 282]. Afterload in the perfusion system was 50 mmHg for the mouse hearts and 80 mmHg for the rat hearts. Depending on the experiment two perfusion protocols were followed. In the first, hearts were aerobically perfused for 60 min with 100 μU/ml insulin added to the perfusate 30 min into perfusion. Samples were taken every 10 min to determine $^{3}H_2O$ and $^{14}CO_2$ production. In the second protocol, hearts were aerobically perfused for 72 min. Vehicle or increasing amounts of insulin glargine were added to the perfusion buffer every 18 min. Samples were taken every 6 min to determine $^{3}H_2O$ and $^{14}CO_2$ production. The amount of $^{3}H_2O$ produced was determined using the $^{3}H_2O$ extraction assay. $^{14}CO_2$ in the perfusate and released as gas was measured to determine the entire amount produced. $^{14}CO_2$ trapped inside the perfusion buffer was released by adding a buffer sample to a vial with sulphuric acid. The released $^{14}CO_2$ bound to hyamine soaked filter paper placed inside scintillation vials at the top of these glass vials. The gaseous $^{14}CO_2$ exited the perfusion system via an exhaust line and was captured in a tube containing hyamine hydroxide. Hearts were immediately frozen in liquid N_2 at the end of the perfusion period and were stored at -80°C [20, 282].

2.3.7 Measurement of mechanical function in the isolated working heart

The mechanical function of the isolated working heart was assessed using a Powerlab acquisition system (to measure heart rate and pressures) and a Transonic flow meter and probes (to assess cardiac flow). A Harvard apparatus pressure transducer attached to the aortic outflow line was used to measure the heart rate and aortic pressure. Transonic probes placed in the preload and afterload lines were used to measure cardiac output and aortic flow. To calculate cardiac work (joules/min/g dry weight), preload pressure subtracted from peak systolic pressure was multiplied by cardiac output and then normalized against the dry weight of the heart. Aortic flow (ml/min) was subtracted from cardiac output (ml/min) to calculate coronary flow (ml/min).

2.3.8 Measurement of energy metabolism in cells

Assays were performed on cells grown in T25 flasks. The cell culture media was replaced with Krebs Henseleit buffer supplemented with at least 0.55 mM fatty acid free BSA, 5 mM glucose, and the appropriate radioactive labeled oleate, palmitate, or glucose. The presence of 0.4 mM oleate and/or 0.4 mM palmitate (bound to the BSA) in the Krebs Henseleit buffer is indicated for each experiment in Chapters 3 and 4. In order to measure glycolysis, [5-^{3}H]glucose was added to the Krebs Henseleit buffer and the $^{3}H_2O$ production was measured at 37°C for 2 hr. For glucose oxidation, palmitate oxidation, and oleate oxidation [U-

^{14}C]glucose, [1-^{14}C]palmitate, or [1-^{14}C]oleate, respectively, were added to the Krebs Henseleit buffer and $^{14}CO_2$ production was measured at 37°C for 3 hr. To do this T25 flasks were attached to glass CO_2 capture devices containing hyamine hydroxide soaked filter paper [136]. At the end of the 3 hr incubation $^{14}CO_2$ trapped in the buffer was released by addition of 9 M sulphuric acid to the T25 flask. To measure fatty acid uptake cells were instead washed 3X with phosphate buffered saline (PBS), lysed, and the cell lysate was counted in the scintillation counter. All filter papers were placed in scintillation vials, scintillation fluid was added, and vials were counted in a scintillation counter.

2.3.9 Measurement of $^{3}H_2O$ production to measure palmitate oxidation and glycolysis

The $^{3}H_2O$ extraction assay was used to determine the amount $^{3}H_2O$ produced. Duplicate 200 µl samples of buffer were taken from each flask (cell culture) or from the perfusate in the working heart perfusion and placed into 1.5 ml capless centrifuge tubes set inside scintillation vials with 500 µl of double distilled H_2O at the bottom of the vial. $^{3}H_2O$ standard and un-metabolized buffer (buffer not added to a flask or perfusion) were also added in parallel. Scintillation vials were capped and then left at 50°C for 24 hr. Vials were immediately transferred to 4°C overnight. Care was taken to keep any H_2O on the side of each centrifuge tube inside the vials. In duplicate, 200 µl of either $^{3}H_2O$ standard or un-metabolized buffer were also placed inside empty scintillation vials in order to

calculate specific activity and transfer efficiency. Scintillation fluid was added and radioactivity was counted in the scintillation counter.

2.3.10 Calculation of proton production

Proton production was calculated by subtracting the glucose oxidation rate from glycolysis rate and then multiplying the result by 2.

2.3.11 Calculation of ATP production

The metabolic rates measured using the methods described above were used to determine ATP production. For glycolysis and glucose oxidation the calculations were based on the assumption that 2 ATP and 31 ATP, respectively, are produced from one glucose metabolized. For lactate oxidation it is assumed that 14.5 ATP are produced. For palmitate and oleate oxidation 105 and 119 ATP are produced, respectively. Based on the amount of ATP that would be expected to be produced from oxidation of the individual amino acids added to the buffer and the respective concentrations used I assumed that BCAA oxidation produced 20.7 ATP per BCAA oxidized.

2.3.12 Homogenate preparation

Frozen tissue was homogenized on ice for 30 sec and left on ice for at least 10 min. Homogenization buffer contained 50 mM Tris HCl, 10% glycerol, 1 mM ethylene diamine tetraacetic acid (EDTA), 0.02% Brij-35, 1 mM dithiothreitol

(DTT), and protease and phosphatase inhibitors (Sigma). Supernatant was obtained by centrifuging tissue homogenates at 10,000 x g for 20 min. Cells were lysed in cell lysis buffer (20 mM Tris HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 M sucrose, 1 mM DTT, and protease and phosphatase inhibitors (Sigma)) on ice. After sitting on ice for 15 min cell lysates were centrifuged at 1,000xrpm for 10 min. Tissue and cell supernatants were stored at -80°C. The protein concentration of these supernatants was measured using the Bradford protein assay.

2.3.13 Western blot

Standard western blotting protocol was followed. Samples were loaded into 5%, 8%, 10% or 15% Tris-HCl gels and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run initially for 10 min at 60 Volts and then moved up to 120 Volts. Protein was then transferred from these gels onto a 0.45 μm nitrocellulose membrane for 2 hr at 90 Volts. Membranes were then stained with ponceau red to confirm an even protein transfer. If protein did not transfer properly the nitrocellulose membranes were discarded. Membranes were blocked for 1 hr in 5% non-fat dry milk (NFDM) dissolved in Tris Buffered Saline with Tween (TBST) at room temperature. Membranes were then probed overnight with the appropriate primary antibody in 5% BSA in TBST at 4°C: pBCKD Ser293 (Bethyl Laboratories, 1:1000), BCATm (Thermo Fisher Scientific, 1:1000), LDHA (Santa Cruz, 1:1000), GLUT1 (Santa

Cruz, 1:1000), GLUT4 (Santa Cruz, 1:1000), cytochrome c (Santa Cruz, 1:1000), IDH (Abcam, 1:1000), HADH (Abcam, 1:1000), LCAD (Abcam, 1:1000), BCKD (Abcam, 1:1000), BCKD kinase (Abcam, 1:1000), PP2Cm (Abcam, 1:1000), PPARα (Abcam, 1:1000), PGAM1 (Cell Signaling, 1:1000), PDH (Cell Signaling, 1:1000), AMPK (Cell Signaling, 1:1000), pAMPK Threonine 172 (Cell Signaling, 1:1000), p70S6K (Cell Signaling, 1:1000) p-p70S6K Threonine 389 (Cell Signaling, 1:1000), pAkt Serine 473 (Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), mTOR (Cell Signaling, 1:1000), p-mTOR Serine 2448 (Cell Signaling, 1:1000), GSK3β (Cell Signaling, 1:1000), pGSK3α/β Serine 21/9 (Cell Signaling, 1:1000), pACC Serine 79 (Millipore, 1:1000), IRS1 (Millipore, 1:500), pIRS1 Serine 307 (Millipore, 1:500), peroxidase labeled streptavidin used for ACC (Jackson Immunoresearch, 1:2000), or pPDH Serine 293 (1:1000). The next day the membranes were washed 4 x 5 min with TBST and then probed with the appropriate secondary antibody in 1% non-fat dry milk in TBST for 1 hr at room temperature: goat anti rabbit, goat anti mouse, or donkey anti goat antibodies (Santa Cruz, 1:2000). Membranes were then washed again with TBST 4 x 5 min. Protein bands were detected using ECL and autoradiography film and analyzed with Image J software.

2.3.14 Echocardiography

Rodents were anesthetized with isoflurane. *In vivo* diastolic and systolic cardiac function was measured with the Vevo 770 high resolution

echocardiography imaging system (VisualSonics, Toronto) and 30-MHz transducer [320]. Doppler and tissue doppler imaging were used to assess these parameters of diastolic function: E'/A', E', E/A, E'/E, and isovolumetic relaxation time (IVRT). % Ejection fraction (%EF) and % Fractional shortening (%FS) was measured by M-mode echocardiography and used to assess systolic function. M-mode echocardiography was also used to assess left ventricle (LV) wall measurements (Interventricular septum end diastole (IVSd), LV internal diameter end diastole (LVIDd), LV posterior wall thickness end diastole (LVPWd), Interventricular septum end systole (IVSs), LV internal diameter end systole (LVIDs), and LV posterior wall thickness end systole (LVPWs)), LV diameter and volume in both systole and diastole (left ventricular end diastolic diameter, left ventricular end systolic diameter, LV volume end diastole (LV Vol;d), LV volume end systole (LV Vol;s)), and corrected LV mass which we used as a measurement of cardiac hypertrophy. Left ventricular wall measurements were also made using M-mode echocardiography. The following equations were used to calculate %EF, %FS, Cardiac output (CO), and Tei index:

%FS= ((left ventricular end diastolic diameter – left ventricular end systolic diameter)/left ventricular end diastolic diameter) X 100

%EF= ((left ventricular end diastolic volume – left ventricular end systolic volume)/left ventricular end diastolic volume) X 100

CO= heart rate X stroke volume

Tei index= (isovolumetric relaxation time + isovolumetric contraction time)/ ejection time

2.3.15 Immunofluorescence

Standard immunofluorescence methods were used and images were taken with a confocal microscope, Zeiss LSM 510 NLO. Fixed BMMSCs were stained with BrDU antibody (TUNEL assay) and Ki67 as described previously [213]. Nuclei were also stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). For the TUNEL assay, deoxyribonucleic acid (DNA) of fixed cells that was cleaved were labeled with 5-Bromo-2′-Deoxyuridine 5′-Triphosphate and terminal deoxynucleotidyl transferase. Incorporation into DNA was detected with the BrDU antibody. Live BMMSCs were stained with TMRM, as described previously [25]. TMRM and Hoechst stain were added to the medium to measure mitochondrial membrane potential and stain nuclei, respectively. After 30 min images were taken of the cells with the confocal microscope At least 3 separate images were taken randomly of each coverslip (TUNEL and Ki67 staining) or coverslip bottom dish (TMRM staining for assessment of mitochondrial membrane potential). At least four separate observations were made for each parameter assessed. Zeiss LSM 510 software was used to analyze images. The average intensity of mitochondrial membrane potential or nuclear staining with

TUNEL and Ki67 was assessed using Zeiss LSM 510 software. All cells in which the entire nucleus (for assessment of Ki57 or TUNEL staining) or entire cell (TMRM staining for assessment of mitochondrial membrane potential) were visible in the image were quantified.

2.3.16 MTT assay

A standard MTT assay protocol was followed. Briefly, wells were aspirated and 0.5 mg/ml MTT (Invitrogen M6494) solution was added to the aspirated wells. After keeping the plate for 2 hr at 37°C wells were again aspirated and rinsed with PBS. The product, formazan, was then dissolved in DMSO and 200 µl was transferred to a 96 well plate. Sample absorbance was measured at 550 nm and values normalized based on the negative control (DMSO).

2.3.17 Caspase activity assay

An Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) (Sigma A1086) kit was used to assess caspase activity. The assay procedure provided by Sigma was followed. Briefly, after treatment was finished cells grown in 96 well plates were washed with PBS and then were lysed with lysis buffer for 20 min on ice. After adding the provided assay buffer fluorescence (excitation 360 nm emission 460 nm) of the samples was measured every 10 min for 60 min at 37°C. Caspase activity was calculated based on the AMC standard curve.

2.3.18 BCAA assay

BCAA levels were assessed using the BCAA kit from Sigma. The product produced during the assay was measured at 450 nm.

2.3.19 Oral glucose tolerance test

Whole body glucose tolerance was assessed in *db/db* mice fasted overnight for 12 hr. Before starting the test body weight and blood glucose levels were measured. Mice were orally gavaged with 2 g glucose/kg body weight. Blood glucose levels were then assessed 15, 30, 60, 90, and 120 min after glucose administration.

2.3.20 Short chain CoA analysis

Standard procedure was followed to assess short chain CoAs using HPLC, as described previously [281, 282]. Short chain CoAs were extracted by homogenizing frozen heart tissue in ice cold 6% (v/v) perchloric acid for 30 seconds. After sitting on ice for 15 min, lysates centrifuged at 12,000xg for 5 min, 4°C. The supernatant was subjected to HPLC to determine tissue short chain CoA levels.

2.3.21 Statistical analysis

One-way analysis of variance (ANOVA) or 2-way ANOVA followed by Bonferroni's Multiple Comparison Post Hoc Test, Kruskal-Wallis test with Dunn's Multiple Comparison test, or t-test was used to determine statistical significance

($p<0.05$) using Graphpad Prism software. Values in all studies are presented as mean ± standard error of mean (SEM).

CHAPTER 3

Effect of fatty acids on human bone marrow mesenchymal stem cell energy metabolism and survival.

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Jun Mori assisted with glycolysis measurements. Roxane Paulin and Alois Haromy performed the microscopy imaging. I performed the rest of the experiments as well as experimental design, statistical analysis, and writing the manuscript.

CHAPTER 3

Effect of fatty acids on human bone marrow mesenchymal stem cell energy metabolism and survival.

3.1 Abstract

Successful stem cell therapy requires the optimal proliferation, engraftment, and differentiation of stem cells into the desired cell lineage of tissues. However, stem cell therapy clinical trials to date have had limited success, suggesting that a better understanding of stem cell biology is needed. This includes a better understanding of stem cell energy metabolism because of the importance of energy metabolism in stem cell proliferation and differentiation. We report here the first direct evidence that human bone marrow mesenchymal stem cell (BMMSC) energy metabolism is highly glycolytic with low rates of mitochondrial oxidative metabolism. The contribution of glycolysis to ATP production is greater than 97% in undifferentiated BMMSCs, while glucose and fatty acid oxidation combined only contribute 3% of ATP production. We also assessed the effect of physiological levels of fatty acids on human BMMSC survival and energy metabolism. We found that the saturated fatty acid palmitate induces BMMSC apoptosis and decreases proliferation, an effect prevented by the unsaturated fatty acid oleate. Interestingly, chronic exposure of human BMMSCs to physiological levels of palmitate (for 24 hr) reduces palmitate oxidation rates.

This decrease in palmitate oxidation is prevented by chronic exposure of the BMMSCs to oleate. These results suggest that reducing saturated fatty acid oxidation can decrease human BMMSC proliferation and cause cell death. These results also suggest that saturated fatty acids may be involved in the long-term impairment of BMMSC survival *in vivo*.

3.2 Introduction

The potential for stem cell therapy to regenerate injured tissue has recently generated considerable interest. Two of the major problems facing stem cell heart therapy include low stem cell survival *in vivo* and negligible stem cell-to-target cell differentiation *in vivo* [22, 57, 146, 149, 169, 276]. The development of strategies to solve these problems should be facilitated by a better understanding of stem cell biology. One aspect of this biology that we believe will be particularly important to better understand is the regulation of energy metabolism because of its potential importance in differentiation and cell proliferation [25, 50, 51, 215, 271, 294]. Despite the evidence that energy metabolism is important in cardiac myocyte differentiation not much is known about stem cell energy metabolism either basally or during cardiac myocyte differentiation. In this study we, therefore, chose to focus on advancing our understanding of stem cell energy metabolism.

There is evidence that glycolysis may be a major contributor to ATP production in stem cells. A major piece of evidence to suggest this is the important role of glycolysis in proliferation, a key characteristic of stem cells. Glycolysis is believed to promote proliferation by providing the cell with substrates needed to maintain high rates of macromolecular synthesis. For example, lipogenesis requires nicotinamide adenine dinucleotide phosphate (NADPH), which is produced by the pentose phosphate pathway which uses glucose-6-phosphate. NADPH production and its use in lipogenesis appears to be essential for cancer cell proliferation [37, 305]. In addition, a key transcription factor regulating glycolysis, hypoxia inducible factor (HIF)1α, enhances macromolecular synthesis by increasing the protein expression of isocitrate dehydrogenase (IDH) 2 [300]. IDH2 helps convert α ketoglutarate back to citrate which can be transported out of the mitochondria and used in lipogenesis.

There is also evidence, however, that fatty acid oxidation may be important in proliferation. For example, stimulation of fatty acid oxidation protects glioblastoma cells, which are normally dependent on Akt for anaerobic glycolysis and survival, from death induced by glucose deprivation [36]. It has also been shown that expression of carnitine palmitoyltransferase 1c, a protein involved in mitochondrial fatty acid transport, promotes the growth of human breast cancer cells implanted into nude mice [314]. Even though this capacity for fatty acid oxidation to maintain cell proliferation may be unique to cancer cells,

these findings suggest that oxidative metabolism, and specifically fatty acid oxidation, does not always hinder proliferative cell survival.

The purpose of this study was to characterize stem cell energy metabolism. We report here the first direct energy metabolic rate profile of bone marrow mesenchymal stem cells (BMMSC), confirming that BMMSCs are highly glycolytic. We also examined what effect physiological levels of fatty acids present in the circulation have on BMMSC glucose and fatty acid metabolism. During the course of these experiments we discovered that fatty acids induce BMMSC death *in vitro.* This suggests that fatty acids may be involved in the low survival observed in stem cells *in vivo* and that an understanding of the effect of fatty acids on BMMSC metabolism is important in developing strategies to successfully augment stem cell survival.

3.3 Materials and methods

Human BMMSCs were used in all experiments in this study. Standard cell culture procedures were used and are described in Chapter 2 of this thesis. Unless indicated otherwise, the Krebs Henseleit buffer used in the measurement of energy metabolism (glycolysis, glucose oxidation, and fatty acid oxidation) was supplemented with 0.55 mM fatty acid free bovine serum albumin (BSA) (Equitech-Bio Inc BAH66) bound to 0.4 mM palmitate, 5 mM glucose, and the appropriate radioactive labeled fatty acid or glucose. Glycolysis, glucose

oxidation, and fatty acid oxidation were measured as described in Chapter 2 of this thesis. The acute effect of palmitate and oleate on BMMSC energy metabolism was assessed by supplementing the Krebs Henseleit buffer with 5 mM glucose and 0.55 mM BSA bound to either no fatty acids, 0.4 mM palmitate, 0.4 mM oleate, or 0.4 mM palmitate and 0.4 mM oleate. The presence of 0.4 mM palmitate (Sigma P0500) and/or 0.4 mM oleate (Fluka Analytical 60420) in the Krebs Henseleit buffer is indicated in the figure legends.

The chronic effects of fatty acids on energy metabolism, proliferation, and survival of BMMSCs were also assessed. The cell culture media was supplemented with 0.55 mM BSA (Equitech-Bio Inc BAH66) or 0.55 mM BSA bound to palmitate and/or oleate or stearate (the type and concentration of fatty acid is indicated in the respective figure and figure legend). BMMSCs treated with stearate were only used in the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The length of chronic treatment (24-72 hr) is also indicated in the figure legends for the individual experiments. Assays used to examine the effect of fatty acids on proliferation and survival include the caspase activity assay, MTT assay, and immunofluorescence (Ki67 expression and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) and Tetramethylrhodamine Methyl Ester (TMRM) staining). These assays are described in Chapter 2 of this thesis.

Details regarding western blot and statistical analysis are provided in Chapter 2 of this thesis.

3.4 Results

3.4.1 Profile of human BMMSC energy metabolism.

We report here the first direct measurements of energy metabolic rates in BMMSCs (Table 3-1). These studies were performed under experimental conditions in which cells were incubated with concentrations of glucose (5 mM), fatty acids (0.4 mM palmitate), and albumin (0.55 mM). Glucose and albumin levels mimic the concentrations of these substrates normally present in the blood. The normal circulating level of fatty acids under non-fasting conditions is 0.4 mM while the concentration of the fatty acid palmitate is about 0.2 mM. Palmitate was used as the representative fatty acid in this assay. In human BMMSCs glycolysis rates (494±33 nmol/mg protein/hr) are extremely high compared to glucose oxidation (0.79±0.22 nmol/mg protein/hr) and palmitate oxidation rates (0.02±0.005 nmol/mg protein/hr). From these rates ATP production from each of these pathways was calculated: glycolysis (988.2±66.7 nmol ATP/mg protein/hr), glucose oxidation (23.8±6.5 nmol ATP/mg protein/hr), and palmitate oxidation (2.07±0.51 nmol ATP/mg protein/hr) (Table 3-1). Based on these rates, 97.5% of ATP in the BMMSCs is derived from glycolysis, 2.2% of ATP from glucose oxidation, and 0.2% of ATP from palmitate oxidation. This confirms the previous assumptions that stem cells are primarily deriving their energy from glycolysis.

3.4.2 Fatty acids affect BMMSC survival.

When stem cells are introduced into the target organ they become exposed to the blood. Some of the blood's contents include glucose, fatty acids, and albumin. Surprisingly, a survey of cell culture media indicate that the level of fatty acids and albumin are much lower than what is present in the circulation [26]. We, therefore, were interested in what effect fatty acids might have on BMMSC viability. When we exposed BMMSCs to media supplemented with levels of palmitate (0.05-0.4 mM) and albumin (0.55 mM) normally present in the blood, we noticed that palmitate in a concentration and time dependent manner decreased BMMSC viability (Figure 3-1). In contrast, exposure of the BMMSCs to only 0.55 mM albumin did not result in any major decrease in cell viability (Figure 3-1). Importantly, not only pathological levels (0.4 mM), but also normal circulating levels of palmitate (0.2 mM) decreased BMMSC viability (Figure 3-1). Further, another saturated fatty acid, stearate, also decreased BMMSC viability (Figure 3-1C). Because both unsaturated and saturated fatty acids are present in the circulation, we also examined what effect the unsaturated fatty acid oleate, the most abundant fatty acid in the circulation, has on BMMSC viability. When we treated BMMSCs with physiologically relevant levels of the unsaturated fatty acid oleate (bound to 0.55 mM albumin) we did not observe a change in BMMSC viability (Figure 3-1B). Further, if palmitate treated BMMSCs were also exposed to oleate, BMMSC viability was protected (Figure 3-1). Oleate also protected against stearate-induced BMMSC death. These results indicate that

saturated fatty acids could be a contributing factor in the loss in viability observed when stem cells are introduced into the body for therapeutic purposes.

3.4.3 Palmitate induces apoptosis and decreases proliferation.

To confirm that palmitate induces BMMSC death and to assess whether the type of cell death involves apoptosis, we measured mitochondrial membrane potential, performed TUNEL staining, and assessed caspase 3 activity. Chronic exposure of BMMSCs to palmitate (48 hr exposure) resulted in an increase in caspase 3 activity (Figure 3-2A). Palmitate also increased TUNEL staining after 24 hr (Figure 3-2B,C) and decreased mitochondrial membrane potential (Figure 3-3A,B).

We also investigated the effect of palmitate on BMMSC proliferation. To do this, we assessed the nuclear expression of Ki67, a marker of proliferation. There is a lower percentage of Ki67 positive human BMMSC nuclei following palmitate exposure (Figure 3-2D).

3.4.4 Oleate inhibits palmitate-induced human BMMSC apoptosis and reduction in proliferation.

To examine the relationship between saturated and unsaturated fatty acids on BMMSC viability we treated BMMSCs with varying ratios of palmitate and oleate up to a normal physiological range (0.2 mM palmitate and 0.3 mM oleate) during non-fasting conditions. When BMMSCs are exposed to equal or greater

amounts of oleate BMMSC viability is preserved (Figure 3-1B,C and Figure 3-2A,C). At the lower ratio tested (0.1 mM oleate and 0.2 mM palmitate) oleate is only partially protective. In addition, oleate prevents the increase in caspase activity and TUNEL positive nuclei induced by palmitate treatment (Figure 3-2A,B,C). Oleate also protects against the drop in mitochondrial membrane potential induced by palmitate (Figure 3-3A,B). Finally, oleate prevents the drop in Ki67 positive human BMMSCs following 24 hr of palmitate treatment (Figure 3-2D).

3.4.5 Acute effect of the fatty acids palmitate and oleate on BMMSC energy metabolism.

To determine whether alterations in energy metabolism could be involved in palmitate-induced cell death we assessed the acute effects of palmitate and oleate on BMMSC energy metabolism. Glycolysis, glucose oxidation and fatty acid oxidation were measured in BMMSCs treated with 5 mM glucose and either 0.55 mM BSA, 0.4 mM palmitate bound to 0.55 mM BSA, 0.4 mM oleate bound to 0.55 mM BSA, or 0.4 mM palmitate and 0.4 mM oleate bound to 0.55 mM BSA. For assessment of the acute effect of fatty acids on BMMSC energy metabolism, BMMSCs were only given these treatments while energy metabolism was being measured. Up until these assays began BMMSCs were only exposed to standard cell culture media. Palmitate or oleate alone did not affect glycolysis, glucose oxidation, or oleate oxidation rates (Figure 3-4). However, combined

treatment with palmitate and oleate did significantly reduce glucose oxidation rates (Figure 3-4B), a condition known to be associated with increased cell proliferation [25]. As expected, palmitate and oleate inhibited each other's uptake (Figure 3-4D,F), although neither inhibited each other's oxidation (Figure 3-4C,E). This suggests that oleate resulted in a better coupling of palmitate uptake to palmitate oxidation, resulting in less palmitate entering other cellular pathways.

3.4.6 Chronic effects of the fatty acids palmitate and oleate on BMMSC energy metabolism.

Since chronic exposure of BMMSCs results in decreased cell viability, we also investigated what effect exposure of human BMMSCs to fatty acids had on energy metabolism (Figure 3-5). Since 72 hr exposure of BMMSCs to palmitate resulted in a substantial decrease in cell viability (Figure 3-1), cells were treated with palmitate for 24 hr prior to measurements of energy metabolism, a time period where no decrease in cell viability was observed. BMMSCs were treated for 24 hr with 5 mM glucose and either 0.55 mM BSA, 0.4 mM palmitate bound to 0.55 mM BSA, 0.4 mM oleate bound to 0.55 mM BSA, or 0.4 mM palmitate and 0.4 mM oleate bound to 0.55 mM BSA. Interestingly, chronic palmitate treatment decreases palmitate oxidation rates (Figure 3-5A), without altering palmitate uptake rates (Figure 3-5B). Chronic exposure to oleate prevents the decrease in palmitate oxidation without altering palmitate uptake rates (Figure 3-5A,B), thereby improving the coupling between palmitate uptake and oxidation.

Neither chronic palmitate and/or oleate treatment affects BMMSC glucose oxidation or glycolysis (Figure 3-5C,D). Overall, glycolysis remains the major source of ATP production in BMMSCs which are chronically exposed to palmitate and/or oleate (Figure 3-5E,F).

3.4.7 Chronic effects of palmitate and/or oleate on BMMSC expression of proteins involved in glycolysis and oxidative metabolism.

To further investigate the effect of fatty acids on BMMSC energy metabolism we assessed the effect of 24 hr treatment with palmitate and/or oleate on the expression of proteins involved in glycolysis and oxidative metabolism. We chose this length of treatment because it was long enough to potentially observe changes in protein expression but soon enough that there would still be cells present to make measurements in. No significant changes occurred in the protein expression of phosphoglycerate mutase (PGAM)1 and lactate dehydrogenase (LDH)-A, two proteins involved in glycolysis (Figure 3-6B,C). Interestingly, HIF1α protein expression, a key transcription factor involved in regulating glycolysis, trends towards being reduced in both groups treated with oleate but palmitate alone did not affect its expression (Figure 3-6D). Isocitrate dehydrogenase, an enzyme involve in the tricarboxylic acid (TCA) cycle, was not affected by any of the treatments (Figure 3-6E). There were also no changes in acetyl CoA carboxylase (ACC) expression or phosphorylation of ACC (Figure 3-6F), a key enzyme involved in the synthesis of malonyl CoA, which is a potent

inhibitor of mitochondrial fatty acid uptake and oxidation. This suggests that regulation of ACC is not an explanation for the reduction in palmitate oxidation observed following 24 hr treatment with palmitate.

3.5 Discussion

This is the first study to directly determine the energy metabolic rate profile of human BMMSCs. We confirmed the previous assumption that BMMSCs derive most of their ATP from glycolysis (>97%) (Table 3-1). This finding is in agreement with indirect measurements of energy metabolism including those showing elevated lactate levels and low oxygen consumption rates in several types of stem cells including mesenchymal, embryonic, and induced pluripotent stem cells [43, 51, 212, 289]. In support of high rates of glycolysis being important for pluripotency, studies have shown that osteogenic differentiation of mesenchymal stem cells and embryonic stem cell-to-cardiomyocyte differentiation are accompanied by a decline in lactate production [43, 51]. We also examined the effect of various fatty acids on the energy substrate metabolism, survival, and proliferation of human BMMSCs. We show that physiologically relevant levels of saturated fatty acids induce BMMSC death and decrease BMMSC proliferation, effects which are prevented by the unsaturated fatty acid oleate. These experiments were designed to assess the effect of levels of fatty acids present in the circulation on BMMSCs. It will be

interesting in the future to also assess the effect of the level of fatty acids present in the bone marrow on BMMSC survival. We also show that decreasing saturated fatty acid oxidation may induce BMMSC death. This has important implications on the therapeutic strategy of using BMMSCs for tissue regeneration, and suggests that strategies should be implemented that minimize circulating saturated fatty acid levels during the therapy.

Fatty acids have previously been reported to affect cell survival. Saturated fatty acids have specifically been reported to induce death in many cell types, including BMMSCs [150, 162, 180]. However, many of these studies used a level of albumin that is much lower than that present in the circulation (0.55 mM). The use of this low level of albumin results in cells used in such studies being exposed to an artificially high level of palmitate [260]. Therefore, in our experiments the level of albumin we always used was 0.55 mM. We found that physiologically relevant levels of palmitate ranging from levels present under fed to fasting conditions induce human BMMSC death while oleate, an unsaturated fatty acid, does not (Figure 3-1 and 3-2). These results disagree with a previous study by Smith et al [258] that reported that oleate induces BMMSC death. In fact, we show that oleate can actually protect BMMSCs from palmitate-induced cell death. It is possible that the discrepancy in Smith et al's findings and ours are simply due to Smith et al exposing BMMSCs to relatively higher levels of oleate (due to the fact that the albumin concentration used was low) [258]. Regardless, the data highlight the need to carefully consider both the fatty acid concentration and

albumin concentration to which the BMMSC is exposed during any attempts at stem cell therapy.

Fatty acids can regulate flux through energy metabolic pathways, and may thereby regulate cell survival. The survival and proliferation of cells with high glycolytic rates tends to be positively correlated with glycolysis [25, 287]. In other cell types a process referred to as the glucose-fatty acid cycle, or the Randle cycle, has been observed, where increased fatty acid oxidation can inhibit glucose oxidation and glycolysis [227]. However, in the heart it has frequently been reported that elevating fatty acid oxidation results in uncoupling of glycolysis from glucose oxidation, due to a greater inhibition of glucose oxidation than glycolysis [159]. In agreement with this, inhibiting fatty acid oxidation via malonyl CoA decarboxylase (MCD) inhibition results in pulmonary artery smooth muscle cell apoptosis and decreased proliferation [271]. This is probably detrimental to these cells because decreasing palmitate oxidation likely results in an improved coupling of glycolysis to glucose oxidation. Therefore, this link between fatty acid oxidation and glucose metabolism could explain why fatty acid oxidation seems to regulate cell proliferation and survival.

An alternative explanation for the effects of fatty acids on cell survival is that fatty acid oxidation could be beneficial independent of its effects on glycolysis. It has been suggested that under conditions where glycolysis is reduced fatty acid oxidation can be used by cancer cells for energy production [36]. We therefore decided to determine whether fatty acids inhibit human

BMMSC glucose metabolism and induce BMMSC death via modulation of glucose and fatty acid energy metabolism. Acute exposure to palmitate and/or oleate did not affect glycolysis or fatty acid oxidation rates (Figure 3-4). However, combined acute exposure to palmitate and oleate did reduce glucose oxidation (Figure 3-4B). These results indicate that the Randle Cycle exists at least to some extent in human BMMSCs. Following chronic treatment with palmitate and/or oleate we observed that only palmitate exposure reduced palmitate oxidation rates (Figure 3-5). Interestingly, combined treatment with oleate, which prevented palmitate-induced death, prevented this reduction in palmitate oxidation (Figure 3-5). Neither palmitate or oleate affected the expression of proteins involved in oxidative metabolism or glycolysis that we measured (Figure 3-6). These palmitate oxidation results agree with a previous report showing that 20 hr exposure of neonatal cardiac myocytes to palmitate induced apoptosis and decreased palmitate oxidation rates [104]. This suggests that palmitate induces BMMSC death via inhibition of palmitate oxidation and that oleate is protective because it prevents palmitate oxidation from decreasing. This is supported by a previous study in BMMSCs in which 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (an activator of 5' AMP-activated protein kinase (AMPK) and fatty acid oxidation) prevented palmitate-induced death [162]. However, we did not observe a change in phosphorylation of ACC, an indicator of AMPK activity and an important pathway by which AMPK increases fatty acid oxidation. This may not actually be that surprising, since AMPK

activation can decrease proliferation but as we show here oleate protects against the drop in proliferation induced by palmitate [60]. It is still a possibility, however, that a reduction in glycolysis may be involved in palmitate-induced BMMSC death, but changes in glycolysis that occur in response to 24 hr treatment with palmitate and/or oleate were masked by switching all groups to the same buffer during the measurement of glycolysis rates. We also may not have detected changes in glycolysis possibly because of a difference in glycogen contribution to glycolysis or a change in the pentose phosphate pathway (during the pentose phosphate pathway $^{3}H_2O$ can be produced from the [5-^{3}H]glucose we use to measure glycolysis).

Oleate protected against palmitate-induced BMMSC death. This may have occurred secondary to inhibiting palmitate uptake. Acutely, oleate and palmitate reduced each other's uptake (Figure 3-4). However, after 24 hr of exposure to palmitate and/or oleate, palmitate uptake was not different between groups (Figure 3-5). It is still possible, however, that oleate did in fact reduce palmitate uptake at 24 hr but it was an acute effect and therefore was not measured (since during the assay the cells in all groups were exposed to 0.4 mM palmitate). Therefore, oleate may be at least partially protecting against palmitate-induced cell death by reducing intracellular palmitate levels by decreasing palmitate uptake.

Another potential mechanism for palmitate-induced cell death in the BMMSCs is the potential involvement of ceramides. Elevated levels of

ceramides are able to induce death in a number of different cell types [133]. The fact that the saturated fatty acids palmitate and stearate (ceramide substrates) induce BMMSC death while the unsaturated fatty acid oleate (not a ceramide substrate) does not induce BMMSC death suggests a potential role for ceramides in saturated fatty acid-induced BMMSC death. In addition, chronic exposure to palmitate reduces fatty acid oxidation, which could result in a redirection of palmitate into ceramides. Further, oleate prevented this drop in fatty acid oxidation and decreased palmitate uptake, which could decrease ceramide production by reducing the amount of palmitate present to be used in ceramide production. In fact, elevated ceramide levels accompany the palmitate-induced reduction in fatty acid oxidation in neonatal cardiac myocytes [104]. Unfortunately, experimental conditions precluded us from measuring ceramide levels in these cells. However, there is evidence that palmitate at least does not always work through ceramides to induce cell death [133, 150, 180].

We demonstrate that human BMMSC energy production is predominantly derived from glycolysis, and we show that modulation of energy metabolism is important in the proliferation and survival of human BMMSCs. In particular, physiologically relevant levels of saturated fatty acids reduce BMMSC proliferation and induce BMMSC apoptosis, all effects that can be prevented by oleate. The decrease in saturated fatty acid oxidation induced by chronic exposure to palmitate may be involved in these deleterious effects of palmitate on BMMSCs. These observations indicate that saturated fatty acids could be

contributing to the low *in vivo* survival of BMMSCs, and therefore to the disappointing results of stem cell therapy clinical trials including those focused on treating heart and pulmonary diseases [22, 52, 57, 146, 149, 169, 198, 276, 279].

Figure 3-1. Oleate prevents palmitate-induced human BMMSC death. A) Effect of palmitate on human bone marrow mesenchymal stem cell (BMMSC) viability was measured by the MTT assay after 48 hr treatment with indicated treatments. B) Effect of the ratio of palmitate and oleate on palmitate-induced BMMSC death after 72 hr treatment was measured by MTT assay. C) Effect of 72 hr treatment with stearate and/or oleate on the amount of viable cells was measured by the MTT assay. The bovine serum albumin (BSA) group was treated with media supplemented with 0.55 mM albumin. All fatty acid treated groups were also treated with media supplemented with 0.55 mM albumin in addition to the type and amount of fatty acid indicated in the figures. The Control group was treated with media identical to the BSA group minus supplementation with albumin. n=7-12 * Significantly different from Control group. # Significantly less than BSA group. ^ Significantly less than 0.2 mM Palmitate Group. ** Groups are significantly different. Values are shown as the mean ± standard error of mean (SEM).

