Role of Lysine Acetylation in the Control of Cardiac Energy Metabolism

by

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Abstract

Cardiac Fatty acids β-oxidation (FAO) markedly increases during maturation and become the major source of energy for the adult heart. However, excessive rates of FAO can compromise cardiac function in obesity, diabetes, and heart failure. Lysine acetylation has recently been identified as a potentially important pathway involved in the control of energy metabolism. In mitochondria, this post-translational acetylation is catalyzed by general control of amino acid synthesis 5-like 1 (GCN5L1), while SIRT3 is a major deacetylase. Despite the fact that a number of FAO enzymes can be acetylated, whether this post-translational modification activates or inhibits FAO enzymes is a matter of debate. We therefore examined the role of lysine acetylation on cardiac FAO during maturation (1-day, 7-day, and 21-day old rabbit), and in hearts from high fat (HF) diet-induced obese mice, and abdominal aortic constriction (AAC)-induced HF obese mice. Cardiac FAO rates were significantly increased during maturation. Activities of the FAO enzymes, long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were increased in hearts from 7-, and 21-day vs 1-day old rabbits, and were associated with LCAD and β-HAD hyperacetylation. Increased overall myocardial protein acetylation during maturation was associated with increased mitochondrial acetyltransferase GCN5L1 expression, while expression of the mitochondrial deacetylase, SIRT3, did not change. Increased expression of the nuclear deacetylase SIRT1 and

a decrease in cardiac SIRT6 expression during maturation was accompanied by decreased acetylation of peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α) and increased ATP production. Acetylation of the glycolytic enzymes hexokinase (HK) and phosphoglycerate mutase (PGM) were increased; scenarios consistent with the decrease in glycolytic activity seen in 7-, and 21-day vs. 1-day old hearts. High FAO rates in isolated working hearts from diet-induced obese mice was found to be positively correlated with an increase in activity of the FAO enzymes, LCAD and β-HAD. An increase in LCAD and β-HAD acetylation was also observed, which was associated with a decrease in SIRT3 expression and no change in the expression of GCN5L1. To further examine the role of acetylation in regulating FAO, we used SIRT3 KO mice to investigate the role of lysine acetylation on cardiac FAO. Although cardiac work was similar, cardiac FAO rates were increased in SIRT3 KO versus WT mice (422±29 vs 291±17 nmol.g dry $wt^{-1}.min^{-1}$, respectively, $p<0.05$). This was accompanied by a decrease in glucose oxidation rates in SIRT3 KO versus WT mice (1262±121 vs 1983±174 nmol.g dry $wt^{-1}.min^{-1}$, respectively, $p<0.05$). Cardiac lysine acetylation was increased in SIRT3 KO mice compared to WT mice, which included an increased acetylation and activity of LCAD and β-HAD. Obesity and heart failure were induced in mice by feeding a HF diet (60% kcal from fat) for 4 weeks and producing an AAC. At 4 wk post-AAC, mice were either switched to a low-

fat (LF) diet (4% kcal from fat; HF AAC LF) or maintained on a HF Diet (HF AAC HF) for a further 10 wk period. After 18 weeks, HF AAC HF mice weighed significantly higher than HF AAC LF mice. Cardiac hypertrophy evident in HF AAC HF mice was associated with SIRT1 mediated inhibition of FoxO1 while activation of FoxO1 and AMPK blunted this effect in HF AAC LF mice. In isolated working hearts, insulin stimulated glucose oxidation rates were also significantly increased in HF AAC LF hearts, compared to HF AAC HF hearts. Activity of the fatty acid β-oxidation enzyme, LCAD was significantly increased in hearts from HF AAC HF vs HF AAC LF mice, and was associated with LCAD hyperacetylation. Heart failure observed in vivo by echocardiography in HF AAC HF was significantly improved in HF AAC LF mice. These results suggest a key regulatory role of acetylation in controlling cardiac energy metabolism. However, we conclude that lysine acetylation increases, rather than decreases, FAO in the heart.

Dedication

I dedicate this thesis to my parents, Yousef and Heyam, to my beautiful wife, Maryam, and our little angel, Rital. This thesis is also dedicated to my sisters, Asma, Tamador, Isra and brothers, Mohammed and Asem.

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List of Abbreviations

FAO	**Fatty Acid Oxidation**
CoA	**Coenzyme A**
ATP	**Adenosine Triphosphate**
ADP	**Adenosine Diphosphate**
AMP	**Adenosine Monophosphate**
HFD	**High Fat Diet**
AAC	**Abdominal Aortic Constriction**
LCAD	**Long Chain Acyl CoA Dehydrogenase**
β-HAD	**Beta-Hydroxy Acyl CoA Dehydrogenase**
PGC1α	**Peroxisome proliferator-activated receptor gamma coactivator 1 alpha**
GLUT4	**Glucose Transporter Type 4**
HK	**Hexokinase**
PGM	**Phosphoglycerate Mutase**
LDH	**Lactate Dehydrogenase**
HIF1α	**Hypoxia Inducible Factor 1 Alpha**
MPC	**Mitochondrial Pyruvate Carrier**
PDH	**Pyruvate Dehydrogenase**
PDK	**Pyruvate Dehydrogenase Kinase**
PDHP	**Pyruvate Dehydrogenase Phosphatase**
IR	**Insulin Receptor**
IRS	**Insulin Receptor Substrate**
PI3K	**Phosphatidylinositol-4,5-bisphosphate 3-kinase**
PIP3	**Phosphatidylinositol 3-phosphate**
PH	**Pleckstrin Homology**
PDK1	**Phosphoinositide dependent protein kinase 1**
Akt (PKB)	**Protein Kinase B**
PPARs	**Peroxisome Proliferator Activated Receptors**
FACS	**Fatty Acyl CoA Synthetase**
CPT1	**Carnitine Palmitoyltransferase 1**

3-KAT	**3-ketoacyl CoA Thiolase**
PFK1	**Phosphofructokinase 1**
FFA	**Free Fatty Acids**
FABP	**Fatty Acids Binding Protein**
FATP	**Fatty Acids Transporter Protein**
ACS	**Acyl CoA Synthase**
CrAT	**Carnitine Acetyltransferase**
VLDL	**Very Density Lipoproteins**
TAG	**Triacylglycerol**
LPL	**Lipoprotein Lipase**
ATGL	**Adipose Triglyceride Lipase**
HSL	**Hormone Sensitive Lipase**
MGL	**Monoglyceride Lipase**
MAG	**Monoacylglycerol**
HSLAS	**Health Sciences Lab Animal Services**
OGTT	**Oral Glucose Tolerance Test**
AUC	**Area Under the Curve**
LV	**Left Ventricle**
AMPK	**Adenosine Monophosphate Activated Protein Kinase**
MCD	**Malonyl CoA Decarboxylase**
$MCD^{-/-}$	**Malonyl CoA Decarboxylase Knockout**
GSK3β	**Glycogen Synthase 3 Beta**
ERRα	**Estrogen Related Receptor Alpha**
PKC	**Protein Kinase C**
P70S6K	**Ribosomal Protein S6 Kinase**
mTOR	**Mammalian Target of Rapamycin**
FoxO	**Forkhead box O**
AICAR	**Aminoimidazole-4-Carboxamide Ribonucleotide**
P38MAPK	**P38 Mitogen Activated Protein Kinase**
TCA	**Tricarboxylic Acid**
ETC	**Electron Transport Chain**

FAD	**Flavin Adenine Dinucleotide**
GCN5	**General Control of Amino Acids 5**
GCN5L1	**General Control of Amino Acids 5 Like Protein 1**
SIRT	**Silent mating type Information Regulation 2 homolog**
HDAC	**Histone Deacetylase**
HAT	**Histone Acetyl Transferase**
NAD	**Nicotinamide Adenine Dinucleotide**
TSA	**Trichostatin A**
NAM	**Nicotinamide**
DIO	**Diet Induced Obesity**
TAC	**Transverse Aortic Constriction**
AAC	**Abdominal Aortic Constriction**
HF-AAC-HF	**High Fat- Abdominal Aortic Constriction-High Fat**
HF-AAC-LF	**High Fat- Abdominal Aortic Constriction-Low Fat**
BMI	**Body Mass Index**
TDI	**Tissue Doppler Imaging**
E/A	**Early to Late ventricular filling**
E'	**Mitral annulus early diastolic velocity**
A'	**Late diastolic mitral annulus velocity**
HW	**Heart Weight**
TL	**Tibia Length**

CHAPTER 1.

INTRODUCTION

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1. INTRODUCTION

1.1 Energy Metabolism in the Heart

Heart is a high energy demand organ which needs continuous supply of adenosine triphosphate (ATP) to maintain contractile function (1). Since no ATP reserves exist in the heart, contractile function of the heart would fail within 5 beats if myocardial ATP synthesis ceased. The continuous synthesis of ATP in the heart is primarily met by the metabolism of fatty acids and carbohydrates (1,2). However, depending on availability the heart can also use ketones and amino acids as energy sources (3). In the adult heart over 90% of the ATP supply is generated from mitochondrial oxidative phosphorylation, with the remainder originating from glycolysis (1,2). The majority of this mitochondrial ATP production normally originates from fatty acid β-oxidation (1,4). The reminder of ATP production is derived from glycolysis, and the oxidation of glucose, lactate, and ketone bodies (Figure 1-1). However, the heart has the ability to switch between these fuel sources depending on a number of factors, which includes contractile demand, nutritional status, transcriptional/translational and post-translational control of energy metabolic pathways, and the presence of underlying cardiac disease(s) (1, 2,5,6). Interestingly, this energy substrate preference differs between the adult and neonatal heart. During the fetal life levels of fatty acids are very

low and the heart relies mostly on glycolysis and lactate oxidation to meet its energy demands (5,7,8). Shortly after birth, major changes in energy metabolism occur, which is characterized by a shift in substrate preference from glycolysis to a rapid increase in fatty acid β-oxidation (5,9). Because fatty acid β-oxidation is the major source of energy in the heart, a thorough description of fatty acid β-oxidation pathway will be first described.

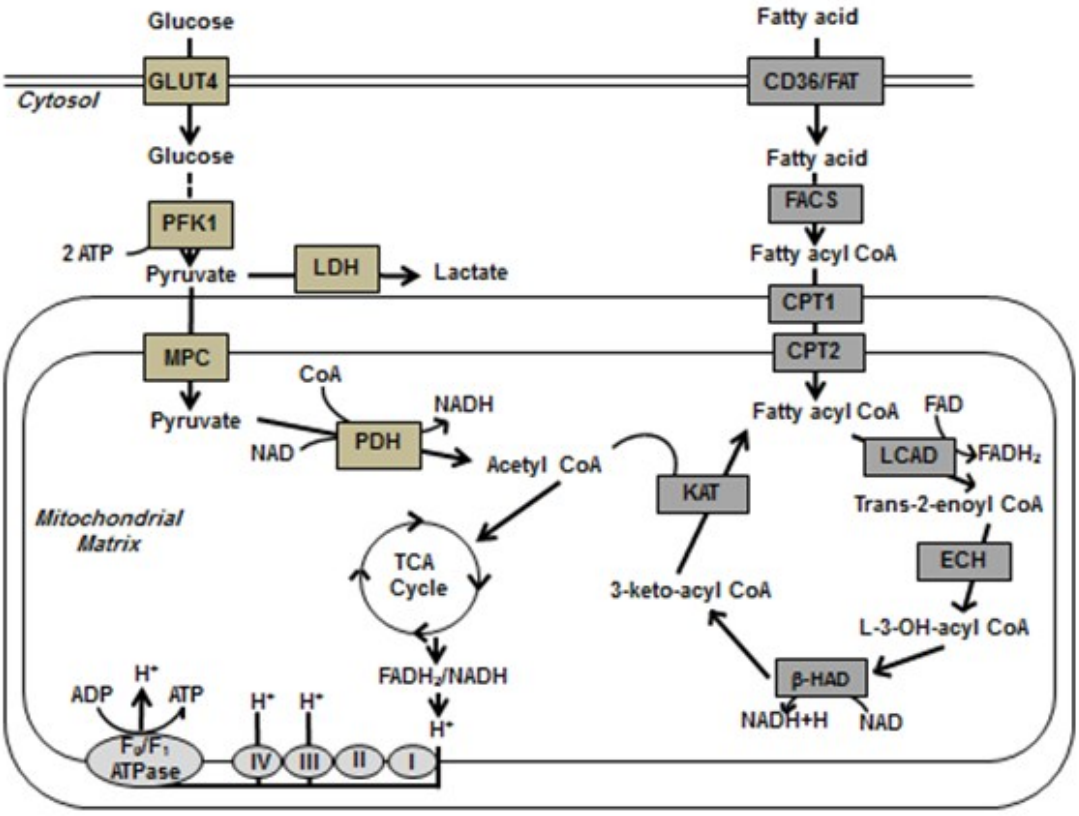


Figure 1-1: Summary of major metabolic pathways in the heart.

Fatty acids and glucose are the two key substrates that provide ATP for the functioning of the heart. Fatty acids are transported into the cytosol by CD36 or fatty acid transport proteins (FAT). Subsequently fatty acids are converted to fatty acyl CoA esters by fatty acyl CoA synthetase (FACS). Carnitine palmitoyl transferase 1 (CPT1) is the key enzyme in mitochondria that catalyzes the conversion of fatty acyl CoA to fatty acyl carnitine that is converted back to fatty acyl CoA by CPT2. The metabolism of fatty acyl CoA in the mitochondria occurs via the β-oxidation

pathway involving the sequential metabolism of acyl CoAs by acyl CoA dehydrogenase (LCAD), enoyl CoA hydratase (ECH), L-3-hydroxyacyl CoA dehydrogenase (β-HAD), and 3-ketoacyl CoA thiolase (3-KAT). Insulin dependent glucose uptake into the cytosol is mediated by GLUT4. Glucose subsequently undergoes glycolysis to generate pyruvate, the rate of which is regulated by phosphofructokinase 1 (PFK1). Pyruvate enters the mitochondria via the mitochondrial pyruvate carrier (MPC) which undergoes oxidation by the enzyme pyruvate dehydrogenase (PDH). Acetyl CoA thus generated from fatty acids and glucose enter the tricarboxylic acid (TCA) cycle to generate reducing equivalents (NADH and $FADH_2$) which then enters the electron transport chain. The electron transport chain and oxidative phosphorylation are coupled by a proton gradient across the inner mitochondrial membrane. The efflux of protons from the mitochondrial matrix creates an electrochemical gradient (proton gradient). This gradient is used by the F_0/F_1 ATP synthase complex to make ATP via oxidative phosphorylation.

1.2 Myocardial Fatty Acid Metabolism

1.2.1 Mitochondrial Fatty Acid Uptake

Circulating free fatty acids (FFAs) either bound to albumin or released by the cell surface enzyme lipoprotein lipase (LPL) from triacylglycerol (TAG) contained in very density lipoproteins (VLDL) or chylomicrons enter the cardiomyocyte either by a carrier mediated pathway or facilitated diffusion (10-12). TAG catabolism is catalyzed by a series of lipolytic reactions that are initiated by the primary endogenous tissue lipase, adipose triglyceride lipase (ATGL) which converts TAG to diacylglycerol (DAG) (290). ATGL is suggested as a crucial enzyme for myocardial TAG lipolysis and cardiac function (291). In agreement, global ATGL deletion in mice resulted in a pronounced increase in

intramyocardial TAG pool, which eventually led to a decline in heart function and premature death (292). Similarly, patients with ATGL gene mutations showed systemic TAG accumulation, and developed sever cardiomyopathy (293). Hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) complete the lipolytic reactions by hydrolyzing DAG and monoacylglycerol (MAG), respectively (290). Various membrane proteins that facilitate cellular fatty acid uptake have been identified. These include the membrane associated fatty acid transporters CD36/FAT, the plasma membrane fatty acid binding protein (FABPpm), and fatty acid transport proteins (FATPs), with various molecular weights and different degrees of post-translational modifications (13-15). Once in the cytoplasm, FFAs are converted into fatty acyl CoA esters by fatty acyl CoA synthetase 1 (1). The fatty acid moiety from fatty acyl CoA is then transferred to carnitine and is taken up into the mitochondria by the carnitine shuttle. Carnitine palmitoyltransferase-1 (CPT-1) is the rate-limiting enzyme of mitochondrial fatty acid uptake (16,17). Malonyl-CoA, a potent allosteric inhibitor of CPT-1 is synthesized by the enzyme acetyl-CoA carboxylase (ACC) (18-20). Two isoforms of ACC have been identified, ACC1 and ACC2, with the later being predominant in the heart (18,21,22). Degradation of malonyl-CoA is mediated by malonyl-CoA decarboxylase (MCD) (20,23,24). Once in the matrix, acylcarnitine is converted back to fatty acyl CoA by the inner

mitochondrial membrane enzyme CPT2 (17,25,26). Once inside the mitochondria, fatty acids undergo β-oxidation.

1.2.2 Fatty Acid β-Oxidation

Fatty acid β-oxidation is the process of breaking down a long chain acyl-CoA molecule to acetyl-CoA in the mitochondrial matrix. The number of molecules of acetyl-CoA produced depends on the carbon length of the fatty acid being oxidized. This process involves a variety of enzymes, with the four main enzymes involved in fatty acid β-oxidation being, in order, acyl-CoA dehydrogenase, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and ketoacyl-CoA thiolase (1,27). At the end of each β-oxidation cycle, two new molecules are formed, an acetyl-CoA and an acyl-CoA that is two carbons shorter. Additionally, during β-oxidation flavin adenine dinucleotide ($FADH_2$) and nicotinamide adenine dinucleotide (NADH) are formed. One $FADH_2$ is produced during the reaction catalyzed by acyl-CoA dehydrogenase. An NADH is produced during the reaction catalyzed by hydroxyacyl-CoA dehydrogenase. The $FADH_2$ and NADH produced during the process of fatty acid β-oxidation are used by the electron transport chain to produce ATP. There are different isoforms of these enzymes of β-oxidation, which have different affinities depending on the length of fatty acid chain. For example, there is a very long chain acyl-CoA dehydrogenase, a long chain acyl-CoA dehydrogenase, a medium chain acyl-CoA dehydrogenase, and a short chain acyl-CoA

dehydrogenase. Interestingly, the enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and ketoacyl-CoA isoforms specific for long chain fatty acids form an enzyme complex on the inner mitochondrial membrane (1, 27).

1.2.3 Regulation of Fatty Acid β-Oxidation

The activity of the enzymes of fatty acid β-oxidation is affected by the level of the products of their reactions (28). Each of the β-oxidation enzymes are inhibited by the specific fatty acyl-CoA intermediate it produces. Interestingly, 3-ketoacyl-CoA can also inhibit enoyl-CoA hydratase and acyl-CoA dehydrogenase (29). β-oxidation can also be allosterically regulated by the ratio of $NADH/NAD^+$ and acetyl-CoA/CoA level. A rise in the $NADH/NAD^+$ or acetyl-CoA/CoA ratios results in inhibition of fatty acid β-oxidation (294). Increases in the acetyl-CoA/CoA ratio have specifically been shown to lead to feedback inhibition of ketoacyl-CoA thiolase (1,30).