Figure 3-1

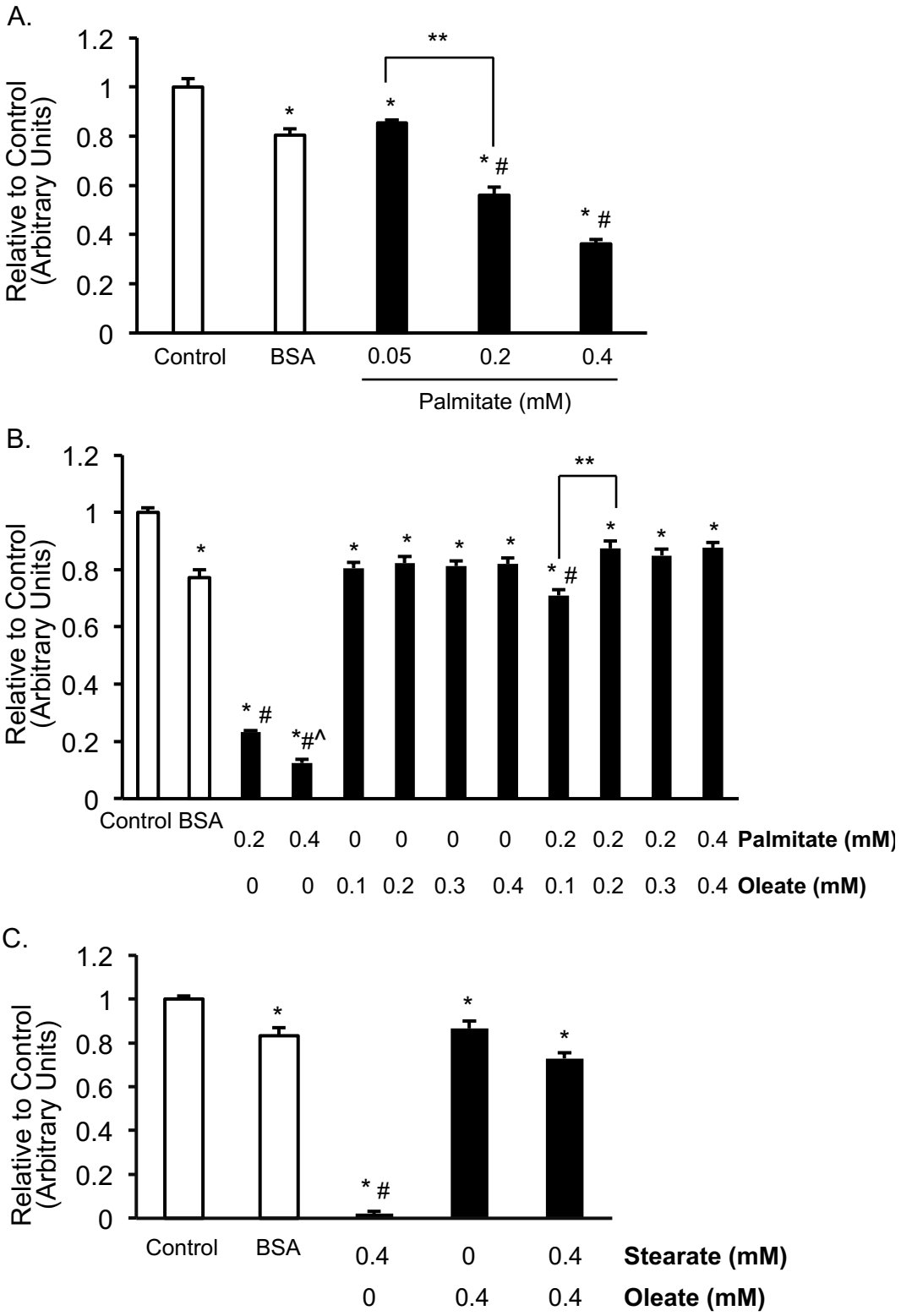

A.
Relative to Control (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
**
*
*
*#
*#
Control
BSA
0.05
0.2
0.4
Palmitate (mM)
B.
Relative to Control (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
**
*#
*#^
Control BSA
0.2 0.4 0 0 0 0 0.2 0.2 0.2 0.4 Palmitate (mM)
0 0 0.1 0.2 0.3 0.4 0.1 0.2 0.3 0.4 Oleate (mM)
C.
Relative to Control (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*#
Control
BSA
0.4 0 0.4 Stearate (mM)
0 0.4 0.4 Oleate (mM)

Figure 3-2. Oleate prevents palmitate-induced human BMMSC apoptosis and reduction in proliferation. A) Caspase activity after 48 hr treatment with indicated treatments. B) Images of TUNEL and Ki67 staining of bone marrow mesenchymal stem cells (BMMSCs) treated for 24 hr with indicated treatments. Image is 28X. C) % nuclei positive for TUNEL. D) % nuclei positive for Ki67. n=5-7 The bovine serum albumin (BSA) group was treated with media supplemented with 0.55 mM albumin. All fatty acid treated groups were also treated with media supplemented with 0.55 mM albumin in addition to the type and amount of fatty acid indicated in the figures. * Significantly different from BSA group. # Significantly different from 0.2 mM Palmitate and 0.4 mM Palmitate groups. ** Significantly different from all groups. Values are shown as the mean ± SEM.

Figure 3-2

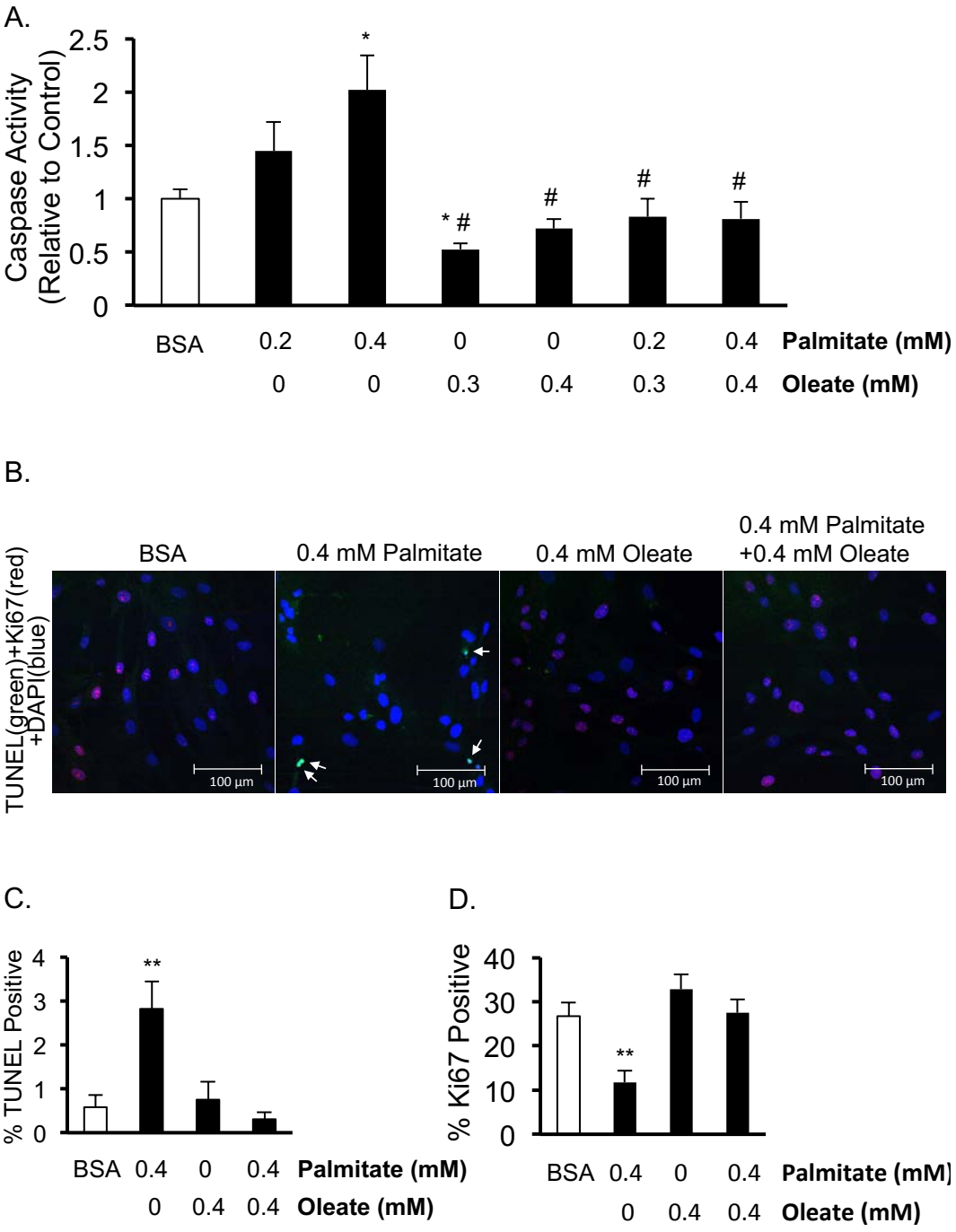

A.
Caspase Activity (Relative to Control)
2.5
2
1.5
1
0.5
0
*
*#
#
#
#
BSA 0.2 0.4 0 0 0.2 0.4 Palmitate (mM)
0 0 0.3 0.4 0.3 0.4 Oleate (mM)
B.
BSA
0.4 mM Palmitate
0.4 mM Oleate
0.4 mM Palmitate +0.4 mM Oleate
TUNEL(green)+Ki67(red) +DAPI(blue)
100 µm
C.
% TUNEL Positive
4
3
2
1
0
**
BSA 0.4 0 0.4 Palmitate (mM)
0 0.4 0.4 Oleate (mM)
D.
% Ki67 Positive
40
30
20
10
0
**
BSA 0.4 0 0.4 Palmitate (mM)
0 0.4 0.4 Oleate (mM)

Figure 3-3. Effect of 24 hr exposure to palmitate and oleate on human BMMSC mitochondrial membrane potential. A) Images of tetramethylrhodamine methyl ester (TMRM) stained bone marrow mesenchymal stem cells (BMMSCs) treated for 24 hr with indicated treatments. B) Relative TMRM levels. BMMSCs were treated for 24 hr with 0.55 mM bovine serum albumin (BSA) alone or palmitate and/or oleate bound to 0.55 mM albumin. TMRM and Hoechst stain were added to the medium to measure mitochondrial membrane potential and stain nuclei, respectively, and images were taken. All fatty acid treated groups were also treated with media supplemented with 0.55 mM albumin in addition to the type and amount of fatty acid indicated in the figures. 4 separate observations. Values are shown as the mean ± SEM. * Significantly different from the BSA group.

Figure 3-3

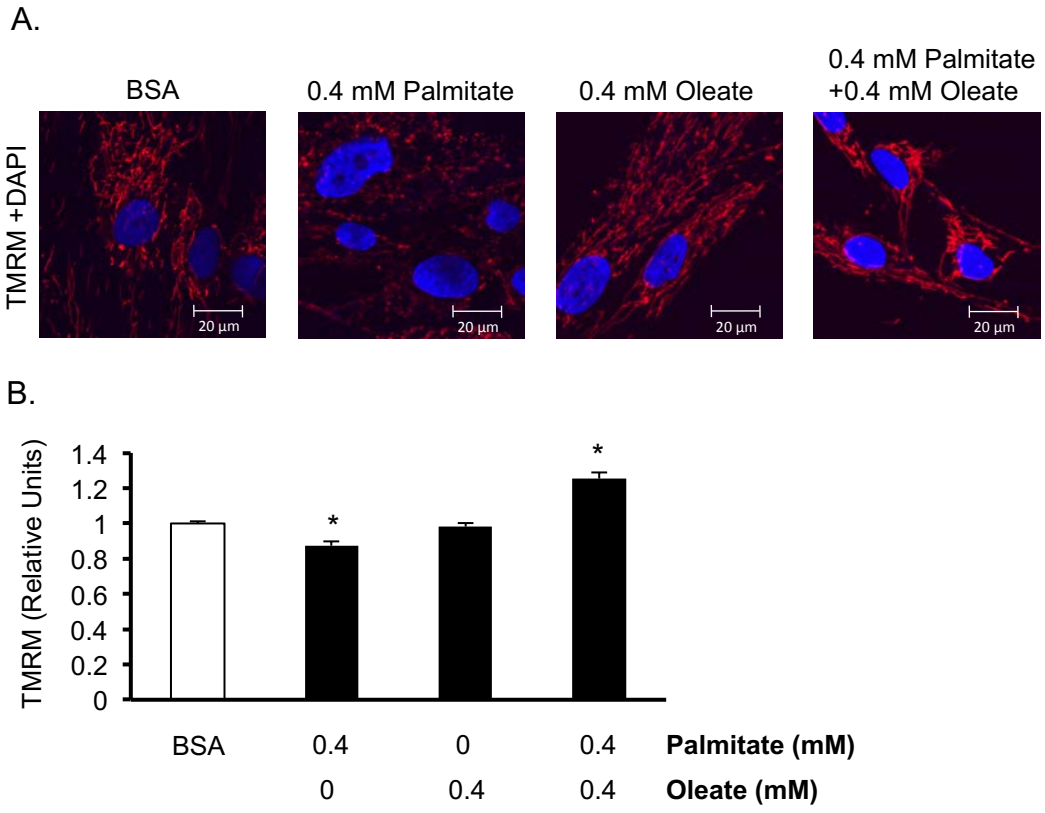

A.
BSA
0.4 mM Palmitate
0.4 mM Oleate
0.4 mM Palmitate
+0.4 mM Oleate
TMRM +DAPI
20 µm
20 µm
20 µm
20 µm
B.
TMRM (Relative Units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)

Figure 3-4. Effect of acute exposure to fatty acids on human BMMSC energy metabolism. A) Glycolysis, B) glucose oxidation, C) palmitate oxidation, D) palmitate uptake, E) oleate oxidation, and F) oleate uptake were measured in untreated human bone marrow mesenchymal stem cells (BMMSCs). n=5-7 During each assay Krebs Henseleit buffer was supplemented with 5 mM glucose and, as indicated in each graph, either 0.55 mM albumin (BSA group) or 0.55 mM albumin bound to 0.4 mM palmitate and/or 0.4 mM oleate. In addition, the Krebs Henseleit buffer was supplemented with either [U-^{14}C]glucose, [1-^{14}C]palmitate, [1-^{14}C]oleate, or [5-^{3}H]glucose for the measurement of glucose oxidation, palmitate oxidation and uptake, oleate oxidation and uptake, or glycolysis, respectively. Since these experiments assessed the acute effect of palmitate and oleate, BMMSCs were maintained in cell culture media used to culture these immediately up to the start of each assay when the media was switched to Krebs Henseleit buffer supplemented with fatty acids. The levels and type of fatty acid BMMSCs were exposed to is indicated on the x-axis of the figures. * Significantly different from all groups. Values are shown as the mean ± SEM.

Figure 3-4

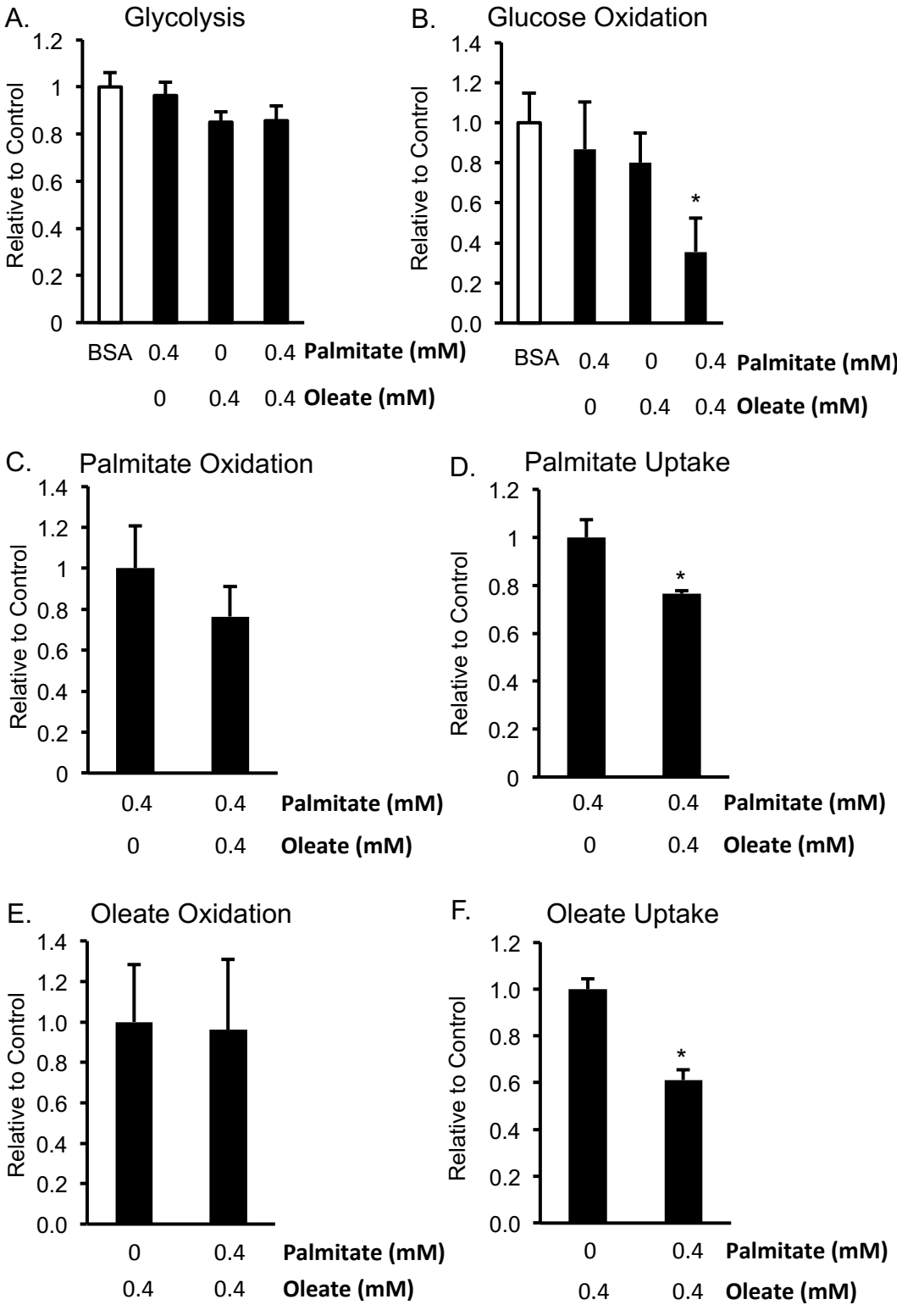

A.
Glycolysis
Relative to Control
1.2
1
0.8
0.6
0.4
0.2
0
BSA 0.4 0 0.4 Palmitate (mM)
0 0.4 0.4 Oleate (mM)
B.
Glucose Oxidation
Relative to Control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
BSA 0.4 0 0.4 Palmitate (mM)
0 0.4 0.4 Oleate (mM)
C.
Palmitate Oxidation
Relative to Control
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.4 0.4 Palmitate (mM)
0 0.4 Oleate (mM)
D.
Palmitate Uptake
Relative to Control
1.2
1
0.8
0.6
0.4
0.2
0
*
0.4 0.4 Palmitate (mM)
0 0.4 Oleate (mM)
E.
Oleate Oxidation
Relative to Control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 0.4 Palmitate (mM)
0.4 0.4 Oleate (mM)
F.
Oleate Uptake
Relative to Control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0 0.4 Palmitate (mM)
0.4 0.4 Oleate (mM)

Figure 3-5. Effect of 24 hr exposure to fatty acids on human BMMSC energy metabolism. A) Palmitate oxidation, B) palmitate uptake, C) glycolysis, and D) glucose oxidation were measured in human BMMSCs that had been treated for 24 hr with either 0.55 mM albumin (BSA group) or 0.55 mM albumin and 0.4 mM palmitate and/or 0.4 mM oleate prior to these metabolism measurements being made. n=5-8 The graphs indicate which groups were exposed to these different treatments for the 24 hr prior to the metabolism measurements. During each assay all groups were given Krebs buffer supplemented with 5 mM glucose and 0.4 mM palmitate bound to 0.55 mM albumin. In addition, the Krebs buffer was supplemented with either [U-^{14}C]glucose, [1-^{14}C]palmitate, or [5-^{3}H]glucose for the measurement of glucose oxidation, palmitate oxidation and uptake, or glycolysis, respectively. E) The contribution of metabolic pathways to ATP production were calculated from the metabolic rate results. * Significantly different from all groups. Values are shown as mean ± SEM.

Figure 3-5

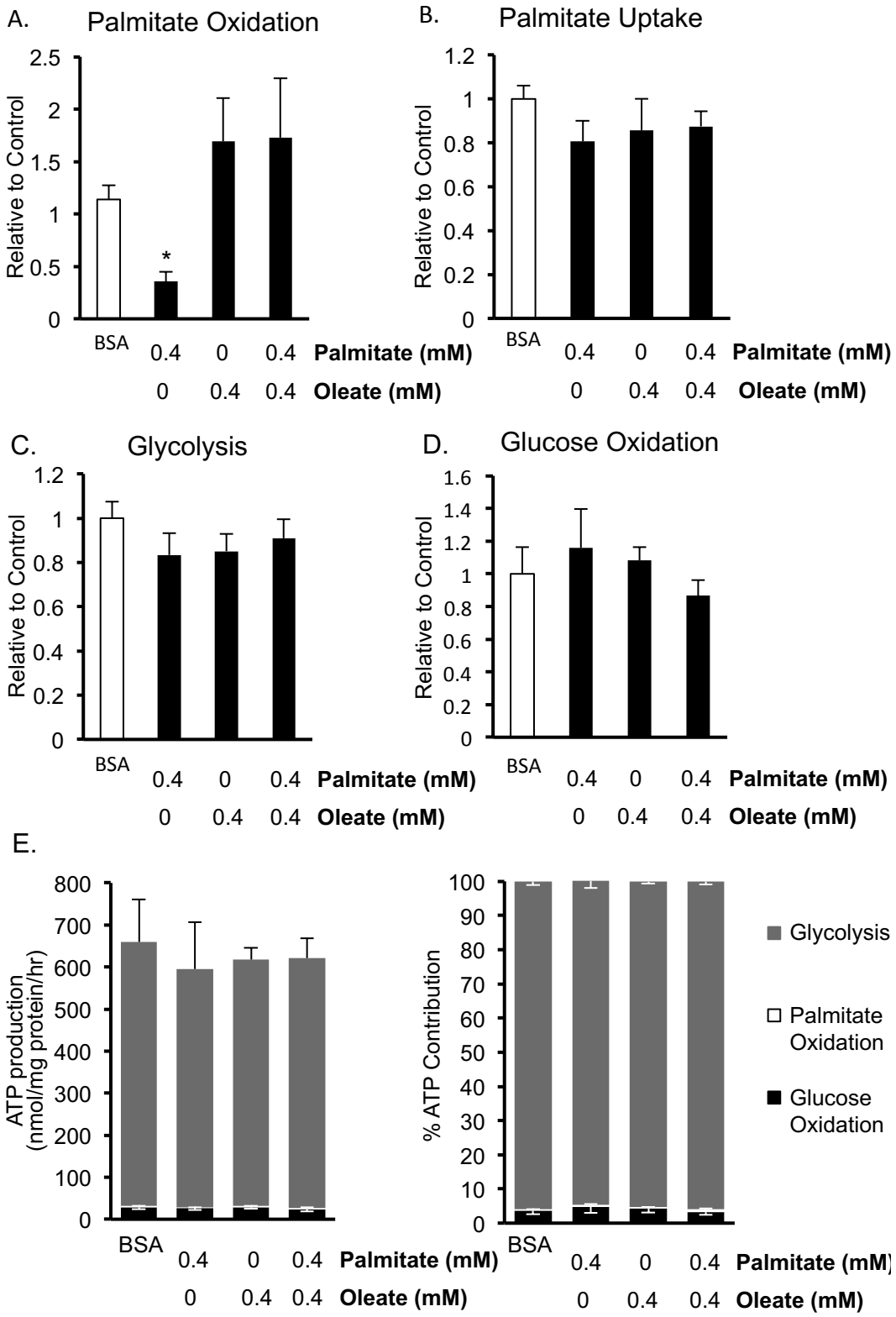

A.
Palmitate Oxidation
Relative to Control
2.5
2
1.5
1
0.5
0
*
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)
B.
Palmitate Uptake
Relative to Control
1.2
1
0.8
0.6
0.4
0.2
0
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)
C.
Glycolysis
Relative to Control
1.2
1
0.8
0.6
0.4
0.2
0
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)
D.
Glucose Oxidation
Relative to Control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)
E.
ATP production (nmol/mg protein/hr)
800
700
600
500
400
300
200
100
0
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)
% ATP Contribution
100
90
80
70
60
50
40
30
20
10
0
BSA
0.4
0
0.4
Palmitate (mM)
0
0.4
0.4
Oleate (mM)
Glycolysis
Palmitate Oxidation
Glucose Oxidation

Figure 3-6. Effect of 24 hr exposure to fatty acids on expression of proteins involved in energy metabolism. A) Representative western blots, B) Lactate dehydrogenase A (LDHA), C) Phosphoglycerate mutase 1 (PGAM1), D) Hypoxia inducible factor 1α (HIF1α), E) Isocitrate dehydrogenase (IDH), F) phospho Acetyl CoA carboxylase (ACC)/ACC protein expression. n=5-6 Protein expression was measured in human bone marrow mesenchymal stem cells (BMMSCs) treated for 24 hr with indicated treatments. The BSA group was treated with media supplemented with 0.55 mM albumin. All fatty acid treated groups were also treated with media supplemented with 0.55 mM albumin in addition to the type and amount of fatty acid indicated in the figures. Values are shown as mean ± SEM.

Figure 3-6

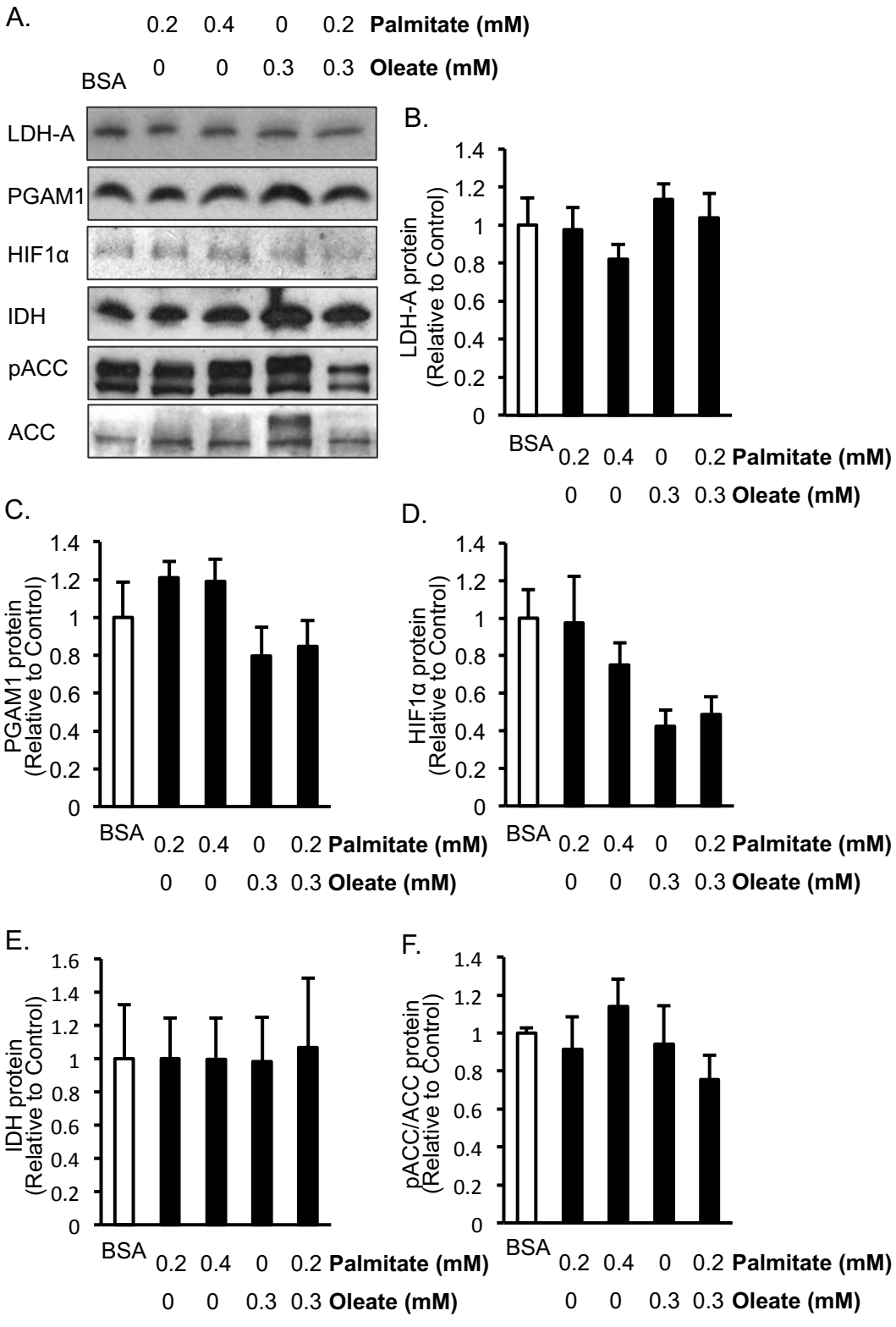

A.
0.2 0.4 0 0.2 Palmitate (mM)
0 0 0.3 0.3 Oleate (mM)
BSA
LDH-A
PGAM1
HIF1α
IDH
pACC
ACC
B.
LDH-A protein (Relative to Control)
C.
PGAM1 protein (Relative to Control)
D.
HIF1α protein (Relative to Control)
E.
IDH protein (Relative to Control)
F.
pACC/ACC protein (Relative to Control)

Table 3-1. Contribution of energy metabolism pathways to ATP production in human BMMSCs.

	Metabolism Rate nmol/mg protein/hr	**ATP Production** nmol/mg protein/hr	**ATP Production** %
Glycolysis	494±33	988.2 ± 66.67	97.5 ± 0.67
Glucose Oxidation	0.79±0.22	23.8 ± 6.50	2.2 ± 0.63
Palmitate Oxidation	0.02±0.005	2.07 ± 0.51	0.2 ± 0.05

During each assay Krebs Henseleit buffer was supplemented with 5 mM glucose and 0.4 mM palmitate bound to 0.55 mM albumin. In addition, the Krebs Henseleit buffer was supplemented with either [U-^{14}C]glucose, [1-^{14}C]palmitate, or [5-^{3}H]glucose in order to measure glucose oxidation, palmitate oxidation, or glycolysis, respectively. Bone marrow mesenchymal stem cells (BMMSCs) were exposed to normal cell culture media immediately up to the point it was switched to this Krebs Henseleit buffer at the start of each assay. Calculations to determine ATP production and % ATP production were made from the metabolic rate results. n=5-8 Values are shown as the mean ± SEM.

CHAPTER 4

Glucose oxidation and glycolysis during cardiac myocyte differentiation.

A manuscript is currently in preparation to be submitted to AJP- Heart and Circulatory Physiology.

I performed all experiments in this chapter.

CHAPTER 4

Glucose oxidation and glycolysis during cardiac myocyte differentiation.

4.1 Abstract

Heart stem cell therapy is hindered by several factors, including negligible rates of stem cell-to-cardiac myocyte differentiation. I believe that a better understanding of energy metabolism during cardiac myocyte differentiation will be important to improve stem cell-to-cardiac myocyte differentiation and stem cell heart therapy efficacy. It has been reported that inhibition of oxidative metabolism inhibits embryonic stem cell (ESC)-to-cardiac myocyte differentiation. Further, cardiac myocyte differentiation is associated with a restructuring of the glycolytic pathway and mitochondrial biogenesis. This indicates that an increase in mitochondrial oxidative metabolism is important in cardiac myocyte differentiation. However, relatively little is known about the specific changes in glucose oxidation, glycolysis, and fatty acid oxidation that occur during cardiac myocyte differentiation. In this study I examined whether changes in glucose oxidation, oleate oxidation, and glycolysis occur when H9C2 cells are differentiated toward cardiac myocytes. Differentiation significantly increased the expression of cytochrome c, pyruvate dehydrogenase (PDH), and the rates of glucose oxidation. Interestingly, there was no significant change in fatty acid

oxidation. The lack of change in glycolysis suggests that differentiation may also improve the coupling of glycolysis and glucose oxidation. While these results suggest that glucose oxidation may be important in cardiac myocyte differentiation, more work is needed to determine if stimulating glucose oxidation can promote cardiac myocyte differentiation.

4.2 Introduction

In the last decade stem cell therapy has received a considerable amount of interest as a potentially promising therapy for heart disease. However, success of heart stem cell therapy is hindered by several factors, including differentiation of stem cells into cardiac myocytes, a process that is necessary for the regeneration of damaged cardiac muscle [19, 67, 83, 122, 257, 303, 304]. For example, mesenchymal stem cells, one of the promising cell types for this therapy, have low rates of differentiation *in vitro* (≤30%) and negligible cardiac myocyte differentiation *in vivo* [67, 122, 257, 303, 304].

There is evidence that metabolism may be a key factor in cardiac myocyte differentiation. Energy metabolism has been implicated in heart maturation and embryonic stem cell (ESC)-to-cardiac myocyte differentiation [50, 51, 156, 160]. In general, changes in energy metabolism during cardiac myocyte differentiation are believed to involve a rise in mitochondrial oxidative metabolism and a decline in glycolysis. However, much of the evidence to support this is based on indirect

measurements. For example, mitochondrial maturation is observed during ESC- and bone marrow mesenchymal stem cell (BMMSC)-to-cardiac myocyte differentiation [22, 51]. ESC-to-cardiac myocyte differentiation is also associated with a restructuring of the glycolytic pathway towards a decrease in glycolysis and glycolytic capacity [50]. This also indicates that the coupling of glycolysis and glucose oxidation may be improved during cardiac myocyte differentiation. Further, experiments conducted with induced pluripotent stem cell (iPSC) cells indicated glycolysis is important in maturation. iPSC generation was associated with a shift to a more glycolytic and lower oxidative metabolism similar to that of ESCs [87, 224, 289]. Further, dichloroacetate (DCA), a drug that stimulates glucose oxidation, inhibited generation of iPSC cells [87].

The importance of these changes in energy metabolism in cardiac myocyte differentiation is not well understood. There is evidence that a rise in oxidative metabolism is particularly important in this process. Inhibition of oxidative metabolism inhibits ESC-to-cardiac myocyte differentiation [51]. In addition, treatment of ESCs with agonists of peroxisome proliferator activated receptor (PPAR)α, a transcription factor that regulates fatty acid oxidation and mitochondrial biogenesis, enhances cardiac myocyte differentiation [65, 66]. Further, inhibition of the electron transport chain promotes pluripotency and partially inhibits ESC-to-cardiac myocyte differentiation [51, 288].

In this study I focused on determining what changes in glucose and fatty acid energy metabolism occur during cardiac myocyte differentiation. In

particular, I observed an increase in glucose oxidation without a change in fatty acid oxidation in response to cardiac myocyte differentiation. These findings may prove valuable in the development of strategies to promote cardiac myocyte differentiation via modulation of energy metabolism.

4.3 Materials and methods

Human BMMSCs and H9C2 cells were used in this study. Details regarding the culture of H9C2 cells and human BMMSCs are provided in Chapter 2 of this thesis. Human BMMSCs were subjected to a number of different cardiac myocyte differentiation protocols. In one set of differentiation protocols passage 2 or 3 BMMSCs were grown to confluence and then cells were treated with 10 μM azacytidine for 24 hr. For the remaining 4 wk the standard cell culture media contained either 16%, 5%, or 1% fetal bovine serum (FBS). In addition, a set of BMMSCs were also treated with 5 mM trichostatin for 24 hr immediately following azacytidine treatment. These cells were then treated with standard cell culture media for the rest of the 4 wk period. In another differentiation protocol BMMSCs were instead grown in dishes with a special extracellular matrix (Celprogen D36044-15-T75 Flasks) and treated for 2 wk with differentiation media purchased from Celprogen (Celprogen M36044-15DS). During this differentiation protocol, cells were passaged once they reached 80% confluency throughout the 2 wk period. At the end of 2 wk BMMSCs were seeded onto glass

coverslips for immunofluorescence and T25 flasks for measurement of palmitate oxidation and glucose oxidation. The BMMSCs seeded onto the glass coverslips were fixed and stained with either α actinin or troponin I using standard immunofluorescence methods described previously [213]. Glucose oxidation and palmitate oxidation were measured as described in Chapter 2 of this thesis. The Krebs-Henseleit buffer was supplemented with 5 mM glucose and 0.4 mM palmitate bound to 0.55 mM bovine serum albumin (BSA) as well as [U-^{14}C] glucose or [1-^{14}C] palmitate for measuring glucose oxidation or palmitate oxidation, respectively.