The enzymes involved in fatty acid β-oxidation are under a precise control by transcription factors. There are a number of transcription factors that regulate the expression of these proteins. The peroxisome proliferator-activated receptors (PPARs) and a transcription factor coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) are the most well-known transcriptional regulators of fatty acid β-oxidation (31). Examples of proteins involved in fatty acid β-

oxidation that are transcriptionally regulated by the PPARs include FATP, acyl-CoA synthetase 1 (ACS1), CD36/FAT, MCD, CPT1, long chain acyl-CoA dehydrogenase (LCAD), and medium chain acyl-CoA dehydrogenase (MCAD) (31-33). Ligands that bind to and modulate the activity of PPARα, δ, and γ include fatty acids. The genes regulated by each of the PPARs vary between tissue types. For example, skeletal muscles PPARδ, but not PPARα, upregulates the protein expression of CPT1 (34). PPAR isoforms are also differentially expressed between tissue types. While PPARδ protein tends to be ubiquitously expressed, PPARα is predominantly expressed in highly metabolic tissues (i.e. heart, skeletal muscle, and liver) and PPARγ is predominantly expressed in tissues such as adipose tissue (35-37). Until recently, PPARγ was not believed to play a significant role in regulating fatty acid β-oxidation. However, recent knockout and over-expression studies have suggested that PPARγ may have a role in regulating fatty acid β-oxidation rates. Overexpressing PPARγ in cardiac muscle results in increased mRNA levels for fatty acid β-oxidation proteins (38,39).

The transcriptional co-activator PGC-1α binds to and increases the activity of PPARs to regulate fatty acid β-oxidation (40,41). PGC-1α upregulates expression of enzymes involved in fatty acid uptake and oxidation (31,32,41). PGC-1α is regulated at both the gene and protein level. AMP-activated protein kinase (AMPK) activates PGC-1α by phosphorylating PGC-1α on threonine and serine residues (42). It was

suggested that AMPK increases PGC-1α mRNA levels by regulating the binding of transcription factors to specific sequences located in the PGC-1α gene promoter (1,31,42). Recently, regulation of fatty acid β-oxidation enzymes by lysine acetylation emerged as a key post-translational control, and will be discussed in detail later in this thesis.

1.3 Myocardial Glucose Metabolism

1.3.1 Glucose Uptake and Glycolysis

Glucose used by the heart originates either from endogenous glycogen stores, or is transported from the blood into cardiac myocytes via glucose transporters (GLUT), specifically isoforms 1 and 4. GLUT1 is constitutively active being responsible for maintaining basal glucose uptake, whereas GLUT4 is translocated to the sarcolemmal membrane in response to insulin, and increased work load (15,43-46). Once inside the cardiomyocyte, glucose is phosphorylated by hexokinase (HK) into glucose-6-phosphate which undergoes a series of enzymatic reactions, until sequentially converted into two pyruvate molecules in a process known as aerobic glycolysis (47,48). Phosphofructokinase 1 (PFK1) is the rate limiting enzyme of glycolysis which catalyzes the conversion of fructose-6 phosphate to fructose 1,6-bisphosphate (44). PFK1 is activated by adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inhibited by ATP and citrate (2,49). PFK1 can also be stimulated

allosterically by fructose-2,6-bisphosphate generated by the enzyme PFK2 (50-52). fructose 1,6-bisphosphate is spliced by aldolase to two trios sugars, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, with later rapidly interconverted into glyceraldehyde 3-phosphate by the enzyme triosephosphate isomerase. The trios sugars are then dehydrogenated by the enzyme glyceraldehyde 3-phosphate dehydrogenase forming 1,3-bisphosphoglycerate. Phosphate group is then transferred from 1,3-bisphosphoglycerate to ADP by phosphoglycerate kinase, producing ATP and 3-bisphosphoglycerate. Phosphoglycerate mutase converted 3-bisphosphoglycerate to 2-bisphosphoglycerate, which further converted to phosphoenolpyruvate by enolase. Final step of glycolysis is catalyzed by pyruvate kinase which phosphorylate phosphoenolpyruvate producing ATP and pyruvate (47,48). At the transcriptional level, hypoxia inducible factor-1α (HIF-1α) is a master transcriptional up-regulator of enzymes involved in glycolysis (53). HIF-1α may be of particular importance in the fetal heart, which resides in a low oxygen environment, as well as the immediate newborn heart, which is highly dependent on glycolysis for ATP generation. HIF-1α protein levels are high in fetal hearts, but rapidly decrease following birth and during maturation (54,55). In the absence of oxygen, pyruvate derived from glycolysis can be converted to lactate by the enzyme lactate dehydrogense (LDH) (12,56). In times of cardiac stress, lactate and

protons produced by anaerobic glycolysis can accumulate and compromise cardiac work and efficiency (57-59).

1.3.2 Glucose Oxidation

The oxidation of pyruvate, derived either from glycolysis (glucose oxidation) or from lactate (lactate oxidation), accounts for the majority of carbohydrate-derived ATP (1,60,61). Pyruvate is transported into mitochondria via the mitochondrial pyruvate carrier (MPC) (62-64). Under normal aerobic conditions, pyruvate is oxidized to acetyl CoA by pyruvate dehydrogenase (PDH) complex, the rate-limiting enzyme of glucose oxidation, which consists of PDH, PDH kinase (PDK), and PDH phosphatase (PDHP) enzymes (65,66). PDH is regulated both by substrate/product ratios and by covalent modifications. PDH activity is decreased by increased ratios of mitochondrial NADH/NAD+ and acetyl-CoA/CoA (67-70). Covalent modifications that regulate PDH activity include phosphorylation and acetylation (as recently suggested), which will be discussed in detail later in this thesis. Dephosphorylation of PDH is positively related to PDH activity, whereas phosphorylation of PDH decreases its activity, thereby restricting the oxidation of pyruvate. The heart expresses three PDK isoforms with the PDK4 isoform widely expressed in heart and skeletal muscles (66,68,70). Increased PDK protein expression, which results in increased PDH phosphorylation, is a

key mechanism involved in reduced PDH flux and glucose oxidation (66,67,71).

1.3.3 Cardiac Insulin Signaling

Insulin is a major hormone central to carbohydrate and lipid metabolism. In the fed state, high blood glucose levels stimulate pancreatic β-cells to secrete insulin. Once in blood, insulin circulates and binds to its receptor on target tissues. Insulin binding to the α-subunit of insulin receptor (IR) results in autophosphorylation and activation of IR kinase activity. The activated IR phosphorylates and activates its downstream substrate, the insulin receptor substrate (IRS) (72). Tyrosine phosphorylated IRS interacts with various protein substrates. Among them, a key substrate for glucose uptake and metabolism is the phosphatidylinositol-3-kinase (PI3K). The activated PI3K increases the production of phosphatidylinositol trisphosphate (PIP3) which binds the pleckstrin homology (PH) domains of phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (Akt) (73). Once the PH domain of Akt binds to PIP3, PDK1 phosphorylate Akt on T308 leading to a robust increase in its activity (74). Activated Akt increases the translocation of GLUT4 vesicle to the cellular membrane leading to increases in glucose uptake (75,76). In addition, activated Akt induces glycogen synthesis through phosphorylation and inactivation of glycogen synthase kinase 3α/β (GSK3α/β), which decreases glycogen synthase phosphorylation

levels and increases its enzymatic activity (77). The net insulin effects on glucose metabolism are increased cellular glucose uptake and glycogen synthesis.

1.4 Neonatal Heart Energy Metabolism

Heart is a highly energy demand organ which depends mainly on oxidation of fatty acids to produce adenosine triphosphate (ATP) required for maintenance of it is function (1). The reminder of ATP production is derived from glycolysis and oxidation of glucose, lactate, and ketone bodies (2,4). Interestingly, this energy substrate preference differs between the adult and neonatal heart (5). During the fetal life levels of fatty acids are very low (less than 0.1 mM in the fetal circulation) and the heart relies mostly on glycolysis and lactate oxidation to meet its energy demands (6). Shortly after birth, major changes in energy metabolism occur, which characterized by a shift in substrate preference from glycolysis to a rapid increase in fatty acid β-oxidation (Figure 1-2) (5,7). The increase in fatty acid β-oxidation is related to the rapid drop of malonyl CoA level days post birth (9). Malonyl CoA is a strong inhibitor of CPT-1, a mitochondrial fatty acid uptake enzyme (1). Both a decreased synthesis by ACC (21) and increased degradation by MCD (25) are responsible for this drop in malonyl CoA level. Moreover, PGC-1α is important transcriptional regulator of genes involved in fatty acid metabolism, with activation is lead

to increase in expression of genes involved in fatty acid uptake, storage, and oxidation (1,2). The expression of PGC-1α is increased with maturation which can lead to increase in fatty acid β-oxidation (78).

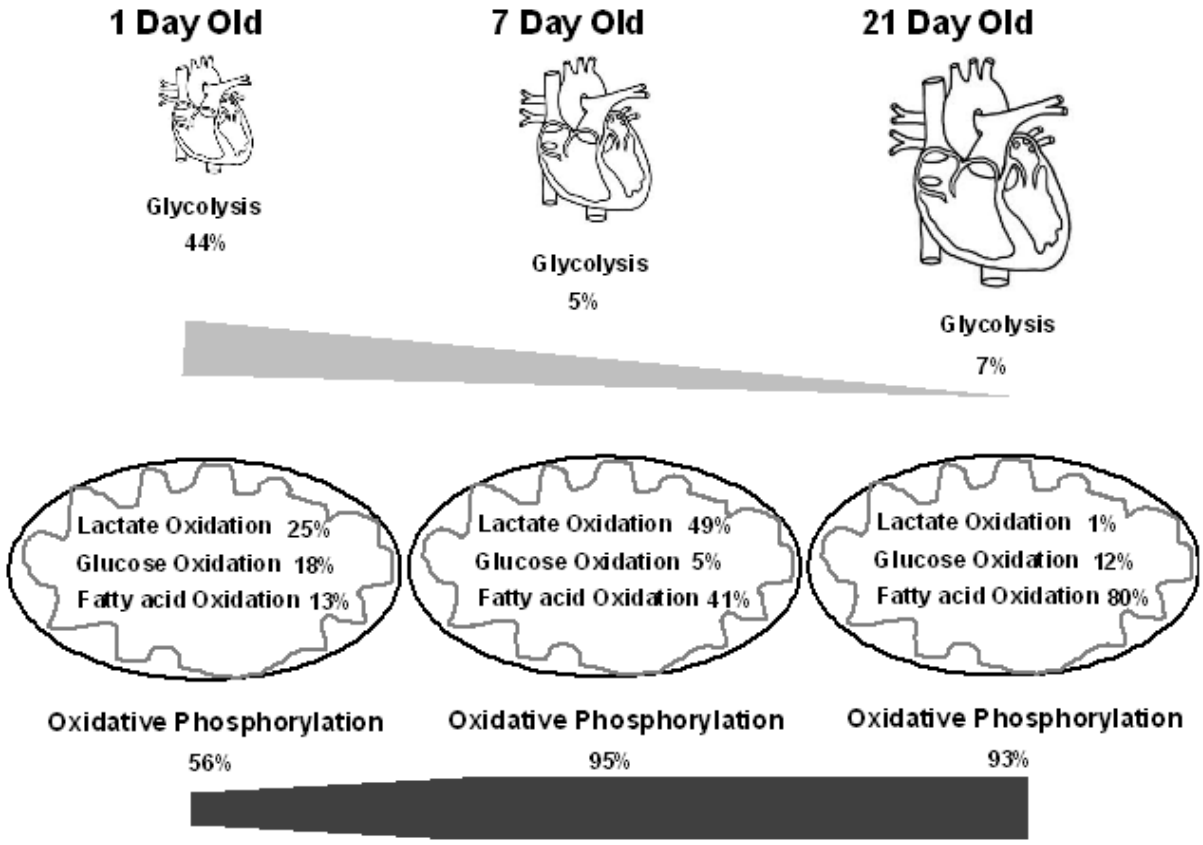


Figure 1-2: Overview of myocardial energy metabolism during maturation.

Percent contributions of glycolysis and oxidative metabolism including lactate, glucose, and fatty acid oxidation to cardiac ATP production during maturation in the isolated working rabbit heart preparation. The 1-day heart relies predominantly on glycolysis and lactate oxidation for ATP production, with minor contributions from the oxidation of glucose and fatty acids. 7 days post-birth, glycolytic rates significantly decline and account for only 5% of myocardial ATP production. By 21 days, cardiac fatty acid β-oxidation predominates and accounts to about 80% of the ATP produced. In summary, during maturation the heart switches from glycolysis to oxidative energy metabolism primarily fatty acid β-oxidation.

One of PGC-1α targets is estrogen receptor related alpha (ERRα), which can increase the expression of PDK4, a negative regulator of the glucose oxidation rate limiting enzyme PDH complex, limiting the oxidation of glucose in neonatal period (79). HIF-1α is a transcription factor regulates the expression of genes involved in anaerobic glycolysis. Hexokinase is under the control of HIF-1α, with activation of HIF1-α upregulate its expression (80). HIF-1α level of expression is significantly decreases post birth (54), which leads to a significant drop in glycolysis during cardiac maturation. In addition, HIF-1α can also influence fatty acid metabolism. In vivo, hypoxic conditions are accompanied by a down-regulation of genes involved in fatty acid β-oxidation, in particular PPARα, MCD, and CPT1(81). Interestingly, HIF-1α itself may be involved in this process as it inhibits the DNA-binding activity of PPARα (82).

1.5 Energy Metabolism in the Diseased Heart

Obesity and diabetes are major health concerns that have reached epidemic proportions in Canada and the world (83). The presence of obesity and diabetes is usually accompanied by cardiovascular complications. Among the many complications is the high risk of ischemic heart diseases and heart failure (HF) (84,85). Although classical pharmacological and non-pharmacological treatment strategies can improve cardiac function and prolong survival in patients with heart

diseases, these attempts are ultimately inadequate to prevent disease progression (86,87). Given the large number of patients suffering from obesity and diabetes, it is important to find novel therapeutic strategies to treat cardiovascular diseases associated obesity and diabetes. Nonetheless, to maximize this potential therapeutic approach for treating obesity-related cardiovascular diseases, it is important to have a better understanding of how energy metabolism is controlled in the heart, which is the primary focus of this thesis.

1.5.1 Cardiac Energy Metabolism in Obesity

Fatty acids are a major energy source for the heart that is used to sustain contractile function. To meet the high energy demands of cardiac muscle, fatty acid uptake and subsequent mitochondrial fatty acid β-oxidation must be coordinately regulated in order to ensure adequate, but not excessive supply, for cardiac energetic requirements. The presence of obesity or insulin-resistance can result in dramatic alterations in cardiac energy metabolism. One such alteration is a switch from using carbohydrates to fatty acids as a source of energy (17,23,61,88,89). The increase in fatty acid β-oxidation is occurring primarily at the expense of glucose oxidation. This inverse relationship between glucose and fatty acid oxidation was first described by Philip Randle in 1960s (90). In humans, positron emission tomography and ^{11}C-palmitate imaging confirm the increase in cardiac fatty acid β-oxidation in the obese and diabetic

patients (91,92). Studies from our laboratory and others have shown that fatty acid β-oxidation rates in the heart are increased in rats and mice subjected to diet-induced obesity (DIO), in db/db mice, and in ob/ob mice, with the increase in fatty acid β-oxidation occurring primarily at the expense of glucose oxidation (21,28,57,88,93,94). Increases in fatty acid β-oxidation that exceed the ability of the mitochondria to metabolize its downstream products can lead to the failure of the muscles to completely oxidize fatty acids, leading to the accumulation of acid soluble metabolites as a markers of incomplete β-oxidation. Indeed, increased fatty acid β-oxidation in the skeletal muscle and heart during high fat feeding contributes to the mismatch between β-oxidation and tricarboxylic acid (TCA) cycle activity, leading to incomplete fatty acid β-oxidation (58,60, 95-97). Increased activity of fatty acid β-oxidative enzymes and their upstream transcriptional regulators such as PPARα contribute to the increased fatty acid β-oxidation seen in obesity and insulin resistance (35-37,58,63,93,98). Furthermore, obesity-induced changes in the acetylation levels of enzymes involved in fatty acid metabolism is proofed to be important regulator of fatty acid β-oxidation and will be discussed extensively later in this thesis.

1.5.2 Cardiac Hypertrophy

Cardiac hypertrophy is an adaptive response characterized by increased heart mass and volume to increase cardiac workload. Cardiac

hypertrophy is associated with numerous protein expression changes which are conducive to metabolic remodeling from adult to a fetal metabolic profile whereby the heart switches from oxidative metabolism to glycolysis (1,2). The mammalian target of rapamycin (mTOR) is an atypical serine/threonine kinase that plays a key role in the regulation of cell homeostasis and stress response (99). mTOR forms a distinct multiprotein complex known as mTORC1 that is a master regulator of protein synthesis, cell growth, and metabolism. mTORC1 activity can be regulated by growth factors and in response to stress. Akt and AMPK are well known regulators of mTORC1 that are in turn activated by growth factors and stress respectively. Growth factors such as insulin, insulin-like growth factor -1 (IGF-1), epidermal growth factor activate Akt and in turn phosphorylates and activates mTOR (100-103). On the otherhand, activation of AMPK in response to energy deprivation, activates tuberous sclerosis protein complex that in turn inhibits Rheb (Ras homolog enriched in brain), an activator of mTORC1 (104). mTORC1 activity is also increased in response to β-adrenergic signaling (105), angiotensin – II (232) and growth factors such as IGF-1 (106) and results in cardiac hypertrophy. The total deletion of mTOR was not accompanied by compensatory hypertrophy in mice subjected to pressure overload (107). However, it impaired the ability of the heart to adapt to pressure overload stress leading to ventricular dilatation and rapid cardiac dysfunction. On the other hand, partial deletion or inhibition of mTORC1 prevents

pathological cardiac hypertrophy in response to pressure overload while the heart develops compensatory increase in work to overcome the increase in afterload (108). It is interesting to note that, while mTORC1 activation is necessary for the development of cardiac hypertrophy, it is not sufficient to produce cardiac hypertrophy suggesting that it may be exerting its effects through multiple signaling pathways.