Details regarding the cardiac myocyte differentiation protocol for H9C2 cells are provided in Chapter 2 of this thesis. Measurements were made in undifferentiated H9C2 cells and H9C2 cells differentiated toward a cardiac myocyte for 7 days. Glucose oxidation, oleate oxidation, and glycolysis were measured as described in Chapter 2 of this thesis. The Krebs-Henseleit buffer was supplemented with 5 mM glucose and 0.4 mM oleate bound to 0.55 mM bovine serum albumin (BSA) as well as [U-^{14}C] glucose, [1-^{14}C] oleate, or [5-^{3}H] glucose for measuring glucose oxidation, oleate oxidation, or glycolysis, respectively.

Protocols for western blot and statistical analysis are provided in Chapter 2 of this thesis.

4.4 Results

4.4.1 Expression of cardiac myocyte proteins did not increase in human BMMSCs subjected to cardiac myocyte differentiation protocols.

In this study I was interested in better understanding the changes in glucose and fatty acid oxidation that occur during cardiac myocyte differentiation. Initially, I attempted to differentiate human BMMSCs to study the changes in energy metabolism during cardiac myocyte differentiation. I used a number of different strategies to try to differentiate BMMSCs toward cardiac myocytes but were not able to observe evidence of a consistent or significant degree of cardiac myocyte differentiation using any of the protocols tested. Some of these differentiation protocols included azacytidine treatment, which has frequently been reported in the literature to induce mesenchymal stem cell-to-cardiac myocyte differentiation [14, 255, 293, 303, 304, 308], or a purchased differentiation media from Celprogen. There was also no difference in the expression of two cardiac myocyte markers, α actinin and troponin I, between control BMMSCs and BMMSCs treated for 2 wk with a commercially available cardiac myocyte differentiation media (Figure 4-1)

Due to our lack of success at differentiating BMMSCs toward cardiac myocytes I decided to switch to the H9C2 cell line, which can be readily differentiated toward a cardiac myocyte [30, 54, 177, 217], for determining what happens to glucose oxidation and fatty acid oxidation during cardiac myocyte

differentiation. As expected, I observed an increase in α actinin protein expression in H9C2 cells differentiated for 7 days (Figure 4-1).

4.4.2 Glucose oxidation increases during cardiac myocyte differentiation.

We then compared fatty acid oxidation, glucose oxidation, and glycolysis in undifferentiated and differentiated H9C2 cells. These measurements indicated there was an increase in overall mitochondrial oxidative metabolism (Figure 4-3C). This finding is in agreement with previous reports that mitochondrial biogenesis accompanies cardiac myocyte differentiation [22, 51, 54, 207]. Interestingly, there was an increase in glucose oxidation but no significant changed in fatty acid oxidation after 7 days of H9C2-to-cardiac myocyte differentiation (Figure 4-3). There was also no significant change in glycolysis in these differentiated cells (Figure 4-4). Overall the contribution of glucose oxidation to ATP production increased significantly in differentiated H9C2 cells (Figure 4-5). These changes in energy metabolism were not observed in BMMSCs treated with the ineffective commercial cardiac myocyte differentiation media described earlier (Figure 4-1 and 4-2).

4.4.3 Increased PDH expression may contribute to the elevated glucose oxidation during cardiac myocyte differentiation.

We then examined what happens to the expression of proteins involved in glucose oxidation and glycolysis during cardiac myocyte differentiation. Pyruvate

dehydrogenase (PDH) protein expression was dramatically increased in differentiated H9C2 cells, which could be a contributing factor to the elevation in glucose oxidation observed in differentiated H9C2 cells (Figure 4-6). I also observed an increase in the expression of the glycolytic proteins hexokinase 1 and phosphoglycerate mutase (PGAM)1 during cardiac myocyte differentiation of H9C2 cells (Figure 4-6).

4.4.4 Fatty acid oxidation enzyme expression is reduced during H9C2-to-cardiac myocyte differentiation.

As mentioned previously, there was no significant change in fatty acid oxidation during H9C2-to-cardiac myocyte differentiation despite evidence of mitochondrial biogenesis. When I measured the protein expression of two fatty acid oxidation enzymes, long chain acyl CoA dehydrogenase (LCAD) and hydroxyacyl CoA dehydrogenase (HADH), I found that they were significantly decreased after 7 days of H9C2-to-cardiac myocyte differentiation (Figure 4-7). These results raise the question of whether mitochondria levels are decreased in the differentiated H9C2 cells. However, cytochrome c protein expression was also increased in these differentiated H9C2 cells indicating that mitochondria levels are actually increased in these cells (Figure 4-7)

4.5 Discussion

Cardiac myocyte differentiation is a complex process that is only partially understood. During cardiac myocyte differentiation a stem cell must change fundamentally, from a highly proliferative cell into a nonproliferative cell with the capability to produce enough energy to fuel contraction. In this study I examined what changes in glucose and fatty acid oxidation and glycolysis occur during cardiac myocyte differentiation. Unfortunately I was not able to assess this in BMMSCs as I had initially planned because in our hands the BMMSC-to-cardiac myocyte differentiation protocols I tested did not induce differentiation. This indicates that a better understanding of differentiation is needed in order to develop more effective strategies of BMMSC-to-cardiac myocyte differentiation. It also suggests that BMMSCs are not as optimal for cardiac myocyte differentiation as I had initially believed and that other cell types may need to be used for both basic studies focused on cardiac myocyte differentiation and in therapies aimed at regenerating the heart. In support of this, a few articles have recently suggested that mesenchymal stem cells do not have a high potential to undergo cardiac myocyte differentiation [93, 122, 257]. I therefore decided to switch to H9C2 cells for examining the changes in glucose oxidation and fatty acid oxidation during cardiac myocyte differentiation. I observed a significant increase in glucose oxidation in response to differentiation, but, interestingly, there was only a trend for an increase in fatty acid oxidation and no change in glycolysis.

Previous studies have suggested that energy metabolism is not just correlated with cardiac myocyte development but is important in the process of differentiation [50, 51, 156, 160]. For example there is evidence of mitochondrial maturation during both ESC and BMMSC differentiation toward a cardiac myocyte [22, 51]. Impairing stem cell mitochondrial oxidative metabolism has been shown to inhibit cardiac myocyte differentiation [51, 207]. In this study I also provide evidence that mitochondrial oxidative metabolism is elevated during cardiac myocyte differentiation. Specifically I observed an increase in glucose oxidation without significant changes in glycolysis or fatty acid oxidation. These results provide unique insight into changes in energy metabolism that occur early on in the process of cardiac myocyte differentiation.

There is evidence to suggest that glucose oxidation may be important in cardiac myocyte differentiation. A decrease in lactate production during ESC-to-cardiac myocyte differentiation indicates an increase in the coupling of glycolysis and glucose oxidation [51]. Our observed rise in glucose oxidation rates could potentially contribute to this decrease in lactate production [51]. Further, studies conducted in iPSC cells also indicate that glucose oxidation may regulate differentiation [87, 224, 289]. DCA, which stimulates glucose oxidation, blunts the increase in the expression of pluripotent markers during iPSC generation [87].

As the cardiac myocyte matures further I hypothesize that changes in energy metabolism may be similar to those reported in the maturing newborn rabbit heart. Immediately after birth the newborn rabbit heart is still highly

glycolytic, but immediately post birth glycolysis is dramatically reduced and oxidative metabolism is increased, especially fatty acid oxidation [156, 160]. In fact, there is already a trend for a rise in fatty acid oxidation and decrease in glycolysis in the differentiated H9C2 cells in this study. Previous studies have reported that fatty acid oxidation may be important in cardiac myocyte differentiation. PPARα agonists enhance the ability of ESCs to undergo cardiac myocyte differentiation [65, 66]. Interestingly, a recent study showed that Let-7 microRNA stimulates fatty acid metabolism and promotes cardiac myocyte maturation but does not affect early stages of cardiac myocyte differentiation [138].

We also examined the potential cause for the changes in energy metabolism observed during H9C2-to-cardiac myocyte differentiation. In particular, I observed a dramatic increase in PDH expression during cardiac myocyte differentiation. This could be contributing to the increase in glucose oxidation that I observed in cardiac myocyte differentiation. Interestingly, I also observed an increase in glycolytic enzymes, including hexokinase 1 and PGAM1 which might explain why glycolysis rates are not significantly reduced in the differentiated cells. Another interesting finding was a reduction in fatty acid oxidation protein expression despite evidence of increased mitochondrial oxidative metabolism. These changes in fatty acid oxidation enzyme expression could be contributing to the lack of a significant increase in fatty acid oxidation observed with differentiation despite evidence of increased overall mitochondrial

levels and mitochondrial oxidative metabolism. In fact, other studies have provided evidence supporting an increase in mitochondrial levels in differentiated H9C2 cells [30, 54, 217].

In conclusion, this study provides novel information regarding fatty acid and glucose metabolism during cardiac myocyte differentiation. There was a specific increase in glucose oxidation with no change in glycolysis or fatty acid oxidation in the differentiated H9C2 cells. As far as I am aware this is the first study to measure glucose oxidation, fatty acid oxidation, and glycolysis during an early stage of cardiac myocyte differentiation. Further work will be necessary to determine if glucose oxidation is important in cardiac myocyte differentiation and can be modulated to promote cardiac myocyte differentiation.

Figure 4-1. Expression of cardiac myocyte proteins did not increase in human BMMSCs subjected to a cardiac myocyte differentiation protocol. Images of A. α actinin B. troponin I staining of normal human bone marrow mesenchymal stem cells (BMMSCs) (Control) or BMMSCs treated for 2 wk with a cardiac myocyte differentiation media purchased from Celprogen (Differentiation Protocol). C. α actinin protein expression in undifferentiated H9C2 cells and H9C2 cells differentiated toward a cardiac myocyte for 7 days. * $p<0.05$ compared to Undifferentiated.

Figure 4-1

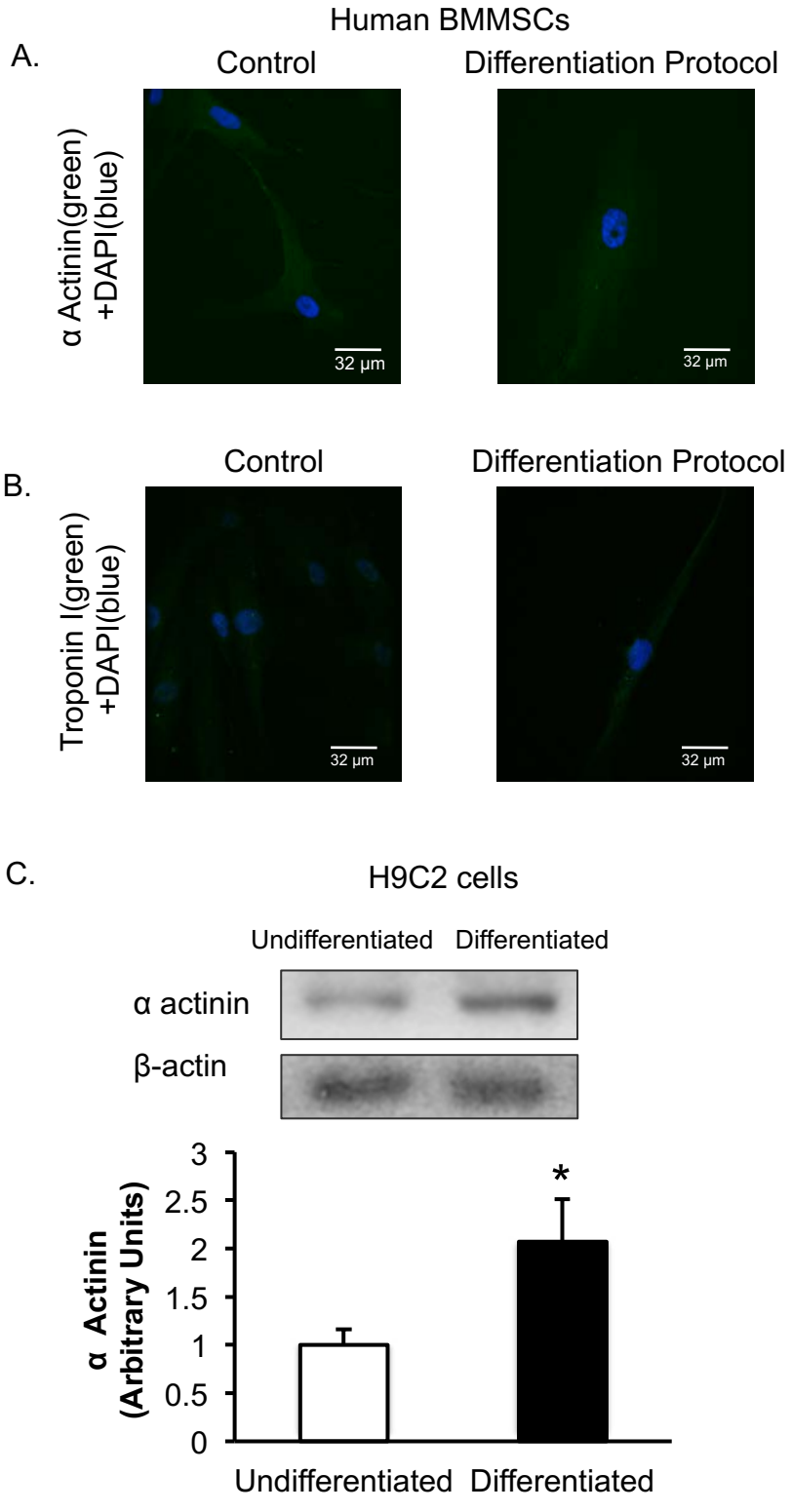

Human BMMSCs
A.
Control
Differentiation Protocol
α Actinin(green)
+DAPI(blue)
32 μm
32 μm
B.
Control
Differentiation Protocol
Troponin I(green)
+DAPI(blue)
32 μm
32 μm
C.
H9C2 cells
Undifferentiated
Differentiated
α actinin
β-actin
α Actinin
(Arbitrary Units)
3
2.5
2
1.5
1
0.5
0
*
Undifferentiated
Differentiated

Figure 4-2. Effect of the differentiation protocol using the Celprogen cardiac myocyte differentiation medium and culture dishes on human BMMSC glucose oxidation and fatty acid oxidation. A. Glucose oxidation and B. Palmitate oxidation were measured in normal human bone marrow mesenchymal stem cells (BMMSCs) (Control) or BMMSCs treated for 2 wk with a cardiac myocyte differentiation media purchased from Celprogen (Differentiation Protocol).

Figure 4-2

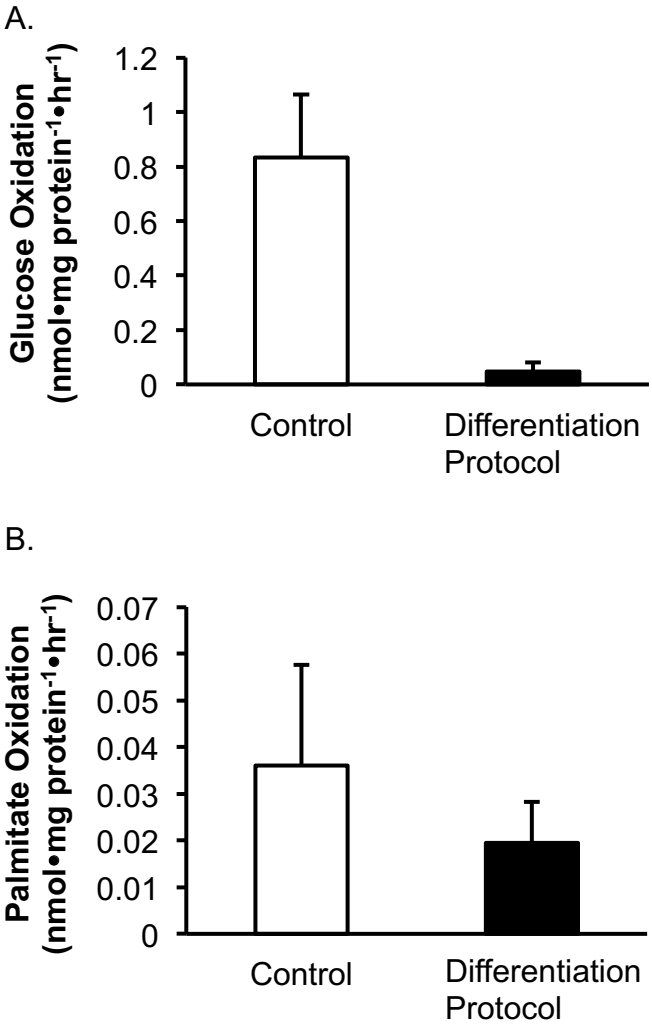

A.
Glucose Oxidation (nmol•mg protein-1•hr-1)
1.2
1
0.8
0.6
0.4
0.2
0
Control
Differentiation Protocol
B.
Palmitate Oxidation (nmol•mg protein-1•hr-1)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
Differentiation Protocol

Figure 4-3. Effect of cardiac myocyte differentiation on glucose oxidation and fatty acid oxidation. A. Glucose Oxidation and B. Oleate oxidation were measured in undifferentiated H9C2 cells and H9C2 cells differentiated toward a cardiac myocyte. C. TCA acetyl CoA production was calculated from these glucose oxidation and oleate oxidation measurements. * $p<0.05$ compared to Undifferentiated.

Figure 4-3

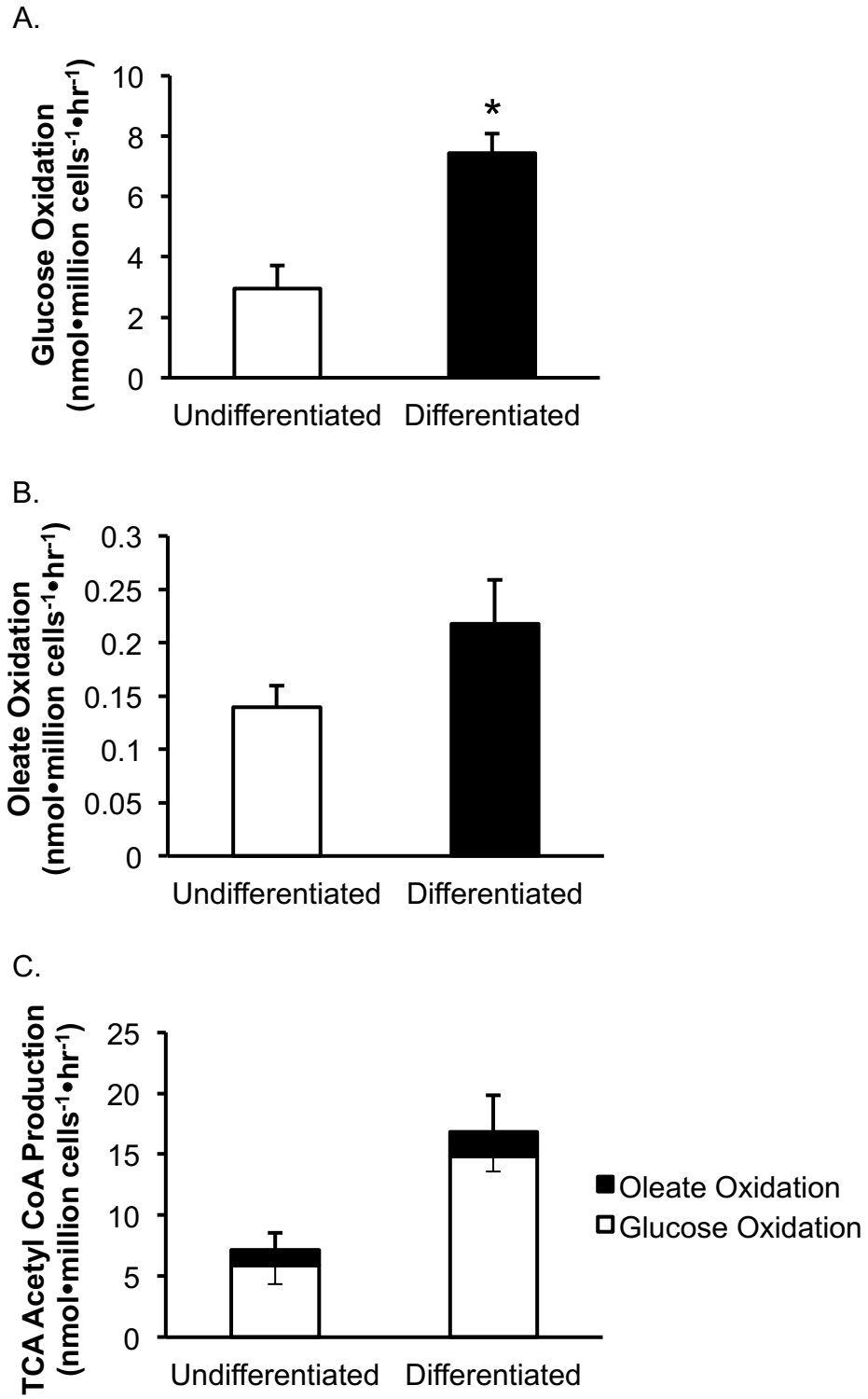

A.
Glucose Oxidation (nmol•million cells-1•hr-1)
10
8
6
4
2
0
*
Undifferentiated
Differentiated
B.
Oleate Oxidation (nmol•million cells-1•hr-1)
0.3
0.25
0.2
0.15
0.1
0.05
0
Undifferentiated
Differentiated
C.
TCA Acetyl CoA Production (nmol•million cells-1•hr-1)
25
20
15
10
5
0
Undifferentiated
Differentiated
Oleate Oxidation
Glucose Oxidation

Figure 4-4. Effect of cardiac myocyte differentiation on glycolysis. Glycolysis was measured in undifferentiated H9C2 cells and H9C2 cells differentiated toward a cardiac myocyte. * $p<0.05$ compared to Undifferentiated.

Figure 4-4

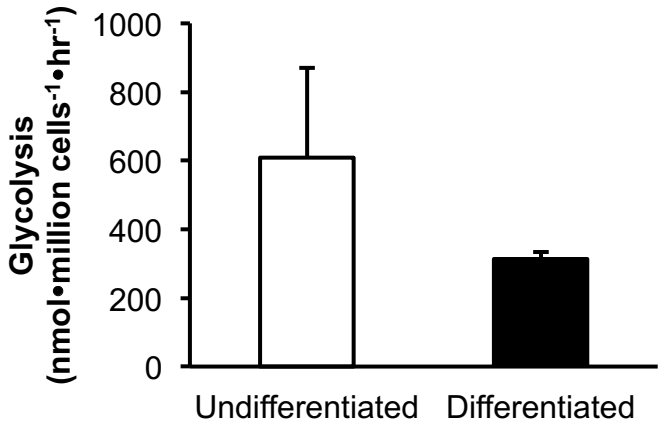

Glycolysis
(nmol•million cells-1•hr-1)
1000
800
600
400
200
0
Undifferentiated
Differentiated

Figure 4-5. Effect of cardiac myocyte differentiation on the contribution of glucose oxidation, oleate oxidation, and glycolysis to ATP production. % ATP Production was compared between undifferentiated H9C2 cells and H9C2 cell differentiated toward a cardiac myocyte. * $p<0.05$ compared to Undifferentiated.

Figure 4-5

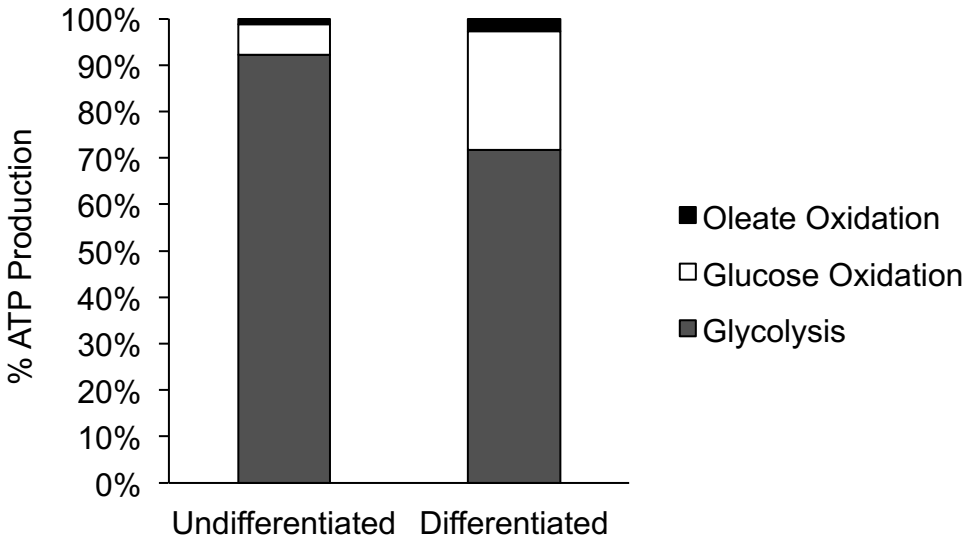

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% ATP Production
Undifferentiated
Differentiated
Oleate Oxidation
Glucose Oxidation
Glycolysis

Figure 4-6. Effect of cardiac myocyte differentiation on glucose metabolism. A. Pyruvate dehydrogenase (PDH), B. Hexokinase 1, and C. Phosphoglycerate mutase 1 (PGAM1) protein expression. D. Representative western blots. Undifferentiated H9C2 cells and H9C2 cells differentiated toward a cardiac myocyte were compared. * $p<0.05$ compared to Undifferentiated.

Figure 4-6

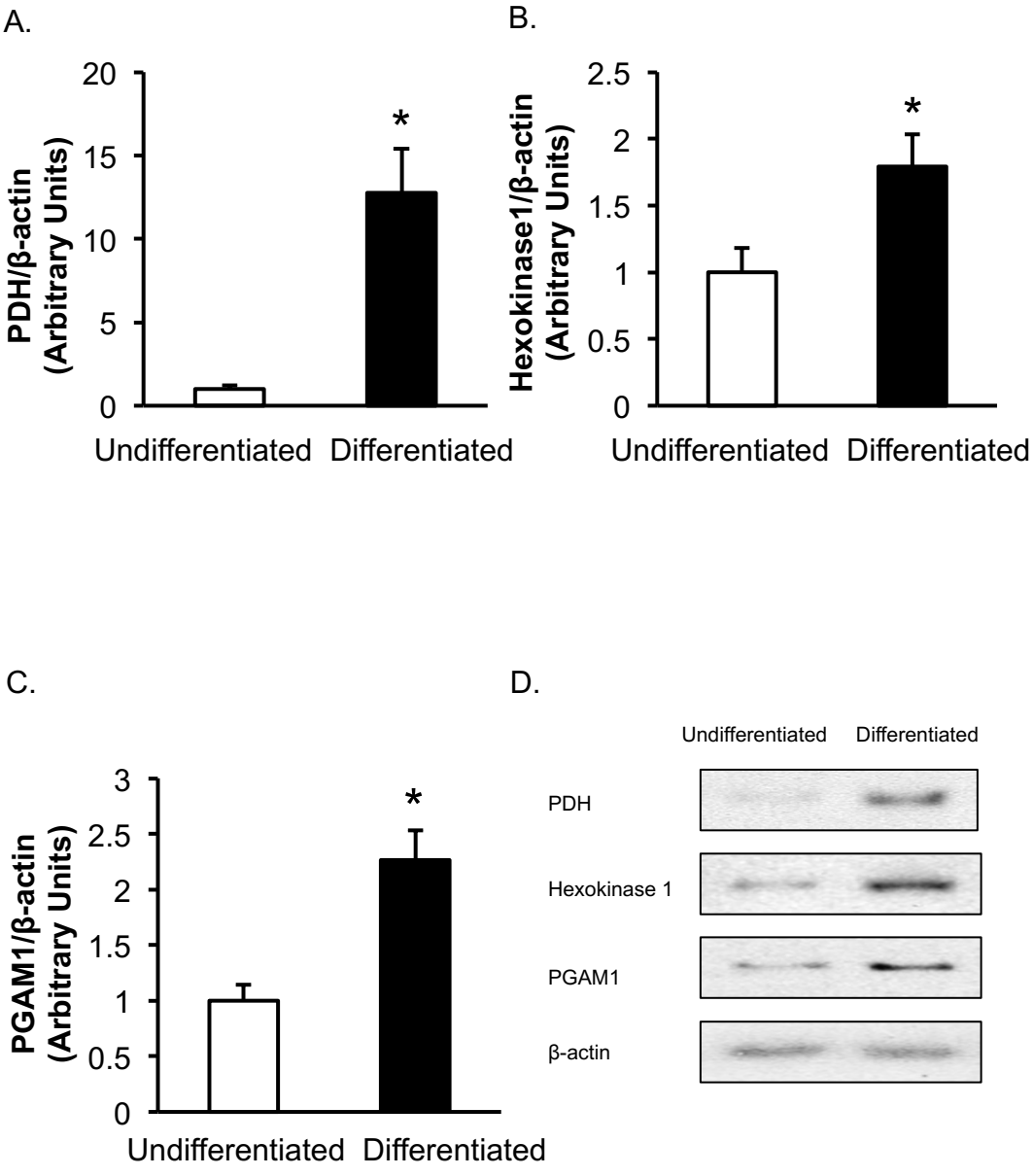
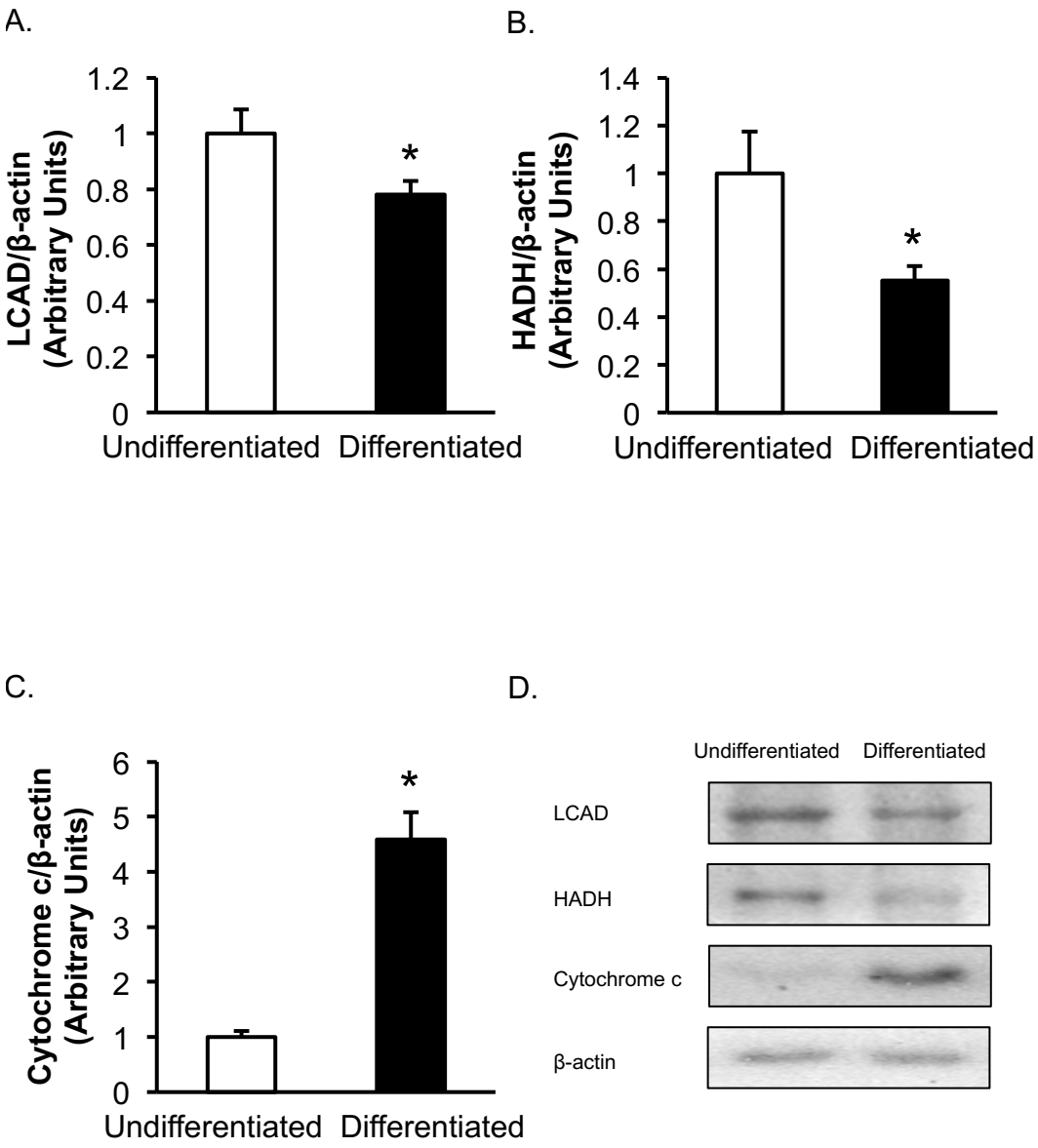

A.
PDH/β-actin (Arbitrary Units)
20
15
10
5
0
*
Undifferentiated
Differentiated
B.
Hexokinase1/β-actin (Arbitrary Units)
2.5
2
1.5
1
0.5
0
*
Undifferentiated
Differentiated
C.
PGAM1/β-actin (Arbitrary Units)
3
2.5
2
1.5
1
0.5
0
*
Undifferentiated
Differentiated
D.
Undifferentiated
Differentiated
PDH
Hexokinase 1
PGAM1
β-actin

Figure 4-7. Effect of cardiac myocyte differentiation on fatty acid oxidation and mitochondrial oxidative metabolism. A. Long chain acyl CoA dehydrogenase (LCAD), B. Hydroxyacyl CoA dehydrogenase (HADH), and C. Cytochrome c protein expression. D. Representative western blots. Undifferentiated H9C2 cells and H9C2 cells differentiated toward a cardiac myocyte were compared. * $p<0.05$ compared to Undifferentiated.

Figure 4-7


A.
LCAD/β-actin (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
*
Undifferentiated
Differentiated
B.
HADH/β-actin (Arbitrary Units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Undifferentiated
Differentiated
C.
Cytochrome c/β-actin (Arbitrary Units)
6
5
4
3
2
1
0
*
Undifferentiated
Differentiated
D.
Undifferentiated
Differentiated
LCAD
HADH
Cytochrome c
β-actin

CHAPTER 5

Insulin glargine stimulates glucose oxidation and improves *db/db* mouse heart function.

Two manuscripts containing portions of the research presented in this chapter are in preparation to be submitted to Diabetes and Cardiovascular Research.

Cory Wagg performed the isolated working heart perfusions. Ken Milner helped with the western blots. Donna Beker performed the echocardiography. Liyan Zhang prepared the HPLC samples and Kenneth Strynadka performed the HPLC measurements. I treated the mice, performed the rest of the experiments including the majority of the western blots as well as the experimental design, statistical analysis, and writing the manuscript.

CHAPTER 5

Insulin glargine stimulates glucose oxidation and improves *db/db* mouse heart function.

5.1 Abstract

In diabetes, the heart has decreased glucose oxidation rates and increased fatty acid oxidation rates. These changes in energy metabolism decrease cardiac efficiency and are believed to contribute to the decreased cardiac function observed in diabetes. The ability of insulin to stimulate glucose oxidation and inhibit fatty acid oxidation is believed to be beneficial in the heart, especially in diabetes. In this study we examined the long-term effects of insulin treatment on cardiac function. In order to determine the long-term effect of long acting insulin on cardiovascular function and energy metabolism, 18 wk old *db/db* mice were subcutaneously injected with vehicle or insulin glargine every day for 4 wk. As expected, insulin glargine improved whole body glucose tolerance. Echocardiographic analysis showed that insulin glargine improved cardiac output and stroke volume. However, no changes in cardiac glucose oxidation or fatty acid oxidation were seen in *ex vivo* perfused hearts. However, acute addition of insulin glargine to *ex vivo* perfused hearts did stimulate cardiac glucose oxidation, inhibit fatty acid oxidation, and improve cardiac efficiency. The ability of insulin glargine to acutely stimulate glucose oxidation may explain why chronic

treatment with insulin glargine improved the *in vivo* cardiac function of *db/db* mice. Overall, these results support the concept that stimulating glucose oxidation is a promising strategy to improve cardiac function and suggest that insulin glargine may reduce cardiac dysfunction in diabetic patients.

5.2 Introduction

Despite there being a general consensus that alterations in energy metabolism are involved in heart disease, there is a lack of consensus on the role of altered glucose oxidation in contributing to cardiac dysfunction. However, it has been reported that improving cardiac efficiency, either by stimulating glucose oxidation or by inhibiting fatty acid oxidation, improves the function of diseased hearts [70, 71, 120, 154, 170, 262, 283, 305]. In diabetes, the heart becomes insulin resistant and glucose oxidation decreases [28, 33, 39, 78, 80, 126, 158, 282, 318]. Further, the failing heart is also insulin resistant [117, 170, 182, 183, 315, 316] and this contributes to the severity of heart failure [159, 190, 240, 245, 261, 316].

There is evidence to indicate that increasing glucose oxidation can improve cardiac function in the failing heart [120, 126, 151, 152, 159, 182, 240, 282]. For example, it has been reported that dichloroacetate (DCA) improves cardiac function in both right ventricle hypertrophy and during ischemia/reperfusion [152, 221, 222, 273]. DCA increases pyruvate

dehydrogenase (PDH) activity (the rate limiting enzyme of glucose oxidation) and glucose oxidation by inhibiting pyruvate dehydrogenase kinase (PDK) activity. Further, DCA can decrease circulating lactate levels, improve diastolic function, and increase overall survival of Dahl salt-sensitive rats on a high salt diet [120]. Stimulating cardiac glucose oxidation, via inhibition of fatty acid oxidation, also improves cardiac function [44, 70]. For example, malonyl CoA decarboxylase knockout (MCD-/-) mice subjected to coronary artery ligation (CAL) have better cardiac function and lower proton production than their wild type counterparts [170]. Inhibiting fatty acid oxidation with a MCD inhibitor also improves the coupling of glycolysis and glucose oxidation and function of pig hearts during ischemia/reperfusion [70].

In this study we examined whether stimulating cardiac glucose oxidation improves cardiac function in insulin resistant hearts. To do this we treated *db/db* mice, a well established model of diabetes and diastolic dysfunction, with vehicle or insulin glargine for 4 wk. We also examined the acute effect of insulin glargine on cardiac energy metabolism in the isolated working heart. Chronic treatment with insulin glargine, which was effective at acutely stimulating cardiac glucose oxidation, improved *db/db* mouse *in vivo* cardiac function. Overall, the results in this study indicate that stimulating glucose oxidation may be a promising strategy to improve the function of insulin resistant hearts.

5.3 Materials and methods

The University of Alberta Health Sciences Animal Welfare Committee approved all animal care and procedures. Mice were fed a standard chow diet *ad libitum* and kept in a 12 hr light:12 hr dark cycle. 10 wk old C57bl6 or *db/db* mouse isolated working hearts were treated acutely with vehicle or increasing amounts of insulin glargine. Glucose oxidation, lactate oxidation, palmitate oxidation, glycolysis and *ex vivo* cardiac function were assessed in these hearts using the isolated working heart. Information pertaining to the isolated working heart and insulin glargine treatment is provided in Chapter 2 of this thesis.

In a second set of experiments, 18 wk old *db/db* mice were subcutaneously injected with either vehicle (Saline) or insulin glargine (150 U/kg body weight) every day for 4 wk. At the end of the treatment period, glucose tolerance, and *in vivo* cardiac function were assessed. Protocols for the oral glucose tolerance test and echocardiography are provided in Chapter 2 of this thesis. Cardiac glucose oxidation, glycolysis, lactate oxidation, palmitate oxidation, and cardiac function were then measured via the isolated working heart perfusion. 100 μU/ml insulin was added to the perfusate 30 min into the 60 min aerobic perfusion. These measurements are described in Chapter 2 of this thesis.

At the end of the isolated working heart perfusions the hearts were immediately snap frozen in liquid N_2 and stored at -80°C. The heart tissue was used for biochemical measurements including assessment of protein expression

and short chain CoA levels. Protocols for these measurements and statistical analysis are provided in Chapter 2 of this thesis.

5.4 Results

5.4.1 Acute treatment with insulin glargine stimulates cardiac glucose oxidation.

The first thing we did in this study was to determine the effect of insulin glargine on cardiac energy metabolism. Insulin glargine stimulated glucose oxidation and inhibited palmitate oxidation in both C57bl/6 and *db/db* mouse hearts (Figure 5-1, 5-2). This resulted in an increase in the % ATP from glucose oxidation in both C57bl/6 (vehicle, 13%; insulin glargine, 30%) and *db/db* (vehicle, 5%; insulin glargine, 14%) mouse hearts. Insulin glargine also significantly improved the cardiac efficiency of C57bl/6 mice (Figure 5-3).

5.4.2 Chronic treatment with insulin glargine improves whole body glucose tolerance.

We then treated *db/db* mice, a diabetic model that develops diastolic dysfunction, with vehicle or insulin glargine daily for 4 wk. At the end of the 4 wk treatment period whole body glucose tolerance was assessed. We observed a significant improvement in glucose tolerance in *db/db* mice treated with insulin glargine compared to vehicle (Figure 5-4). Body weight did not appear to be a

factor in this improvement in glucose tolerance since it was not significantly different between groups (Figure 5-4).

5.4.3 Chronic insulin glargine administration improves *in vivo* cardiac function in *db/db* mice.

We also assessed cardiac function at the end of the treatment period. Echocardiography results indicated that insulin glargine does not impair *db/db* mouse cardiac function (Figure 5-5 and Table 5-1). Further, chronic treatment of *db/db* mice with insulin glargine significantly increased cardiac output and stroke volume (Figure 5-5). We also did not observe negative effects when we assessed the *in vitro* function of hearts either treated acutely with insulin glargine or after the 4 wk treatment period (Figure 5-5).

5.4.4 Chronic treatment with long-acting insulin does not impair cardiac energy metabolism of *db/db* mice.

Cardiac energy metabolism was also measured in the *db/db* mice treated chronically with vehicle or insulin glargine. We did not observe significant changes in any of the parameters assessed, which included glucose oxidation, palmitate oxidation, glycolysis, and lactate oxidation (Figure 5-6). In addition, insulin glargine did not have any effect on the ability of insulin to stimulate glucose oxidation, or inhibit fatty acid oxidation (Figure 5-6). When we assessed the phosphorylation status of key proteins involved in insulin signaling (phospho

glycogen synthase kinase 3β (pGSK3β), pAkt, pPDH) we did not observe any difference between hearts from mice treated chronically with vehicle and insulin glargine (Figure 5-7). We also did not observe a significant acute or chronic effect of insulin glargine on cardiac malonyl CoA levels (Table 5-2, 5-3, 5-4) or 5' AMP-activated protein kinase (AMPK) phosphorylation (Figure 5-8).

5.4.5 Chronic treatment with insulin glargine decreases cardiac CD36 expression in *db/db* mice.

We also assessed peroxisome proliferator activated receptor (PPAR)α signaling in an attempt to elucidate the mechanisms responsible for the beneficial effects observed in *db/db* mice treated for 4 wk with insulin glargine. Cardiac protein expression of CD36, which is regulated by PPARα, was reduced in the insulin glargine treated mice (Figure 5-9). This change in CD36 expression was accompanied by a reduction in PPARα protein expression (Figure 5-9).

5.5 Discussion

In this study we assessed the effect of long acting insulin on cardiac energy metabolism and function. The major goal for initiating this study was to assess the effect of stimulating cardiac glucose oxidation on cardiac function. In the first part of this study we examined the acute effect of insulin glargine on cardiac energy metabolism. We then treated *db/db* mice, a diabetic model that

develops diastolic dysfunction [78, 158, 261], with insulin glargine for 4 wk. We did not observe any detrimental effects of insulin glargine on *db/db* mouse cardiac function or energy metabolism. Further, chronic treatment with insulin glargine, which stimulated cardiac glucose oxidation, improved *db/db* mouse cardiac function.

One of the metabolic changes that frequently accompanies heart failure is a decrease in the coupling of glycolysis and glucose oxidation. For example, CAL decreases the coupling of glycolysis and glucose oxidation in mouse hearts [170]. Further, Dahl salt-sensitive rat hearts with heart failure with preserved ejection fraction (HFpEF) have elevated cardiac glucose uptake and plasma lactate levels [120]. There is also evidence that lessening this uncoupling of glycolysis and glucose oxidation improves cardiac function. For example, DCA improves cardiac function in both right ventricle hypertrophy and during ischemia/reperfusion [152, 221, 222, 273]. DCA increases PDH activity and glucose oxidation by inhibiting PDK activity. In Dahl salt-sensitive rats DCA decreases circulating lactate levels, improved diastolic function, and increased overall survival of Dahl salt-sensitive rats on a high salt diet (36).

We hypothesized that stimulating glucose oxidation with insulin may improve cardiac function in the setting of diabetic cardiomyopathy. We determined the acute effect of insulin glargine on cardiac energy metabolism. In C57bl6 and *db/db* mouse hearts insulin glargine significantly increased glucose oxidation and decreased palmitate oxidation. However, acute treatment with

insulin glargine only significantly improved cardiac efficiency in the C57bl6 mice. Insulin glargine may not have stimulated glucose oxidation and inhibited palmitate oxidation sufficiently to improve *db/db* mouse cardiac efficiency. The beneficial effects of insulin glargine on cardiac energy metabolism indicate that this drug does not pose a cardiovascular risk. This is in agreement with the Outcome Reduction with Insulin Glargine Intervention (ORIGIN) trial and its long term follow up which showed that insulin glargine treatment does not increase the risk of cardiovascular events [205, 206].

To examine what long term effects stimulating glucose oxidation has on cardiac function and energy metabolism, 18 wk old *db/db* mice were treated for 4 wk with insulin glargine. 18 wk old *db/db* mice were used because by this age they have a clear diastolic dysfunction (32-34). This allowed us to examine whether insulin glargine can improve cardiac function. Echocardiographic analysis of these hearts at the end of the treatment period indicated that chronic treatment with insulin glargine improved *db/db* mouse *in vivo* cardiac function. This improvement in cardiac function was not accompanied by a change in diastolic function. Based on the current information it is unclear what parameters insulin glargine is improving to increase stroke volume and cardiac output in these *db/db* mice. The absence of differences in cardiac energy metabolism and function *ex vivo* suggest that the benefit of insulin glargine on cardiac function is probably acute. However, an improvement in cardiac efficiency may not have been a contributing factor to this improved cardiac function in response to insulin

glargine treatment since insulin glargine was not effective at acutely improving cardiac efficiency in *db/db* mouse hearts *ex vivo*.

Despite this evidence that chronic effects are not responsible for the beneficial effect on insulin glargine on in vivo cardiac function, we observed a decrease in PPARα and CD36 expression that would be expected to be beneficial in diabetes. PPARα has been previously implicated in the elevation of cardiac fatty acid oxidation and fatty acid uptake observed in diabetes [33, 81]. While cardiac PPARα expression is not elevated in 15-18 wk old *db/db* mice, the expression of its target genes (eg CD36, long chain acyl CoA dehydrogenase) are elevated [159]. In fact, PPARα transgenic mice develop a phenotype resembling Type 2 diabetes [81, 211]. We were, therefore, interested in whether insulin glargine regulates CD36 expression. Chronic treatment with insulin glargine reduced cardiac expression of CD36 protein. This was accompanied by a decrease in PPARα expression, which suggests that insulin glargine decreases PPARα signaling. Since PPARα signaling can impair cardiac function [243], these results suggest that a reduction in PPARα signaling may also contribute to the improvement in cardiac function observed with insulin glargine treatment. Interestingly, protein O-GlcNAcylation has been shown to increase cardiac fatty acid oxidation, potentially due to an elevation in CD36 expression [139]. Therefore, it is possible that insulin glargine decreased CD36 expression in the *db/db* mouse hearts by inhibiting the hexosamine biosynthesis pathway and protein O-GlcNAcylation. However, it would be expected that if a decrease

in PPARα signaling contributed to the improved cardiac function, cardiac function would have also been improved in *ex vivo* measurements made in working heart perfusions.

In summary, we demonstrate that chronic treatment with insulin glargine improves *db/db* mouse cardiac function. However, this was not associated with an improvement in *ex vivo* cardiac function or energy metabolism. Insulin glargine does acutely stimulate cardiac glucose oxidation. This suggests that the insulin glargine induced improvement in *in vivo* cardiac function is due to an acute affect of insulin glargine on glucose oxidation. While further work is necessary to confirm this, these findings suggest that stimulating glucose oxidation may be a promising strategy for treating cardiac dysfunction.

Figure 5-1. Insulin glargine stimulates cardiac glucose oxidation. A. Glucose oxidation and B. glycolysis was measured in 10 wk old C57bl6 mouse hearts treated acutely with vehicle or insulin glargine during the working heart perfusion (concentrations indicated in the figure). C. Glucose oxidation and D. glycolysis was measured in 10 wk old *db/db* mouse hearts treated acutely with vehicle or insulin glargine during the working heart perfusion (concentrations indicated in the figure). n=6 * $p<0.05$ compared to Vehicle. ** $p<0.05$ between groups. Values shown as mean ± SEM.

Figure 5-1

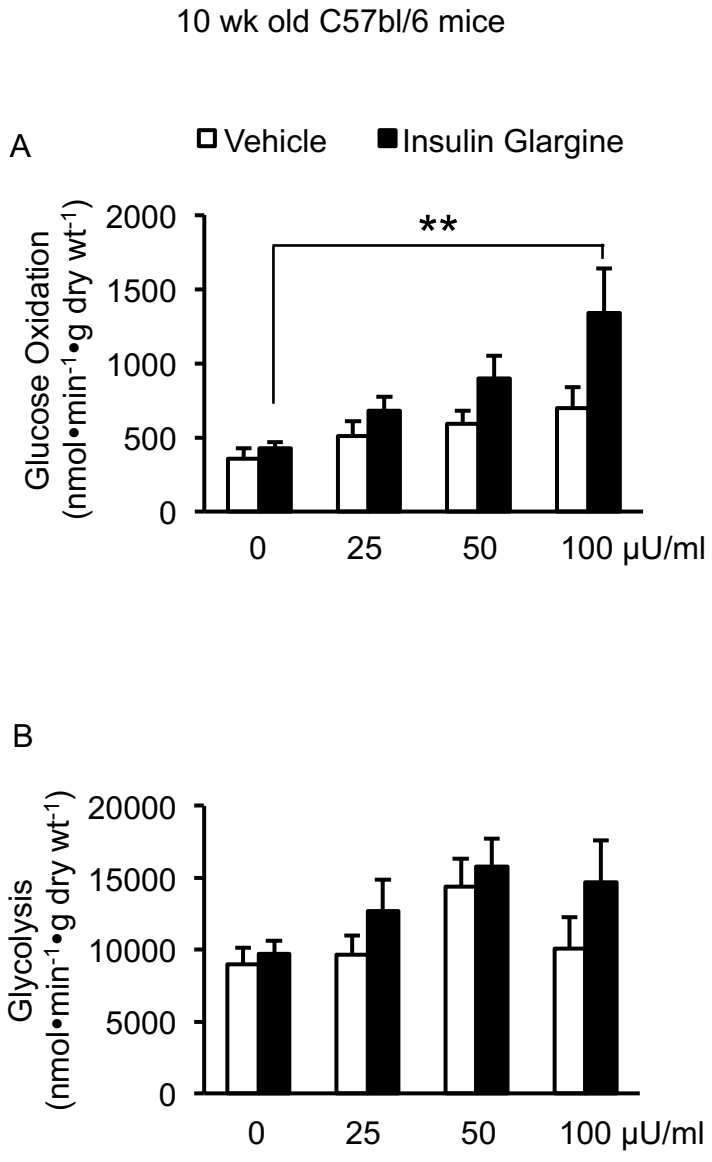

10 wk old C57bl/6 mice
A
Vehicle
Insulin Glargine
Glucose Oxidation (nmol•min-1•g dry wt-1)
2000
1500
1000
500
0
**
0
25
50
100 µU/ml
B
Glycolysis (nmol•min-1•g dry wt-1)
20000
15000
10000
5000
0
0
25
50
100 µU/ml

Figure 5-1

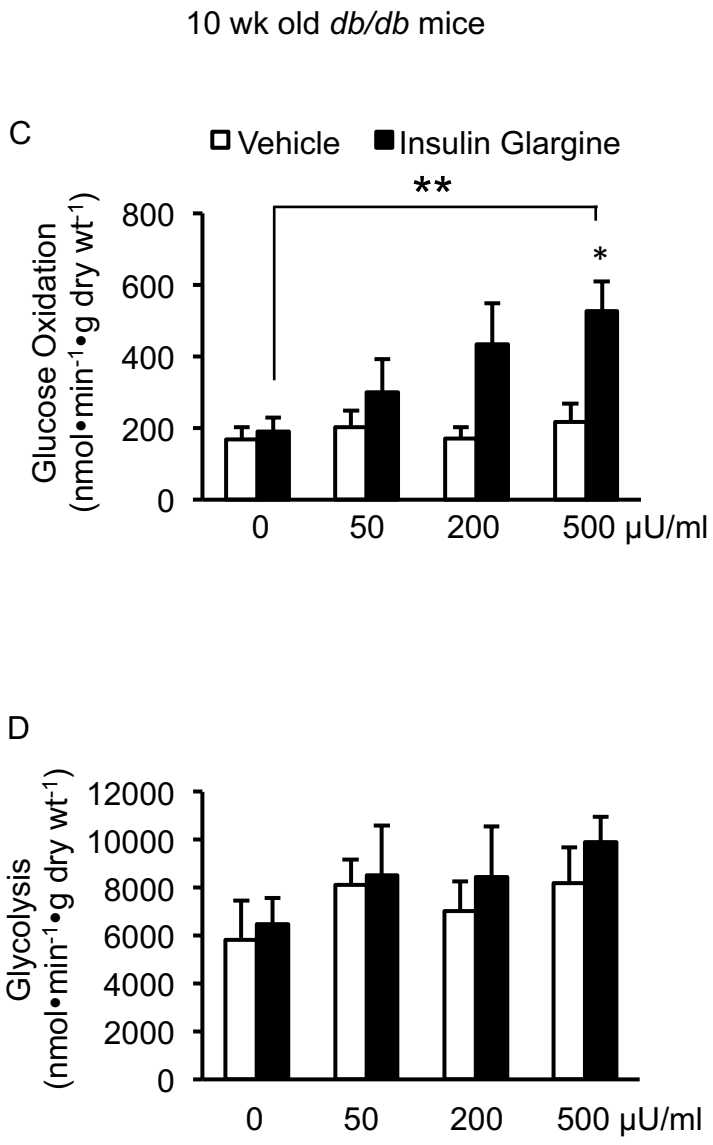

10 wk old *db/db* mice
C
Vehicle
Insulin Glargine
**
*
Glucose Oxidation
(nmol•min-1•g dry wt-1)
800
600
400
200
0
0
50
200
500 µU/ml
D
Glycolysis
(nmol•min-1•g dry wt-1)
12000
10000
8000
6000
4000
2000
0
0
50
200
500 µU/ml

Figure 5-2. Insulin glargine reduces cardiac palmitate oxidation. A. Palmitate oxidation and B. lactate oxidation were measured in 10 wk old C57bl6 mouse hearts treated acutely with vehicle or insulin glargine during the working heart perfusion (concentrations indicated in the figure). C. Palmitate oxidation and D. lactate oxidation were measured in 10 wk old *db/db* mouse hearts treated acutely with vehicle or insulin glargine during the working heart perfusion (concentrations indicated in the figure). n=6 * $p<0.05$ compared to Vehicle. ** $p<0.05$ between groups. Values shown as mean ± SEM.

Figure 5-2

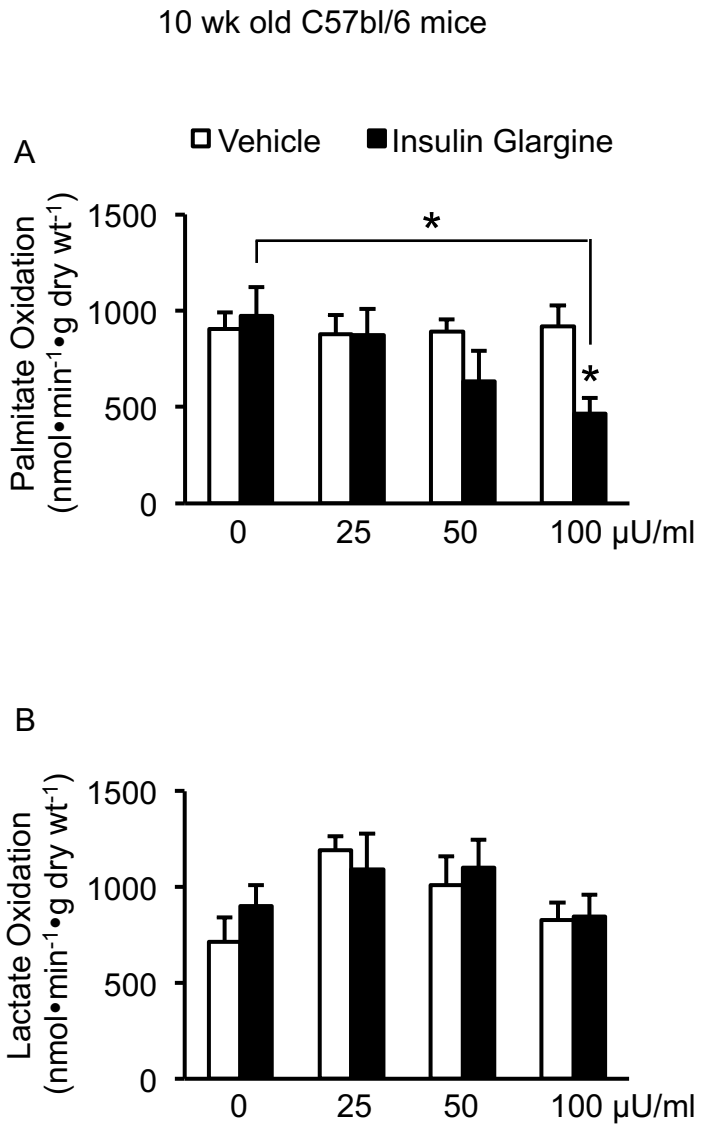

10 wk old C57bl/6 mice
A
Vehicle
Insulin Glargine
Palmitate Oxidation (nmol•min-1•g dry wt-1)
1500
1000
500
0
*
*
0
25
50
100 µU/ml
B
Lactate Oxidation (nmol•min-1•g dry wt-1)
1500
1000
500
0
0
25
50
100 µU/ml

Figure 5-2

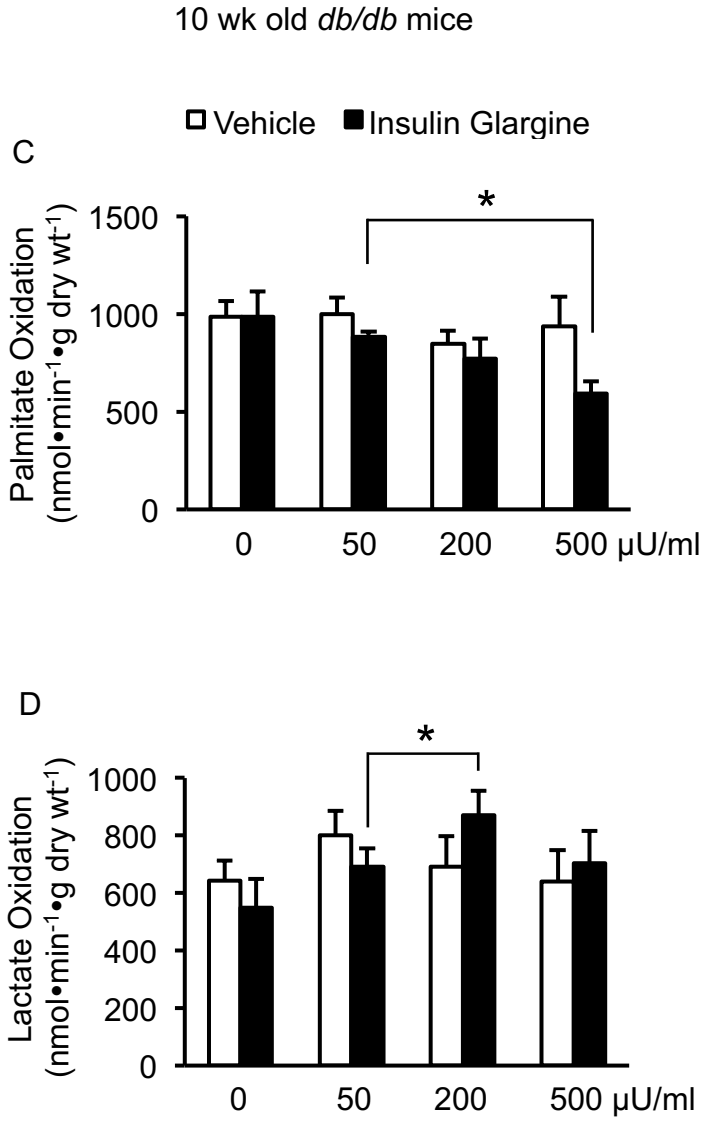

10 wk old *db/db* mice
Vehicle
Insulin Glargine
C
Palmitate Oxidation
(nmol•min-1•g dry wt-1)
1500
1000
500
0
*
0
50
200
500 µU/ml
D
Lactate Oxidation
(nmol•min-1•g dry wt-1)
1000
800
600
400
200
0
*
0
50
200
500 µU/ml

Figure 5-3. Insulin glargine improves cardiac efficiency. A. Cardiac efficiency and B. proton production were measured in 10 wk old C57bl6 mouse hearts treated acutely with vehicle or insulin glargine during the working heart perfusion (concentrations indicated in the figure). C. Cardiac efficiency and D. proton production were measured in 10 wk old *db/db* mouse hearts treated acutely with vehicle or insulin glargine during the working heart perfusion (concentrations indicated in the figure). Proton production was calculated based on glucose oxidation and glycolysis rates. n=5-6* $p<0.05$ compared to Vehicle. ** $p<0.05$ between groups. Values shown as mean ± SEM.

Figure 5-3

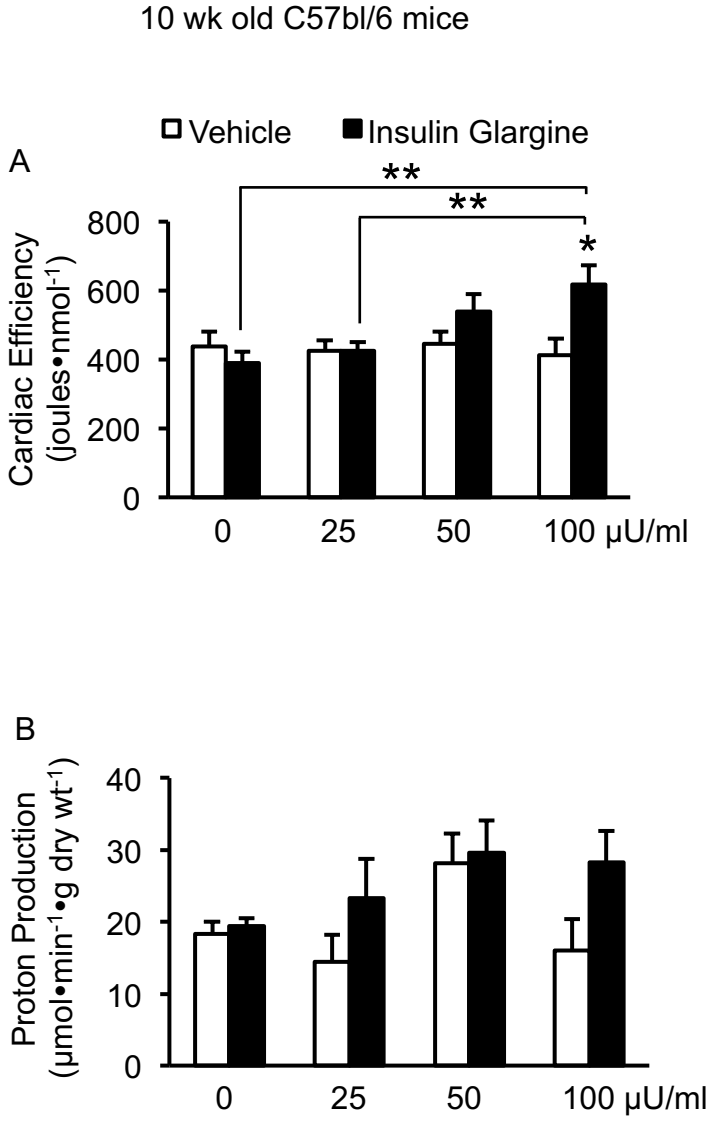

10 wk old C57bl/6 mice
Vehicle
Insulin Glargine
A
**
**
*
Cardiac Efficiency (joules•nmol-1)
800
600
400
200
0
0
25
50
100 µU/ml
B
Proton Production (µmol•min-1•g dry wt-1)
40
30
20
10
0
0
25
50
100 µU/ml

Figure 5-3

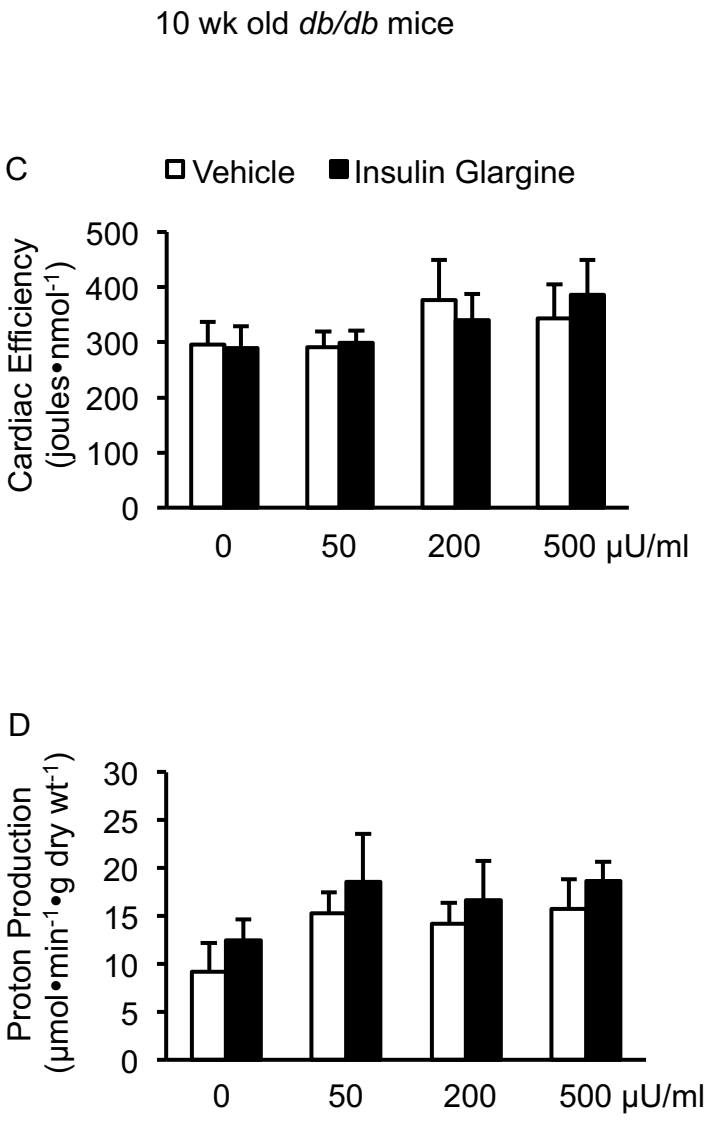

10 wk old *db/db* mice
C
Vehicle
Insulin Glargine
Cardiac Efficiency (joules•nmol-1)
500
400
300
200
100
0
0
50
200
500 µU/ml
D
Proton Production (µmol•min-1•g dry wt-1)
30
25
20
15
10
5
0
0
50
200
500 µU/ml

Figure 5-4. Chronic treatment with long acting insulin improves whole body glucose tolerance in *db/db* mice. A. Whole body glucose tolerance and B. body weight were assessed in the *db/db* mice at the end of the 4 wk treatment with either vehicle or insulin glargine. n=6 * $p<0.05$ compared to Vehicle. Values shown as mean ± standard error of mean (SEM)

Figure 5-4

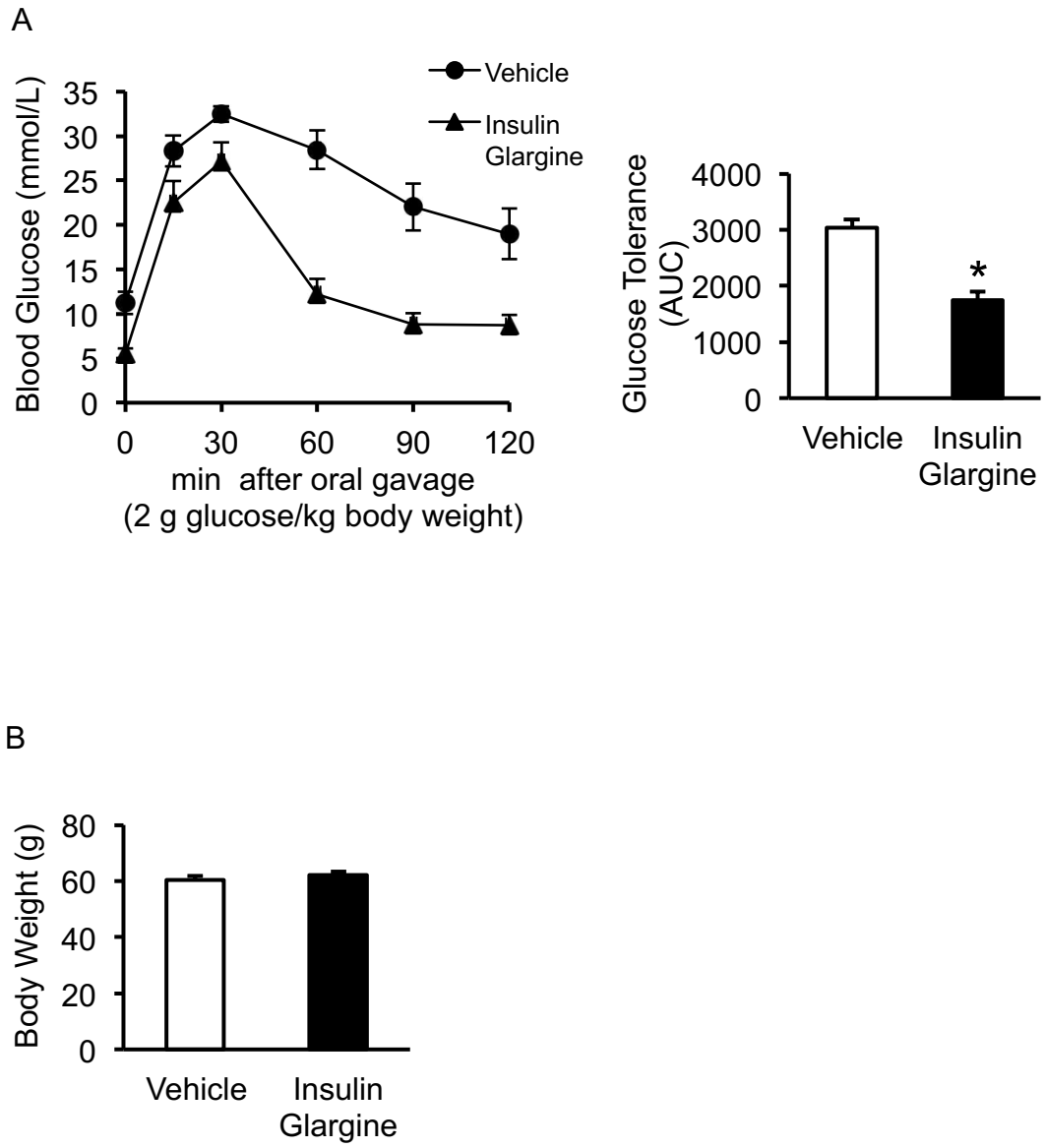

A
Vehicle
Insulin Glargine
Blood Glucose (mmol/L)
35
30
25
20
15
10
5
0
0
30
60
90
120
min after oral gavage
(2 g glucose/kg body weight)
Glucose Tolerance (AUC)
4000
3000
2000
1000
0
*
Vehicle
Insulin Glargine
B
Body Weight (g)
80
60
40
20
0
Vehicle
Insulin Glargine

Figure 5-5. Insulin glargine improves *in vivo* cardiac function in *db/db* mice. A. Cardiac output, B. stroke volume, C. % ejection fraction (EF), and D. E'/A' were assessed via echocardiography in *db/db* mice treated for 4 wk with vehicle or insulin glargine. The acute affect of insulin glargine on cardiac work in E. 10 wk old C57bl/6 mice and F. 10 wk old *db/db* mice was assessed during the isolated working heart perfusion. G. Cardiac work was also assessed in hearts from *db/db* mice treated for 4 wk with vehicle or insulin glargine during the isolated working heart perfusion. n=9-10 * $p<0.05$ compared to Vehicle. Values shown as mean ± SEM

Figure 5-5

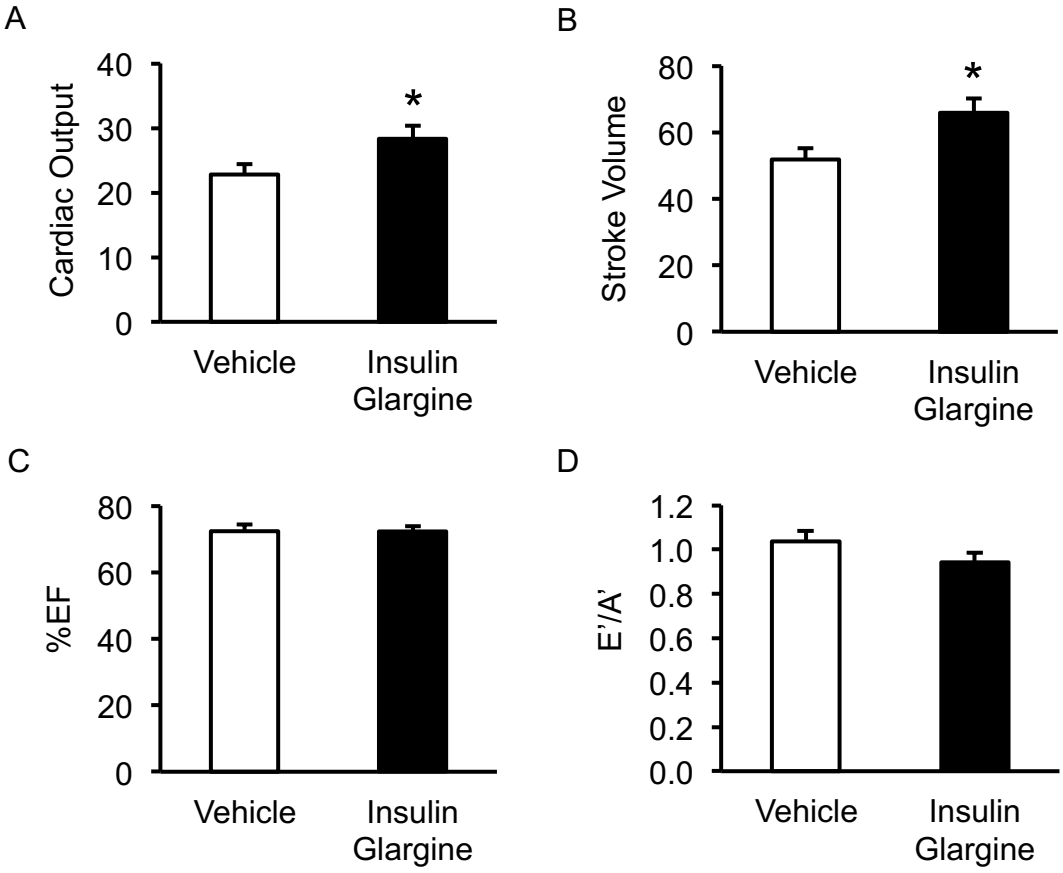

A
Cardiac Output
40
30
20
10
0
*
Vehicle
Insulin Glargine
B
Stroke Volume
80
60
40
20
0
*
Vehicle
Insulin Glargine
C
%EF
80
60
40
20
0
Vehicle
Insulin Glargine
D
E'/A'
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
Insulin Glargine

Figure 5-5

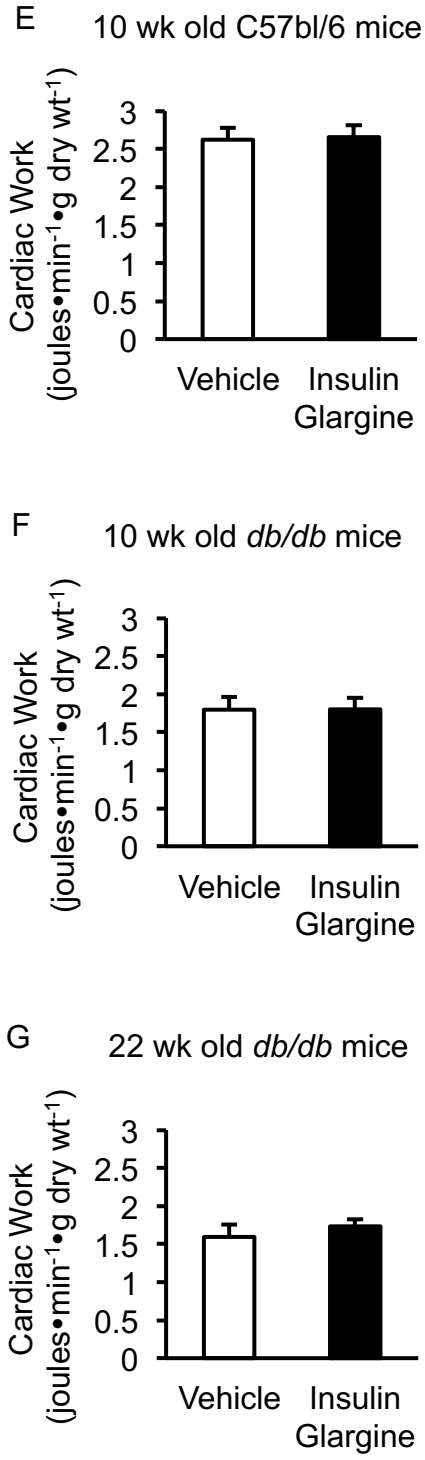

E
10 wk old C57bl/6 mice
Cardiac Work
(joules•min-1•g dry wt-1)
3
2.5
2
1.5
1
0.5
0
Vehicle
Insulin Glargine
F
10 wk old db/db mice
Cardiac Work
(joules•min-1•g dry wt-1)
3
2.5
2
1.5
1
0.5
0
Vehicle
Insulin Glargine
G
22 wk old db/db mice
Cardiac Work
(joules•min-1•g dry wt-1)
3
2.5
2
1.5
1
0.5
0
Vehicle
Insulin Glargine

Figure 5-6. Chronic treatment with long acting insulin does not impair cardiac energy metabolism in *db/db* mice. A. Glucose oxidation, B. glycolysis, C. palmitate oxidation, D. lactate oxidation, and E. cardiac efficiency were assessed in hearts from *db/db* mice treated for 4 wk with vehicle or insulin glargine. n=5 Values shown as mean ± SEM.