The Forkhead families of transcription factors are characterized by the presence of a conserved DNA binding domain known as the Forkhead box (Fox) (109). Three members of the FoxO subfamily – FoxO1, FoxO3, FoxO4 play important roles in maintaining cardiac function. The transcriptional activity of FoxO is inhibited by PI3K-Akt signaling (110). The phosphorylation of Akt by PI3K causes Akt to translocate to the nucleus where it subsequently phosphorylates FoxO (111). This results in transport of FoxO from the nucleus to the cytoplasm resulting in its inhibition. In addition to Akt, serum and glucocorticoid induced kinase 1 (SGK1) can also phosphorylate and inhibit FoxO3 activity (112). On the otherhand, phosphorylation of FoxO3 by AMPK results in its activation (113).

FoxO exerts its anti-hypertrophic activity through its target gene products, the muscle specific ubiquitin ligases atrogin-1 (MAFbx) and MuRF1 (114). Atrogin-1 targets calcineurin for degradation decreases NFAT-dependent transcription and prevents calcineurin-dependent cardiac hypertrophy (115). Atrogin-1 overexpression has been shown to

decrease Akt-mediated FoxO1/3 phosphorylation resulting in activation of FoxO (116). However, the IGF-Akt signaling cascade promotes cardiac hypertrophy by inhibiting FoxO activity (117). In addition to its role in cardiac hypertrophy, FoxOs also play a role in cardiac energy metabolism. FoxO binding sites are found in several metabolic genes such as the acetyl Co A carboxylase 2 (ACC2), fatty acid transporter (CD36), and Glut 4 (118). FoxO proteins also exert metabolic effects through the transcription of PGC-1α that activate genes involved in mitochondrial biogenesis, and fatty acid β-oxidation (119). Activation of FoxO can activate the transcription of PDK4 resulting in suppression of glucose oxidation (120).

1.5.3 Cardiac Energy Metabolism in Heart Failure

Heart failure (HF) is a condition characterized by the inability of the ventricles to fill with or eject blood to meet body requirements (121). The metabolic phenotype of the failing heart appears to be, at least partially, dependent on the stage/severity of the syndrome. Mitochondrial oxidative metabolism and ATP generation are depressed in HF (122-124). There is not a clear consensus as to what changes in fatty acid and carbohydrate oxidation occur in HF. Fatty acid oxidation rates have been shown to be increased (125), decreased (126), or unchanged (127) in patients with HF. Studies involving HF secondary to pressure overload also show variable results. Overall oxidative metabolism was decreased characterized by

decreases in both fatty acid and glucose oxidation in rats with pressure-overload HF (128,129). This contrasts with findings in the canine model of severe HF induced by rapid ventricular pacing, where decreased rates of fatty β-oxidation and increased rates of glucose oxidation are observed (130-132). Furthermore, hypertrophic hearts from spontaneously hypertensive rats have decreased mitochondrial oxidation and increased glucose uptake (suggesting increased glycolysis) (133,134). A study in which fatty acid β-oxidation was decreased by heterologous deletion of CPT-1β in mice was also associated with a worsening of cardiac hypertrophy and function following a transverse aortic constriction (TAC) (135). A recent study by Rong Tian's group also showed that cardiac deletion of ACC2 (which decreases malonyl CoA and increases fatty acid β-oxidation) could also improve cardiac energetics and function in TAC mice (136). Both glucose oxidation and fatty acid β-oxidation decreased very early during the development of cardiac hypertrophy in mice subjected to an abdominal aortic constriction (AAC), leaving the heart in an energy deficient state (137). Recent studies from our group have shown a selective decrease in glucose oxidation in TAC (138) and angiotensin – II induced HF mouse models (58,59,97). Collectively, these findings support the concept that targeting the balance between fatty acid β-oxidation and glucose oxidation is a promising therapeutic strategy to improve cardiac efficiency in HF.

1.6 Acetylation Control of Cardiac Energy Metabolism

Coenzyme A (CoA) and CoA derivatives have a critical role in regulating cardiac energy metabolism (276). This includes a key role as a substrate and product in the energy metabolic pathways, as well as serving as an allosteric regulator of cardiac energy metabolism. The product of both fatty acid and glucose oxidation is acetyl CoA, which then feeds into the TCA cycle (1). CoA and CoA derivatives do not readily cross cellular membranes, and a mechanism is required for shuttling acetyl groups out of the mitochondria. One potential pathway may involve the conversion of mitochondrial acetyl CoA to citrate, which would then be shuttled out of the mitochondria via tricarboxylic anion transporters, which is then converted back to acetyl CoA by a cytoplasmic ATP-citrate lyase (295). While this pathway is the major mechanism by which acetyl groups are shuttled out of the mitochondria in liver, both the tricarboxylic anion transporter and ATP-citrate lyase are expressed at much lower levels in heart versus liver. However, this does not necessarily rule out this pathway as a key mechanism by which acetyl groups are shuttled out of heart mitochondria. A second potential pathway by which acetyl groups may be shuttled out of the mitochondria is via carnitine acetyltransferase (CrAT). CrAT is highly expressed in the heart (296,297), and catalyzes the transfer of acetyl groups from acetyl CoA to acetylcarnitine (296,297). This acetylcarnitine can then be readily transported out of the mitochondria via

a carnitine translocase, where a cytoplasmic CrAT transfers the acetyl groups back to acetyl CoA, which can be used as a substrate for lysine acetylation.

Lysine acetylation is an important dynamic/reversible post-translational modification that occurs on proteins involved in many cellular processes, including nuclear transcription, cell survival, apoptosis, and mitochondrial function (6,139-142). Interestingly, almost every enzyme in glycolysis, glucose, and fatty acid oxidation are acetylated at least on one lysine residue (Figure 1-3). This post translational modification is mediated by histone acetyltransferases which catalyze the transfer of the acetyl moiety from acetyl coenzyme A to the ε-amino group of lysine residue, resulting in neutralization of lysine positive charge and eventually alteration of protein structure and interactions (139,141,143). This reaction is reversed by histone deacetylases, which remove the acetyl moiety from lysine residue, producing deacetylated protein, O-Acetyl Adenosine Diphosphate ribose, and nicotinamide (144,145). Sirtuins (SIRTs) are nicotinamide adenine dinucleotide (NAD^+)-dependent class III histone deacetylases which have been shown to regulate key processes in metabolism (6,142,146-148). SIRT1, SIRT6, and SIRT7 are localized in the nucleus (149-153), SIRT2 is localized in the cytoplasm (154,155), while SIRT3, SIRT4, and SIRT5 are localized in the mitochondria (6,140, 142,146,151,152). This thesis will focus on the role of deacetylases in controlling cardiac energy metabolism.

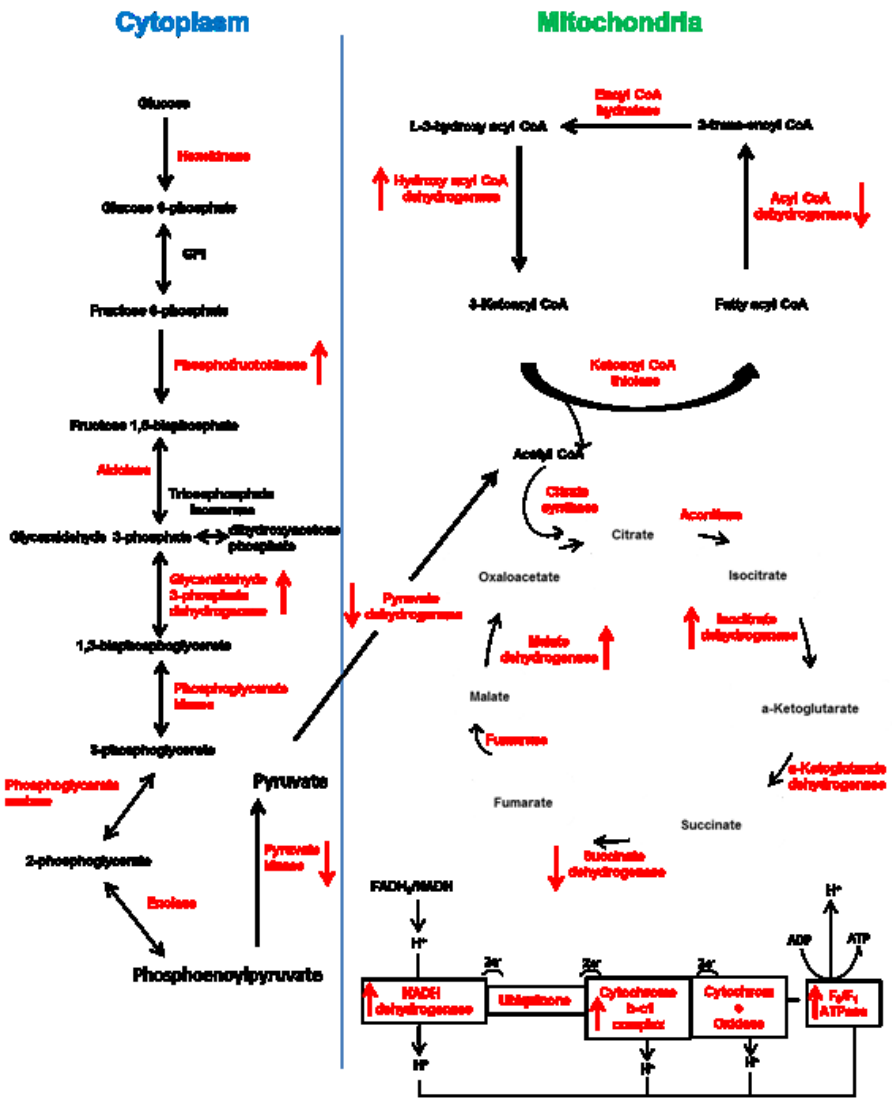


Figure 1-3: Acetylation control of key enzymes involved in cardiac energy metabolism.

Lysine acetylation is a novel post-translational modification that controls the activity of enzymes involved in glucose and fatty acid metabolism. Almost every enzyme in glycolysis, glucose oxidation, fatty acid β-oxidation, TCA cycle, and electron transport chain is acetylated at least on one lysine residue. Metabolic enzymes in red color are known to be acetylated. Red arrows facing up or down indicate stimulatory or inhibitory effects of acetylation on enzyme activity, respectively.

1.6.1 Acetylation Regulation of Cytoplasmic Proteins

SIRT2 is a major cytoplasmic deacetylase and a key regulator of heart necrosis and metabolism (155-157). SIRT2 Knockout (KO) mice have no basal cardiac abnormalities. However, it was noted that SIRT KO mice are protected from cardiac ischemia-reperfusion injury (156). In rat cardiac tissue, SIRT2 was detected exclusively in the cytoplasm (158). However, it was also found in the nucleus of H9C2 cardiomyocytes (158). Multiple enzymes in glycolysis are targets for acetylation (158,159,160). However, there is no consensus about the impact of lysine acetylation on the activity of enzymes involved in glycolysis. In human embryonic kidney (HEK) cells, acetylation of the glycolytic enzymes; phosphoglycerate mutase-1 and glyceraldehyde-3-phosphate dehydrogenase enhances enzymes activity (161,162). In contrast, Xiong *et al* recently showed that acetylation of the glycolytic enzyme; pyruvate kinase at lysine 305 inhibits its activity (163). Moreover, proteomic analysis of other glycolytic enzymes including hexokinase, phosphoglycerate kinase, and enolase revealed that those enzymes are heavily acetylated (159,160); however, no further investigations regarding the impact of this post-translational modification on the enzyme activity was performed.

Insulin signaling pathway is also under acetylation control. Akt is an important component of the insulin signaling pathway and is regulated by phosphorylation. However, in recent years Akt was identified as a target for acetylation (141). A recent study by Rardin *et al* identified lysine

acetylation as important regulator of Akt activity in cardiac hypertrophy (164). Furthermore, recent findings showed that deacetylation of lysine residues 14 and 20 in the PH domain of Akt is a prerequisite for its activation (164), and that the activation of SIRT1 will enhance insulin signaling in obesity and diabetes (141,164-166). Interestingly, SIRT2 is also identified as a key regulator of Akt activity in HEK cells (157). SIRT2 binds and activates Akt in insulin responsive cells, whereas SIRT2 inhibition or genetic downregulation diminishes AKT activation by insulin (157). In embryonic stem cells, knockdown of SIRT2 leads to the activation of GSK3β through decreased serine 9 phosphorylation (167). Studies undertaken in this thesis will demonstrate the acetylation control of glycolytic and insulin signaling enzymes in different myocardial disease states.

1.6.2 Acetylation Regulation of Nuclear Proteins

Nuclear lysine acetylation has been extensively studied, and is linked to active gene transcription. This post translational modification is mediated by histone acetyltransferases (HATs) and is reversed by histone deacetylases (HDACs) (168). Both class 1 and class 2 HDACs play important roles in cardiac hypertrophy (169,170). Nuclear acetylation has an important role in regulating cardiac energy metabolism. For instance, PGC-1α and HIF-1α, important transcriptional regulators of genes involved in mitochondrial oxidative metabolism and glycolysis, are both under

acetylation control (171,172). SIRT1 is the best characterized and most widely studied family member. SIRT1 can directly interact with and regulate the activity of peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α), a transcriptional regulator of genes that encode enzymes involved in mitochondrial biogenesis and fatty acid oxidation (171-173). There are about 13 lysine residues within the amino acid chain regions 200-400 of PGC-1α that can be reversibly acetylated (171,174,175). SIRT1 deacetylates and activates PGC-1α (148,171,174). Interestingly, phosphorylation of PGC-1α by AMPK is a prerequisite for deacetylation and activation by SIRT1 (176). Moreover, it was recently demonstrated that SIRT1 activates AMPK to improve mitochondrial function (177). Previous studies showed that acetylation of PGC-1α by the nuclear acetyltransferase general control of amino acid synthesis 5 (GCN5), results in an inhibition of PGC-1α transcriptional activity (178, 179). Interestingly, SIRT6 binds to and activates GCN5 thereby increasing PGC-1α acetylation (179). Peroxisome proliferator-activated receptors (PPARs) are transcription factors that regulate the activity of enzymes involved in fatty acids uptake and oxidation (180). PPARγ is deacetylated by SIRT1 (181). In white adipose tissue, SIRT1 represses PPARγ activity to promote fat mobilization (182). However, there is no direct evidence that deacetylation of PPARγ by SIRT1 inhibits its activity. Unlike PPARγ, PPARα is not a target for SIRT1. However, SIRT1 enhances PPARα activity in hepatic cells (183) .

HIF-1α, a transcription factor that upregulates the expression of genes involved in anaerobic glycolysis is another target for acetylation control (53,80). Recently SIRT1 was reported as an upstream regulator of HIF-1α activity (172,184). SIRT1 deacetylates and represses HIF-1α activity (185). Interestingly, SIRT6 was also reported as a corepressor of HIF-1α thereby inhibiting glycolysis (186).

FoxO1, a transcription factor that plays a key role in regulating the expression of proteins involved in cell growth and differentiation is under acetylation control. FoxO1 is known to be deacetylated and inhibited by SIRT1 (187,188). In skeletal muscles, SIRT1 deacetylate FoxO1 and inhibits induction of atrophy induced by fasting (189). Interestingly, overexpression of FoxO1 increases SIRT1 protein expressions in HEK293 cells (190). This result suggests that FoxO1 is a positive transcriptional regulator of SIRT1.

1.6.3 Acetylation Regulation of Mitochondrial Proteins

Data from large scale proteomic surveys suggested that about 20% of mitochondrial proteins are acetylated (142,191-195). Out of the seven sirtuins, SIRT3, 4, and 5 are localized in the mitochondria. However, SIRT3 appears to be the major deacetylase in mitochondria (194,191, 192,196-199). SIRT3 targets include key enzymes of fatty acid oxidation such as LCAD and β-HAD (159,192,196,200), PDH, the rate limiting enzyme of glucose oxidation (201,202), the TCA cycle (199,203), and the

ETC (204,205). It is generally considered that acetylation of fatty acid β-oxidation enzymes inhibits fatty acid β-oxidation. However, conflicting information has emerged regarding the role of acetylation in liver and muscle cell fatty acid β-oxidation. For instance, Hirschey *et al* proposed that acetylation of the fatty acid β-oxidation enzyme; LCAD inhibits liver fatty acid β-oxidation (196,200). Under conditions such as fasting, acetylation of LCAD was increased. Furthermore, in SIRT3 KO mice, lower hepatic fatty acid β-oxidation rates were observed compared to wild-type mice (196). LCAD was de-acetylated in wild-type mice under fasted conditions by SIRT3. In contrast, hyper-acetylation of LCAD reduced its enzymatic activity. This suggests that deacetylation of fatty acid oxidative enzymes by SIRT3 accelerates fatty acid β-oxidation. Moreover, chemical acetylation of LCAD on lysines 318/322 reduced enzymatic activity, while deacetylation with recombinant SIRT3 restored catalytic activity (206). In contrast, Zhao *et al* showed that acetylation of the fatty acid β-oxidative enzyme, enoyl CoA hydratase/3-hydroxyacyl CoA dehydrogenase in cardiac myocytes, results in an activation of enzyme activity (159). Interestingly, mitochondria from hindlimb muscles of fasted mice were shown to have an increased proteins acetylation that was associated with increased fatty acid β-oxidation rates (201). Diaphragm muscle of SIRT3 KO also showed mitochondrial proteins hyperacetylation and increased fatty acid β-oxidation rates (201). In addition, it is well established that obesity and diabetes, both of which are associated with high fatty acid β-

oxidation rates (61,96,137,207), lead to a decrease in SIRT3 protein expression (196,208,209). Studies undertaken in this thesis will also show that acetylation is actually associated with increased fatty acid β-oxidation rates in the heart. In support of this notion, a recent study suggested a positive correlation between acetylation and fatty acid β-oxidation in mitochondria and peroxisomes isolated from human and mouse hepatic cells (210). GCN5-like 1 (GCN5L1) was recently identified as an essential component of the mitochondrial acetyltransferase machinery which plays important role in mitochondrial protein acetylation, respiration, and metabolism (211). Liver GCN5L1 protein expression was increased with aging in rats (212). In a recent study, GCN5L1 has shown to enhance cell cycling by activating the master regulator of autophagy transcription factor EB (TFEB) (213). Very little is known about any changes in GCN5L1 expression patterns and if it has any role in controlling cardiac energy metabolism in health and disease.

Nasrin *et al* recently demonstrated that knocking down SIRT4 in mouse hepatocytes or myoblasts results in an increase in expression of many fatty acid oxidation enzymes, which was accompanied by an increase in fatty acid β-oxidation (214). While a deacetylase activity for SIRT4 had not previously been detected (215), a recent study showed that malonyl CoA decarboxylase (MCD), an enzyme primarily responsible for malonyl CoA degradation, was a target of SIRT4 (216). SIRT4 deacetylates and represses MCD activity. SIRT4 knockdown in liver and

muscle results in a significant upregulation of MCD activity, as well as increased fatty acid β-oxidation (216). This suggests that SIRT4 is a negative regulator of fatty acid β-oxidation. Furthermore, recent results from the same lab suggested that SIRT4 inhibits hepatic fatty acid β-oxidation by repressing PPARα activity (217). These results suggested a key role for SIRT4 in regulating lipid metabolism.