Figure 5-6

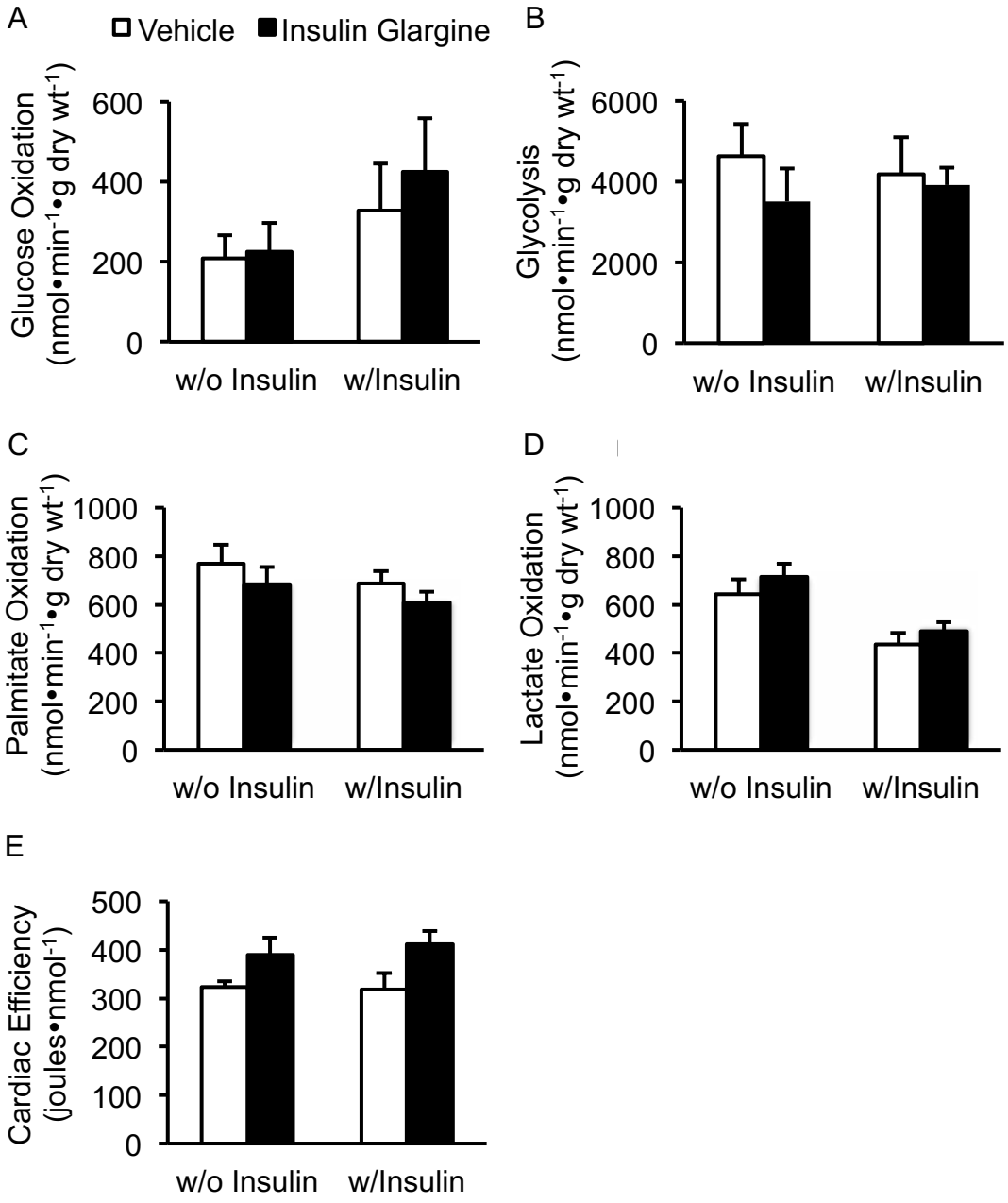

A
Vehicle
Insulin Glargine
Glucose Oxidation (nmol•min-1•g dry wt-1)
600
400
200
0
w/o Insulin
w/Insulin
B
Glycolysis (nmol•min-1•g dry wt-1)
6000
4000
2000
0
w/o Insulin
w/Insulin
C
Palmitate Oxidation (nmol•min-1•g dry wt-1)
1000
800
600
400
200
0
w/o Insulin
w/Insulin
D
Lactate Oxidation (nmol•min-1•g dry wt-1)
1000
800
600
400
200
0
w/o Insulin
w/Insulin
E
Cardiac Efficiency (joules•nmol-1)
500
400
300
200
100
0
w/o Insulin
w/Insulin

Figure 5-7. Chronic treatment with long acting insulin does not impair cardiac insulin signaling. A. pAkt, B. pGSK3β, and C. pPDH were measured in hearts from *db/db* mice treated for 4 wk with either vehicle or insulin glargine. n=5-6 Values shown as mean ± SEM.

Figure 5-7

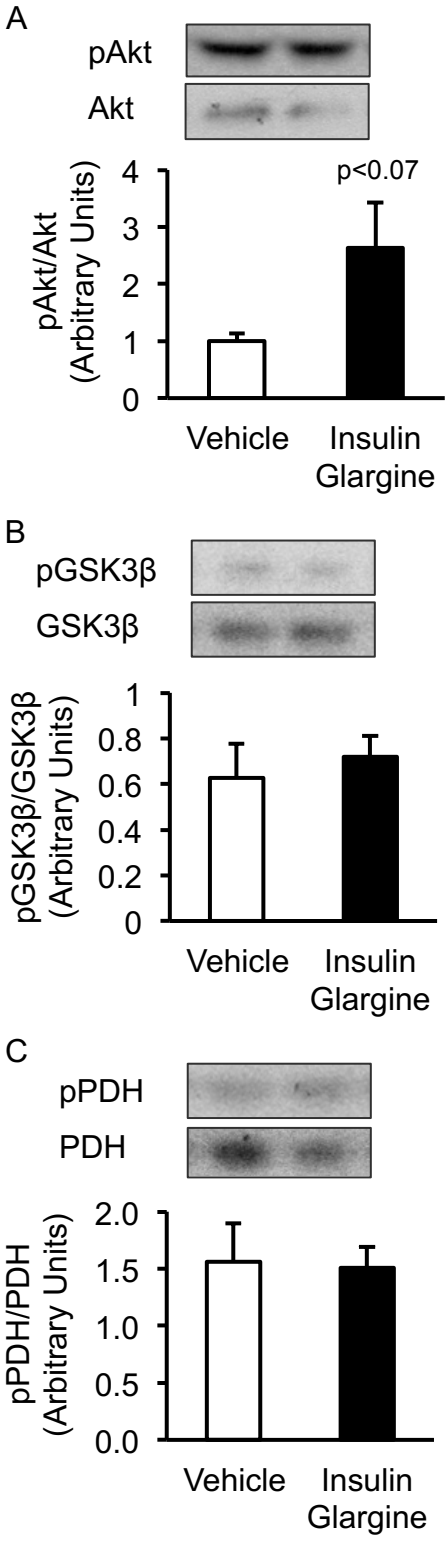

A
pAkt
Akt
pAkt/Akt (Arbitrary Units)
p<0.07
4
3
2
1
0
Vehicle
Insulin Glargine
B
pGSK3β
GSK3β
pGSK3β/GSK3β (Arbitrary Units)
1
0.8
0.6
0.4
0.2
0
Vehicle
Insulin Glargine
C
pPDH
PDH
pPDH/PDH (Arbitrary Units)
2.0
1.5
1.0
0.5
0.0
Vehicle
Insulin Glargine

Figure 5-8. Insulin glargine does not impair AMPK phosphorylation. pAMPK/AMPK was measured in hearts from A. *db/db* mice treated with vehicle or insulin glargine for 4 wk and B. C57bl6 mice and C. *db/db* mice treated acutely with vehicle or insulin glargine. n=4-6 Values shown as mean ± SEM.

Figure 5-8

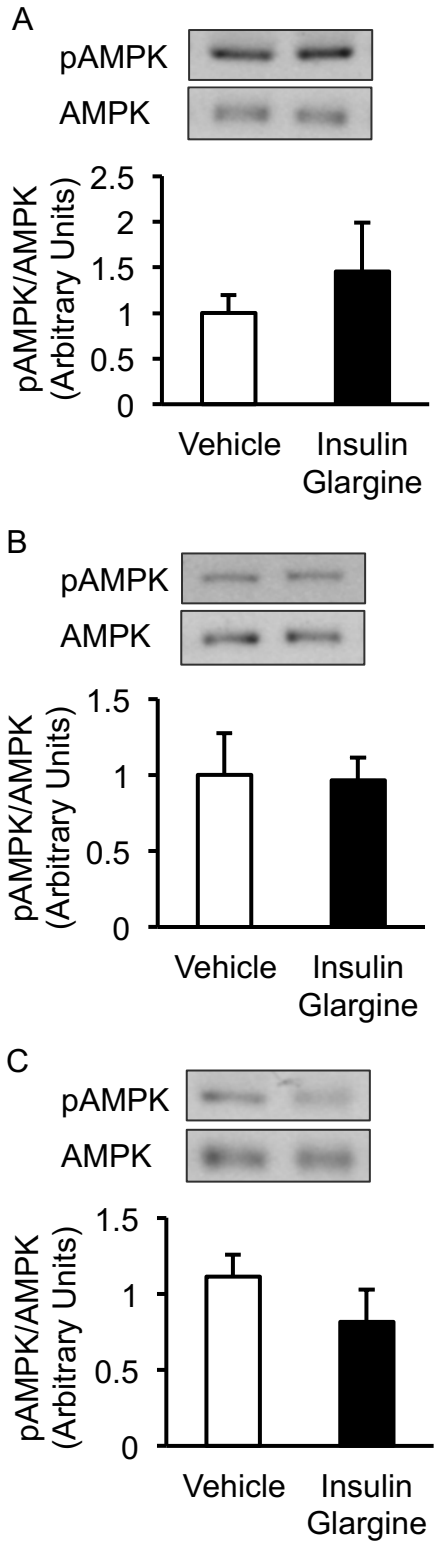

A
pAMPK
AMPK
pAMPK/AMPK (Arbitrary Units)
2.5
2
1.5
1
0.5
0
Vehicle
Insulin Glargine
B
pAMPK
AMPK
pAMPK/AMPK (Arbitrary Units)
1.5
1
0.5
0
Vehicle
Insulin Glargine
C
pAMPK
AMPK
pAMPK/AMPK (Arbitrary Units)
1.5
1
0.5
0
Vehicle
Insulin Glargine

Figure 5-9. Chronic treatment with insulin glargine decreases PPAR signaling. A. CD36 and B. peroxisome proliferator activated receptor (PPAR)α protein expression were measured in hearts from *db/db* mice treated with vehicle or insulin glargine for 4 wk. n=4-6 * $p<0.05$ compared to Vehicle. Values shown as mean ± SEM.

Figure 5-9

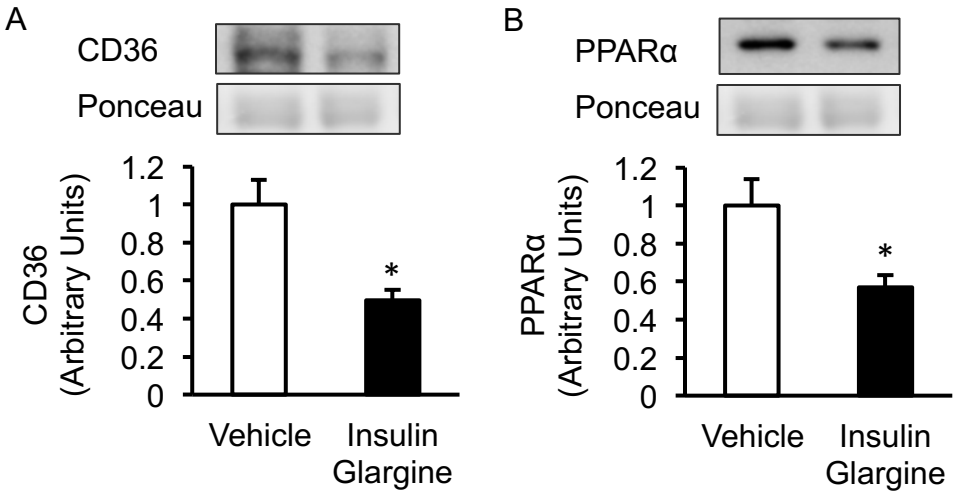

A
CD36
Ponceau
CD36 (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
*
Vehicle
Insulin Glargine
B
PPARα
Ponceau
PPARα (Arbitrary Units)
1.2
1
0.8
0.6
0.4
0.2
0
*
Vehicle
Insulin Glargine

Table 5-1. Effect of insulin glargine on *in viv*o cardiac function in *db/db* mice.

	Vehicle	Insulin Glargine
EF (%)	72±2.1	72±1.5
FS (%)	41±1.8	42±1.3
LV Mass Corrected	102±6.7	118±4.3
IVSd (mm)	0.86±0.04	0.87±0.02
LVIDd (mm)	4.0±0.16	4.4±0.13
LVPWd (mm)	0.82±0.03	0.83±0.02
IVSs (mm)	1.3±0.05	1.4±0.05
LVIDs (mm)	2.4±0.15	2.6±0.1
LVPWs (mm)	1.3±0.05	1.3±0.03
LV Vol;d	73±5.9	91±6.0
LV Vol;s	22±3.3	24.7±2.4
E/E'	35±3.8	31±1.6
E/A	1.4±0.07	1.4±0.10
E'/A'	1.0±0.05	0.9±0.04
Tei Index	0.68±0.02	0.70±0.02
E'	22±2.0	25±1.5
IVRT (ms)	20±0.7	20±0.7
IVCT (ms)	13±0.6	15±1.1

These results are from *db/db* mice treated with vehicle or insulin glargine for 4 wk. In vivo cardiac function was measured via echocardiography. n=9-11 * $p<0.05$. Values shown as mean ± SEM

Table 5-2. Effect of long term treatment with insulin glargine on short chain CoA levels in *db/db* mouse hearts.

	Vehicle	Insulin Glargine
CoA	98±5.4	92±15.7
Malonyl CoA	5.2±0.6	4.6±0.3
Acetyl CoA	23±2.5	31±3.3
Succinyl CoA	17±2.5	17±1.5

Short CoA levels (nmol/g wet wt) were measured in hearts from *db/db* mice treated with vehicle or insulin glargine for 4 wk. n=7 * $p<0.05$. Values shown as mean ± SEM

Table 5-3. Acute effect of insulin glargine on short chain CoA levels in C57bl6 mouse hearts.

	Vehicle	Insulin Glargine
CoA	50±13	46±8
Malonyl CoA	4.6±0.8	5.5±0.7
Acetyl CoA	18±1.7	23±3.7
Succinyl CoA	16±1.9	19±2.2

Short CoA levels (nmol/g wet wt) were measured in hearts from C57bl6 mice treated acutely with vehicle or insulin glargine. n=6-7 * $p<0.05$. Values shown as mean ± SEM

Table 5-4. Acute effect of insulin glargine on short chain CoA levels in *db/db* mouse hearts.

	Vehicle	Insulin Glargine
CoA	58±10	50±12
Malonyl CoA	6.5±1.1	6.5±0.5
Acetyl CoA	22±4.4	16±1.6
Succinyl CoA	22±2.1	18±1.6

Short CoA levels (nmol/g wet wt) were measured in hearts from *db/db* mice treated acutely with vehicle or insulin glargine. n=6-7 * $p<0.05$. Values shown as mean ± SEM

CHAPTER 6

Uncoupling of glycolysis and glucose oxidation accompanies the development of heart failure with preserved ejection fraction in Dahl salt-sensitive rats.

A manuscript is currently is preparation to be submitted to AJP- Heart and Circulatory Physiology: Natasha Fillmore, Jody L Levasseur, Arata Fukushima, Cory S Wagg, Wei Wang, Jason R Dyck, Gary D Lopaschuk. "Uncoupling of Glycolysis and Glucose Oxidation Accompanies the Development of Diastolic Heart Failure in Dahl Salt Sensitive Rats."

Jody Levasseur took care of the rats. Cory Wagg performed the isolated working heart perfusions. Donna Beker performed the echocardiography. My role in this work involved performing the rest of the experiments, statistical analysis, and writing the manuscript.

CHAPTER 6

Uncoupling of glycolysis and glucose oxidation accompanies the development of heart failure with preserved ejection fraction in Dahl salt-sensitive rats.

6.1 Abstract

Alterations in cardiac energy metabolism are believed to contribute to the development of heart failure. In severe heart failure, overall mitochondrial oxidative metabolism is significantly reduced, resulting in a reduced energy reserve. In addition, it is generally believed that there is an overall increase in cardiac glucose metabolism. However, there is evidence to indicate that there is a specific increase in glycolysis with glucose oxidation actually being unchanged or decreased. This results in increased uncoupling of glycolysis and glucose oxidation, which may contribute to the decline in cardiac function observed in heart failure. In this study we assessed cardiac energy metabolism during the development of heart failure with preserved ejection fraction (HFpEF) in Dahl salt-sensitive rats fed a high salt diet (HSD). Over the course of 9 wk, the HSD increased glycolysis but did not alter glucose oxidation, resulting in increased uncoupling of glycolysis and glucose oxidation. The HSD also decreased palmitate oxidation. While we did not observe changes in the protein expression of enzymes involved in glucose oxidation or fatty acid oxidation, we did observe a significant increase in GLUT1 expression, which may account for the elevation

in cardiac glycolysis after 6 wk on the HSD. Overall, these results show that the coupling of glycolysis and glucose oxidation is reduced during the development of HFpEF in the Dahl salt-sensitive rat. They also suggest that this uncoupling of glycolysis and glucose oxidation may contribute to the development of heart failure and may be a promising target for the treatment of heart disease.

6.2 Introduction

An abundance of evidence indicates that alterations in energy metabolism contributes to the severity of heart failure [21, 33, 55, 61, 62, 120, 126, 142, 151, 152, 159, 182, 188, 193, 240, 275, 282]. Prior research has established that in severe heart failure there is a decline in overall cardiac mitochondrial oxidative metabolism [21, 55, 159, 188, 193, 275]. However, there is not a consensus on the importance of the specific metabolic pathways to the development of heart failure. In particular, it is generally believed that there is an increase in overall glucose metabolism, which is an important contributor to the decline in cardiac function in the failing heart [113]. However, it turns out that there is actually a specific increase in glycolysis in heart failure [142, 159, 170]. This results in an increase in the uncoupling of glycolysis and glucose oxidation. However, much of this work has focused on heart failure with reduced ejection fraction (HFrEF). In this study we further examined the role of energy metabolism in heart failure, focusing

on the changes in cardiac energy metabolism that accompany the development of heart failure with preserved ejection fraction (HFpEF).

The coupling of glycolysis and glucose oxidation may be important in the development of heart failure. Several studies have indicated that an increase in the uncoupling of glycolysis and glucose oxidation decreases cardiac efficiency [21, 55, 61, 62, 88, 120, 142, 152, 159, 188, 193, 275]. This decrease in cardiac efficiency is believed to contribute to the decreased function of failing hearts [21, 55, 61, 62, 70, 71, 88, 120, 142, 152, 154, 159, 170, 188, 193, 262, 275, 283, 305]. Further, improvement of cardiac efficiency through strategies that increase glucose oxidation can improve cardiac function [70, 71, 120, 154, 170, 262, 283, 305]. Stimulation of glucose oxidation by inhibition of pyruvate dehydrogenase kinase (PDK) has been found to improve cardiac function in multiple conditions including ischemia, cardiac hypertrophy, and diastolic dysfunction [120, 151].

In this study we examined the changes in energy metabolism that occur during the development of HFpEF. The Dahl salt-sensitive rat, a well characterized model of HFpEF, was utilized in this study. We assessed the changes in cardiac energy metabolism that occur after 3 wk, 6 wk, or 9 wk of rats on a high salt diet (HSD). We observed an increase in the uncoupling of glycolysis and glucose oxidation as diastolic dysfunction developed. These results show that uncoupling of glycolysis and glucose oxidation accompanies the development of diastolic dysfunction and suggest that this uncoupling may contribute to the development of diastolic dysfunction.

6.3 Materials and methods

All animal care and procedures were approved by the University of Alberta Health Sciences Animal Welfare Committee. 8 wk old Dahl salt-sensitive rats were either fed a standard low salt diet (Research Diets, D10012G) or a HSD (Research Diets, D11021901) containing 8% NaCl. Control rats were kept on the low salt diet while treatment groups were fed a HSD for 3, 6, or 9 wk. Food and water were provided *ad libitum*. Rats were kept in 12 hr light: 12 hr dark cycle. At the end of the feeding time period *in vivo* cardiac function was assessed via echocardiography (as described in Chapter 2). Cardiac glucose oxidation, glycolysis, lactate oxidation, palmitate oxidation, and cardiac function were then measured via the isolated working heart perfusion, as described in Chapter 2. 100 μU/mL insulin was added to the perfusate 30 min into the 60 min aerobic perfusion. At the end of the aerobic perfusion, hearts were immediately frozen in liquid N_2 and stored at -80°C for future biochemical analysis [20, 282]. Western blot and statistical analysis are described in Chapter 2 of this thesis.

6.4 Results

6.4.1 The development of diastolic dysfunction is accompanied by decreased cardiac oxidative metabolism in Dahl salt-sensitive rats.

Dahl salt-sensitive rats had reduced diastolic function when fed a HSD for either 6 wk or 9 wk. At the end of the treatment period we assessed *in vivo*

cardiac function via echocardiography and observed a progressive decline in diastolic function and increase in cardiac hypertrophy (Table 6-1, Figure 6-1). The decline in E'/A' observed after 6 wk of HSD is due to a decrease in the ratio of the rate of movement of the mitral valve during early diastole (E') and late diastole (A'). This indicates that the ability of the left ventricle to relax is impaired.

We then assessed the cardiac energy metabolism in these hearts and did not find evidence of reduced mitochondrial oxidative metabolism until the rats had been fed the HSD for 9 wk. By 9 wk on the HSD there was a significant decline in fatty acid oxidation rates but no change in glucose oxidation (Figure 6-2). It is important to note that the HSD does not have a significant effect on fatty acid oxidation rates if fatty acid oxidation rates are normalized for differences in cardiac work (Table 6-2).

6.4.2 The coupling of glycolysis and glucose oxidation is reduced in hearts from Dahl salt-sensitive rats fed a HSD.

Measurements of glycolysis and glucose oxidation indicated an elevation in the uncoupling of glycolysis and glucose oxidation during the development of HFpEF. We found that the HSD induced a progressive rise in glycolysis (Figure 6-2 and Table 6-2). Due to the lack of change in glucose oxidation this rise in glycolysis resulted in an increased uncoupling of glycolysis and glucose oxidation and rise in proton production (Figure 6-2). Further, the fact that this uncoupling

of glycolysis and glucose oxidation precedes the development of diastolic dysfunction suggests a causal link between the uncoupling of glycolysis and glucose oxidation and HFpEF.

6.4.3 Increased GLUT1 expression may contribute to the increased uncoupling of glycolysis from glucose oxidation observed in diastolic dysfunction.

Examination of the expression of glycolytic proteins indicated that a change in glucose transport may contribute to the rise in glycolysis. While phosphoglycerate mutase (PGAM)1 and glucose transporter (GLUT)4 protein expression were not significantly altered by the HSD, lactate dehydrogenase (LDH)A was significantly increased after 3 wk on the HSD. This suggests that LDHA may contribute to the initial decrease in coupling of glycolysis and glucose oxidation observed in response to the HSD (Figure 6-4). Further, after 9 wk on the HSD, GLUT1 protein expression was significantly elevated in Dahl salt-sensitive rat hearts (Figure 6-4), which suggests that GLUT1 could be involved in maintaining the elevation in glycolysis. Hypoxia inducible factor (HIF)1α, a transcription factor regulating glycolysis, was not altered by the HSD (Figure 6-4).

We also did not find evidence for changes in mitochondrial oxidative metabolism enzyme expression that might contribute to the alterations in cardiac energy metabolism observed in response to the HSD. The HSD had no significant

effect on PDH phosphorylation or expression (Figure 6-5). In addition, cytochrome c protein expression was not significantly altered.

6.5 Discussion

Energy metabolism is a promising target for the treatment of heart failure. In particular, studies suggest that a decline in overall mitochondrial oxidative metabolism and increased uncoupling of glycolysis and glucose oxidation are associated with and may be involved in the progression of heart failure [120, 151, 152, 159, 316]. In this study we provide evidence that an uncoupling of glycolysis and glucose oxidation is an early change in energy metabolism during the development of HFpEF in the Dahl salt-sensitive rat. Decreased coupling of glycolysis and glucose oxidation preceded the decline in diastolic function. This suggests that the decrease in cardiac efficiency caused by an uncoupling of glycolysis and glucose oxidation may be an important factor in the initial development of diastolic dysfunction. This hypothesis is supported by previous reports that the coupling of glycolysis and glucose oxidation is decreased in other more severe models of heart failure. For example, the coupling of glycolysis and glucose oxidation is reduced in mouse hearts subjected to coronary artery ligation [170].

Although we found that the coupling of glycolysis and glucose oxidation is decreased in heart failure, the mechanisms at work remain unclear. One

possibility is the decline in overall mitochondrial oxidative metabolism [21, 120, 170, 192, 316]. In the Dahl salt-sensitive rat, the rise in glycolysis, independent of changes in glucose oxidation, (Figure 6-2) could be due to the decline in mitochondrial oxidative metabolism, particularly a decline in PDH activity. However, we found no change in the phosphorylation of PDH in hearts from Dahl salt-sensitive rats fed a HSD for up to 9 wk (Figure 6-5), a finding that agrees with other studies [120]. Another potential explanation for the increased uncoupling of glycolysis and glucose oxidation is an increased capacity for glycolysis or increased glucose transport. Although we did not observe a change in the expression of the glycolytic enzyme PGAM1, we did find an increase in LDHA protein expression after 3 wk on the HSD and a later rise in GLUT1 protein expression (Figure 6-4). Since LDH isoforms that contain LDHA are more likely to convert pyruvate to lactate, as opposed to catalyzing the opposite reaction, this increase in protein expression could contribute to the initial increase in the uncoupling of glycolysis and glucose oxidation induced by the HSD.

The increase in cardiac GLUT1 expression may also contribute to the rise in glycolysis, and, potentially, the development of HFpEF in the Dahl salt-sensitive rat. GLUT1 has previously been shown to regulate cardiac glycolysis. Moreover, glycolysis is elevated in hearts overexpressing GLUT1 and decreased in hearts lacking GLUT1 [148, 216]. Studies that regulate GLUT1 expression provide mixed results on a role for GLUT1 in the development of heart failure. While overexpression of GLUT1 has been reported to prevent pressure overload

induced heart failure in mouse hearts, deletion of GLUT1 does not affect the rate of development of pressure overload induced heart failure. The GLUT1-/- mouse has elevated fatty acid oxidation rates and reduced glucose oxidation rates, which would be expected to decrease cardiac efficiency and may explain why these hearts are not resistant to pressure overload induced heart failure [216]. However, the results from these two studies do not preclude the possibility that a more acute up-regulation of GLUT1 expression could increase glycolysis and decrease cardiac function.

Based on these results we hypothesize that stimulating glucose oxidation may be a promising strategy for treating and potentially preventing the development of HFpEF. As mentioned earlier, stimulating cardiac glucose oxidation has been associated with an increase in cardiac efficiency and an improvement in cardiac function [70, 71, 120, 154, 170, 262, 283, 305]. Treatment of Dahl salt-sensitive rats on a HSD with DCA was effective at activating PDH, decreasing circulating lactate levels, and improving cardiac function and overall survival [120]. However, in this study intervention with DCA was at a later stage of heart failure development, which was also associated with changes in systolic function. It will be important to determine if stimulating glucose oxidation with more specific drugs can improve or even prevent the development of HFpEF.

In conclusion, this study increases our understanding of the role of energy metabolism in HFpEF. We show that one of the earlier changes in cardiac energy

metabolism that occurs during the development of HFpEF is a decrease in the coupling of glycolysis and glucose oxidation. Our findings combined with previous work suggest that the coupling of glycolysis and glucose oxidation is important in maintaining normal cardiac function. While these results suggest that increasing the coupling of glycolysis and glucose oxidation may be a promising strategy for the treatment of heart failure, more work is needed to determine if therapeutically improving the coupling of glycolysis and glucose oxidation improves diastolic function.

Figure 6-1. Time dependent effects of HSD on Dahl salt-sensitive rat cardiac function and hypertrophy. Diastolic dysfunction was measured by A. E'/A' and cardiac hypertrophy was measured by B. corrected LV mass and C. LVPWd using echocardiography on Dahl salt-sensitive rats fed a low salt diet, 0.3% NaCl (Control) or a HSD, 8% NaCl, for 3, 6, or 9 wk. D. Cardiac p-p70S6K/p70S6K protein expression was also assessed in these hearts. n= 5-8 * $p<0.05$ compared to Control. # $p<0.05$ compared to 3 wk. Values shown as mean ± SEM

Figure 6-1

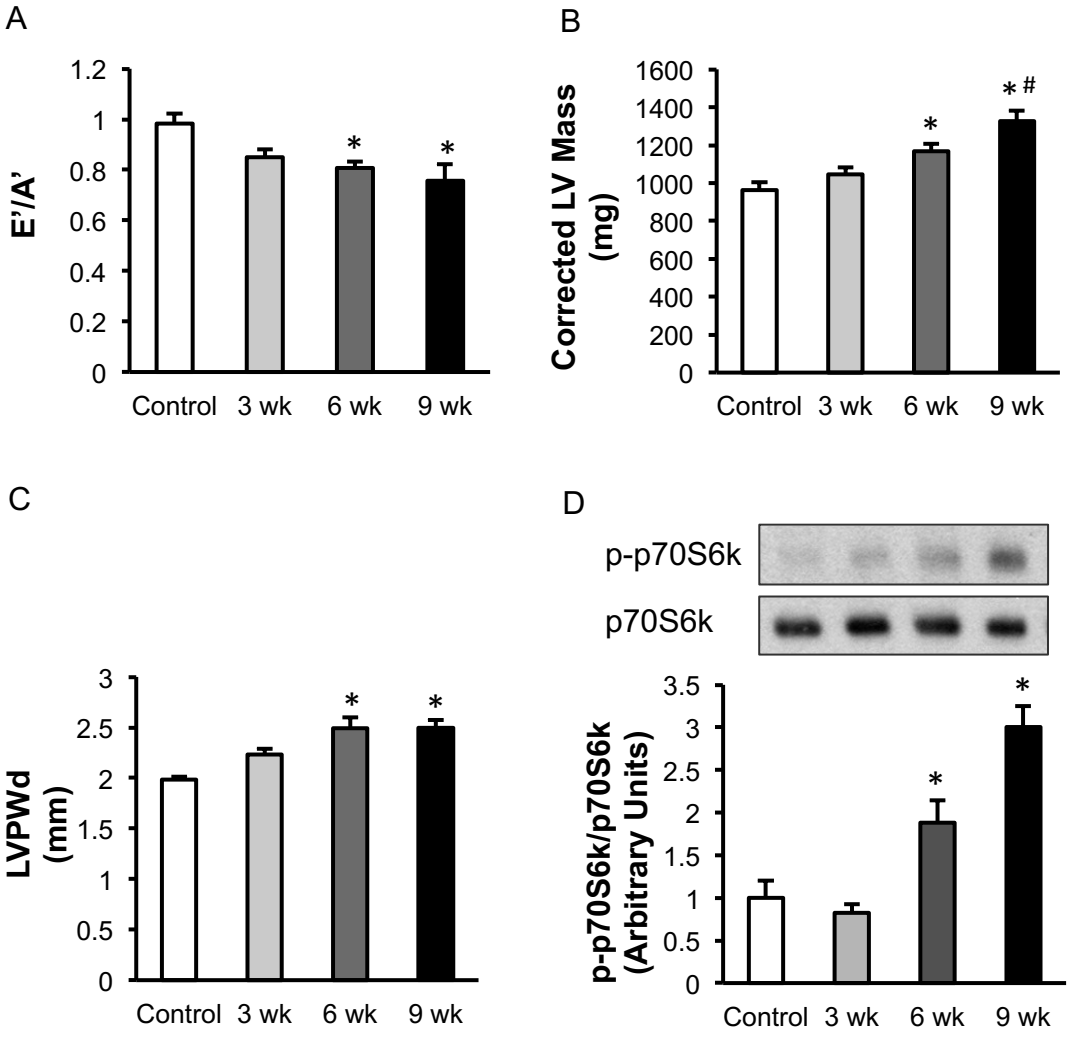

A
E'/A'
1.2
1
0.8
0.6
0.4
0.2
0
Control
3 wk
6 wk
9 wk
B
Corrected LV Mass (mg)
1600
1400
1200
1000
800
600
400
200
0
C
LVPWd (mm)
3
2.5
2
1.5
1
0.5
0
D
p-p70S6k
p70S6k
p-p70S6k/p70S6k (Arbitrary Units)
3.5
3
2.5
2
1.5
1
0.5
0

Figure 6-2. Time dependent effects of HSD on palmitate oxidation, glucose oxidation, glycolysis, and lactate oxidation in Dahl salt-sensitive rat hearts. A. Glycolysis, B. glucose oxidation, C. palmitate oxidation, D. lactate Oxidation, and E. proton production were measured in hearts from Dahl salt-sensitive rats fed a low salt diet, 0.3% NaCl (Control) or a HSD, 8% NaCl, for 3, 6, or 9 wk. Energy metabolic rates were assessed via the isolated working heart perfusion. Proton production was calculated based on glycolysis and glucose oxidation rates. n=3-5 * $p<0.05$ compared to Control. ** $p<0.05$ between compared groups. Values shown as mean ± SEM

Figure 6-2

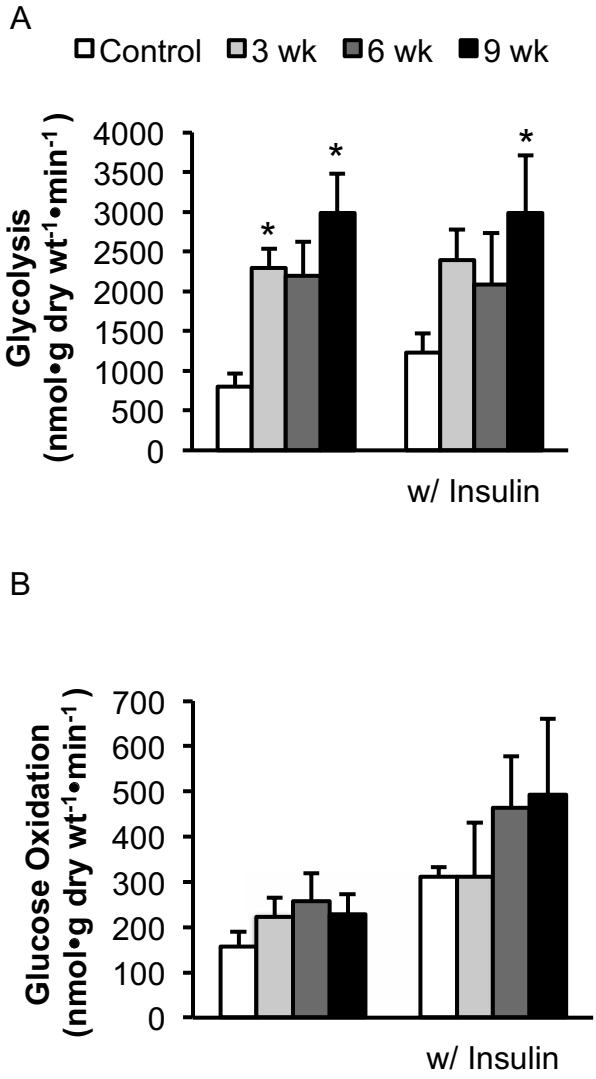

A
Control
3 wk
6 wk
9 wk
Glycolysis (nmol•g dry wt-1•min-1)
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
*
w/ Insulin
B
Glucose Oxidation (nmol•g dry wt-1•min-1)
700
600
500
400
300
200
100
0
w/ Insulin

Figure 6-2

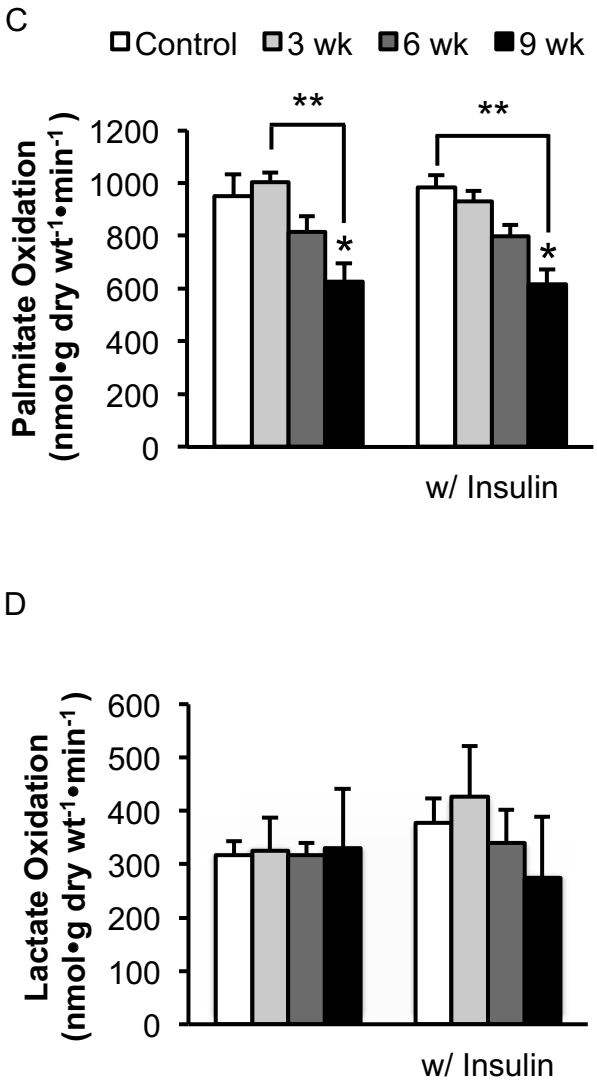

C
Control
3 wk
6 wk
9 wk
**
**
*
*
Palmitate Oxidation
(nmol•g dry wt-1•min-1)
1200
1000
800
600
400
200
0
w/ Insulin
D
Lactate Oxidation
(nmol•g dry wt-1•min-1)
600
500
400
300
200
100
0
w/ Insulin

Figure 6-2

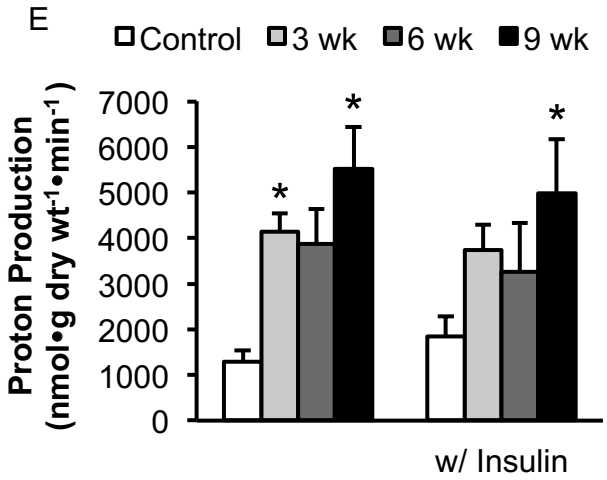

E
Control
3 wk
6 wk
9 wk
Proton Production
(nmol•g dry wt-1•min-1)
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
w/ Insulin

Figure 6-3. Time dependent effects of HSD on Dahl salt-sensitive rat heart ATP production. A. Contribution to ATP production and B. % contribution to ATP production were calculated from the metabolic rates assessed via the isolated working heart perfusion in Dahl salt-sensitive rats. n=3-5 Values shown as mean ± SEM

Figure 6-3

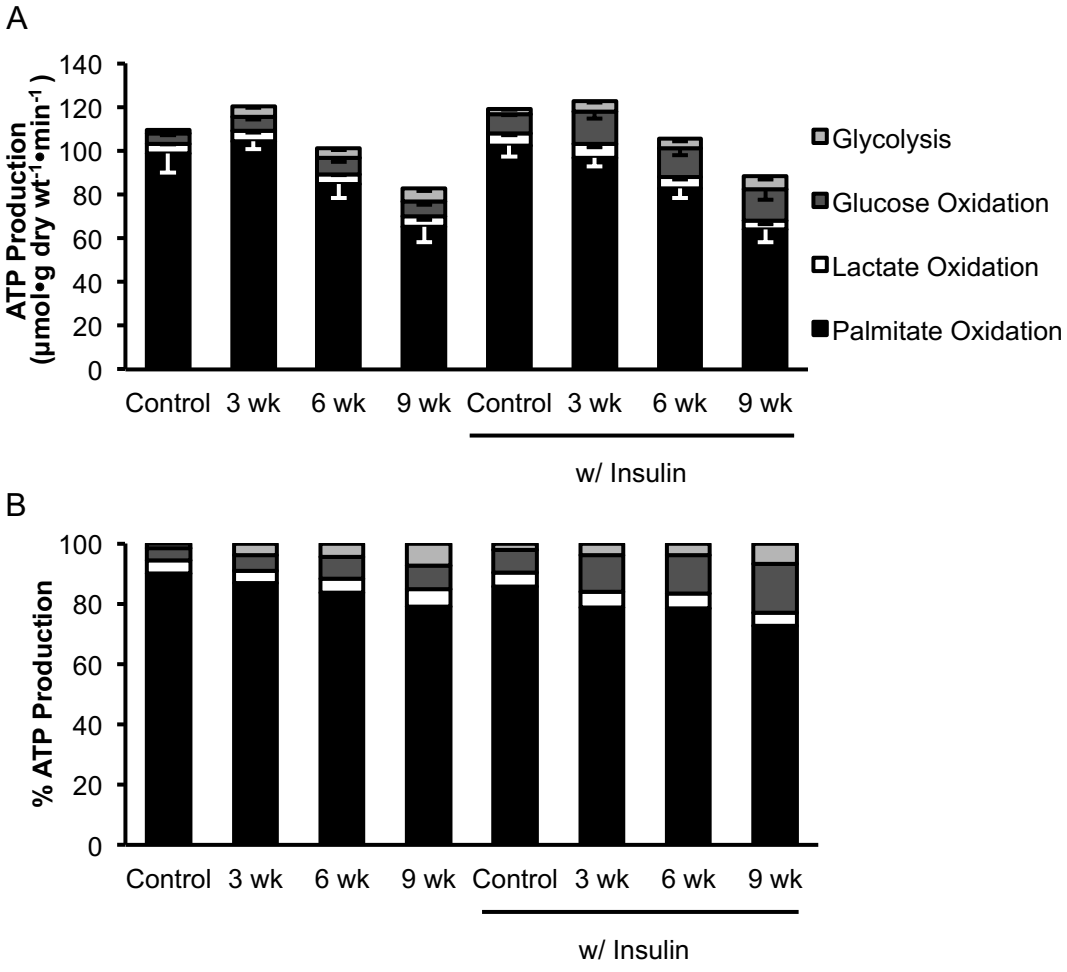

A
ATP Production
(µmol•g dry wt-1•min-1)
140
120
100
80
60
40
20
0
Control
3 wk
6 wk
9 wk
Control
3 wk
6 wk
9 wk
w/ Insulin
Glycolysis
Glucose Oxidation
Lactate Oxidation
Palmitate Oxidation
B
% ATP Production
100
80
60
40
20
0
Control
3 wk
6 wk
9 wk
Control
3 wk
6 wk
9 wk
w/ Insulin

Figure 6-4. Effect of HSD on Dahl salt-sensitive rat heart glucose metabolic enzymes. A. Representative western blots. B. GLUT1, C. GLUT4, D. PGAM1, E. LDHA expression, and F. HIF1α protein expression was measured in hearts from Dahl salt-sensitive rats fed either a low salt diet, 0.3% NaCl (Control) or a HSD, 8% NaCl, for 3, 6, or 9 wk. n=4-9 * $p<0.05$ compared to Control. ** $p<0.05$ between compared groups. Values shown as mean ± SEM

Figure 6-4

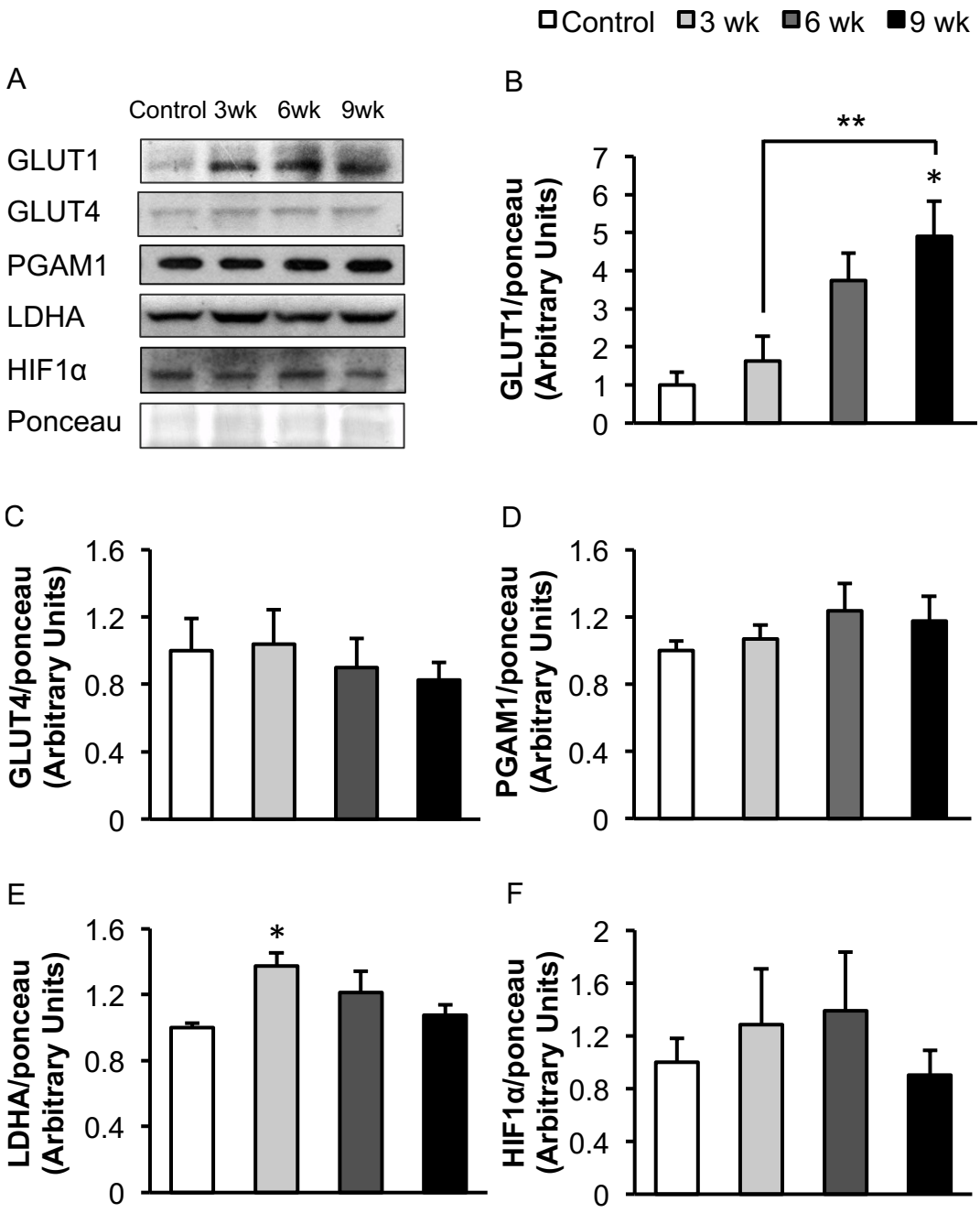

Control
3 wk
6 wk
9 wk
A
Control 3wk 6wk 9wk
GLUT1
GLUT4
PGAM1
LDHA
HIF1α
Ponceau
B
GLUT1/ponceau (Arbitrary Units)
**
*
C
GLUT4/ponceau (Arbitrary Units)
D
PGAM1/ponceau (Arbitrary Units)
E
LDHA/ponceau (Arbitrary Units)
*
F
HIF1α/ponceau (Arbitrary Units)

Figure 6-5. Effect of HSD on the cardiac expression of proteins involved in oxidative metabolism. A. Representative western blots. B. pPDH Ser293/PDH, C. HADH, and D. cytochrome c protein expression was measured in hearts from Dahl salt-sensitive rats fed a low salt diet, 0.3% NaCl (Control) or a HSD, 8% NaCl, for 3, 6, or 9 wk. n= 4-9 * $p<0.05$ compared to Control. Values shown as mean ± SEM

Figure 6-5

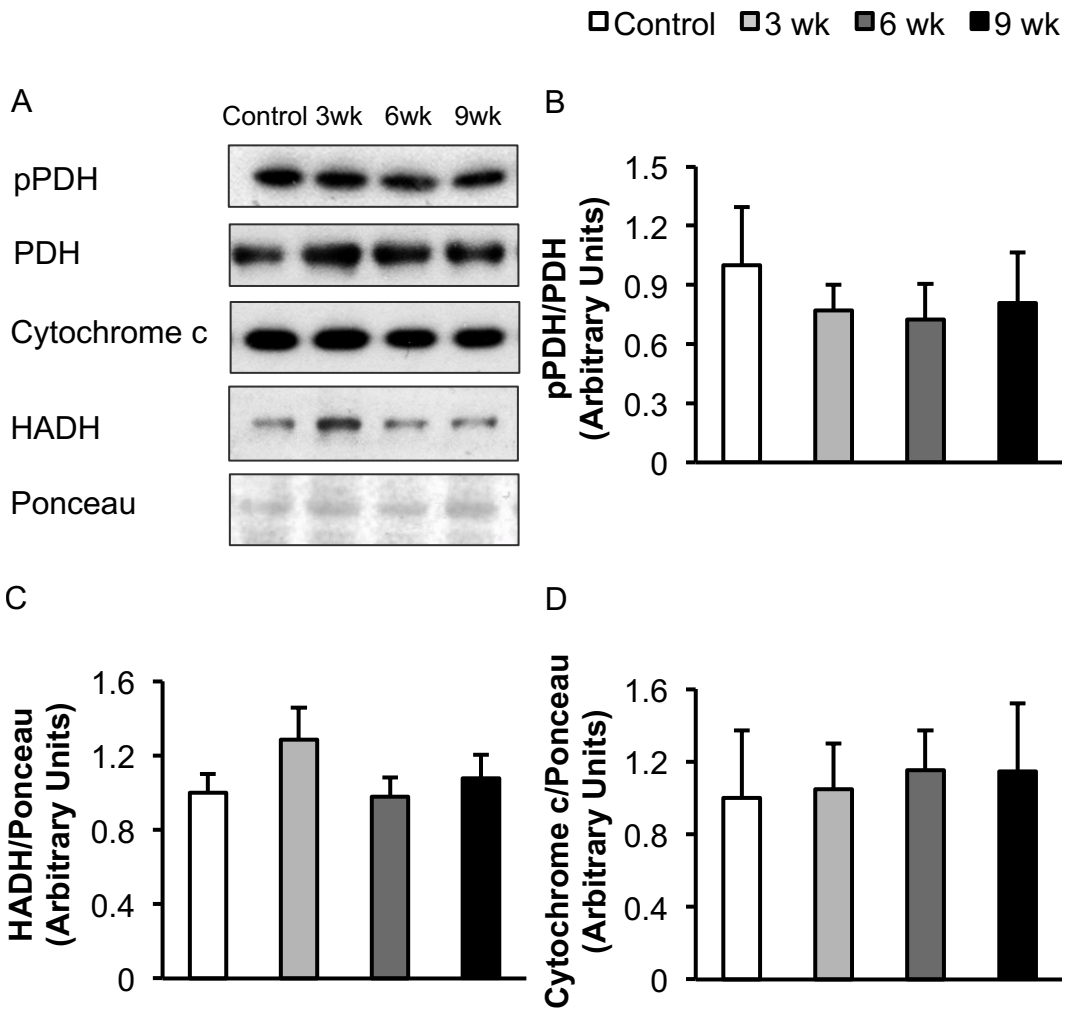

Control
3 wk
6 wk
9 wk
A
Control 3wk 6wk 9wk
pPDH
PDH
Cytochrome c
HADH
Ponceau
B
pPDH/PDH (Arbitrary Units)
1.5
1.2
0.9
0.6
0.3
0
C
HADH/Ponceau (Arbitrary Units)
1.6
1.2
0.8
0.4
0
D
Cytochrome c/Ponceau (Arbitrary Units)
1.6
1.2
0.8
0.4
0

Table 6-1. Effect of HSD on *in vivo* cardiac function in Dahl salt-sensitive rats.

	Control	3 wk	6 wk	9 wk
EF (%)	83.79±1.44	82.21±2.50	84.91±1.14	76.21±2.19 ^
FS (%)	54.60±1.69	53.12±2.57	54.45±2.39	47.64±2.34
LV Mass Corrected	962.88±43.45	1045.85±36.11	1167.85±38.96 *	1328.94±52.37 *#
IVSd (mm)	1.97±0.05	2.23±0.06 *	2.40±0.07 *	2.50±0.07 *#
LVIDd (mm)	7.91±0.12	7.32±0.14 *	7.45±0.09	7.52±0.20
LVPWd (mm)	1.98±0.03	2.23±0.06	2.49±0.10 *	2.50±0.08 *
IVSs (mm)	3.40±0.05	3.67±0.10	3.68±0.09	3.68±0.15
LVIDs (mm)	3.87±0.18	3.46±0.23	3.40±0.09	4.25±0.08
LVPWs (mm)	3.45±0.12	3.68±0.11	3.77±0.08	3.65±0.13
LV Vol;d	335.75±10.95	283.11±12.35 *	294.62±7.78	301.74±17.16
LV Vol;s	51.99±6.28	45.76±6.96	47.51±3.37	81.30±3.62 *#^
E/E'	19.04±2.18	20.49±1.95	18.82±1.22	18.29±1.51
E/A	1.60±0.11	1.64±0.07	1.33±0.15	1.40±0.20
E'/A'	0.98±0.04	0.85±0.03	0.81±0.02 *	0.76±0.07
Tei Index	0.65±0.03	0.72±0.04	0.78±0.05	0.76±0.05 *
E'	54.23±6.85	54.68±4.61	46.79±2.14	38.26±3.49
IVRT (ms)	24.32±0.60	22.68±0.68	27.97±1.76	28.13±1.63
IVCT (ms)	15.69±0.90	16.88±1.29	15.81±1.48	17.47±1.43

In vivo cardiac function was measured via echocardiography in Dahl salt-sensitive rats fed a low salt diet, 0.3% NaCl (Control) or a HSD, 8% NaCl, for 3, 6, or 9 wk. n= 5-8 * $p<0.05$ compared to Control. * $p<0.05$ compared to Control. # $p<0.05$ compared to 3 wk. ^ $p<0.05$ compared to 6 wk. Values shown as mean ± SEM

Table 6-2. Effect of HSD on metabolic rates normalized to cardiac work.

	w/o 100 µU/ml Insulin				w/ 100 µU/ml Insulin			
	Control	**3 wk**	**6 wk**	**9 wk**	**Control**	**3 wk**	**6 wk**	**9 wk**
Cardiac Work (joules•min^{-1}• g dry wt^{-1})	2.21±0.17	1.89±0.17	1.61±0.07*	1.54±0.20*	2.02±0.09	1.83±0.17	1.50±0.09*	1.50±0.10*
Metabolic rates normalized to cardiac work								
Glycolysis (nmol•joules^{-1})	412±85.5	1296±86.4	1014±166.5	2593±1013.2*	648±124.9	1454±161.7	1018±197.3	3985±2340.4
Glucose Oxidation (nmol•joules^{-1})	81.2±18.0	121.4±17.6	112.7±15.8	199.3±78.6	165.0±19.3	310.2±65.3	244.5±27.1	666.6±382.2
Palmitate Oxidation (nmol•joules^{-1})	410.4±41.8	498.5±38.0	502.2±31.4	388.0±16.1	468.7±72.1	456.0±17.7	513.7±30.4	410.7±45.6
Lactate Oxidation (nmol•joules^{-1})	137.1±15.9	170.1±48.6	198.4±21.3	199.3±60.8	177.1±15.1	205.7±44.0	217.2±31.8	180.4±71.2

Values shown as mean ± SEM Cardiac work and metabolic rates were measured during the isolated working heart perfusion. n=3-5 * $p<0.05$ compared to Control.

CHAPTER 7

Elevation in branched-chain amino acid oxidation is not responsible for high fat diet-induced cardiac insulin resistance.

The manuscript is currently in preparation to be submitted to Diabetes: Natasha Fillmore, Cory S Wagg, Liyan Zhang, Arata Fukushima, Gary D Lopaschuk. "Elevation in Branched-Chain Amino Acid Oxidation is Not Responsible for High Fat Diet-Induced Cardiac Insulin Resistance."

Liyan Zhang and Arata Fukushima treated the mice and helped with the western blots. Cory Wagg performed the isolated working heart perfusions. My role in this work involved performing the rest of the experiments including the majority of the western blots as well as the experimental design, statistical analysis, and writing of the manuscript.

CHAPTER 7

Elevation in branched-chain amino acid oxidation is not responsible for high fat diet-induced cardiac insulin resistance.

7.1 Abstract

Recent studies have proposed that elevated branched-chain amino acids (BCAAs) induce insulin resistance in muscle secondary to increased branched chain amino acid (BCAA) oxidation inhibiting glucose oxidation and fatty acid oxidation. However, BCAA oxidation rates have not been assessed in muscle insulin resistance and in cardiac muscle fatty acid oxidation is actually elevated in obesity-associated cardiac insulin resistance. I therefore examined the role of BCAA oxidation in cardiac insulin resistance. Mice were fed a low fat diet (LFD) or high fat diet (HFD) for 10 wk and BCAA oxidation, glucose oxidation, glycolysis, and fatty acid oxidation were measured in isolated working hearts. Mice on a HFD had significantly reduced cardiac BCAA oxidation rates, which was accompanied by a rise in circulating BCAAs, and an impairment in cardiac insulin-stimulated glucose oxidation. BCAA oxidation contributed less than 1% of the ATP production in hearts, and were actually decreased in HFD mice, indicating that changes in BCAA oxidation could not significantly impact glucose or fatty acid oxidation in insulin resistant hearts. Further, cardiac insulin signaling was not impaired when BCAA oxidation was stimulated by treating mice with a

branched chain ketoacid dehydrogenase (BCKD) kinase inhibitor for 1 wk. These results suggest that cardiac insulin resistance is not due to BCAA oxidation inhibition of glucose or fatty acid oxidation. Rather, I hypothesize that reduced BCAA oxidation contributes to insulin resistance by leading to increased BCAA levels which may be stimulating pathways that impair insulin sensitivity.

7.2 Introduction

Insulin resistance is believed to contribute to cardiac dysfunction in disease states including obesity, diabetes, and heart failure [28, 33, 114, 119, 124, 218, 235, 245, 316]. The ability of insulin to stimulate glucose oxidation is impaired in insulin resistant hearts [33, 40, 102, 155, 158, 172, 184, 218, 282, 306, 319]. There is also an overall decrease in glucose oxidation and increase in fatty oxidation in insulin resistant hearts in both obesity and diabetes [28, 33, 39, 78, 80, 126, 158, 282, 318]. This decrease in glucose oxidation and increase in fatty acid oxidation reduces cardiac efficiency, which contributes to the decreased cardiac function seen in diabetes and heart failure [33, 109, 151, 152, 159, 190, 204, 235, 240, 245, 254, 261, 312, 316]. In this study I was interested in how insulin stimulated glucose oxidation becomes impaired in cardiac insulin resistance. I focused on the potential role of branched chain amino acid (BCAA) oxidation in regulating cardiac insulin resistance.

Increased plasma and tissue branched chain amino acids (BCAAs) has been shown to correlate with muscle insulin resistance [9, 76, 111, 164, 195]. It has been suggested that increased BCAA levels results in elevated BCAA oxidation which inhibits glucose oxidation and fatty acid oxidation, leading to insulin resistance [194]. However, cardiac insulin resistance in the setting of obesity is actually associated with an elevation in fatty acid oxidation [102, 155, 184, 282, 306, 319]. Furthermore, it is not known if BCAAs can compete with glucose or fatty acids as a source of muscle tricarboxylic acid (TCA) cycle acetyl CoA. While work is still needed to confirm this proposed mechanism, there is a strong positive association between BCAAs and whole body insulin resistance in obesity, diabetes, and heart failure [9, 76, 120, 164, 195, 246]. Further, increasing BCAAs in the diet exacerbates insulin resistance by impairing insulin signaling in liver and skeletal muscle [195, 277]. In contrast, whole body insulin sensitivity is improved by removing BCAAs from the diet of *db/db* mice [302].

In this study I examined the effect of insulin resistance on BCAA oxidation and its contribution to overall ATP production. Because I utilized the working heart perfusion to measure flux through the glucose, fatty acid, and BCAA metabolic pathways this study also provides novel information on the role of BCAA oxidation in cardiac insulin resistance. I first fed mice a high fat diet (HFD) for 10 wk to induce insulin resistance and then measured cardiac BCAA oxidation. I then treated a second set of mice on a HFD with a branched chain ketoacid dehydrogenase (BCKD) kinase inhibitor in order to assess the effect of

stimulating BCAA oxidation on cardiac insulin sensitivity. Together, our results indicate that BCAA oxidation is reduced in cardiac insulin resistance. Further, a rise in BCAA oxidation does not impair insulin sensitivity, but instead may lessen insulin resistance.

7.3 Materials and methods

All animal care and procedures were approved by the University of Alberta Health Sciences Animal Welfare Committee. Mice were kept in 12 hr light: 12 hr dark cycle and either fed a standard low fat diet (LFD) or HFD *ad libitum*. Mice were fed a HFD for 10 wk or 5 wk. The set of mice fed a LFD or HFD for 5 wk also received intraperitoneal injections with vehicle (5% DMSO, 10% cremophor EL, and 85% 0.1 M sodium bicarbonate, pH 9.0) or a BCKD kinase inhibitor (20mg/kg/day; Sigma, L499110) [278] daily during their 5^{th} wk on the HFD. At the end of the treatment period mice were anesthetized and hearts were used in the isolated working heart perfusion to assess BCAA oxidation, glucose oxidation, palmitate oxidation, and glycolysis. The details on these measurements are provided in Chapter 2 of this thesis. Gastrocnemius muscle, liver, and plasma were also frozen in liquid nitrogen, and stored at -80°C for future biochemical analysis.

Hearts from untreated mice were also treated acutely with the BCKD kinase inhibitor. The BCKD kinase inhibitor (200 μM) was added to the perfusate

used in the isolated working heart perfusion. The drug was either dissolved in DMSO or a chremophore solution (5% DMSO, 10% Chremophore, 85% 0.1 M Sodium bicardonate, pH=9.0). The Krebs Henseleit solution used in all three sets of hearts perfusions was supplemented with 5 mM glucose, 0.8 mM palmitate bound to 3% bovine serum albumin (BSA), 0.15 mM leucine, 0.15 mM isoleucine, 0.2 mM valine, and the appropriate radioactive labeled glucose, palmitate, or BCAA. Hearts from mice fed a LFD or HFD for 5 or 10 wk underwent aerobic perfusion for 60 min with 100 μU/ml insulin added to the Krebs buffer 30 min into the perfusion. The other set of hearts underwent aerobic perfusion with 100 μU/mL insulin and either vehicle or BCKD kinase inhibitor (200 μM) included in the buffer during the entire 60 min aerobic perfusion. Immediately after perfusion, hearts were frozen in liquid N_2 and stored at -80°C for future biochemical analysis.

Protocols for western blot, measurement of BCAA levels, and statistical analysis are provided in Chapter 2 of this thesis.

7.4 Results

7.4.1 BCAA oxidation is reduced, not elevated in insulin resistant hearts.

It has been suggested that BCAA oxidation impairs insulin sensitivity by inhibiting glucose and fatty acid oxidation [9, 76, 164, 194, 195]. While much of this work has been done in skeletal muscle, this mechanism has also been

suggested to contribute to cardiac insulin resistance. However, BCAA oxidation has not been directly measured in the setting of insulin resistance. Therefore, I characterized cardiac BCAA oxidation in the setting of HFD-induced insulin resistance. Mice were fed a HFD for 10 wk to induce cardiac insulin resistance (Figure 7-1 and 7-2 and Table 7-1). After 10 wk on the HFD, cardiac BCAA oxidation was significantly reduced (Figure 7-1 and Table 7-1). This indicates that in the insulin resistant state cardiac BCAA oxidation does not inhibit fatty acid oxidation any further than it would be under normal conditions. Further, BCAA oxidation contributes such a small proportion of the overall ATP production (approximately 1%) that a change in BCAA oxidation would not be expected to have any significant effect on fatty acid oxidation or glucose oxidation (Figure 7-3). Another interesting finding is that cardiac BCAA oxidation is not acutely regulated by insulin. This is true both in LFD and HFD hearts (Figure 7-1 and Table 7-1).

7.4.2 The decrease in cardiac BCAA oxidation observed in HFD-induced insulin resistance may be due to a decrease in BCKD expression.

In order to better understand why BCAA oxidation is reduced in insulin resistant hearts I assessed the expression of enzymes involved in the BCAA oxidation pathway. While I did not observe changes in the expression of mitochondrial branched chain amino transferase (BCATm) or BCKD kinase and mitochondrial protein phosphatase 2C (PP2Cm), which regulate BCKD

phosphorylation, I did observe a significantly lower BCKD protein expression in HFD versus LFD hearts (Figure 7-4). Interestingly, effects of HFD on the BCAA oxidation pathway appear to be tissue specific. BCKD phosphorylation, BCKD expression, and BCATm expression were all not significantly different in gastrocnemius from LFD and HFD mice (Figure 7-5). However, HFD significantly increased BCKD phosphorylation but did not alter BCKD expression in the liver (Figure 7-5).

7.4.3 Inhibition of BCKD kinase increases BCAA oxidation.

In order to investigate the effect of stimulating BCAA oxidation on cardiac insulin resistance I conducted a series of experiments utilizing a BCKD kinase inhibitor. First, I treated hearts in the working heart perfusion with a BCKD kinase inhibitor to determine the effect of BCKD kinase inhibition on cardiac BCAA oxidation. This experiment confirmed that this BCKD kinase inhibitor increases cardiac BCAA oxidation (Figure 7-6).

7.4.4 Stimulation of BCAA oxidation does not impair cardiac insulin sensitivity.

We then treated mice with a BCKD kinase inhibitor during the 5th wk on a HFD. The BCKD kinase inhibitor significantly reduced circulating BCAA levels (Figure 7-6). Measurements of glucose oxidation, fatty acid oxidation, and pAkt

all indicate that stimulation of BCAA oxidation does not exacerbate HFD-induced cardiac insulin resistance (Figure 7-6 and Table 7-2).

7.5 Discussion

Recent studies suggest that BCAAs contribute to insulin resistance [76, 95, 164, 194, 195]. It has been proposed that the resulting rise in tissue BCAAs results in increased BCAA oxidation and may increase BCAA oxidation intermediate levels (suggesting elevated BCAA oxidation) and inhibition of fatty acid and glucose oxidation in skeletal muscle [194]. This hypothesis has been suggested to also be involved in cardiac insulin resistance despite the fact that BCAA oxidation has not yet been measured in insulin resistant hearts. I therefore decided in this study to examine the contribution BCAA oxidation may have in the development of cardiac insulin resistance.

In order to test this mechanism I first assessed the effect of insulin resistance on cardiac BCAA oxidation. To do this mice were fed a LFD or HFD for 10 wk, a treatment that induces a pronounced insulin resistance in the heart. I was surprised to find that BCAA oxidation was reduced in hearts from mice fed the HFD (Figure 7-1 and Table 7-1). This indicates that an elevation in BCAA oxidation does not contribute to the cardiac insulin resistance that accompanies diet induced obesity.

We also show that BCAA oxidation also only contributes a small proportion (<1%) of overall ATP production in the heart. This is very similar to what was reported by Ichihari *et al* who calculated, under conditions where only BCAAs were present as a substrate, that BCAA oxidation only provides 3.3% ATP production in the heart [112]. I show that the low contribution of BCAAs to ATP production is seen in both healthy and insulin resistant mice (Figure 7-3). Since BCAA oxidation has such a low contribution to ATP production, changes in BCAA oxidation would not be expected to have a significant impact on glucose oxidation or fatty acid oxidation rates. In fact, I did not observe a lower fatty acid oxidation in insulin resistant hearts (Figure 7-1 and Table 7-1). This is in agreement with past studies which have shown that insulin resistance in the setting of obesity is usually associated with an elevation in cardiac fatty acid oxidation [102, 155, 184, 282, 306, 319].