The rate-limiting step for glucose and lactate oxidation is flux through the PDH complex. PDH is regulated both by substrate/product ratios and by covalent modifications (180). Covalent modifications include phosphorylation and the recently suggested acetylation (194,201,202). Acetylation of lysine residues in the E1α subunit of PDH leads to suppressed enzymatic activity (201). Inhibition of SIRT3 in skeletal muscles or myoblasts induced hyperacetylation and inactivation of PDH, suggesting PDH as a major target of SIRT3 (201). However, the critical lysine residues targeted for acetylation could not be determined in this study (201) . In hearts from mice treated with angiotensin II, a reduction in SIRT3 protein level was associated with hyperacetylation and inactivation of PDH (59). A recent study by Fan *et al* suggested that acetylation at lysine 321 of PDH is an important regulator of PDH activity in different types of human cancer cells (202). SIRT3 knockdown results in increased lysine 321 acetylation and decreased PDH flux rate in cancer cells (202). A key component of this thesis will explore the impact of lysine acetylation on activity of key enzymes involved in glucose and fatty acid oxidation.

1.6.4 Acetylation Control of Energy Metabolism in Diabetes and Heart Failure

Accumulating body of evidence supports a role for sirtuins in the pathology of metabolic disorders, including diabetes and heart failure. Although alterations in histone acetylation and histone deacetylases are the best characterized examples of protein acetylation in diabetes, insulin resistance, and heart failure, emerging evidence also indicates potentially important effects of non-histone acetylation in these pathological states (218,219). For example, aging and high fat-feeding associated with insulin resistance results in a reduced expression of liver SIRT1 (220-222). *In vivo*, SIRT1 over-expression in mice results in a decrease in diet-induced obesity insulin resistance (165). Reduced SIRT1 also leads to reduced Akt activation and insulin-induced IRS-2 tyrosine phosphorylation (223). Activating SIRT1 can improve insulin sensitivity and glucose homeostasis in animal models of obesity and diabetes (165,166,171,177). In parallel, SIRT3 level was decreased in obesity and diabetes (196,208,209). Jonscher *et al* demonstrated that exposure to high fat feeding led to hyperacetylation of enzymes involved with mitochondrial oxidative phosphorylation and stress response (197). Hepatic SIRT3 activity was significantly reduced with no change in SIRT1 activity. Exposure of SIRT3 deleted mice to high fat diet led to further increase in acetylation of liver proteins that was associated with a significant decrease in the activity of respiratory complexes III and IV. This suggests that SIRT3 is a dominant

regulator of mitochondrial functions and protects against hepatic lipotoxicity under conditions of high fat feeding (196,197). Furthermore, SIRT3 deletion impairs insulin signaling in skeletal muscles by deactivating IRS-1 (208). Several studies have shown a correlation between depletion of SIRTs and heart failure (141,198,224,225). SIRT3 protein was decreased in Dahl salt-sensitive and spontaneously hypertensive heart failure rats (224). Mitochondrial proteins such as LCAD, ATP synthase, and PDH were hyperacetylated (224). Interestingly, mitochondrial complex I deletion promotes mitochondrial protein hyperacetylation and exacerbates pressure overload heart failure (226). Decreased NAD^+/NADH ratio inhibits SIRT3 which leads to protein hyperacetylation and sensitization of the permeability transition pore in mitochondria (226). It has been shown that exogenous NAD^+ supplementation was able to prevent cardiac hypertrophy via activation of SIRT3-LKB1-AMP activated kinase pathway; along with inhibition of pro-hypertrophic Akt1 signaling (227). In contrast, overexpression of SIRT1 induces cardiac hypertrophy and heart failure, whereas decreased levels of SIRT1 protects against age-dependent cardiac hypertrophy (55,228). In agreement, decreased SIRT1 expression in the heart impairs Akt activation and decreased hypertrophy in response to angiotensin treatment or physical exercise (164). Collectively, these results suggested a key role of SIRT1/3 in the regulation of hypertrophy signals. However,

the effects of SIRT1/3 on cardiac energy metabolism in the setting of heart failure are poorly studied.

1.7 Hypothesis and Aims

General Hypotheses

Post-translational modification of lysine acetylation of cardiac energy metabolic enzymes is important in controlling both mitochondrial oxidative metabolism and glycolysis. Increased mitochondrial acetylation in the heart increases fatty acid β-oxidation and decreases glucose oxidation. Furthermore, hyperacetylation of glycolytic enzymes represses glycolysis during maturation. Obesity, and heart failure results in a decrease in SIRT1 and SIRT3 activity which is associated with an increased acetylation of PGC-1α and Akt, leading to a decrease in both mitochondrial oxidative capacity and impaired insulin signaling. Decreased mitochondrial SIRT3 activity will lead to an increase in acetylation and activity of enzymes involved in fatty acid β-oxidation.

Specific Hypothesis

The specific hypotheses of this thesis are described within the individual chapters for each study.

Specific Aim 1

To determine whether changes in cardiac energy metabolism during maturation are accompanied by changes in acetylation levels of key enzymes involved in glycolysis and the oxidation of glucose and fatty acids. Hearts from 1-, 7-, and 21-day old rabbits will be used to measure glycolysis, and the oxidation of lactate, glucose, and fatty acids. Furthermore, acetylation levels of key enzymes involved in fatty acid and glucose metabolism will be assessed.

Specific Aim 2

To determine whether changes in lysine acetylation has any impact on cardiac fatty acid and glucose oxidation in obese and SIRT3 KO mice. Ex vivo cardiac function and metabolism will be measured using isolated working heart perfusions. In addition, we will determine what effect lysine acetylation has on cardiac insulin signaling.

Specific Aim 3

To investigate the role of lysine acetylation in regulating fatty acid β-oxidation and cardiac hypertrophy in volume overload heart failure mouse model. *In vivo* and *ex vivo* cardiac function and metabolism will be measured by ultrasound echocardiography and isolated working heart perfusions, respectively. Furthermore, acetylation levels of main proteins

involved in fatty acid metabolism and cardiac hypertrophy will be determined.

CHAPTER 2.

MATERIALS AND METHODS

CHAPTER 2

2. MATERIALS AND METHODS

2.1 INTRODUCTION

In my thesis, I have addressed the novel mechanisms of regulation of cardiac energy metabolism during maturation of a newborn heart and in disease states such as obesity, diabetes, and HF. To address this relationship, I have utilized a number of approaches varying from whole animal studies to evaluate whole body glucose tolerance, to organ level cardiac function *in vivo* using echocardiography and *ex vivo* to measure flux through metabolic pathways, to tissue level using molecular approaches such as western blot to understand how changes in protein expression from various signaling pathways may involve in the development of cardiac diseases. In addition, immunoprecipitation was used to determine levels of protein acetylation in health and disease states. Overall, my studies have employed a number of up to date technologies at the organ, and cellular levels to understand how cardiac energy metabolism is regulated by lysine acetylation during maturation of a newborn heart and in disease states.

2.2 MATERIALS

Low fat diet was obtained from LabDiet® (St. Louis, Montana), while high fat diet was purchased from Research Diets Inc. (New Brunswick, New Jersey). Radiolabeled glucose [U-^{14}C] and D-[5-^{3}H] were obtained from Amersham Canada Ltd. (Oakville, Ontario). Isotopes [9,10-^{3}H] palmitate and [1-^{14}C] palmitate were obtained from NEN research products (Boston, Massachusetts). L-[^{14}C (U)] lactate was obtained from Perkin Elmer (Waltham, Massachusetts). Free fatty acids were prebound to bovine serum albumin (BSA) obtained from Equitech-Bio Inc. (Kerrville, Texas). Human insulin (Novolin ^{TM} ge Toronto) was provided by University of Alberta hospital stores. Scintillation counting fluid was obtained from MP Biomedicals (Solon, Ohio). Hyamine hydroxide was obtained from NEN Research Products (Boston, Massachusetts). For Western Blotting, Enhance Chemiluminescence (ECL) Supelcosil^{TM} Western blotting detection reagents were purchased from GE Healthcare (Piscataway, New Jersey) and Western Lightning® Chemiluminescence Reagents Plus were obtained from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). Nitrocellulose Trans-Blot® Transfer Medium was obtained from BioRad (Richmond, California). Medical X-ray FUJI films were obtained from Mandel Scientific (Guelph, Ontario). Monoclonal and polyclonal primary antibodies for FoxO1 (9454), GSK3β (9315), p-GSK3β (9331), Akt

(9272), P-Akt (9271), PGC1α (4259), AMPK (5831), P-AMPK (2531) and SIRT1 (2028) were obtained from Cell Signaling Technology (Danvers, Massachusetts). Antibodies for β-Actin (sc-47778), VDAC (sc-32063), PKCα (sc-208), and Atrogin1 (sc-33782) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Antibodies for acetyl-lysine (05-515) were obtained from Millipore (Billerica, Massachusetts). Antibodies for PPARα (ab8934), SIRT3 (ab86671), SIRT6 (ab13697), LCAD (ab128566), and β-HAD (ab93172) were purchased from Abcam (Cambridge, Massachusetts). GCN5L1 was generously provided by Dr. M.N. Sack (NIH, Bethesda, MD). Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). All other chemicals used for buffers preparations were obtained from Sigma-Aldrich (St. Louis, Missouri).

2.3 METHODS

2.3.1 Animals

2.3.1.1 Ethics Approval

The use of animals for research in our study protocol was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. The care of mice conformed to the guidelines of the Canadian Council on Animal Care.

2.3.1.2 Rabbits

Rabbits were housed in groups of 1-2 per cage at the Health Sciences Lab Animal Services (HSLAS) facility, University of Alberta. The room was maintained at ambient temperature (18 – 23 ºC) and humidity (40-70%) and a 12h light/dark cycle was maintained. They were provided food and water ad libitum. They were fed the Laboratory Rabbit Diet (Canadian Lab Diets Inc., Leduc, AB, Canada). One-day (70 g), 7-day (140 g), and 21-day old (400 g) New Zealand White rabbits were used in this study.

2.3.1.3 Mice

Mice were housed in groups of 4-5 per cage at the Health Sciences Lab Animal Services (HSLAS) facility, University of Alberta. The room was maintained at ambient temperature (18 – 23 ºC) and humidity (40-70%) and a 12h light/dark cycle was maintained. They were provided food and water ad libitum. Male C57Bl/6J mice (4-wk of age) were randomly assigned to be fed either standard chow (low fat diet, LFD, 12% Kcal from fat) or high fat diet (HFD, 60% Kcal from fat Cat # D12492; Research Diets Inc., New Brunswick, NJ, USA). Mice in either group underwent a sham or abdominal aortic constriction (AAC) procedure to induce heart failure. Hearts were excised following anesthesia with sodium pentobarbital for isolated working heart perfusion studies.

2.3.1.4 Feeding Protocol

Male C57Bl/6J mice (4-wk of age) were randomly assigned to be fed either standard chow (low fat diet, LFD, 12% Kcal from fat) or high fat diet (HFD, 60% Kcal from fat). The mice were fed their respective diets for 18 weeks before they were sacrificed to obtain hearts for perfusion.

2.3.2 Abdominal Aortic Constriction

Mice were anesthetized with 0.75% isoflurane. A 2 cm medio-lateral incision extending from the level of the 13th rib was made on the ventral side of the left abdominal wall 1.5 cm lateral to the spine. The abdominal aorta was located at the level of the adrenal gland. A titanium vascular clip was applied to constrict the aorta. It was set for a 0.11 mm closure. A silk suture was placed around to constrict the aorta and the titanium clip was removed. Sham operated animals were subjected to an identical surgical procedure except that a clip was not applied to the aorta. The surgical incision was then closed and the animals were allowed to recover under supervision.

2.3.3 Glucose Tolerance Test

In preparation for an oral glucose tolerance test (OGTT) food was removed and mice were introduced into clean cages. They had ad libitum access to water. After an overnight fast for 16h, mice were subjected to OGTT (229). After obtaining a fasting blood glucose sample, mice were

challenged with glucose (2g/Kg body weight) administered by oral gavage. Blood glucose was measured at 15, 30, 60, 90, and 120 min after glucose administration using Accu Check Aviva (Roche Diagnostics). Food was re-introduced at the end of this process.

2.3.4 Magnetic Resonance Imaging

The body composition of fat and lean mass in mice was analyzed after 18 weeks of the feeding protocol using EchoMRI QMNR 4-in-1 Whole Body Composition Analyzer, Echo Medical Systems Inc., Houston, TX, USA.

2.3.5 Echocardiography

Ultrasound echocardiography to assess in vivo cardiac function of the mouse heart was performed using a Visualsonic Vevo 770 high-resolution echocardiography imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada).

Mice were anesthetized with 0.75% isoflurane for the duration of the procedure. M-mode images were obtained for measurements of left ventricular (LV) wall thickness, LV end-diastolic diameter, and LV end-systolic diameter. LV ejection fraction (%EF) and fractional shortening (%FS) were also calculated. Tissue Doppler imaging (TDI) was used to assess diastolic function, where a reduction in E′/A′ and an elevation in E/E′ were considered markers of elevated LV filling pressure and diastolic

dysfunction. TDI was utilized to characterize the inferolateral region in the radial short axis at the base of the LV with the assessment of early (E′) and late diastolic (A′) myocardial velocities.

Diastolic function is determined by flow across the mitral valve (E/A) and tissue doppler (E'/A') as described above. E indicates early diastolic velocity; A, late diastolic mitral flow due to atrial contraction; E′, mitral annulus early diastolic velocity; A′, late diastolic mitral annulus velocity.

2.3.6 Isolated Cardiac Perfusion

2.3.6.1 Rabbit Langendorff Heart Perfusion

One-, 7-, and 21-day old rabbits were anesthetized by intraperitoneal injection with 60mg/kg pentobarbital sodium. After a surgical plane anesthesia achieved, signed by loss of sensation, rabbit's thoracic cavity was opened and the heart was excised and immersed immediately in ice-cold Krebs-Henseleit solution. Hearts were retrogradely perfused with Krebs-Henseleit solution (2.5 mM Ca^{2+}, 5 mM glucose, 0.4 mM palmitate prebound to 3% albumin, 1 mM lactate, and 100 μU/mL insulin) to measure glycolysis, and the oxidation of glucose, lactate, and fatty acids. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. ATP production rates from glycolysis, and the oxidation of glucose, lactate, and palmitate were calculated using the values of 2, 15, 31, and 105 mol ATP/mol of glucose

degraded in glycolysis, and from the oxidation of lactate, glucose, and palmitate, respectively (7-9,21,25,230).

2.3.6.2 Isolated Working Mouse Heart

Mouse hearts were perfused in the working perfused mode as previously described (4,18,231,232). The strain of mice used and treatments with specific diet or any genetic modification are described in the individual chapters. Mice were anesthetized with sodium pentobarbital (60 mg/kg IP), and the hearts were subsequently excised and immersed in ice-cold Krebs-Henseleit bicarbonate solution containing 118 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 5 mM EDTA pH = 7.4, 1.2 mM $MgSO_4 \bullet 7H_2O$, 2.5 mM $CaCl_2 \bullet 2H_2O$, 5 mM glucose and gassed with 95% O_2 and 5% CO_2. The aorta was cannulated with 18-G plastic cannula fixed to the perfusion apparatus and the heart was perfused with Krebs-Henseleit solution (37 ºC) at an initial hydrostatic pressure of 60 mmHg. Heart was trimmed off the excess tissue and the opening to the left atrium was cannulated. After equilibration in the Langendorff mode, hearts were switched to the working mode by clamping off the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. Oxygenated Krebs-Henseleit solution consisting of 0.4 mM (Normal Fat) or 0.8 mM (High Fat) palmitate bound to 3% fatty acid free BSA, palmitate was pre-bound to the albumin as described previously (233) , 5 mM glucose, 2.5 mM Ca^{2+} in the presence or absence of 100 µU/mL insulin

was delivered to the left atrium at a preload pressure of 11.5 mmHg. The perfusate was ejected from spontaneously beating hearts into a compliance chamber and into the aortic outflow line against a hydrostatic afterload pressure of 50 mmHg. The perfusate was recirculated, and pH was maintained at 7.4 by gassing the perfusate in a glass oxygenator with a gas mixture containing 95% O_2 and 5% CO_2. At the end of perfusion, hearts were immediately frozen in liquid N_2 with Wollenberger tongs, and stored at -80 ºC until further experiments were performed.

2.3.7 Mechanical Function Measurements in Isolated Working Mouse Hearts

Heart rate and aortic pressure (mmHg) were measured using a Gould P21 pressure transducer (Harvard Apparatus) connected to the aortic outflow line. Cardiac output and aortic flow (mL/min) were measured with Transonic T206 (Transonic Systems Inc., New York) ultrasonic flow probes in the preload and afterload lines, respectively. Coronary flow (mL/min) was calculated as the difference between cardiac output and aortic flow.

Coronary flow (mL/min) = Cardiac output (CO, mL/min) – Aortic flow (ml/min)

Cardiac work was calculated as the product of cardiac output times peak systolic pressure (PSP) (aortic systolic pressure minus preload pressure) per unit weight of heart tissue

Cardiac work (joules / min / g) = (PSP – 11.5) X 133.322 X CO X 0.000001 X 60 / 60

Data were collected utilizing an MP100 system from AcqKnowledge (BIOPAC Systems Inc., California).

2.3.8 Measurement of Glycolysis, Glucose, Lactate, and Palmitate Oxidation

Glycolysis, glucose, lactate, and palmitate oxidation were measured by perfusing hearts with [5-^{3}H/U-^{14}C] glucose, [U-^{14}C] lactate, and [9,10-^{3}H] palmitate, respectively. The total myocardial $^{3}H_2O$ production and $^{14}CO_2$ production were determined at 10 min intervals during the 60 min aerobic perfusion period. To measure the rates of glycolysis or palmitate from ^{3}H substrate, $^{3}H_2O$ in perfusate samples was separated from [^{3}H] glucose and [^{3}H] palmitate utilizing a vapor transfer method as previously described (233). This method involves the addition of 500 µL of water into a 5 mL scintillation vial, and then placing a capless 1.5mL microcentrifuge tube inside the scintillation vial. A 200 µL perfusate sample was then added to the microcentrifuge tube, and the scintillation vial was capped. Scintillation vials were then stored initially at 50 ºC for 24 hr to allow vaporization and then cooled at 4ºC for 24 hr. Following storage, the microcentrifuge tube was removed, scintillation fluid (Ecolite, ICN) was added, and the vials were counted for radioactivity in a liquid scintillation counter.