While our results indicate that a rise in BCAA oxidation does not contribute to cardiac insulin resistance, there are alternative mechanisms whereby BCAAs may be contributing to insulin resistance. BCAA itself may directly induce insulin resistance via activation of mTOR signaling [165]. Surprisingly, an increase in mTOR signaling was not observed in hears from mice fed a HFD despite the fact that it has previously been reported that a 10 wk HFD stimulates mTOR signaling in the mouse hearts [319]. This may be due to the fact that prior to biochemical analysis both groups of hearts were perfused for an hour with BCAAs. This may have resulted in sufficient stimulation of the mTOR pathway in

the LFD group to make the measurements of mTOR signaling not significantly different between LFD and HFD fed mice. The reduced BCAA oxidation I observed in insulin resistance could actually be further increasing BCAA levels, exacerbating insulin resistance. It has also been suggested that intermediates of BCAA oxidation, such as branched chain keto acids (BCKAs), accumulate and are toxic to mitochondria, contributing to insulin resistance [79, 164, 203]. However, an experiment conducted in BCATm-/- mice indicated that BCKAs must be converted back to BCAAs in order for insulin resistance to occur [321]. Since the decline in BCAA oxidation in insulin resistant hearts could also result in an elevation in BCKAs, further studies will need to be done to assess if BCAAs or a BCAA intermediate impairs insulin sensitivity.

In order to further test whether this BCAA oxidation may regulate insulin sensitivity I fed mice for 5 wk with a HFD and during the 5th wk treated them with a BCKD kinase inhibitor to stimulate BCAA oxidation. While our final results do not indicate that stimulation of BCAA oxidation improves cardiac insulin sensitivity (Figure 7-6 and Table 7-2), they do not support the concept that BCAA oxidation causes insulin resistance. One potential explanation is that there was an improvement in insulin sensitivity upon treatment with the drug but it was not observed during and post heart perfusion because I opted to not include the BCKD kinase inhibitor in the perfusate in order to assess more long term effects of the treatment. Initially these results appear to disagree with our hypothesis that stimulation of BCAA oxidation may be a promising strategy for reducing BCAA

levels and improving insulin sensitivity. Further work will need to be done to determine if stimulation of BCAA oxidation can improve insulin sensitivity and the degree and length of stimulation that is necessary to do so.

BCAAs may also be involved in the development of insulin resistance in other conditions, such as heart failure. Whole body insulin resistance not only increases the chance of developing heart failure but also increases the severity of heart failure [114, 119, 124]. A positive correlation has been reported between BCAA related metabolic clusters and heart disease [110, 252]. Further, BCAAs are elevated in both human and rodent failing hearts [120, 246]. I hypothesize that BCAAs are also causing insulin resistance in the setting of heart failure through direct BCAA stimulation of the mTOR pathway. Further, defects in BCAA oxidation enzymes in diseases such as methylmalonic acidemia are associated with human cardiomyopathy [110].

In conclusion, this study provides novel information regarding the role of BCAA oxidation in cardiac insulin resistance. I demonstrate that: 1) cardiac BCAA oxidation is reduced in obese insulin resistant mice, and does not effectively compete with fatty acid or glucose oxidation for ATP production; 2) cardiac BCAA oxidation is not acutely regulated by insulin; 3) BCAA oxidation makes up a small proportion of ATP production in the heart (≈1%); and 4) stimulation of BCAA oxidation does not impair cardiac insulin sensitivity. These data provide important information regarding the mechanisms regulating cardiac insulin resistance, indicating that a rise in BCAAs, due to a reduction in cardiac

BCAA oxidation, may increase mTOR signaling and reduce insulin sensitivity. While more work needs to be done to follow up on these findings, these results suggest that BCAA oxidation may be a promising target for improving cardiac insulin sensitivity and treatment of diseases associated with insulin resistance.

Figure 7-1. Effect of HFD on BCAA oxidation. A. BCAA oxidation, B. glucose oxidation, C. palmitate oxidation, and D. glycolysis were measured in hearts from mice fed a LFD or HFD for 10 wk. Values are mean ± SEM (n= 6-12) * $p<0.05$ significantly different from LFD

Figure 7-1

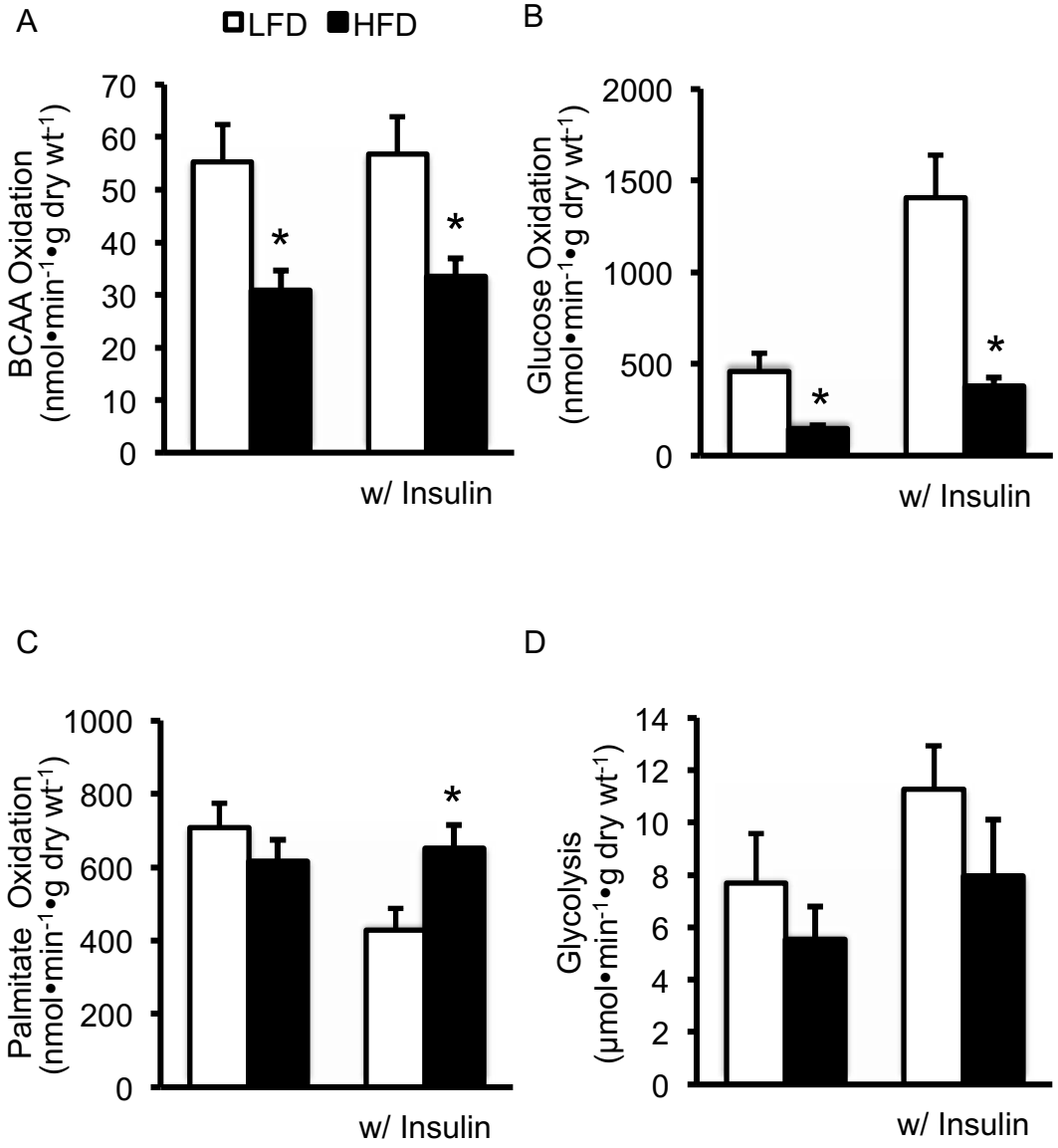

A
LFD
HFD
BCAA Oxidation
(nmol•min-1•g dry wt-1)
70
60
50
40
30
20
10
0
*
*
w/ Insulin
B
Glucose Oxidation
(nmol•min-1•g dry wt-1)
2000
1500
1000
500
0
*
*
w/ Insulin
C
Palmitate Oxidation
(nmol•min-1•g dry wt-1)
1000
800
600
400
200
0
*
w/ Insulin
D
Glycolysis
(µmol•min-1•g dry wt-1)
14
12
10
8
6
4
2
0
w/ Insulin

Figure 7-2. The HFD induced decline in cardiac BCAA oxidation is associated with reduced cardiac insulin signaling. A. pAkt, B. pGSK3β Ser 9, C. pIRS1 Ser307, D. p-p70S6k, E. p-mTOR, and F. pAMPK protein expression were measured in hearts from mice fed a LFD or HFD for 10 wk. Values are mean ± SEM (n= 5-6) * $p<0.05$ significantly different from LFD

Figure 7-2

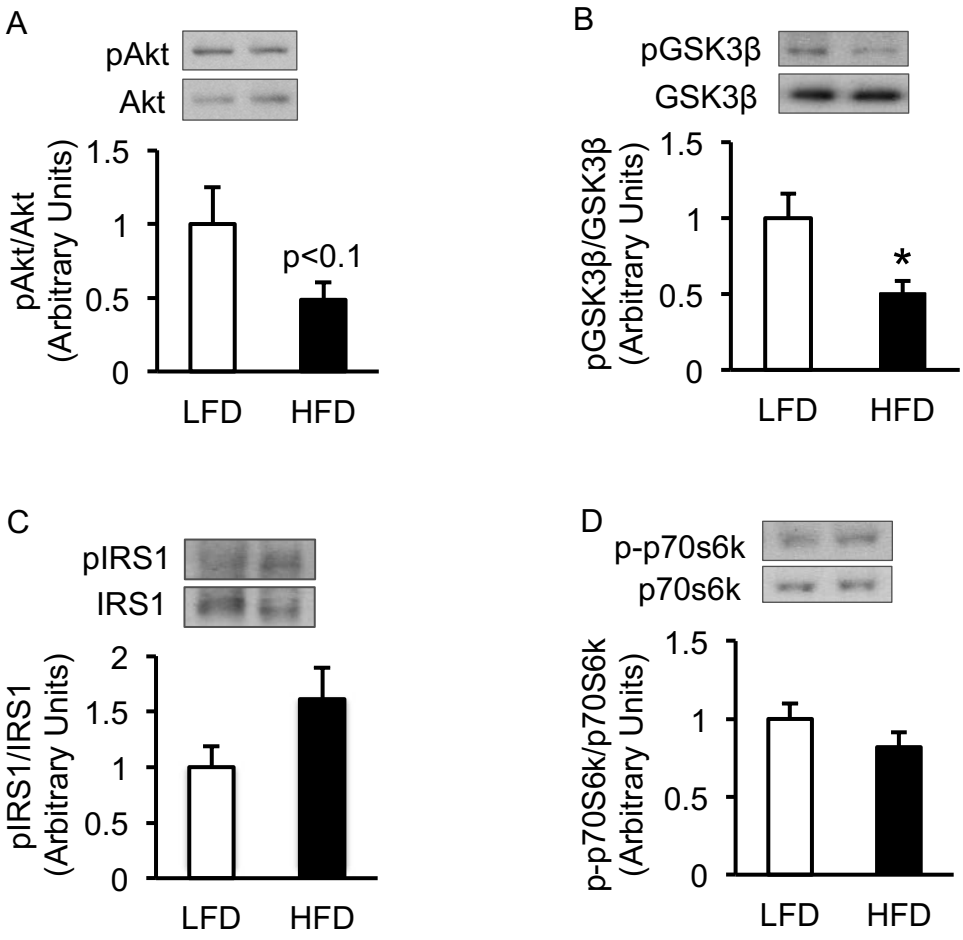

A
pAkt
Akt
pAkt/Akt
(Arbitrary Units)
1.5
1
0.5
0
p<0.1
LFD
HFD
B
pGSK3β
GSK3β
pGSK3β/GSK3β
(Arbitrary Units)
1.5
1
0.5
0
*
LFD
HFD
C
pIRS1
IRS1
pIRS1/IRS1
(Arbitrary Units)
2
1.5
1
0.5
0
LFD
HFD
D
p-p70s6k
p70s6k
p-p70S6k/p70S6k
(Arbitrary Units)
1.5
1
0.5
0
LFD
HFD

Figure 7-2

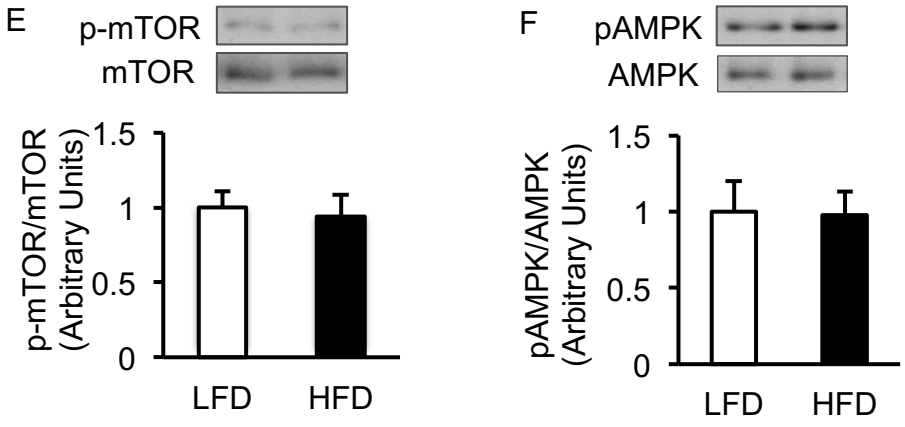

E
p-mTOR
mTOR
p-mTOR/mTOR (Arbitrary Units)
1.5
1
0.5
0
LFD
HFD
F
pAMPK
AMPK
pAMPK/AMPK (Arbitrary Units)
1.5
1
0.5
0
LFD
HFD

Figure 7-3. Contribution of BCAA oxidation to cardiac energy metabolism. % contribution to ATP production was calculated from the metabolic rates assessed via the isolated working heart perfusion in hearts from mice fed a LFD or HFD for 10 wk. Values are mean ± SEM (n= 6-12) * $p<0.05$ significantly different from LFD

Figure 7-3

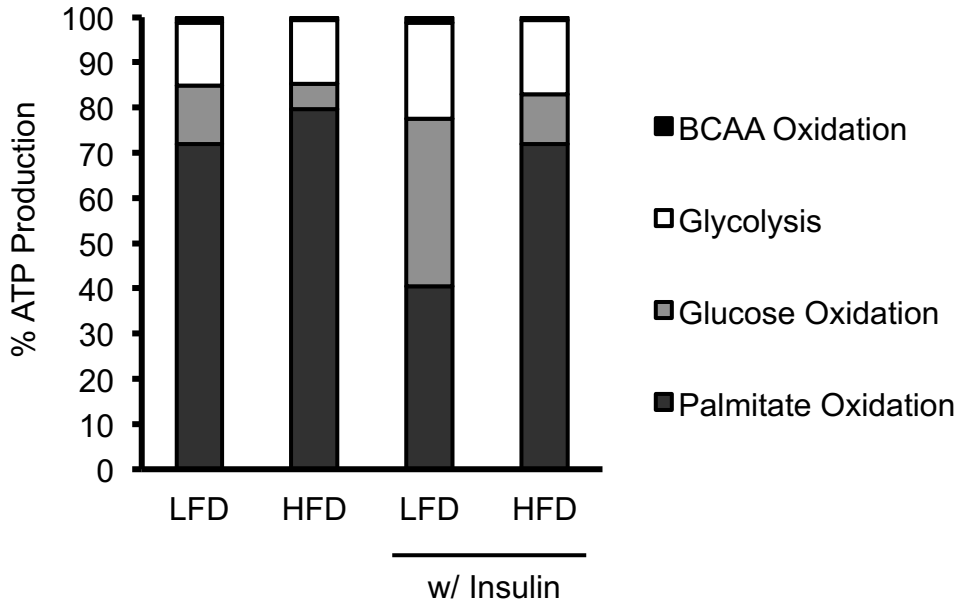

100
90
80
70
60
50
40
30
20
10
0
% ATP Production
LFD
HFD
LFD
HFD
w/ Insulin
BCAA Oxidation
Glycolysis
Glucose Oxidation
Palmitate Oxidation

Figure 7-4. Cardiac BCKD expression is reduced in mice fed a HFD. A. Plasma BCAA levels and cardiac B. phospho BCKD (pBCKD) and total BCKD, C. PP2Cm, D. BCKD kinase, and E. BCATm were measured in mice fed a LFD or HFD for 10 wk. Values are mean ± SEM (n= 4-6) * $p<0.05$ significantly different from LFD

Figure 7-4

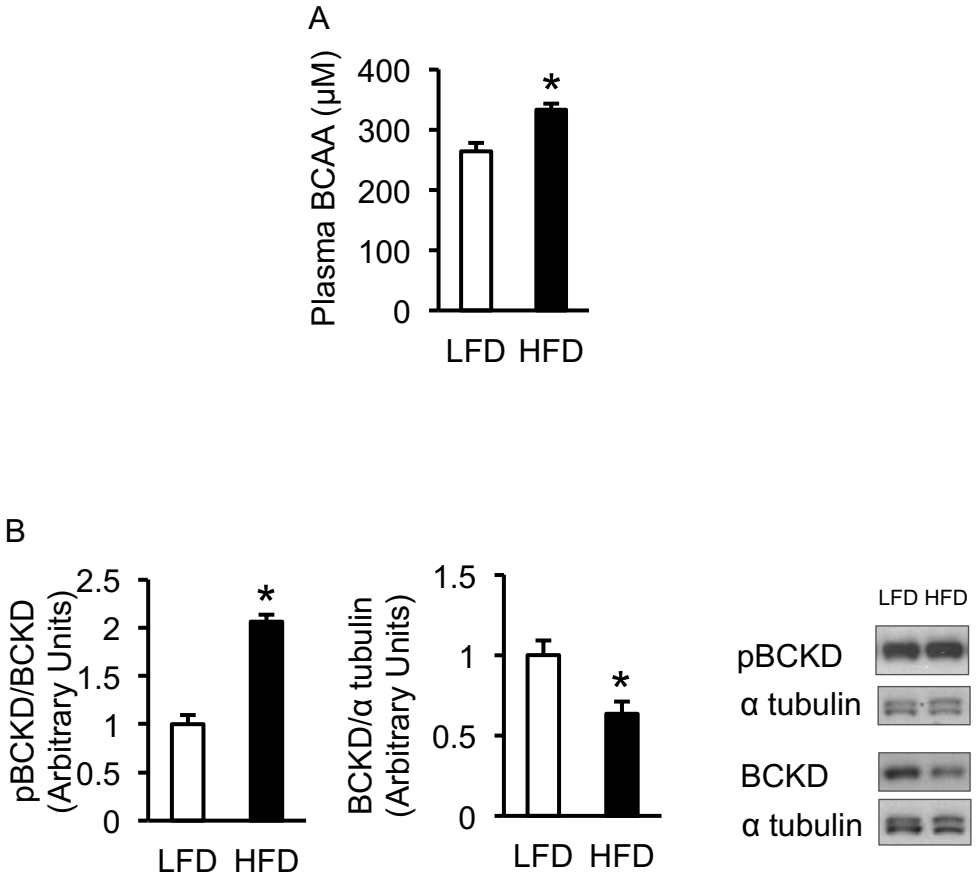

A
Plasma BCAA (μM)
400
300
200
100
0
*
LFD HFD
B
pBCKD/BCKD (Arbitrary Units)
2.5
2
1.5
1
0.5
0
*
LFD HFD
BCKD/α tubulin (Arbitrary Units)
1.5
1
0.5
0
*
LFD HFD
LFD HFD
pBCKD
α tubulin
BCKD
α tubulin

Figure 7-4

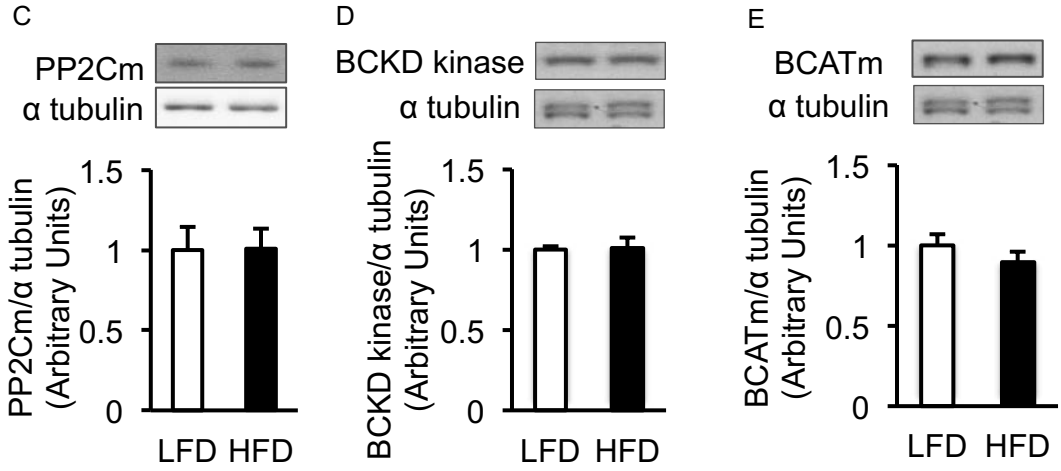

C
PP2Cm
α tubulin
PP2Cm/α tubulin (Arbitrary Units)
1.5
1
0.5
0
LFD HFD
D
BCKD kinase
α tubulin
BCKD kinase/α tubulin (Arbitrary Units)
1.5
1
0.5
0
LFD HFD
E
BCATm
α tubulin
BCATm/α tubulin (Arbitrary Units)
1.5
1
0.5
0
LFD HFD

Figure 7-5. HFD does not alter BCKD or BCATm expression in skeletal muscle or liver. Gastrocnemius A. pBCKD/BCKD, B. BCKD, C. BCATm and liver D. pBCKD and E. BCKD protein expression were measured in mice fed a LFD or HFD for 10 wk. Values are mean ± SEM (n= 6-7) * $p<0.05$ significantly different from LFD

Figure 7-5

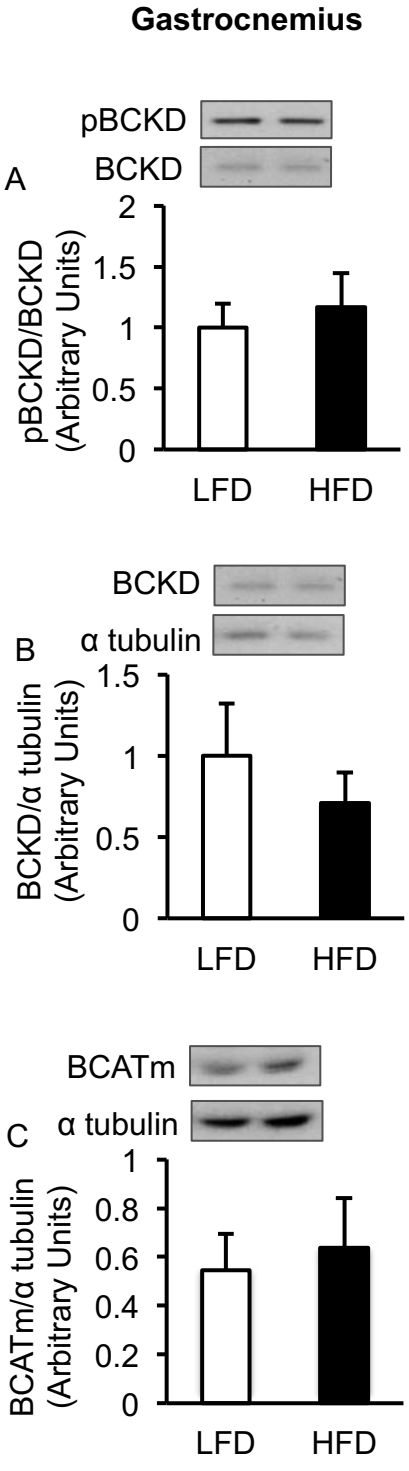

Gastrocnemius
A
pBCKD
BCKD
pBCKD/BCKD (Arbitrary Units)
2
1.5
1
0.5
0
LFD
HFD
B
BCKD
α tubulin
BCKD/α tubulin (Arbitrary Units)
1.5
1
0.5
0
LFD
HFD
C
BCATm
α tubulin
BCATm/α tubulin (Arbitrary Units)
1
0.8
0.6
0.4
0.2
0
LFD
HFD

Figure 7-5

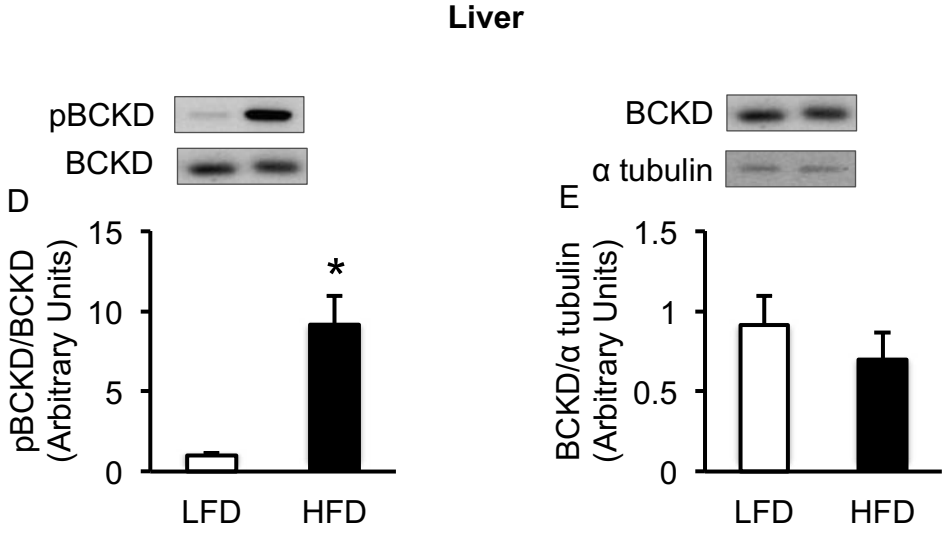

Liver
pBCKD
BCKD
D
pBCKD/BCKD (Arbitrary Units)
15
10
5
0
*
LFD
HFD
BCKD
α tubulin
E
BCKD/α tubulin (Arbitrary Units)
1.5
1
0.5
0
LFD
HFD

Figure 7-6. BCKD kinase inhibitor treatment *in vivo* does not affect *ex vivo* cardiac insulin sensitivity in mice fed a HFD. A. BCAA oxidation was measured in hearts treated with 200 μM BCKD kinase inhibitor or vehicle just during the working heart perfusion. These mice were not given any treatment *in vivo*. (n=4) * $p<0.05$ significantly different from Vehicle. Cardiac B. glucose oxidation, C. palmitate oxidation, D. plasma BCAA levels, and E. cardiac pAkt expression were measured in mice treated with a BCKD kinase inhibitor or vehicle for the final wk on a 5 wk LFD or HFD. Values are mean ± SEM (n= 4-5) * $p<0.05$ significantly different from LFD, ** $p<0.05$ significant effect of BCKD kinase inhibitor treatment. # $p<0.05$ significantly different from LFD BCKD kinase inhibitor. ^ $p<0.05$ significantly different from HFD. LFV, LFD Vehicle; LFI, LFD BCKD kinase inhibitor; HFV, HFD Vehicle; HFI, HFD BCKD kinase inhibitor.

Figure 7-6

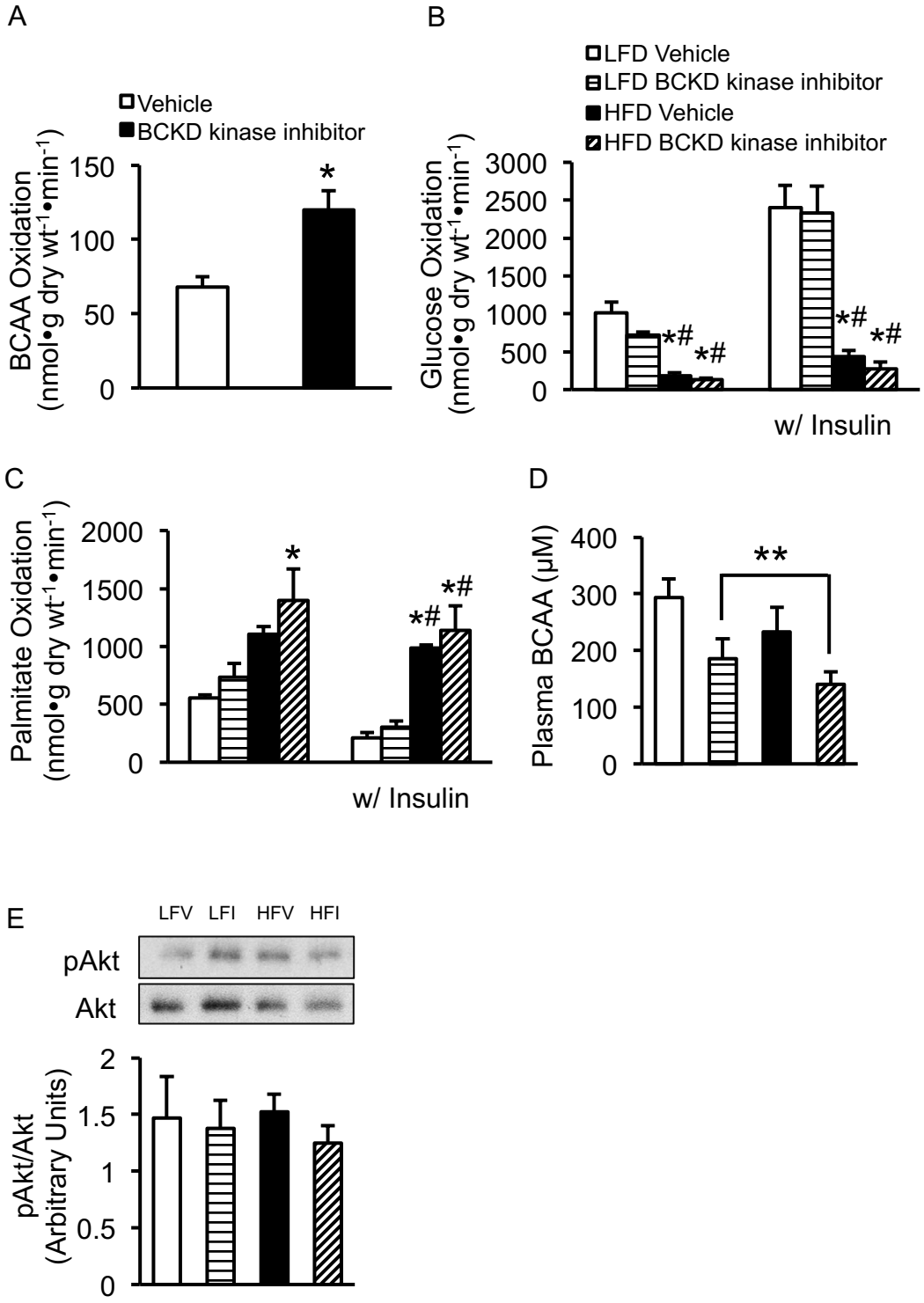

A
Vehicle
BCKD kinase inhibitor
BCAA Oxidation (nmol•g dry wt-1•min-1)
150
100
50
0
*
B
LFD Vehicle
LFD BCKD kinase inhibitor
HFD Vehicle
HFD BCKD kinase inhibitor
Glucose Oxidation (nmol•g dry wt-1•min-1)
3000
2500
2000
1500
1000
500
0
*#
*#
*#
*#
w/ Insulin
C
Palmitate Oxidation (nmol•g dry wt-1•min-1)
2000
1500
1000
500
0
*
*#
*#
w/ Insulin
D
Plasma BCAA (μM)
400
300
200
100
0
**
E
LFV LFI HFV HFI
pAkt
Akt
pAkt/Akt (Arbitrary Units)
2
1.5
1
0.5
0

Table 7-1. Effect of HFD on cardiac BCAA oxidation normalized to cardiac work.

			w/ Insulin	
	LFD	HFD	LFD	HFD
Cardiac Work				
(joules•min^{-1}• g dry wt^{-1})	1.5±0.1	1.2±0.1	1.7±0.2	1.3±0.1
BCAA Oxidation				
(nmol BCAA•joules^{-1})	33.0±2.8	22.2±3.0*	31.3±3.3	25.0±3.4
Glucose Oxidation				
(nmol glucose•joules^{-1})	318.9±51.4	127.1±30.5*	905.7±129.6	291.9±52.2*
Palmitate Oxidation				
(nmol palmitate•joules^{-1})	450.5±66.2	539.9±62.8	231.1±33.0	517.2±49.8*
Glycolysis				
(nmol glucose•joules^{-1})	6042±946	5031±1578	8715±1271	7267±2626

Values are mean ± SEM. Cardiac energy metabolism was assessed in isolated working hearts from mice fed a LFD or HFD for 10 wk (n= 6-12) * $p<0.05$ significantly different from LFD

Table 7-2. Cardiac energy metabolism in BCKD kinase inhibitor treated mice.

					w/ Insulin			
	LFD	LFD+ BCKDKi	HFD	HFD+ BCKDKi	LFD	LFD+ BCKDKi	HFD	HFD+ BCKDKi
Cardiac Work (joules•min^{-1}• g dry wt^{-1})	2.4±0.4	2.3±0.2	2.4±0.2	2.6±0.4	2.7±0.4	2.3±0.1	2.4±0.3	2.2±0.5
Glucose Oxidation (µmol•joules^{-1})	0.4±0.05	0.3±0.05	0.1±0.01*†	0.1±0.01*†	0.9±0.09	1.0±0.16	0.2±0.05*†	0.1±0.02*†
Palmitate Oxidation (µmol•joules^{-1})	0.3±0.02	0.3±0.05	0.5±0.06*	0.5±0.04$^{*\#}$	0.1±0.02	0.1±0.02	0.4±0.03*†	0.5±0.04*†‡

Values are mean ± SEM. Cardiac energy metabolism was assessed in isolated working hearts from mice fed a LFD or HFD for 5 wk and treated with either vehicle or a BCKD kinase inhibitor (BCKDKi) during the 5th wk. (n=4-5) * $p<0.05$ significantly different from LFD # $p<0.05$ significantly different from LFD BCKDKi ‡ $p<0.05$ significantly different from HFD

CHAPTER 8

Discussion and Conclusions

CHAPTER 8

Discussion and conclusions

8.1 Summary

The regulation of glucose oxidation in the heart has been examined in this thesis in both cardiac myocyte differentiation and heart failure. As part of better understanding energy metabolism in cardiac myocyte differentiation we spent some time examining the energy metabolism of one of the promising stem cell types for heart therapy, bone marrow mesenchymal stem cells (BMMSC). We have characterized the metabolic profile of BMMSCs and have shown direct evidence that the Warburg effect occurs in these stem cells. We also discovered that fatty acids regulate BMMSC survival, proliferation, and energy metabolism, a finding that suggests that fatty acids may also be involved in the low survival of transplanted stem cells. We then examined the changes in energy metabolism that occur during cardiac myocyte differentiation, finding that there is an increase in glucose oxidation and hypothesized that glucose oxidation may be a promising target for enhancing heart regeneration. We also examined the changes in energy metabolism that occur in heart failure with preserved ejection fraction (HFpEF), discovering that the coupling of glycolysis and glucose oxidation is reduced due to a rise on glycolysis. We hypothesized that improving this coupling may be an effective strategy for the treatment of heart failure. Prior to this research there were reports that insulin resistance promotes heart failure. Based on this we

focused a substantial amount of effort on understanding how cardiac insulin resistance is regulated and whether improving cardiac efficiency in insulin resistant hearts can improve cardiac function. We found that the cardiac function of *db/db* mice was improved when we stimulated glucose oxidation with insulin glargine for 4 wk. We also found that cardiac branched chain amino acid (BCAA) oxidation is reduced in high fat diet (HFD) induced insulin resistance. This indicates that BCAA oxidation is not contributing to insulin resistance by competing with flux through glucose oxidation. Instead, a decrease in BCAA oxidation could be leading to a rise in BCAA levels in the heart which stimulate mechanistic target of rapamycin (mTOR) signaling and thereby decrease insulin sensitivity. Whether BCAAs stimulate mTOR signaling or another pathway to impair cardiac insulin sensitivity is not clear from the results of this study.

Overall, this research provides novel information regarding the importance of energy metabolism, especially glycolysis and glucose oxidation, in the development and treatment of heart disease. We also provide groundwork knowledge about stem cell energy metabolism that will be important for future work using energy metabolism as a target to improve stem cell heart therapy efficacy and treat heart failure. The rest of this chapter will discuss these findings in more detail as well as some of the limitations of this research and potential future directions for this research.

8.2 BMMSC energy metabolism is predominantly glycolytic.

Glycolysis is important in proliferation. The first evidence that high rates of glycolysis are important for proliferation was reported by Otto Warburg in cancer cells in the 1920s [295-297]. Since then, the Warburg effect, or the high uncoupling of glycolysis and glucose oxidation, has also been associated with stem cells. The Warburg effect has not only been implicated broadly as a way cell growth is regulated, but there is also evidence that it enhances stem cell pluripotency [86, 87, 171, 209, 247]. Despite the apparent importance of energy metabolism in stem cells, when we started this research a metabolic rate profile had not yet been determined for a stem cell. We found that the BMMSC is primarily glycolytic with less than 3% of ATP production coming from mitochondrial oxidative metabolism (glucose and fatty acid oxidation). These results agree with studies that have compared, using less direct methods, the energy metabolism of a stem cell with differentiated cells. Studies using mitochondrial staining and lactate production measurements have suggested that there is an increase in mitochondrial biogenesis and improved coupling of glycolysis and glucose oxidation during cardiac myocyte differentiation [50, 51]. However, using less direct methods Pattappa et al estimated that mitochondrial oxidative metabolism is contributing around 30% of ATP production in BMMSCs [212]. This is a not an insignificant difference between our two studies. We may have overestimated glycolysis due to the fact that release of $^{3}H_2O$ from the radioactive isomer of glucose that we used to measure glycolysis, [5-^{3}H]glucose,

can not only be released at the enolase step of glycolysis but also by transaldolase in the pentose phosphate pathway. This is an important consideration since flux through the pentose phosphate pathway is important for cell proliferation and is affected by the density of BMMSCs, which affects BMMSC proliferation [153, 285]. However, both our data and the results from this study do agree that BMMSCs are primarily glycolytic. While we have only characterized the metabolism of BMMSCs, we hypothesize that a low mitochondrial oxidative metabolism and high glycolysis is a common feature of stem cells.