Glucose or lactate oxidation rates were determined by quantitative measurement of $^{14}CO_2$ production including $^{14}CO_2$ released as a gas in the oxygenation chamber and $^{14}CO_2$ dissolved as $H^{14}CO_3^-$ in perfusate. The gaseous $^{14}CO_2$, which exits the perfusion system via an exhaust line, was trapped in hyamine hydroxide solution. The dissolved $^{14}CO_2$ as $H^{14}CO_3^-$ was released and trapped on filter paper saturated with hyamine hydroxide in the central well of 25 mL stoppered flasks after perfusate samples were acidified by the addition of 1 mL of 9*N* H_2SO_4 (233).

2.3.9 Heart Tissue Preparation

Immediately following perfusion, the hearts were flash frozen in liquid nitrogen. Frozen ventricular tissue was homogenized for 30s with a Polytron homogenizer in a homogenization buffer containing 0.05M Tris-HCL, 10% glycerol, 1mM EDTA, 0.02% Brij-35, and 1mM dithiothreitol in the presence of protease and phosphatase inhibitors (Sigma). Homogenized tissues were then centrifuged at 800 g for 10 min to obtain a supernatant lysate. Protein assay was performed using the Bradford method. Lysates from frozen heart tissue were used to assess level of protein expression and acetylation. Samples were boiled for 5 min in a sample preparation buffer containing (0.062M Tris-HCL, 10% glycerol, 0.003% bromphenol blue, 5% 2-β-mercapto-ethanol, 2% SDS, and 6M Urea), and loaded onto 10% SDS-PAGE gels.

2.3.10 Mitochondrial Isolation

Mice were euthanized with sodium pentobarbital. After opening the thoracic cavity, the heart was carefully removed and place on a petridish containing chilled heart homogenization medium (HHM), containing 0.2 M mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, and 10 mM HEPES (pH 7.4). After briefly rinsing, the heart was minced on ice and homogenized in HHM containing protease inhibitor cocktail, 500 nM trichostatin A (TSA), and 10 mM nicotinamide using a chilled glass dounce. TSA and nicotinamide are class I and class III histone deacetylase inhibitors respectively that prevent the deacetylation of proteins *in vitro*. The heart tissue homogenate was then decanted and stored on ice. All homogenates are first centrifuged for 10 min at 1000g, 4ºC. The supernatant is decanted and then centrifuged for 10 min at 3000g, 4ºC. The resulting supernatant is then aspirated and discarded, leaving a brown mitochondrial pellet. The mitochondrial pellet is then re-suspended in HHM and recentrifuged for 10 min at 3000g, 4ºC. This procedure is repeated for a total of three washes. After the final wash, the mitochondria are re-suspended in the HHM buffer and used for further experiments.

2.3.11 Immunoblot Analysis

Proteins (20 µg/lane) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with

5% skim milk for 1 hour and probed with specific primary antibodies overnight. Membranes were incubated with the appropriate secondary antibodies for 1 hour. Enhanced chemiluminescence (BioRad Inc, Hercules, USA) system was used for band detection. The intensity of band signals was analyzed by Quantity One software (4.4.0).

2.3.12 Immunoprecipitation

One hundred µg of total heart lysate was pre-cleared with 50 µl of protein A-agarose beads and used for immunoprecipitation. Lysates were rotated with acetyl-K antibodies (3µg/100µg lysate) overnight at 4ºC. 50 µl of protein A-agarose beads was added to each sample and incubated on a rotator for 4 hr in a cold room. After 4 hr, samples were washed with a wash buffer containing (0.1% Triton X-100, 50mM Tris.HCL, 300 mM NaCl, and 5 mM EDTA, at pH 7.4) and centrifuged at 16,000 x g for 5 min. This wash was repeated 3 times. After the third wash, proteins were boiled in a sample preparation buffer for 5 min (59).

2.3.13 Assessment of β-hydroxylacyl CoA dehydrogenase (β-HAD) Activity

β-HAD activity was assayed on total heart lysates prepared from frozen heart tissues. Heart lysates were pipetted into a 96 well plate. Each well is brought to a final volume of 190 µL with 160 µL of 50 mM imidazole (pH 7.4) and 20 µL of 1.5 mM NADH. The reaction was initiated by the

addition of 10 µL of 2 mM acetoacetyl CoA and the absorbance at a 340 nM wavelength was followed for 5 min using a spectrophotometer kinetic plate reader, as described previously (17,55).

2.3.14 Assessment of long chain acyl CoA dehydrogenase (LCAD) Activity

LCAD activity was assayed based on the method described by Lehman et al (234). In brief, 20 µg of total heart lysate was added to potassium phosphate buffer containing 200 µM ferriceneum hexafluorophosphate ($Fc^{+}PF6^{-}$), N-ethylmaleimide (500 µM), and EDTA (0.1 µM) at pH 7.2. A base line rate is observed for 3-4 minutes before starting the reaction. The reaction was initiated by the addition of palmitoyl CoA (50 µM) and the absorbance at 300 nM wavelength was followed for 2 min using a spectrophotometer kinetic plate reader.

2.3.15 Statistical Analysis

Data are represented as means ± SE. Comparisons between two groups were performed using the Students t-test. Comparisons between three or more groups were performed using one and two-way ANOVA followed by Bonferroni's multiple-comparison test whenever differences were detected. $P<0.05$ was deemed significant.

CHAPTER 3.

Acetylation of Cardiac Regulatory Proteins Increases Fatty Acid β-Oxidation and Represses Glycolysis During Maturation

My role in this work involved performing all experiments (except those noted below), as well as the writing of the manuscript. Cory Wagg perfused the rabbit's heart, and Sowndramalingam Sankaralingam performed perfusion data analysis.

Manuscript Status: This manuscript is currently in preparation to be submitted to the Journal of Cardiovascular Research for publication as an original article.

CHAPTER 3.

Acetylation of Cardiac Regulatory Proteins Increases Fatty Acid β-Oxidation and Represses Glycolysis During Maturation

Abstract

Aims: Dramatic cardiac developmental changes in energy metabolism occur in neonate, with a shift from glycolytic to mitochondrial oxidative metabolism occurring shortly after birth. Lysine acetylation has recently been identified as a potentially important pathway involved in the control of energy metabolism. We therefore investigated the impact of changes in protein acetylation on the maturational changes in energy metabolism of the newborn rabbit heart.

Methods and results: One-day, 7-day, and 21-day old rabbit hearts were perfused retrogradely with Krebs-Henseleit solution to measure glycolysis, and the oxidation of lactate, glucose, and fatty acids. Cardiac fatty acid β-oxidation rates were significantly increased in 21-day vs 1-, and 7-day old rabbits (555±26 vs 299±10 and 364±24 nmol.g dry wt-1.min-1, $p<0.05$). Activities of the fatty acid β-oxidation enzymes, long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were increased in hearts from 7-, and 21-day vs 1-day old rabbits, and were associated with LCAD and β-HAD hyperacetylation. Increased overall myocardial proteins acetylation during maturation was associated

with increased GCN5L1 expression, while expression of the mitochondrial deacetylase, SIRT3, did not change. Increased expression of the nuclear deacetylase SIRT1 and the decreases in SIRT6 cardiac protein level during maturation was accompanied by decreased acetylation of PGC-1α and increased ATP production. Acetylation of the glycolytic enzymes hexokinase (HK) and phosphoglycerate mutase (PGM) were increased; scenarios consistent with the decrease in glycolytic activity seen in 7-, and 21-day vs. 1-day old hearts.

Conclusions: We conclude that increased lysine acetylation during maturation enhances cardiac fatty acid β-oxidation, and impairs glycolysis.

3.1 INTRODUCTION

Heart is a high energy demand organ which depends mainly on oxidation of fatty acids and glucose to produce adenosine triphosphate (ATP) required for maintenance of it is function (1). The reminder of ATP production is derived from glycolysis, and the oxidation of lactate, and ketone bodies (2). Interestingly, this energy substrate preference differs between the adult and neonatal heart (8). During the fetal life levels of fatty acids are very low and the heart relies mostly on glycolysis and lactate oxidation to meet its energy demands (9,8). Shortly after birth, major changes in energy metabolism occur, which is characterized by a shift in substrate preference from glycolysis to a rapid increase in fatty acid β-oxidation (5). The increase in fatty acid β-oxidation is related to maturational changes of transcription factors, and perhaps changes in posttranslational modifications that control key enzymes of fatty acid β-oxidation. Peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α) is an important transcriptional regulator of genes involved in fatty acid metabolism, with activation is lead to increase in expression of genes involved in fatty acid uptake, storage, and oxidation (1). The expression of PGC-1α is increased with maturation which can lead to increase in fatty acid β-oxidation (78).

Lysine acetylation is an important post-translational modification which has recently been identified as a substantial pathway involved in

regulation of energy metabolism (191,192,194,200,235). Almost every enzyme in glycolysis, glucose oxidation, fatty acid β-oxidation, and tricarboxylic cycle is acetylated at least on one lysine residue (140,159,191,194). However, the impact of this post-translational modification on the activity of key enzymes involved in glucose and fatty acid metabolism is poorly understood. General control of amino acid synthesis 5 like 1(GCN5L1) is recently characterized as a mitochondrial acetyltransferase which plays important role in mitochondrial protein acetylation, respiration, and metabolism (211). Protein deacetylation is mediated by a family of seven enzymes called sirtuins (SIRT1-7) (148,165,177,236). SIRT3 is suggested as a major mitochondrial deacetylase which controls the activity of key enzymes involved in glucose and fatty acid β-oxidation (192,194,199,237). However, conflicting information has emerged regarding the role of acetylation in liver and muscle cell fatty acid β-oxidation. Studies by Hirschey *et al* suggested that deacetylation of fatty acid oxidative enzyme; long chain acyl CoA dehydrogenase (LCAD) by SIRT3 accelerates fatty acid β-oxidation (196,200,206). In contrast, Zhao *et al* showed that acetylation of the fatty acid β-oxidative enzyme; enoyl CoA hydratase/3-hydroxyacyl CoA dehydrogenase, results in an activation of enzyme activity (159). The impact of maturational changes of lysine acetylation on cardiac fatty acid β-oxidation has never been addressed before. SIRT6 plays an important role in glucose homeostasis and a protective role in cardiac hypertrophy

(238). The purpose of this study is to determine the role of lysine acetylation in regulation of cardiac energy metabolism and the contribution of this post-translational modification in the dramatic energy changes seen during maturation.

3.2 MATERIALS AND METHODS

Rabbits received care and were treated according to the guidelines of the Canadian Council on Animal Care, and all procedures performed on animals were approved by the University of Alberta Health Sciences Animal Welfare Committee. One-, 7-, and 21-day old New Zealand White rabbits were used in this study. Rabbits were sacrificed and hearts were retrogradely perfused with Krebs-Henseleit solution (2.5 mM Ca2+, 5 mM glucose, 0.4 mM palmitate prebound to 3% albumin, 1 mM lactate and 100 μU/mL insulin) to measure glycolysis, and the oxidation of glucose, lactate, and fatty acid, as described in details in **chapter 2**. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. Protocols for assessment of β-HAD activity, LCAD activity, immunoprecipitation, immunoblotting, and statistical analysis are described in details in **chapter 2** of this thesis.

3.4 RESULTS

3.4.1 Cardiac metabolic profile during maturation:

Glycolytic rates were significantly decreased in 21- and 7-day compared to 1-day old hearts (Figure 3-1A). In contrast, cardiac palmitate oxidation rates were markedly increased 3 weeks post-birth (Figure 3-1B). Glucose oxidation rates were significantly decreased in 21-day compared to 1-day old hearts (Figure 3-1C). Lactate oxidation rates were initially increased in 7-day old hearts. However, it declined markedly at 21 day old hearts (Figure 3-1D). Interestingly, there was a maturational increase in overall myocardial protein acetylation during the neonatal period 1–21 days of age (Figure 3-1E).

3.4.2 Age-dependent changes in PGC-1α acetylation:

Protein level of SIRT1 was significantly increased in 21-day compared to 1-, and 7-day old rabbit hearts (Figure 3-2A). In contrast, SIRT6 protein expression was significantly decreased with maturation (Figure 3-2B). There were no age-dependent changes in the level of PCG-1α protein expression, a key transcription factor involved in mitochondrial biogenesis (Figure 3-2C). However, levels of PCG-1α acetylation were significantly decreased in 21-day compared to 1-, and 7-day old rabbit hearts (Figure 3-2D). This decrease in PCG-1α acetylation was

accompanied by an overall increase in cardiac ATP production (Figure 3-2E).

3.4.4 Age-dependent changes in β-HAD acetylation and activity:

There were no changes in SIRT3 protein level (Figure 3-3A); however, the protein expression of GCN5L1 (mitochondrial acetyltransferase) was significantly increased with maturation (Figure 3-3B). Because of the marked increase in fatty acid β-oxidation during maturation, we examined whether the acetylation state was altered in hearts from 1-, 7-, and 21-day old rabbits. The levels of β-HAD protein was not changed during maturation (Figure 3-3C). However, the levels of β-HAD acetylation was significantly increased in hearts of 7-day compared to 1-, and 21-day old rabbits (Figure 3-3D) accompanied with age-dependent increase in β-HAD activity (Figure 3-3E).

3.4.5 Age-dependent changes in LCAD acetylation and activity:

Similar to what was observed with β-HAD, the level of LCAD protein expression was the same in all groups (Figure 3-4A). However, a significant age-dependent increase in LCAD acetylation was observed (Figure 3-4B) accompanied by a marked increase in enzyme activity (Figure 3-4C). Collectively, our results suggest that acetylation is activating fatty acid β-oxidation.

3.4.6 Age-dependent changes in PDH acetylation:

Pyruvate dehydrogenase (PDH) is the rate limiting enzyme in the glucose oxidation pathway. Western blot analysis showed that cardiac expression of PDH was not change with maturation (Figure 3-5A). No differences in PDH acetylation/PDH expression were found between hearts from 1-, 7-, and 21-day old rabbits (Figure 3-5B).

3.4.7 Age-dependent changes in Hexokinase (HK) acetylation:

The cytoplasmic deacetylase SIRT2 cardiac protein level was decreased in 7-day old rabbits; however, its level in 21-day old hearts was comparable to 1-day old hearts (Figure 3-6A). Glycolytic rates significantly declined in the heart with maturation. HK is a key enzyme that catalyzes the first step of glycolysis. The level of HK protein expression was not changed between groups (Figure 3-6B). HK acetylation was significantly increased 7- and 21-days post-birth compared to 1-day old hearts (Figure 3-6C).

3.4.8 Age-dependent changes in Phosphoglycerate Mutase (PGM) acetylation:

To further investigate the role of acetylation in glycolysis, we assessed the acetylation level of another glycolytic enzyme PGM. The level of PGM protein expression was similar in all groups (Figure 3-7A). cardiac PGM acetylation levels were significantly increased in 7- and 21-

day compared to 1-day old rabbits (Figure 3-7B). Hypoxia inducible factor one alpha (HIF-1α) is a transcriptional factor which upregulates glycolytic enzyme expression. Interestingly, HIF-1α protein expression was significantly declined 3 weeks post-birth (Figure 3-7C).

Figure 3-1: Steady-state rates of glycolysis, glucose, lactate, and palmitate oxidation in isolated langendorff perfused hearts from 1-, 7-, and 21-day old rabbits.

A) Glycolysis **(B)**, Palmitate oxidation **(C)**, Glucose oxidation **(D)**, Lactate oxidation **(E)**, Total protein acetylation. Values represent mean±SEM; (n=6). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

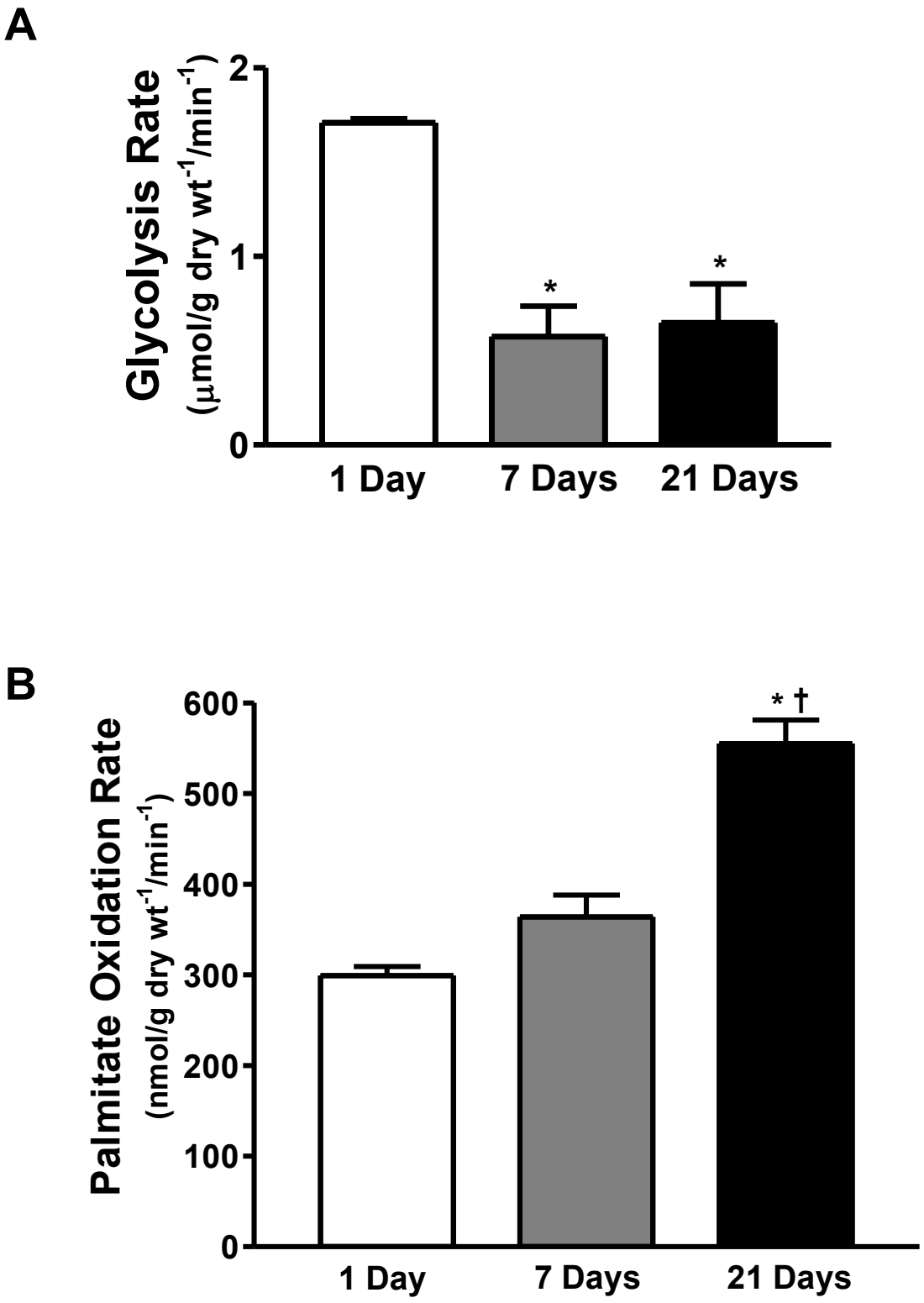
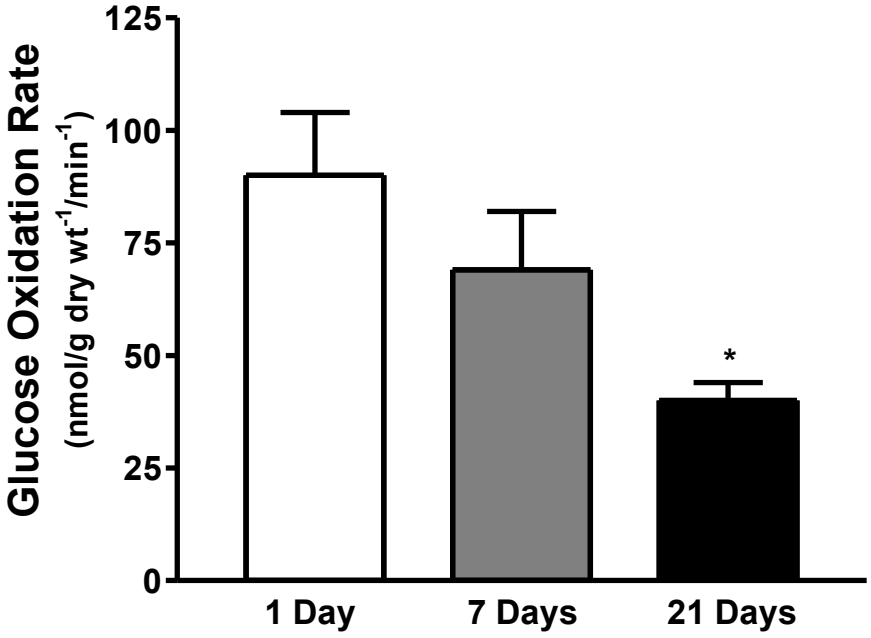