8.3 Changes in energy metabolism during cardiac myocyte differentiation.

While stem cell therapy holds a lot of promise for the treatment of heart disease, these stem cells have been unable to regenerate the heart [10, 19, 22, 67, 93, 122, 265, 284]. This lack of stem cell-to-cardiac myocyte differentiation is likely to be an important factor in this therapy not showing the degree of benefit that was expected. We believe that a better understanding of stem cell biology and in particular energy metabolism, especially during cardiac myocyte differentiation, will be important in developing strategies to enable cardiac regeneration to occur.

In cardiac myocyte differentiation there is evidence, albeit indirect, that changes in energy metabolism do occur. Just comparing the metabolism of a stem cell to that of the adult heart shows that dramatic changes in the metabolic profile

of a stem cell would need to occur for it resemble the metabolism of a functioning cardiac myocyte. While we have shown that a stem cell has high rates of glycolysis with a very low mitochondrial oxidative metabolism, the adult heart derives most of its energy from mitochondrial oxidative metabolism with less than 10% of ATP coming from glycolysis. This increase in mitochondrial oxidative metabolism may be necessary for cardiac myocyte differentiation to occur. Inhibiting mitochondrial oxidative metabolism impedes stem cell-to-cardiac myocyte differentiation [51]. Further, other studies have provided evidence that there is a massive increase in mitochondrial biogenesis and improved coupling of glycolysis and glucose oxidation during cardiac myocyte differentiation through mitochondrial staining and lactate production measurements [50, 51]. We decided, therefore to examine glucose and fatty acid metabolism in cardiac myocyte differentiation. Since there are problems differentiating some of the more promising stem cells for heart therapy, such as BMMSCs, into cardiac myocytes we chose to use the H9C2 cell line in this study [10, 19, 22, 93, 265, 284]. The H9C2 cell line can be consistently differentiated toward cardiac myocytes using a relatively simple medium (media supplemented with 1% FBS and 10 nM retinoic acid) [177]. We found that 7 day differentiation of H9C2 cells specifically enhances glucose oxidation. We observed a trend for an increase in oleate oxidation and no significant change in glycolysis. It will be necessary in the future to determine if these early metabolic changes also occur in other models of cardiac myocyte differentiation.

Future experiments assess energy metabolism in other models of cardiac myocyte differentiation is especially important because the H9C2 cell line is immortalized and is not a true stem cell. The H9C2 cell is already committed to undergoing myocyte differentiation. It will also be important to determine what changes in metabolism occur during the process of early commitment to cardiac myocyte differentiation. Another important limitation of the H9C2 cell is that it does not contract. Since mitochondrial oxidative metabolism is dependent on cardiac work our glucose and fatty acid oxidation rates may be significantly less than what occurs in cells with a similar elevation in cardiac myocyte markers (eg cardiac myocyte specific contractile proteins, transcription factors that promote cardiac myocyte differentiation). It is possible that the metabolic changes we observed in differentiated H9C2 cells will prove to be substantially different from what occurs in cells that can undergo complete differentiation into spontaneously contracting cardiac mycoytes (such as induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs)). Overall, our results suggest that glucose oxidation may be a promising target for stimulating cardiac myocyte differentiation.

8.4 Stimulating glucose oxidation is a promising treatment for heart disease.

In the rest of this thesis we focused on better understanding how energy metabolism could be involved in the development of heart failure. Interestingly, cardiac insulin resistance has been reported to both precede the development of

heart failure [316] and increase the severity of heart failure [117, 159, 170, 182, 183, 190, 240, 245, 261, 315, 316]. This suggests that insulin resistance is one of the early changes that leads to cardiac inefficiency and contributes to the development of heart failure. Improving cardiac efficiency, either by stimulating glucose oxidation or inhibiting fatty acid oxidation, has been reported to improve the function of diseased hearts [70, 71, 120, 154, 170, 262, 283, 305]. Two of the factors that frequently contribute to cardiac inefficiency in insulin resistant hearts is a decreased ability of insulin to stimulate glucose oxidation and a reduced energy substrate preference for glucose oxidation [28, 33, 39, 78, 80, 126, 158, 282, 318].

Stimulation of glucose oxidation has been reported by multiple research groups to improve insulin sensitivity as well as cardiac function. For example, dichloroacetate (DCA) can improve cardiac function in both right ventricle hypertrophy and during ischemia/reperfusion [152, 221, 222, 273]. Further, DCA improves diastolic function and overall survival of Dahl salt-sensitive rats on a high salt diet (HSD) [120]. Malonyl CoA decarboxylase knockout (MCD-/-) mice protection against HFD induced insulin resistance was accompanied by a higher cardiac glucose oxidation. When subjected to coronary artery ligation (CAL) these mice have better cardiac function and lower proton production than their wild type counterparts [170, 282].

In light of these potential benefits of stimulating glucose oxidation in heart disease, we treated *db/db* mice with insulin glargine in an effort to better

understand whether stimulating glucose oxidation can improve cardiac function in insulin resistant hearts. We treated these *db/db* mice with vehicle or insulin glargine daily for 4 wk. *Db/db* mice treated with insulin glargine had better cardiac function than the vehicle treated mice. Despite the fact that 18 wk old *db/db* mouse have started to develop cardiac dysfunction due to a decline in diastolic dysfunction, insulin glargine did not significantly improve the measurements of diastolic dysfunction we assessed using echocardiography. While it is unclear what is responsible for the improvement in stroke volume and cardiac output there was a trend for an increase in E' in the insulin glargine. This suggests that diastolic function may possibly be improved in response to chronic insulin glargine treatment *in vivo*. On the other hand it may be that insulin glargine does not affect diastolic dysfunction and may not be able to treat cardiac dysfunction characterized by severe diastolic dysfunction.

While we observed an improvement in cardiac function *in vivo*, there was no difference in *ex vivo* cardiac function between vehicle and insulin glargine groups. This indicates that the improved cardiac function is due to acute effects of insulin glargine. As part of this study we showed that insulin glargine acutely increases cardiac glucose oxidation and decreases cardiac palmitate oxidation. While it is well known that insulin has these effects in the mouse heart, this was the first study to report the effect of the long acting insulin insulin glargine on cardiac energy metabolism. We hypothesize that this stimulation of glucose oxidation is an important factor in the improvement of cardiac function induced

by insulin glargine. These results suggest that glucose oxidation is a promising strategy to treat cardiac function in diabetic cardiomyopathy. A potential argument against this hypothesis is that in order to stimulate glucose oxidation acutely in *db/db* mouse hearts we had to use 5 times the dose that was sufficient in WT mouse hearts. This was due to the severe insulin resistance of *db/db* mouse hearts. This suggests that we may not have been able to stimulate cardiac glucose oxidation when we treated the *db/db* mice for 4 weeks with insulin glargine (150 U/kg body weight). on the other hand, this dose of insulin glargine is 15 times the dose recommended for diabetic patients and was effective at improving whole body glucose tolerance in the *db/db* mice. Due to the link between insulin resistance and heart failure, this study also adds to a growing body of evidence that stimulating glucose oxidation can treat and potentially prevent the development of heart failure.

8.5 HFpEF is characterized by an increased uncoupling of glycolysis and glucose oxidation.

We then examined the changes in energy metabolism that occur during the development of HFpEF. This is important because while there has been research looking at the changes in energy metabolism during heart failure with reduced ejection fraction (HFrEF), relatively little work has been done to look at it in a model of HFpEF. Work that has been conducted has mainly used less direct

methods such as overall myocardial glucose uptake. In this study we found that HFpEF in the Dahl salt-sensitive rat is characterized by a decreased coupling of glycolysis and glucose oxidation. This uncoupling preceded a significant decrease in diastolic function suggesting that it could be involved in the development of HFpEF. These findings are supported by another study also conducted in Dahl salt-sensitive rats that reported an increase in myocardial glucose uptake and circulating lactate levels in the rats that had developed heart failure [120]. Interestingly, we only observed evidence of a decline in mitochondrial oxidative metabolism, in the form of a decrease in fatty acid oxidation, in rats that had been fed the HSD for 9 wk (which is not significantly different when normalized to cardiac work). This occurred at the same time we observed the first evidence of a decline in systolic function, a slight decline in % Ejection Fraction (%EF). This suggests that a decline in mitochondrial oxidative metabolism may be the difference in metabolism between HFrEF and HFpEF.

We hypothesize that this uncoupling of glycolysis and glucose oxidation could be contributing to the development of HFpEF. Increased uncoupling of glycolysis and glucose oxidation has been observed in a number of other models of heart failure. For example, the coupling of glycolysis and glucose oxidation is reduced in mouse hearts subjected to coronary artery ligation (CAL) [170]. Further, in multiple rat models right ventricular hypertrophy is also associated with elevated glycolysis [221, 222]. In addition, the hexosamine biosynthesis pathway, which may also be increased in hearts from the Dahl-salt sensitive rats

fed the high salt diet, has been associated with hypertrophy and heart failure and may also be a mechanism whereby increased glucose uptake into the heart contributes to heart failure development [64, 163]. It is also believed that the uncoupling of glycolysis and glucose oxidation decreases cardiac efficiency, contributing to a decline in cardiac function [21, 55, 61, 62, 120, 142, 159, 188, 193, 275]. However, one potential argument against this hypothesis is that fetal hearts seem to function just fine despite high rates of glycolysis and uncoupling of glycolysis and glucose oxidation. The fetal heart is more resistant than the adult heart to hypoxia and acidosis [248]. The lower sensitivity of the fetal heart is to acidosis induced decline in troponin sensitivity to calcium is attributed to the expression of slow skeletal muscle troponin I [185, 248]. A drop in pH also reduces contractility of the adult heart by hydrogen ion inhibition of the slow calcium current [45, 263, 292]. However, fetal heart contraction is more reliant on reversal of the Na^+/Ca^{2+} exchange for calcium influx, which contributes to the decreased sensitivity of the fetal heart to acidosis [46, 226, 298].

There is evidence that improving the coupling of glycolysis and glucose oxidation can improve cardiac function. For example, right ventricle hypertrophy and cardiac function can be improved by DCA [221, 222]. DCA also improved the efficiency and function of hearts subjected to ischemia/reperfusion [152, 273]. Further, treatment of Dahl salt-sensitive rats on a HSD with DCA was effective at activating pyruvate dehydrogenase (PDH), decreasing lactate levels, and improving diastolic function and overall survival [120]. In addition, indirectly

stimulating glucose oxidation by inhibiting fatty acid oxidation also improves cardiac function [44, 70]. For example, MCD-/- mice have decreased proton production when subjected to CAL [170]. This inhibitor also improves the coupling of glycolysis and glucose oxidation in pig hearts subjected to ischemia/reperfusion, leading to a decrease in proton production and improved cardiac function [70]. However, further work will be needed to determine if this uncoupling of glycolysis and glucose oxidation is contributing to the development of HFpEF, and if it can be targeted to treat HFpEF.

An alternative explanation for the beneficial effect of stimulating glucose oxidation in heart failure has been suggested by a study published by Kato et al in the Dahl salt-sensitive rat [120]. In this study, an analysis of the pentose phosphate pathway indicated that the pathway is further upregulated when Dahl salt-sensitive rats in chronic heart failure are treated with DCA. This suggests that beneficial effects of glucose oxidation in cardiac function could also be due to changes in the pentose phosphate pathway that could occur as a result of stimulation of glucose oxidation. While the pentose phosphate pathway has been implicated in cell growth, which suggests that it can contribute to cardiac hypertrophy, it also can provide GSG which is an antioxidant and could be beneficial in heart failure.

8.6 BCAA oxidation in cardiac insulin resistance

While a number of studies suggest that BCAAs contribute to insulin resistance, the mechanism involved in cardiac insulin resistance is not well understood. Several potential mechanisms have been suggested to explain how BCAAs contribute to insulin resistance. One of these proposed mechanisms suggests that increased tissue BCAA levels results in increased BCAA oxidation, which inhibits fatty acid and glucose oxidation [194]. This has been extrapolated to the heart despite these studies being conducted in other tissues including skeletal muscle. There is also evidence that BCAAs can induce insulin resistance by stimulating mTOR signaling [9, 68, 145, 164, 165, 195, 242, 319]. Finally, it has been suggested that accumulation of intermediates of BCAA oxidation, such as branched chain ketoacids (BCKA), is toxic to mitochondria and contributes to insulin resistance [79, 164, 203].

In this thesis we tested the hypothesis that increased BCAA oxidation contributes to cardiac insulin resistance. We were surprised to find that cardiac BCAA oxidation is reduced in the insulin resistant hearts from mice fed a HFD. Another surprising finding was that the contribution of BCAA oxidation to % ATP production in the heart is <1%. These two results indicate that BCAA oxidation is not elevated in insulin resistant hearts and that a change in BCAA oxidation is not likely to have a significant effect on flux through glucose or fatty acid oxidation. This reduced BCAA oxidation may exacerbate insulin resistance due to a buildup of BCAAs in the heart. If this hypothesis is correct it would

support a role for BCAA stimulation of mTOR signaling in mediating cardiac insulin resistance. Alternatively, a reduction in BCAA oxidation could result in accumulation of BCAA oxidation intermediates. Accumulation of intermediates of BCAA oxidation, such as BCKA, has been suggested to be toxic to mitochondria and contribute to insulin resistance [79, 164, 203]. However, BCKAs do not induce insulin resistance in mitochondrial branched chain amino transferase (BCATm)-/- mice, indicating that BCKAs must be converted back to BCAAs in order for insulin resistance to occur [321].

We also hypothesized that stimulating BCAA oxidation can reduce insulin resistance. However, we did not observe an improvement of cardiac insulin sensitivity when we stimulated BCAA oxidation with a branched chain ketoacid dehydrogenase (BCKD) kinase inhibitor in HFD fed mice. There are several reasons why this finding does not preclude the possibility that stimulating BCAA oxidation could be used to improve insulin sensitivity. First, insulin stimulated glucose oxidation was measured in the isolated working heart and the drug was not included in the perfusion. The effect on insulin sensitivity may have been lost due to the absence of the drug in the perfusion. Second, the BCKD kinase inhibitor dose might not have stimulated BCAA oxidation sufficiently to improve cardiac insulin sensitivity. Finally, in this experiment mice were fed the HFD for only 5 weeks. A 5 week HFD was not sufficient to increase circulating BCAA levels. It is possible that if we had fed the mice a HFD for 10 wk, as we did in the first part of the study, we would have observed a similar elevation in circulating

BCAA levels and the BCKD kinase inhibitor would have improved cardiac insulin sensitivity. Further work will be required to follow up on these observations and determine if stimulating BCAA oxidation can improve cardiac insulin sensitivity.

A limitation of our work to understand the importance of BCAA oxidation in cardiac insulin resistance is that we do not have direct evidence that the BCKD kinase inhibitor stimulated cardiac BCAA oxidation *in vivo*. The reduction in circulating BCAA levels in the mice treated with the BCKD kinase inhibitor suggests that the inhibitor was effective at stimulating BCAA oxidation. However, we do not have direct evidence that the BCKD kinase inhibitor stimulated cardiac BCAA oxidation *in vivo*. We did provide evidence that the BCKD kinase inhibitor is capable of stimulating cardiac BCAA oxidation. However, in this experiment *ex vivo* isolated working mouse hearts were treated acutely with the BCKD kinase inhibitor. If it in fact was not effective at stimulating cardiac BCAA oxidation that could help explain why we did not observe an improvement in cardiac insulin sensitivity in HFD fed mice treated with the BCKD kinase inhibitor. It remains to be seen if this drug can stimulate cardiac BCAA oxidation rates *in vivo*.

8.7 Justification and limitations of methodology

8.7.1 The isolated working heart

The isolated working heart is an experimental system that can be used to study cardiac function as well as energy metabolism. It allows for cardiac energy metabolism to be assessed under preload and afterload conditions similar to those present *in vivo*. The conditions in perfusate can be adjusted to expose the heart to physiological or pathological substrate levels as required for a specific study. A limitation of this method is that the hearts are no longer exposed to the exact same conditions that were present *in vivo.* This can result in these metabolism measurements not giving an accurate representation of cardiac metabolism *in vivo*. However, this is also a strength of this technique because it allows chronic changes in cardiac metabolism to be assessed without the factors the heart is exposed to during the assay confounding the results. Only two substrates can be assessed in a given isolated working heart perfusion. We therefore used two sets of hearts to measure the four metabolic rates assessed in each of the animal studies (Chapter 5 and 6: glucose oxidation, glycolysis, lactate oxidation, and palmitate oxidation; Chapter 7: glucose oxidation, glycolysis, BCAA oxidation, and palmitate oxidation)

Energy metabolism is tightly regulated by cardiac work. The ability to assess energy metabolism in a heart working at physiologically relevant levels is one of the advantages the isolated working heart perfusion has over some of the

more utilized methods for measuring energy metabolism including whole cell homogenates and mitochondrial preparations. In homogenates and isolated cardiac myocytes oxidative energy metabolism is much lower than that of the isolated working heart, probably due to the absence of work.

8.7.2 Measurement of glucose oxidation, fatty acid oxidation, and glycolysis in cell culture.

Two of the most common methods that are used to measure glucose oxidation, fatty acid oxidation, and glycolysis in cell culture utilize radioactive glucose and fatty acid tracers or the Seahorse flux analyzer. In this thesis we used ^{14}C-glucose, ^{14}C-fatty acid, and ^{3}H-glucose to measure glucose oxidation, fatty acid oxidation, and glycolysis, respectively. Use of radioactive labeled fatty acid and glucose allowed us to directly measure flux through these metabolic pathways and include any metabolic substrates desired in the media. We felt that this was important in our studies in order to make the conditions during the assays more physiologically relevant and therefore give a better idea of energy metabolism *in vivo*. A weakness of using [U-^{14}C]glucose to measure glucose oxidation is that pyruvate, which is produced at the end of glycolysis, can also be carboxylated to malate or oxaloacetate. This technique can not distinguish between $^{14}CO_2$ produced from PDH derived acetyl CoA that enters the tricarboxylic acid (TCA)

cycle and $^{14}CO_2$ that is produced from this malate or oxaloacetate entering the TCA cycle.

The other more frequently used method to measure energy metabolism, especially for stem cells, is the Seahorse flux analyzer. It is essentially based on two measurements, oxygen consumption and extracellular acidification rate. The acidification rate is used as a measurement of glycolysis. However, what is really being measured is the degree of uncoupling of glycolysis and glucose oxidation. Even though this is not directly measuring glycolysis, it is probably a fairly accurate representation of glycolysis in cells that are primarily glycolytic such as cancer cells and stem cells (as we have shown in this thesis for BMMSCs). Glucose oxidation and fatty acid oxidation are also not measured directly. Instead either glucose or fatty acids are added to the media and oxygen consumption is measured. Because only one substrate is present it is assumed that any oxygen that is consumed represents glucose oxidation or fatty acid oxidation. A major problem with this lies in the fact that glucose and fatty acids can not be present together in the media. Since glucose and fatty acid oxidation regulate each other this would impair the ability to extrapolate these results to what might be occurring *in vivo*. However, the absence of radioactivity and the relatively low number of cells required are two of the characteristics that make this a more convenient method to use for measuring cell energy metabolism.

8.7.3 Use of [5-^{3}H]glucose to measure glycolysis

Two radioactive isomers of glucose can be used to directly measure glycolysis, [5-^{3}H]glucose and [2-^{3}H]glucose. ^3H_2O is released at the enolase step when [5-^{3}H]glucose is used, while ^3H_2O is released at the isomerase step when [2-^{3}H]glucose is used. One of the problems with using [2-^{3}H]glucose is that the ^{3}H can shift to the carbon 1 position. This results in an underestimation of glycolytic rates. In addition the step at which ^3H_2O is released from [2-^{3}H]glucose can result in an overestimation of glycolysis rates. For example, in ischemia GAPDH can become the rate limiting step for glycolysis [130]. However, ^3H_2O can still be produced from [2-^{3}H]glucose despite the fact that much of the glucose is not fully passing through glycolysis to pyruvate. On the other hand this does not become a problem when [5-^{3}H]glucose is used because it is downstream of GAPDH.

Another potential concern is that [5-^{3}H]glucose may provide an overestimation of glycolysis rates when there is high flux through the pentose phosphate pathway. It has been suggested that ^3H_2O is released by transaldolase in the pentose phosphate pathway which results in overestimation of glycolysis rates in the heart [96]. However, a subsequent study has shown that in normal hearts glycolysis rates measured from [5-^{3}H]glucose actually matches the rates that would be expected based on glucose oxidation and lactate production [143]. This indicates that [5-^{3}H]glucose does not actually overestimate glycolysis in a healthy adult heart. However, in the conditions where a significant proportion of glucose enters the pentose phosphate cycle this may be a concern and lead to an overestimation of glycolysis. This suggests that we may have overestimated the

glycolytic rates in BMMSCs, H9C2 cells, and in hearts from Dahl salt-sensitive rats fed a high salt diet [120, 285].

8.7.4 Species differences in cardiac energy metabolism

In this thesis we studied cardiac energy metabolism in both the mouse and rat heart. There are some key differences between their cardiac energy metabolism that need to be taken into account. First, rates of fatty acid oxidation are different in mouse and rat hearts. In the isolated working heart perfusion rat hearts have a significantly higher fatty acid oxidation than mouse hearts with equivalent fatty acid concentration in the perfusate [15, 70, 71, 88, 137]. Under similar conditions glucose oxidation and glycolysis rates are higher in mouse hearts than rat hearts [70, 71, 85, 88]. The relative contribution of palmitate and glucose oxidation to ATP production in the human heart is more similar to the rat than the mouse heart [210]. Therefore, we chose to use rats in the study focused on HFpEF in Chapter 6.

Another important difference between the mouse and rat heart is the effect of insulin on energy metabolism. Insulin induces a large increase in glucose oxidation in the mouse [85]. Insulin also significantly reduces fatty acid oxidation in the mouse heart. This parallels the increase in glucose metabolism and decrease in fatty acid utilization observed in human hearts treated with insulin [77]. In the rat heart insulin induces a much more pronounced stimulation of glycolysis with less of a stimulation of glucose oxidation and no reduction in fatty acid oxidation

[240]. These differences in response to insulin are important when designing an experiment to study cardiac insulin resistance. The response of the mouse heart to insulin allows it to be much more easily used to assess differences in the effect of insulin on cardiac glucose oxidation and fatty acid oxidation and assess effects on insulin resistance. However, due to the different responses of mouse and rat hearts to insulin these studies are not likely to be duplicated in rats. The differences in how energy metabolism is regulated between species may even result in different results depending on the species used. We therefore chose in Chapter 5 and 7 to use two mouse models of insulin resistance, *db/db* mice and HFD, respectively. Another important point is that, based on this information, the absence of dramatic effects of insulin on energy metabolism in Dahl salt-sensitive rat hearts in Chapter 6 was not unexpected.

8.7.5 Models of insulin resistance

In this thesis two models of insulin resistance were used, high fat feeding and the *db/db* mouse. The *db/db* mouse, a well characterized model of insulin resistance and diabetic cardiomyopathy, was used in Chapter 6. The *db/db* mouse is homozygous for a spontaneous mutation that results in a nonfunctional leptin receptor. 18 wk old *db/db* mice were used in the experiment in which we treated mice chronically with insulin glargine because at this age *db/db* mice have begun to develop diastolic dysfunction (32-24). Unfortunately we did not have a wild type group in this experiment due to the risk of hypoglycemia in response to

treatment with insulin glargine. An argument frequently made against the use of the *db/db* mouse is that deficiency in the leptin receptor is an uncommon cause of obesity in the human population [197].

We used the HFD to induce cardiac insulin resistance in mice in Chapter 7. We believe that the HFD is one of the models of insulin resistance that most closely resembles insulin resistance in the human population. A limitation of our high fat feeding protocol is the high amount of fat (lard constitutes 60% kcal) compared to a 45% HFD which would more closely resemble the western diet. However, this higher amount of fat mainly increases the rate at which the insulin resistance and obesity develop in response to the diet [94, 118].

8.7.6 Dahl salt-sensitive rat as a model of HFpEF

In this thesis we used the HSD fed Dahl salt-sensitive rat as a model of HFpEF. This Dahl salt-sensitive rat was created by inbreeding [230]. Feeding these rats an 8% HSD has several effects related to the development of hypertension including cardiac hypertrophy, renal failure, and HFpEF [108, 129, 230]. A limitation of this model is the reduced sensitivity of female rats to HSD induced diastolic dysfunction. One of the features that supports the use of this model is the ability to induce heart failure through a HSD instead of surgery. In addition, the progressive development of HFpEF over several weeks allows the development of HFpEF to be examined, a feature taken advantage of in this thesis.

8.8 Final conclusions

The research in this thesis provides important information regarding the role of energy metabolism in stem cell survival/proliferation and cardiac function as well as the changes in energy metabolism that accompany cardiac myocyte differentiation, insulin resistance, and heart failure. These novel findings suggest that glucose oxidation may be a promising target for promoting cardiac myocyte differentiation and treating heart failure (Figure 8-1). We have found that as initially hypothesized bone marrow mesenchymal stem cell energy metabolism is primarily glycolytic (glycolysis contributes more than 97% of the ATP production) and have been able to show that fatty acids regulate BMMSC survival, proliferation, and energy metabolism. We speculate that BMMSC energy metabolism may contribute to the low survival of BMMSCs exposed to saturated fatty acids as well as the low survival of transplanted stem cells. We have also been able to show that during H9C2-to-cardiac myocyte differentiation glucose oxidation increases. This suggests that it may be important in the differentiation process and may be a promising target for stimulating cardiac myocyte differentiation.

We also focused on understanding cardiac energy metabolism in heart failure. When we looked at the changes in energy metabolism during the development of HFpEF we found that there is an increase in cardiac glycolysis which results in increased uncoupling of glycolysis and glucose oxidation in the Dahl Salt-sensitive rat heart. This resembles a shift back toward fetal heart energy

metabolism. We speculate that stimulating glucose oxidation and improving this coupling might improve cardiac function. We also found evidence that stimulating glucose oxidation could potentially be used to prevent the development of cardiac dysfunction. Treatment with insulin glargine, which stimulates glucose oxidation, for 4 wk increases stroke volume and cardiac output in *db/db* mice with cardiac dysfunction. This suggests that stimulating glucose oxidation could be a promising strategy for the treatment of heart failure. Finally, we looked at BCAA oxidation to get a better understanding of the factors that control insulin resistance due to the link between insulin resistance and heart failure. Cardiac BCAA oxidation decreases in response to a HFD and contributes less than 1% ATP production in the heart. This indicates that BCAA oxidation does not contribute to cardiac insulin resistance by competing with flux through glucose and fatty acid oxidation.

8.9 Future directions

A considerable amount of research is still required to follow up on the novel data that has been presented in this thesis. In particular, it remains to be determined if glucose oxidation is important in cardiac myocyte differentiation and if it could be a promising target for promoting cardiac myocyte differentiation and potentially improve efficacy of stem cell heart therapies (Figure 8-1). A significant amount of work will also be required to follow up on findings

regarding the role of glucose oxidation and BCAA oxidation in insulin resistance and the potential to treat heart failure by stimulating these pathways (Figure 8-1).

An important area that requires further investigation is whether glucose oxidation promotes cardiac myocyte differentiation. We reported in this thesis that 7 days of H9C2-to-cardiac myocyte differentiation is accompanied by an increase in glucose oxidation. Modulation of glucose oxidation by pharmacologically or genetically altering overall PDH or mitochondrial pyruvate carrier (MPC) activity would allow the importance of glucose oxidation in cardiac myocyte differentiation to be examined. It will also help to determine whether stimulating glucose oxidation might be an effective strategy to promote cardiac myocyte differentiation, and thereby potentially improve stem cell heart therapy efficacy. However, to get to this point, the research presented in this thesis will need to be tested and expanded upon in cell types that can be used in this type of therapy, such as cardiac stem cells and iPSCs. Currently, there is very little known with regard to the role of energy metabolism in this differentiation process. It has been reported that stimulating glucose oxidation lessens pluripotent marker expression during iPSC generation, indicating that glucose oxidation may promote differentiation [87]. Other studies have also provided evidence that oxidative metabolism is important for cardiac myocyte differentiation [51, 207]. Despite the fact that in the research presented fatty acid oxidation was not elevated during cardiac myocyte differentiation another possibility is that stimulating fatty acid oxidation would promote cardiac myocyte differentiation. Several studies have

reported that increasing the overall activity of PPARs, transcription factors that promote fatty acid oxidation, also increases cardiac myocyte differentiation [65, 66, 256]. In addition, it has been suggested that let-7 microRNA promotes cardiac myocyte maturation, but not early cardiac myocyte differentiation, by increasing fatty acid metabolism [138]. It will remain to be seen which, if any, of the metabolic pathways can be targeted to promote cardiac myocyte differentiation. It will also be interesting to test whether strategies for modulating energy metabolism, that have proven effective at promoting cardiac myocyte differentiation in vitro, can promote regeneration of the injured heart and improve cardiac function.

Another area that will be important to investigate further is the potential modulating energy metabolism has to improve transplanted stem cell survival. In this thesis we show that fatty acids affect survival and proliferation in a fatty acid type specific manner. Decreased survival and proliferation of BMMSCs is accompanied by a decrease in fatty acid oxidation. The effect of directly stimulating or inhibiting fatty acid oxidation on stem cell death and proliferation still needs to be determined. It will also be interesting to see if stimulating fatty acid oxidation could improve the survival of transplanted stem cells. Further, it would be interesting to determine if the ratio of circulating palmitate and oleate contributes to the low survival of transplanted stem cells. This could lead to the development of strategies to improve the survival of transplanted stem cells and potentially improve the efficacy of stem cell therapies.

A pivotal finding in this thesis is that insulin glargine improves *db/db* mouse cardiac function. One of the questions raised by this finding is whether insulin glargine could improve cardiac function in diabetic patients. It would be interesting to determine if treating individuals acutely with long acting insulin could directly improve cardiac function. It will also be interesting to determine if all long acting insulins have similar effects on cardiac function or if this is a specific effect of insulin glargine. The direct application of long acting insulin to treat cardiac dysfunction is limited to individuals with hyperglycemia due to the risk of hypoglycemia. However, this research suggests that stimulating glucose oxidation can improve the function of an insulin resistant heart not just in diabetes but possibly in heart failure as well. This adds to a growing body of evidence suggesting that stimulating glucose oxidation is beneficial in the setting of heart failure [120, 126, 151, 152, 159, 182, 240, 282]. In heart failure this strategy could also improve cardiac efficiency by improving the coupling of glycolysis and glucose oxidation. In fact, in this thesis we showed that there is a rise in the uncoupling of glycolysis and glucose oxidation that accompanies the development of diastolic dysfunction in Dahl salt-sensitive rats. This suggests that stimulating glucose oxidation could also be used to improve this uncoupling and potentially prevent the development of and treat HFpEF. It will be important to determine if directly stimulating glucose oxidation can improve cardiac function in diabetic cardiomyopathy and heart failure. In addition, it will be important to examine

whether stimulating cardiac glucose oxidation can prevent the development of HFpEF and HFrEF.

Finally, this thesis also provides novel information regarding the role of BCAA oxidation in cardiac insulin resistance. This research indicates that instead of an elevation in BCAA oxidation contributing to cardiac insulin, a decrease in BCAA oxidation may be involved. Several aspects related to this research still need to be addressed. First, it needs to be determined if decreased cardiac BCAA oxidation contributes to cardiac insulin resistance as well as whole body insulin resistance. This could be examined by inhibiting BCAA oxidation both pharmacologically and by using genetic models (eg BCKD-/- mice, BCATm-/-) and then examining the effect on whole body insulin sensitivity and cardiac insulin sensitivity. Plans are in progress to start to address this by seeing if BCKD-/- mice are more susceptible to HFD induced cardiac insulin resistance. Second, it still needs to be determined if stimulation of BCAA oxidation can improve cardiac insulin sensitivity. This could be further examined using a BCKD kinase inhibitor in HFD fed insulin resistant mice and examining the effect of insulin administration *in vivo* on cardiac insulin signaling. At the same time it would be useful to determine if stimulating BCAA oxidation improves insulin sensitivity in the heart as well as at the whole body level. Examining mTOR signaling and the ability of insulin to modulate cardiac energy metabolism and upregulate insulin signaling pathways in *ex vivo* hearts (WT hearts or hearts with modified BCAA oxidation due to genetic modifications treated) with the BCKD

kinase inhibitor would also help elucidate the acute and chronic nature of the effects of BCAAs and BCAA oxidation on cardiac insulin sensitivity.

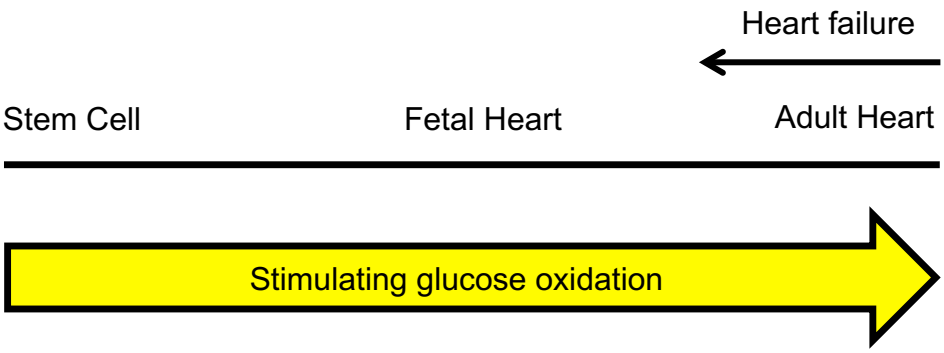
Since there is a substantial link between insulin resistance and heart failure we hypothesize that BCAA oxidation may be involved in the development of heart failure. In fact, a positive correlation has been reported between BCAAs and BCAA related metabolic clusters and heart failure in both human and rodent hearts [110, 120, 246, 252]. We are currently determining whether BCAA oxidation is altered in heart failure and if stimulation of BCAA oxidation can improve insulin sensitivity and cardiac function. To test this we plan to directly measure cardiac BCAA oxidation in mouse hearts subjected to transverse aortic constriction (TAC) surgery. We are also examining the effect of increasing or decreasing BCAA oxidation on insulin sensitivity and function in these failing hearts. One of these approaches will involve stimulating BCAA oxidation pharmacologically (BCKD kinase inhibitor). In addition, mice with alterations in key enzymes involved in BCAA oxidation will be used to assess the effect of stimulating BCAA oxidation (cardiac specific BCKD kinase-/-) and inhibiting BCAA oxidation (tamoxifen inducible cardiac specific BCATm-/-) on cardiac insulin resistance and function in heart failure.

I think it will also be important to examine the relative contribution of BCAAs and BCKAs to cardiac insulin resistance in high fat diet induced obesity, diabetes, and in heart failure. One of the tools that could be used to assess this is BCATm-/- mice. In these mice a decline in BCAA oxidation might result in a

buildup of tissue BCAAs but they would not be converted to BCKAs. If BCATm-/- mice are able to develop cardiac insulin resistance it would support a role for BCAAs not BCKAs in insulin resistance. If on the other hand, these mice are resistant to cardiac insulin resistance that would support a role for BCKAs in regulating cardiac insulin sensitivity. To further examine this it would be beneficial to perform similar experiments in mice without BCKD activity, such as in the BCKD-/- mouse. In these mice a buildup of BCKAs would be expected. If BCATm-/- hearts are resistant to insulin resistance and BCKD-/- hearts are insulin resistant or hypersensitive to conditions that can induce insulin resistance that would support a role for BCKAs instead of BCAAs in the development of cardiac insulin resistance. Understanding the mechanisms involved in BCAA related cardiac insulin resistance is important because it may provide clues to appropriate strategies to regulate cardiac BCAA oxidation in order to improve cardiac insulin sensitivity and potentially improve cardiac function in conditions such as diabetic cardiomyopathy and heart failure.

Figure 8-1. Stimulating glucose oxidation may be promising strategy to treat heart disease. We speculate that stimulating glucose oxidation may be a promising strategy to both promote cardiac myocyte differentiation and treat heart failure. Some important future research directions will be to determine whether stimulating glucose oxidation can both promote cardiac myocyte differentiation as well as treat and potentially prevent the development of heart failure. It will also be important to further examine whether stimulation of BCAA oxidation could improve the ability if insulin to stimulate cardiac glucose and treat cardiac insulin resistance and heart failure.

Figure 8-1


Heart failure
Stem Cell
Fetal Heart
Adult Heart
Stimulating glucose oxidation

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