A
Glycolysis Rate
(μmol/g dry wt-1/min-1)
2
1
0
*
*
1 Day
7 Days
21 Days
B
Palmitate Oxidation Rate
(nmol/g dry wt-1/min-1)
600
500
400
300
200
100
0
* †
1 Day
7 Days
21 Days

Figure 3-1

C


Glucose Oxidation Rate
(nmol/g dry wt-1/min-1)
125
100
75
50
25
0
*
1 Day
7 Days
21 Days

D

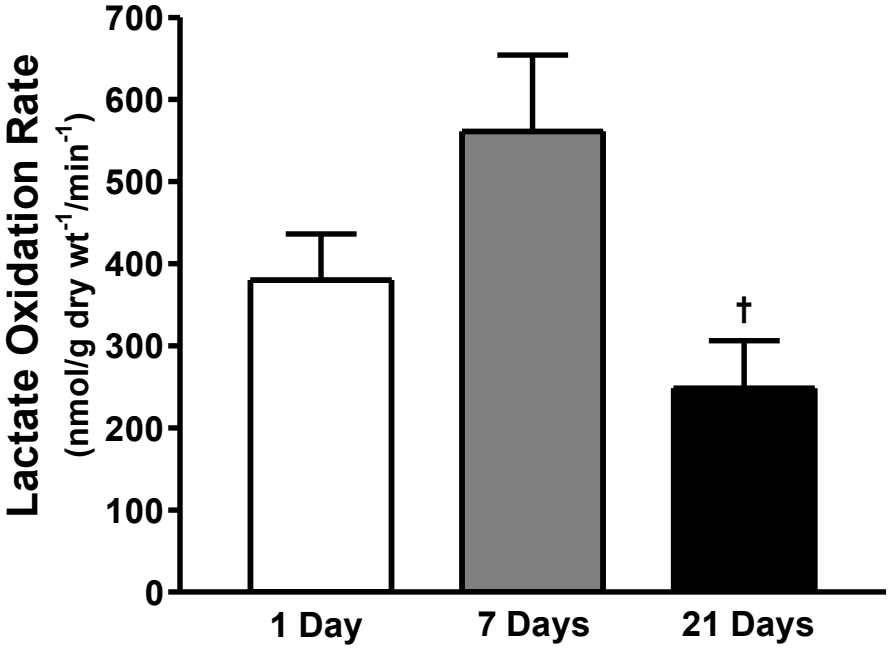

Lactate Oxidation Rate
(nmol/g dry wt-1/min-1)
700
600
500
400
300
200
100
0
†
1 Day
7 Days
21 Days

Figure 3-1

E

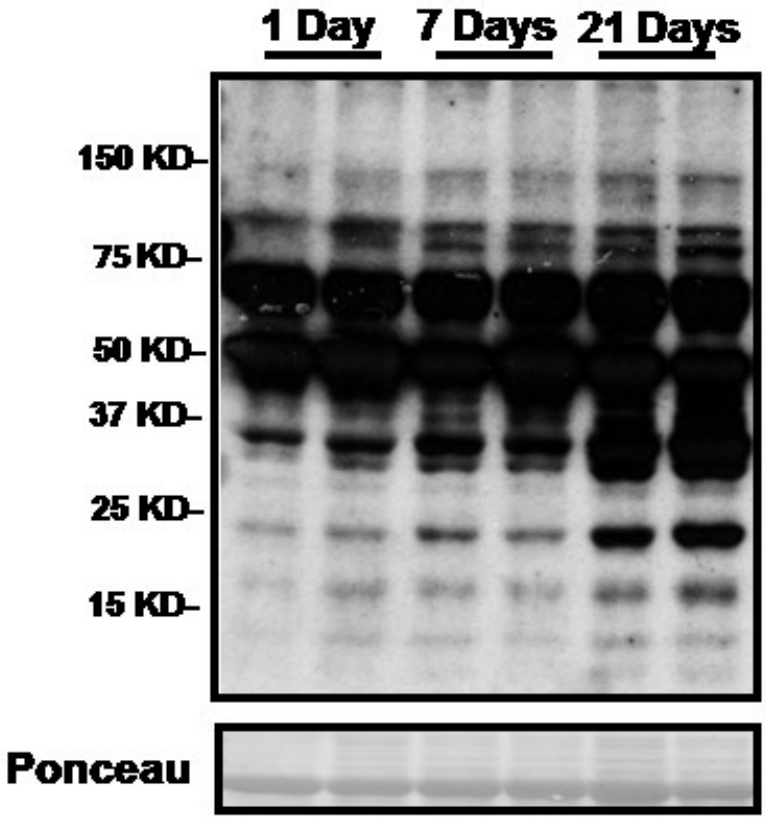


Figure 3-1

Figure 3-2: Level of cardiac PGC-1α acetylation and protein expression of its upstream sirtuins regulator in 1-, 7-, and 21-day old rabbits.

Protein expressions of SIRT1 **(A)**, SIRT6 **(B)**, and PGC-1α **(C)**. **D)** Level of PGC-1α acetylation. Total lysate from 1-, 7-, and 21-day perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGC-1α. **E)** Calculated steady-state ATP production rate (n=6). Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old. Steady-state rates of glycolysis, glucose, lactate, and palmitate oxidation in isolated langendorff perfused hearts from 1-, 7-, and 21-day old rabbits.

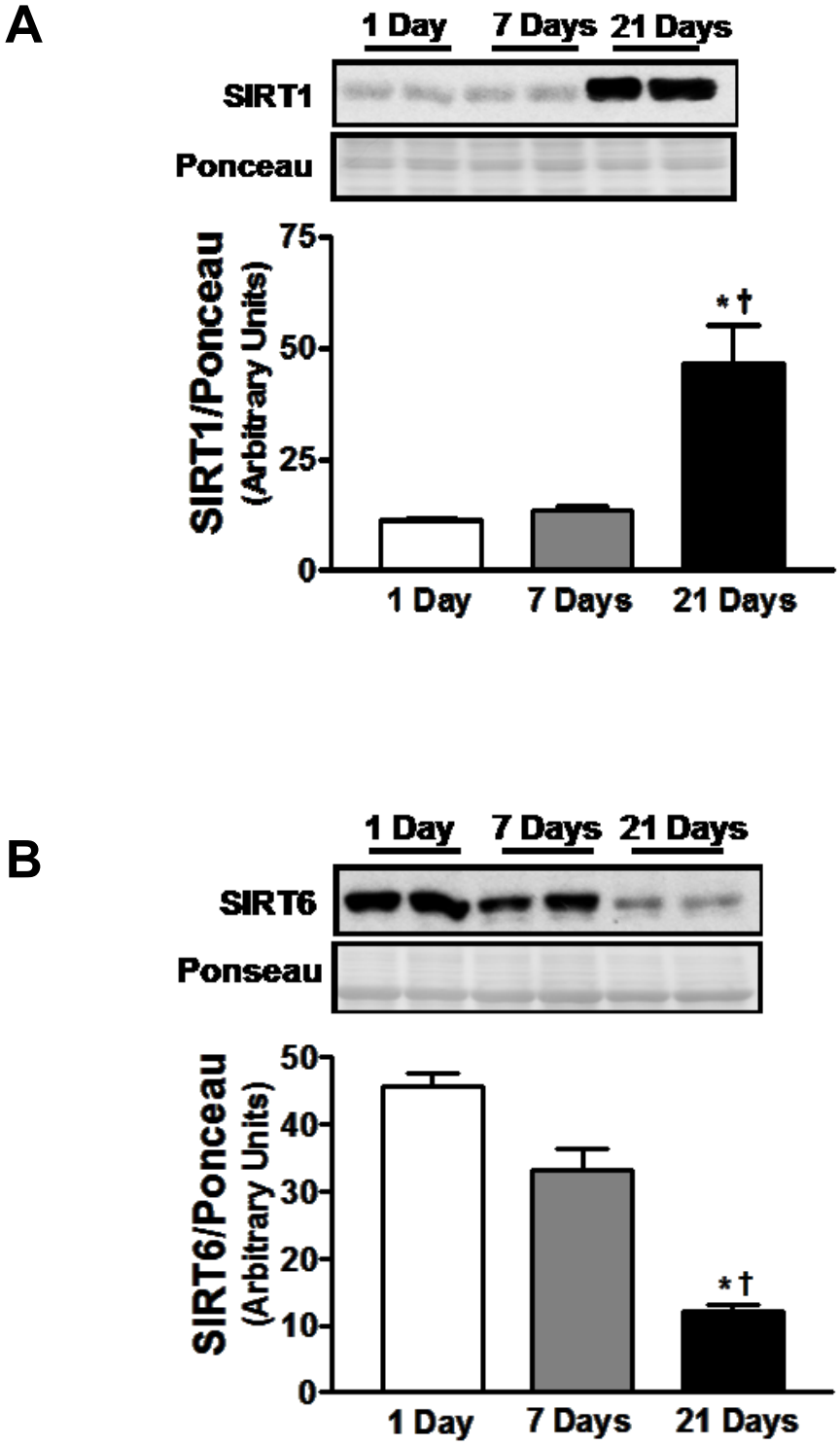
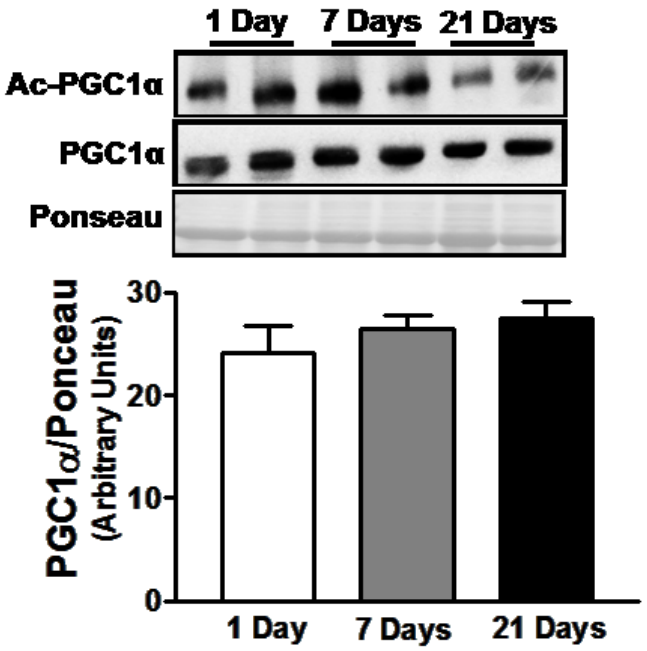

A
1 Day
7 Days
21 Days
SIRT1
Ponceau
SIRT1/Ponceau
(Arbitrary Units)
75
50
25
0
*†
1 Day
7 Days
21 Days
B
1 Day
7 Days
21 Days
SIRT6
Ponseau
SIRT6/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
*†
1 Day
7 Days
21 Days

Figure 3-2

C


1 Day
7 Days
21 Days
Ac-PGC1α
PGC1α
Ponseau
PGC1α/Ponceau
(Arbitrary Units)
30
20
10
0
1 Day
7 Days
21 Days

D

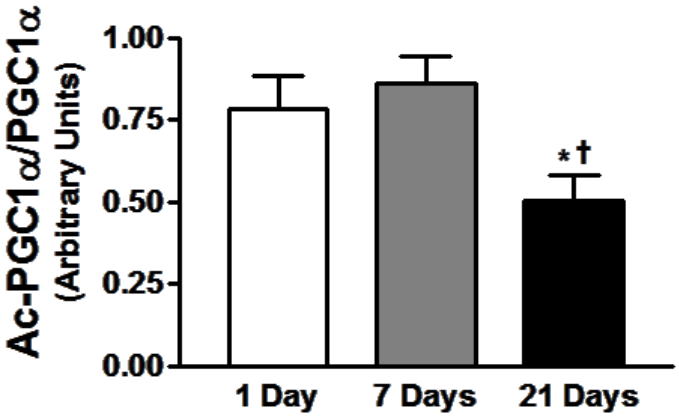

Ac-PGC1α/PGC1α
(Arbitrary Units)
1.00
0.75
0.50
0.25
0.00
*†
1 Day
7 Days
21 Days

Figure 3-2

E

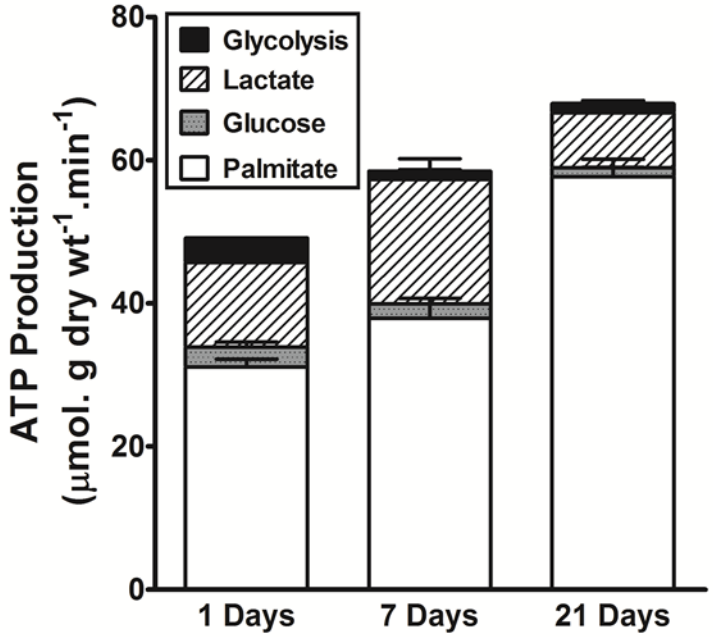

Glycolysis
Lactate
Glucose
Palmitate
ATP Production
(μmol. g dry wt-1.min-1)
80
60
40
20
0
1 Days
7 Days
21 Days

Figure 3-2

Figure 3-3: Protein expression, acetylation, and activity of β-HAD in hearts from 1-, 7-, and 21-day old rabbits.

Protein expressions of SIRT3 **(A)**, GCN5L1 **(B)**, and β-HAD **(C)**. **D)** Level of β-HAD acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for β-HAD. **E)** β-HAD enzyme activity (n=6). Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

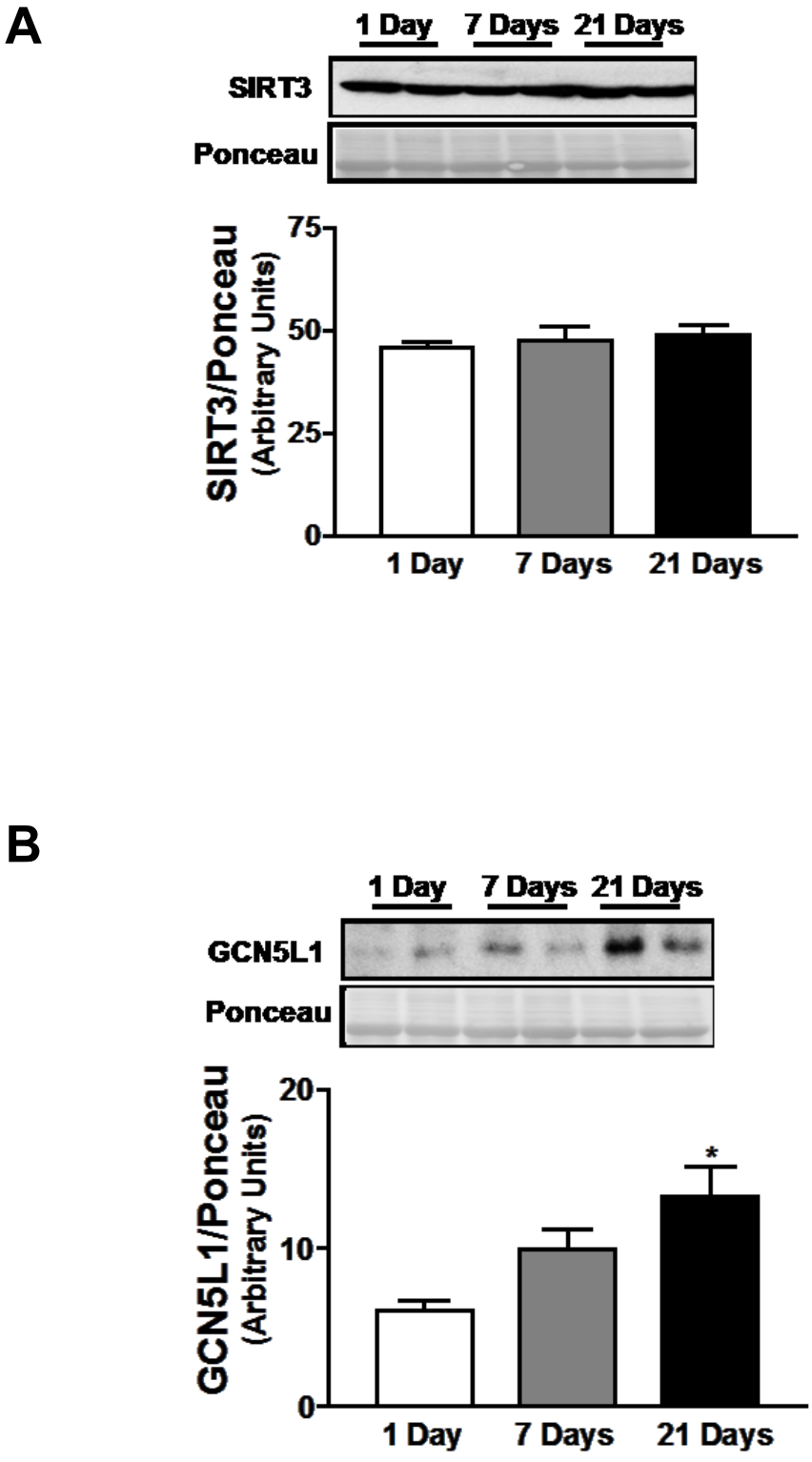
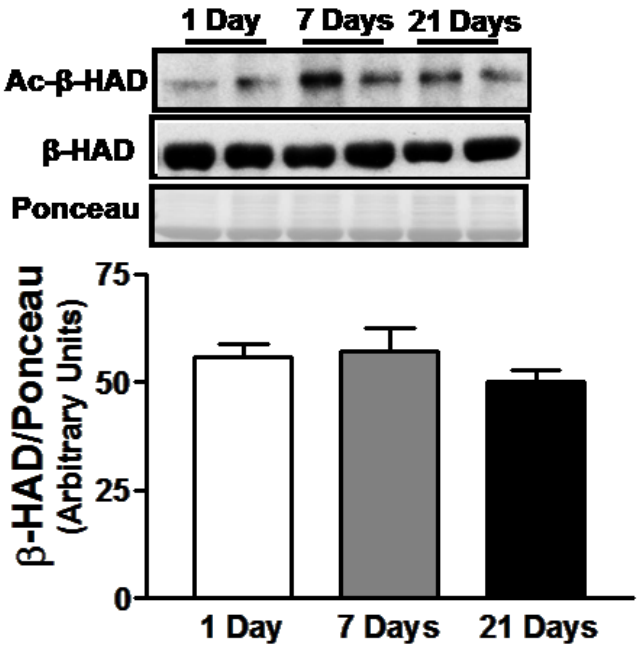

A
1 Day
7 Days
21 Days
SIRT3
Ponceau
SIRT3/Ponceau
(Arbitrary Units)
75
50
25
0
1 Day
7 Days
21 Days
B
1 Day
7 Days
21 Days
GCN5L1
Ponceau
GCN5L1/Ponceau
(Arbitrary Units)
20
10
0
*
1 Day
7 Days
21 Days

Figure 3-3

C


1 Day
7 Days
21 Days
Ac-β-HAD
β-HAD
Ponceau
β-HAD/Ponceau
(Arbitrary Units)
75
50
25
0
1 Day
7 Days
21 Days

D

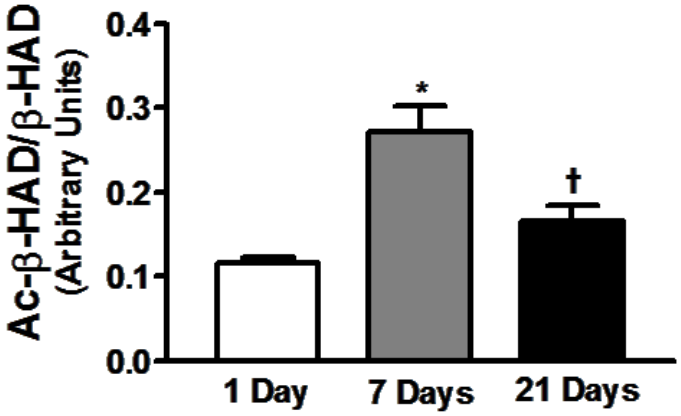

Ac-β-HAD/β-HAD
(Arbitrary Units)
0.4
0.3
0.2
0.1
0.0
*
†
1 Day
7 Days
21 Days

Figure 3-3

E

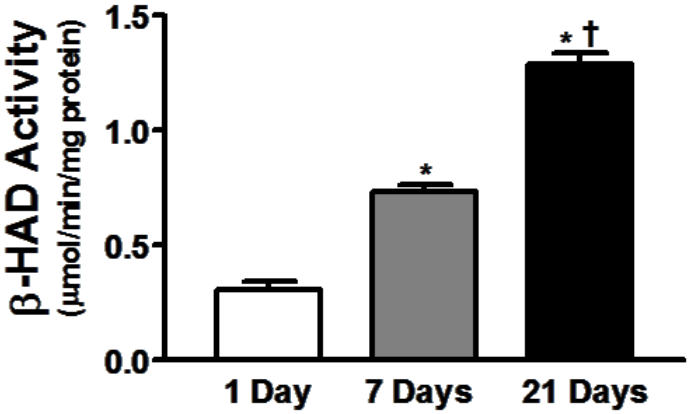
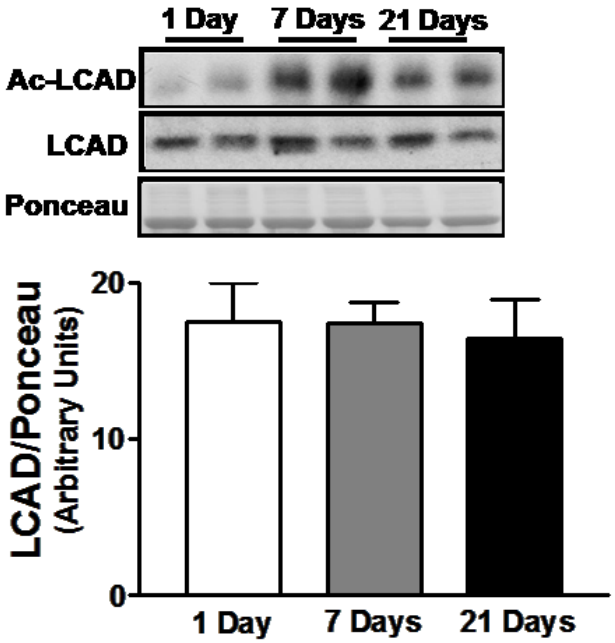

β-HAD Activity
(µmol/min/mg protein)
1.5
1.0
0.5
0.0
*
* †
1 Day
7 Days
21 Days

Figure 3-3

Figure 3-4: Levels of cardiac LCAD acetylation and activity during maturation.

A) LCAD expression. **B)** Level of LCAD acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for LCAD. **C)** LCAD activity (n=6). Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
Ac-LCAD
LCAD
Ponceau
LCAD/Ponceau
(Arbitrary Units)
20
10
0
1 Day
7 Days
21 Days

B

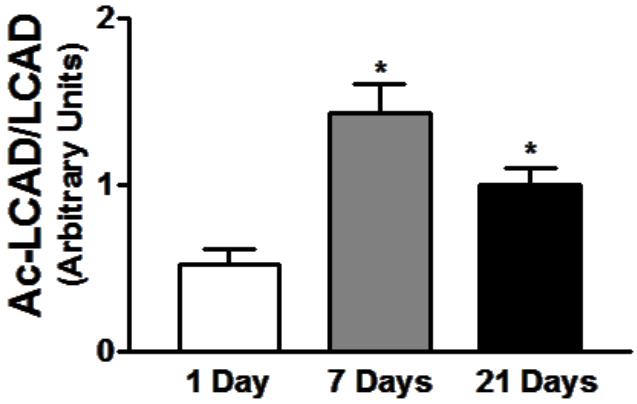

Ac-LCAD/LCAD
(Arbitrary Units)
2
1
0
*
*
1 Day
7 Days
21 Days

Figure 3-4

C

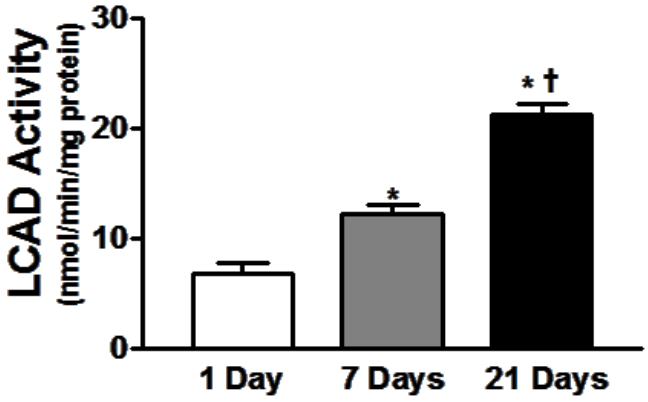

LCAD Activity
(nmol/min/mg protein)
30
20
10
0
*
* †
1 Day
7 Days
21 Days

Figure 3-4

Figure 3-5: PDH acetylation levels in hearts from 1-, 7-, and 21-day old rabbits.

A) PDH expression. **B)** Level of PDH acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PDH. Values represent mean±SEM; (n=4).

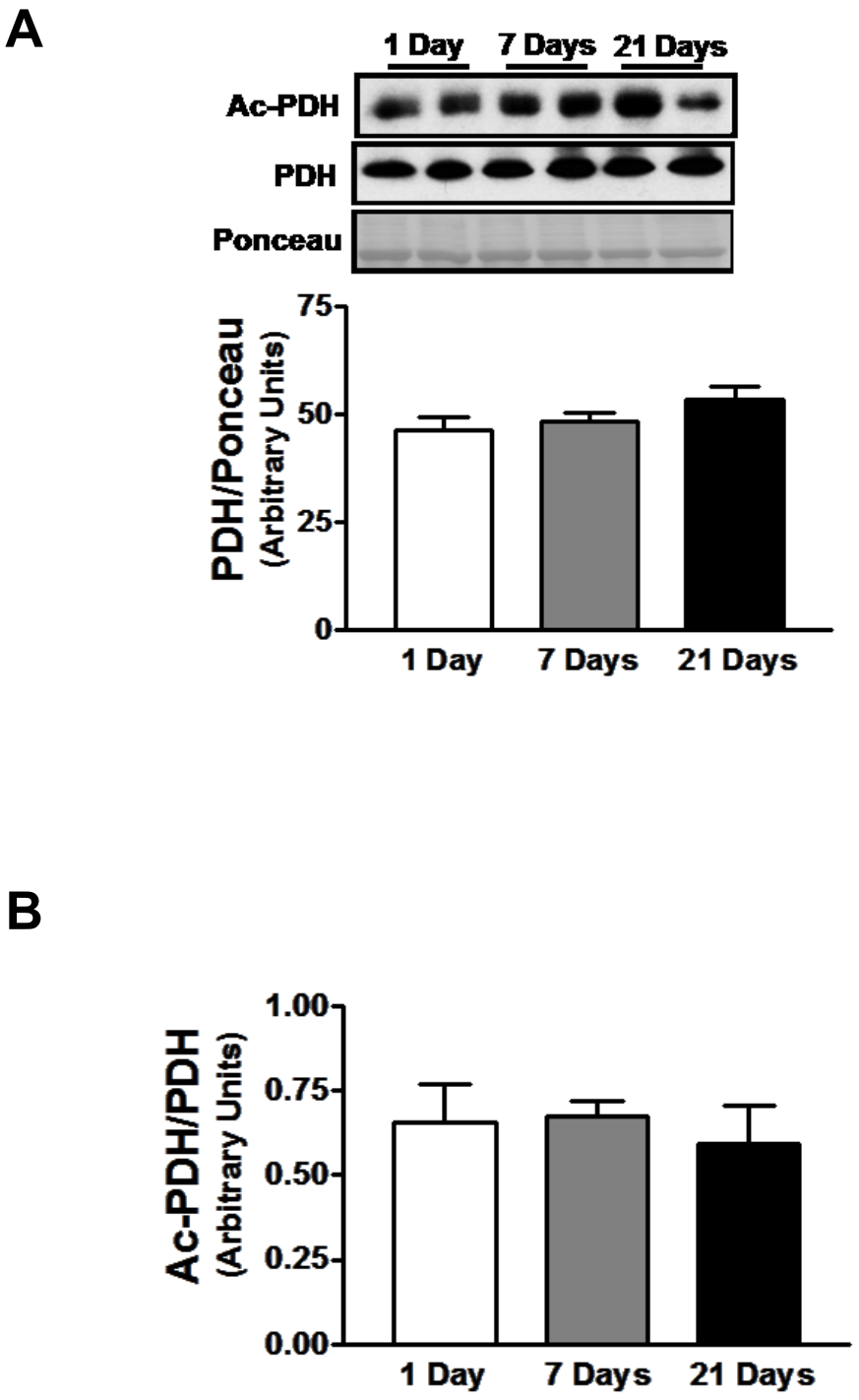
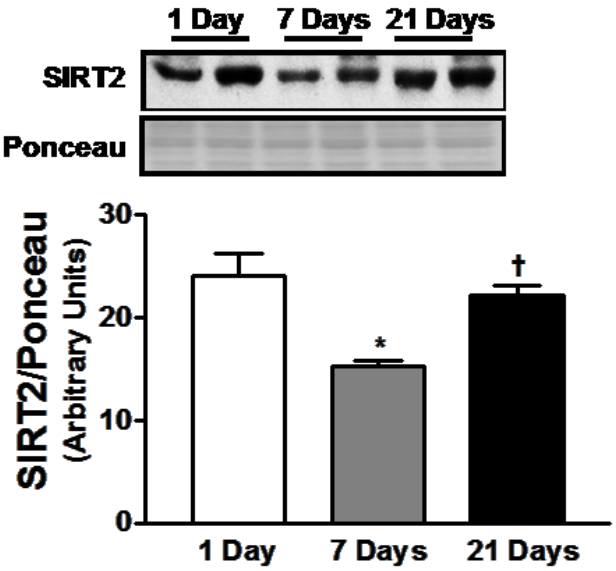

A
1 Day
7 Days
21 Days
Ac-PDH
PDH
Ponceau
PDH/Ponceau
(Arbitrary Units)
75
50
25
0
1 Day
7 Days
21 Days
B
Ac-PDH/PDH
(Arbitrary Units)
1.00
0.75
0.50
0.25
0.00
1 Day
7 Days
21 Days

Figure 3-5

Figure 3-6: Acetylation levels of the glycolytic enzyme HK in hearts from 1-, 7-, and 21-day old rabbits.

Protein expressions of SIRT2 **(A)**, and HK **(B)**. **C)** Level of HK acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for HK. Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
SIRT2
Ponceau
SIRT2/Ponceau
(Arbitrary Units)
30
20
10
0
*
†
1 Day
7 Days
21 Days

B

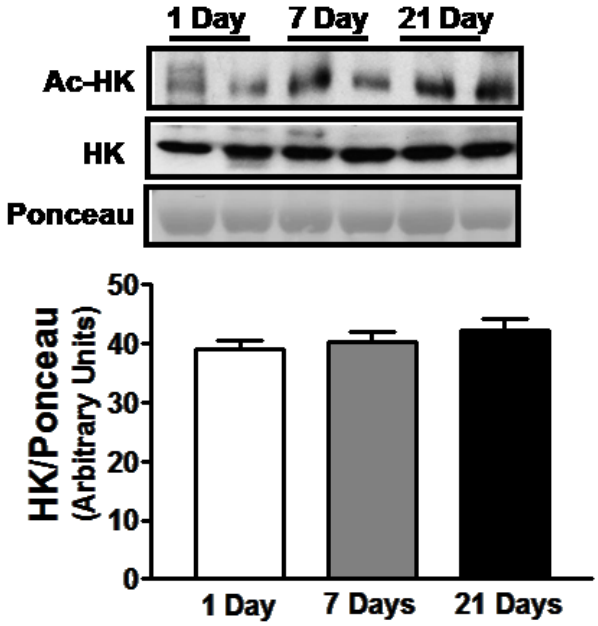

1 Day
7 Day
21 Day
Ac-HK
HK
Ponceau
HK/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
1 Day
7 Days
21 Days

Figure 3-6

C

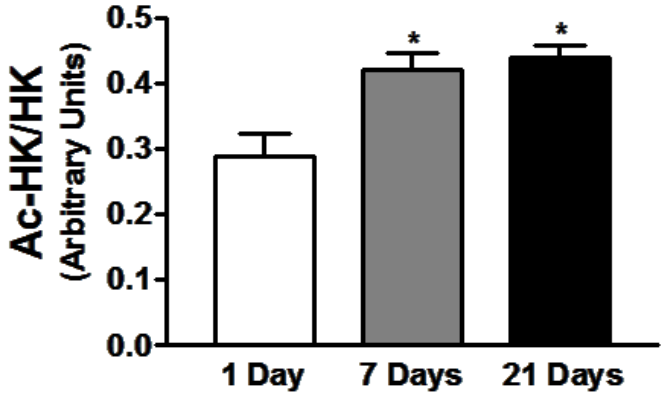
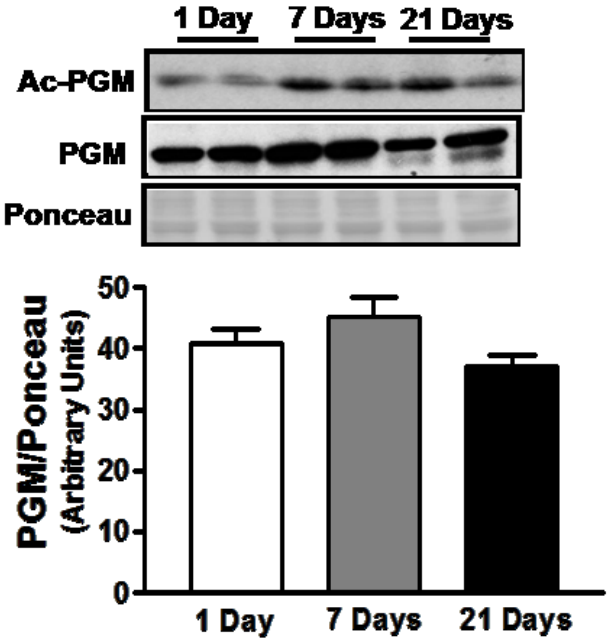


Figure 3-6

Figure 3-7: Acetylation levels of the glycolytic enzyme PGM in hearts from 1-, 7-, and 21-day old rabbits.

A) PGM expression. **B)** Level of PGM acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGM.. **C)** HIF-1α expression. Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
Ac-PGM
PGM
Ponceau
PGM/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
1 Day
7 Days
21 Days

B

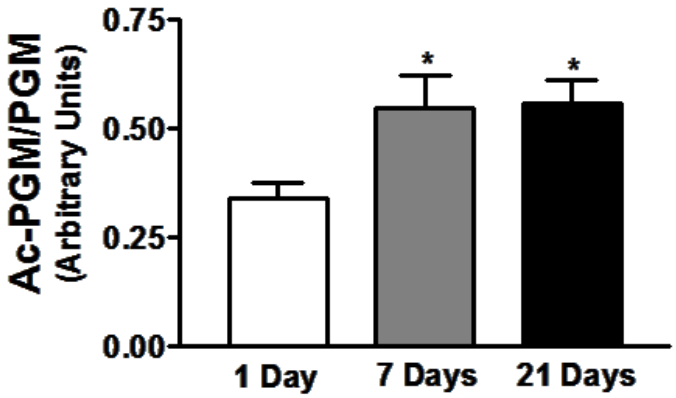

Ac-PGM/PGM
(Arbitrary Units)
0.75
0.50
0.25
0.00
*
*
1 Day
7 Days
21 Days

Figure 3-7

C

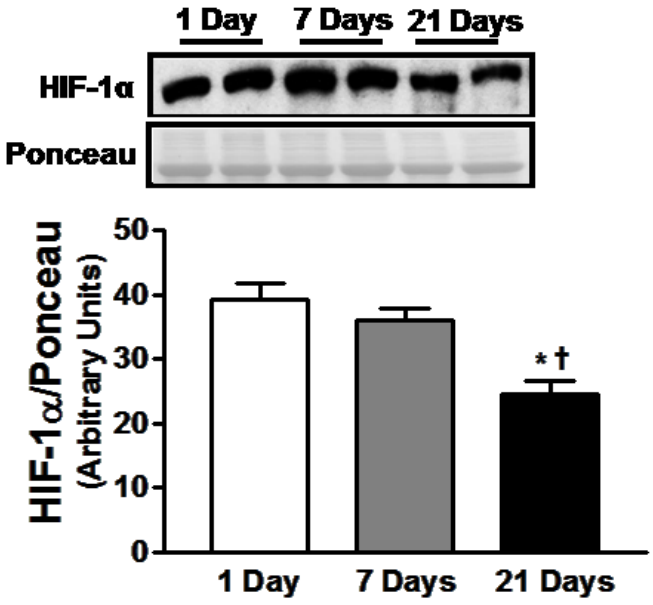

1 Day
7 Days
21 Days
HIF-1α
Ponceau
HIF-1α/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
*†
1 Day
7 Days
21 Days

Figure 3-7

3.5 DISCUSSION

This study investigated the effects of acetylation on activity of enzymes involved in glycolysis, glucose, and fatty acid β-oxidation during maturation of newborn rabbit's heart. Our study provides a number of novel observations regarding age-dependent changes in acetylation. First, there was an age-dependent increase in the overall acetylation of cardiac proteins along with an increase in GCN5L1 and no change in SIRT3 protein expression. Second, acetylation of PGC-1α was significantly decreased with maturation, concomitant with decreased SIRT6 and increased SIRT1 expression. Third, a significant increase in fatty acid β-oxidation during maturation was accompanied by an increased acetylation and activity of the fatty acid β-oxidation enzymes, β-HAD and LCAD. Finally, acetylation of the glycolytic enzymes HK and PGM were increased; scenarios consistent with the decrease in glycolytic activity seen in 21-day vs. 1-, 7-day old hearts. Collectively, these data suggest an important role of lysine acetylation in regulating cardiac energy metabolism during maturation.

Examination of PGC-1α acetylation revealed an age-dependent decrease in acetylation following birth. Previous studies showed that PGC-1α acetylation by the nuclear acetyltransferase GCN5 inhibits its activity (178,190). SIRT1 can deacetylate and activate PGC-1α (33-35). We showed that increased SIRT1 expression 3 weeks post-birth leads to a

robust decrease in PGC-1α acetylation and enhanced mitochondrial oxidative capacity and ATP production. SIRT6 binds to and activates GCN5, a nuclear acetyltransferase that acetylates and inhibits PGC-1α activity (179). Interestingly, cardiac protein level of SIRT6 was significantly decreased in 21-day compared to 1-, and 7-day old rabbits. The decrease in SIRT6 can lead to inhibition of GCN5 and hence decreased PGC-1α acetylation in 21-day old hearts. In acute inflammatory response, SIRT1 and SIRT6 coordinate the switch from glucose to fatty acid β-oxidation (239). Results from these studies support our findings that increased SIRT1 and decreased SIRT6 coordinate PGC-1α acetylation to improve mitochondrial oxidative capacity during maturation (174,176,178,179,190,239).

Fatty acid β-oxidation rates were significantly increased during maturation. There was an age-dependent increase in β-HAD and LCAD acetylation levels. Since hearts from 7-, 21- day have a greater reliance on fatty acid β-oxidation as a source of energy, our results suggest a stimulatory effect of acetylation on β-HAD and LCAD activity. These, effects can be mediated by the upregulation of mitochondrial acetyltransferase GCN5L1. Our findings are in agreement with the study by Zhao *et al* where they showed that lysine acetylation activates fatty acid β-oxidation in muscle cells (159). In liver, enhanced fatty acid β-oxidation increases mitochondrial and peroxisomal protein acetylation (210). The increases in fatty acid β-oxidation during maturation enhanced overall

cardiac protein acetylation. SIRT3 is identified as a major deacetylase and a key regulator of mitochondrial oxidative metabolism (191,192,199,200). The stable expression of SIRT3 during maturation suggested that SIRT3 is pivotal for cardiac development and function. PDH activity can be regulated by covalent modifications, such as phosphorylation and acetylation as identified recently (201,202,234). Hyperacetylation of PDH inhibits enzyme activity in skeletal muscles and human cancer cells (201,202). We demonstrated that neither PDH protein levels, nor PDH acetylation were altered during maturation. Our results suggest that mechanisms other than acetylation primarily regulate PDH activity during cardiac maturation. The increase in fatty acid β-oxidation can predominate and inhibit glucose oxidation during maturation (90). Moreover, increased PGC-1α activity upregulates pyruvate dehydrogenase kinase 4 resulting in hyper-phosphorylation and inactivation of PDH during maturation (79,240).

Lactate is an important fuel during maturation, as fetal hearts can readily extract and oxidize lactate (56). We found that cardiac lactate oxidation was high till 7-day post-birth, however, 3 weeks later rates of lactate oxidation significantly dropped. As circulating lactate levels are very high in the fetus (241), lactate oxidation accounts for the majority of myocardial oxygen consumption (9,241). Following birth, blood levels of lactate decreases significantly, and the contribution of lactate oxidation to ATP production in the neonatal period markedly decreases (8,9,242).

SIRT2 is a cytoplasmic deacetylase and a key regulator of heart necrosis and metabolism (154-156). SIRT2 is a critical regulator for Akt and GSK3β activation in mouse and human embryonic cells (145,167). We showed that SIRT2 protein level was initially decreased 7 days post-birth, and then increased 3 weeks post-birth to baseline. A number of glycolytic enzymes are acetylated, and inhibition of glycolysis is associated with a reduction in SIRT1 expression (172). In contrast, a recent study suggested that SIRT1 activation inhibits the activity of the glycolytic enzyme phosphoglycerate mutase-1, suggesting that acetylation actually stimulates glycolysis (161). We showed that glycolysis rates markedly declined with maturation along with an increase in acetylation of the glycolytic enzymes; HK and PGM. Our data suggest that acetylation of the glycolytic enzymes HK and PGM inhibit glycolysis. Acetylation of the glycolytic enzyme pyruvate kinase at lysine 305 inhibits its activity in muscle (163). At the transcriptional level, Hypoxia inducible factor alpha (HIF-1α) is a transcription factor that upregulates the expression of genes involved in anaerobic glycolysis (80). It was shown previously that HIF-1α level of expression is significantly decreased post-birth (54). Indeed, cardiac protein level of HIF-1α was significantly decreased 3 weeks post-birth. Our data suggest that both, the decrease in HIF-1α expression and increased glycolytic enzymes acetylation could account for the age-dependent decrease in glycolysis.

In conclusion, we demonstrate that decreased acetylation of PGC-1α, and increased acetylation of β-HAD, LCAD, HK, and PGM may account for the dramatic shift in energy metabolism from glycolysis to fatty acid β-oxidation seen during maturation. Our results suggest that lysine acetylation enhances cardiac fatty acid β-oxidation and inhibits glycolysis during maturation.

CHAPTER 4.

Obesity-Induced Lysine Acetylation Increases Cardiac Fatty Acid Oxidation and Impairs Insulin Signaling

My role in this work involved performing all experiments (except those noted below), as well as the writing of the manuscript. Cory Wagg perfused the mouse heart, and Sowndramalingam Sankaralingam performed perfusion data analysis. Amy Barr conducted the MRI for mice body fat composition.

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CHAPTER 4.

Acetylation of Regulatory Proteins in the Heart Increases Fatty Acid Oxidation and Impairs Insulin Signaling

Abstract

Aims: Lysine acetylation has emerged as a novel post-translational pathway that regulates the activities of enzymes involved in both fatty acids and glucose metabolism. We examined whether lysine acetylation controls heart glucose and fatty acid β-oxidation in high fat diet (HFD) obese and SIRT3 KO mice.

Methods and results: C57BL/6 mice were placed on either a HFD (60% fat) or low fat diet (LFD) (4% fat) for 16- or 18-wk. Cardiac fatty acid β-oxidation rates were significantly increased in HFD vs LFD mice (845±76 vs 551±87 nmol.g dry wt-1.min-1, $p<0.05$). Activities of the fatty acid β-oxidation enzymes, long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were increased in hearts from HFD vs LFD mice, and were correlated with LCAD and β-HAD hyperacetylation. Cardiac protein hyperacetylation in HFD fed mice was associated with a decrease in SIRT3 expression, while expression of the mitochondrial acetylase, GCN5L1, did not change. Interestingly, SIRT3 deletion in mice also led to an increase in cardiac fatty acid β-oxidation compared to WT mice (422±29 vs 291±17 nmol.g dry wt-1.min-1, $p<0.05$).

Cardiac lysine acetylation was increased in SIRT3 KO mice compared to WT, including increased acetylation and activity of LCAD and β-HAD. Although the HFD and SIRT3 deletion decreased glucose oxidation, pyruvate dehydrogenase (PDH) acetylation was unaltered. However, the HFD did increase Akt acetylation, while decreasing its phosphorylation and activity.

Conclusions: We conclude that increased lysine acetylation enhances cardiac fatty acid β-oxidation, and impairs myocardial insulin sensitivity.

4.1 INTRODUCTION

Fatty acids β-oxidation is a major energy source for the adult heart (2). However, the presence of obesity, diabetes, and heart failure result in an increased reliance of the heart on fatty acid β-oxidation as a source of energy, which can decrease cardiac efficiency and compromise cardiac function (89). The reasons for the increase in cardiac fatty acid β-oxidation under these conditions are not completely understood, but do involve increases in circulating fatty acids, alterations in transcriptional control of fatty acid oxidative enzymes, and the development of cardiac insulin resistance that decreases glucose oxidation (58). Given the large number of patients with obesity and diabetes, a better understanding of processes that regulate cardiac energy metabolism could lead to novel therapeutic approaches to treat cardiovascular complications associated with these conditions by optimizing cardiac energy metabolism.

Alterations in post-translational control of fatty acid and glucose oxidation are another potential mechanism contributing to the obesity-induced shift in energy metabolism in the heart. Lysine acetylation has recently emerged as a novel important post-translational modification, which can modify the activity of a number of enzymes involved in fatty acid and glucose metabolism (31,94,98,159,165,243). This post-translational modification is controlled, in part, by sirtuins (SIRTs), which are nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases

localized in distinct subcellular compartments (200,208). Three of the SIRT isoforms (SIRT1, SIRT6, and SIRT7) are localized in the nucleus (178), where they can play an important role in regulating acetylation of nuclear proteins involved in transcriptional regulation of genes involved in cardiac energy metabolism. This includes the acetylation of peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α), a transcriptional regulator of genes that encode enzymes involved in fatty acid β-oxidation. Previous studies showed that acetylation of PGC-1α by the nuclear acetyltransferase general control of amino acid synthesis 5 (GCN5), results in an inhibition of PGC-1α transcriptional activity (94,197). Interestingly, SIRT6 can deacetylate and activate GCN5 thereby increasing PGC-1α acetylation (193). On the other hand, SIRT1 deacetylates and activates PGC-1α (244,245).

In mitochondria, SIRT3 appears to be the major deacetylase (140,190) and targets numerous enzymes involved in energy metabolism, including key enzymes involved in fatty acid β-oxidation such as long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) (98,174,179,190,243). However, conflicting information has emerged regarding the role of deacetylation in controlling fatty acid oxidation. Hirschey *et al* proposed that deacetylation of LCAD by SIRT3 accelerates fatty acid β-oxidation in the liver (179,243). Furthermore, chemical acetylation of LCAD on lysines 318/322 reduced enzymatic activity, while deacetylation with recombinant SIRT3 restored

catalytic activity (171). In contrast, Zhao *et al* showed that acetylation of β-HAD results in an activation of enzyme activity in muscle cells (98). Mitochondria from hindlimb muscles of fasted mice were also shown to have an increased acetylation that was associated with an increase in fatty acid β-oxidation rates (194). Diaphragm muscle of SIRT3 knockout mice also showed hyperacetylation and increased fatty acid β-oxidation rates (194). Furthermore, LCAD is hyperacetylated in obese dam offsprings concomitant with a significant decrease in SIRT3 expression and activity (191). Therefore, further studies are needed to clarify the impact of acetylation on fatty acid oxidation.

GCN5-like 1 (GCN5L1) was recently identified as an essential component of the mitochondrial acetyltransferase machinery (196). However, little is known about its role in regulating acetylation levels of mitochondrial proteins in obesity. Obesity and type 2 diabetes have been shown to be associated with cardiac dysfunction and impaired insulin signaling (192,206). In the heart, insulin stimulated glucose oxidation is dramatically decreased in high fat diet (HFD)-induced obesity (201). Disruption of cardiac insulin signaling by inhibiting protein phosphorylation along the insulin/Akt (protein kinase B)/ glycogen synthase kinase 3-β (GSK3β) axis can lead to a reduced cardiac glucose use (201,246). However, in addition to inhibition of Akt phosphorylation, acetylation may play important role in the initiation of insulin signaling. A recent study by Gupta *et al* identified lysine acetylation as an important regulator of Akt

activity during cardiac hypertrophy (211). Despite this, the role of lysine acetylation in regulating cardiac insulin signaling in obesity has not yet been defined. The aim of this study is to identify what role lysine acetylation has in regulating cardiac fatty acid and glucose oxidation in obese and SIRT3 KO mice heart. In addition, we determined what effect lysine acetylation has on cardiac insulin signaling.

4.2 MATERIALS AND METHODS

Mice received care and were treated according to the guidelines of the Canadian Council on Animal Care, and all procedures performed on animals were approved by the University of Alberta Health Sciences Animal Welfare Committee. GTT and MRI were conducted to determine levels of blood glucose and body composition of fat, respectively. Hearts isolated from pentobarbital euthanized mice were perfused as isolated working hearts with Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 5 mM [U-^{14}C]glucose, 0.8 mM [9,10-^{3}H]palmitate prebound to 3% albumin, in the presence or absence of 100 μU/mL insulin, as described in details in **chapter 2**. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. Protocols for assessment of β-HAD activity, LCAD activity, immunoprecipitation, immunoblotting, and statistical analysis are described in details in **chapter 2** of this thesis.

4.3 RESULTS

4.3.1 Characteristics of HFD obese mice:

Body weight and whole body fat mass, as measured by MRI in a wake mice, were significantly increased after 18 wk of HFD feeding compared to LFD fed mice (Table 4-1). Heart weight normalized to tibia length was also increased in mice fed a HFD. Blood glucose levels in overnight-fasted mice, as assessed by OGTT, were higher in HFD obese mice compared to mice on a LFD (Table 4-1).

4.3.2 Cardiac lysine acetylation in HFD obese mice:

Overall myocardial acetylation levels were increased in obese HFD fed mice compared to LFD fed mice (Figure 4-1A). Mitochondrial proteins acetylation were also markedly increased in hearts isolated from HFD fed mice (Figure 4-1B). The effect of a HFD on the expression of cardiac sirtuins (SIRTs, lysine deacetylases), and GCN5L1 (mitochondrial acetyltransferase) are shown in Figure 4-1. There were no changes in SIRT1 (Figure 4-1C) and GCN5L1 expression (Figure 4-1G); however the level of SIRT3 protein expression was significantly decreased in HFD obese mice (Figure 4-1D). In contrast, the level of SIRT6 expression was significantly increased in HFD obese mice (Figure 4-1E), while the expression of SIRT2 was significantly decreased (Figure 4-1F).

4.3.3 Cardiac palmitate oxidation rates in HFD obese mice:

To examine what effect obesity has on cardiac energy metabolic rates; glucose and palmitate oxidation rates were measured in isolated working hearts from mice subjected to either a HFD or a LFD for an 18-wk period. A significant increase in palmitate oxidation rates (Table 4-2) were seen in mice subjected to a HFD, compared to LFD controls. In contrast, rates of glucose oxidation were significantly lower in hearts from HFD obese mice vs LFD mice. Because cardiac work is an important determinant of oxidative metabolism, rates of palmitate and glucose oxidation were also normalized to cardiac work. The increase in palmitate oxidation and decrease in glucose oxidation were confirmed (Table 4-2). Since all hearts were perfused under identical conditions, the data suggests that molecular changes at the level of the heart were responsible for the shift from glucose oxidation to fatty acid oxidation in hearts from HFD obese mice.

4.3.4 Acetylation of fatty acid β-oxidation enzymes in hearts from HFD obese mice:

Because cardiac fatty acid β-oxidation rates were significantly higher in HFD obese mice, we examined whether the expression levels, activity, and acetylation state of fatty acid oxidative enzymes were altered in hearts from HFD obese mice. β-Hydroxyacyl CoA dehydrogenase (β-HAD) and long chain acyl CoA dehydrogenase (LCAD) are two enzymes

of fatty acid β-oxidation pathway that have been shown to be under acetylation control. We therefore assessed the level of acetylation of these enzymes in hearts from obese HFD fed mice. The levels of β-HAD protein were not changed in mice on a HFD or LFD (Figure 4-2A). However, the levels of β-HAD acetylation were significantly increased in hearts of HFD obese mice (Figure 4-2B) accompanied by a significant increase in β-HAD activity (Figure 4-2C).

Similar to what was observed with β-HAD, the level of LCAD protein expression was not different between hearts of HFD obese and LFD mice (Figure 4-3A). However, a significant increase in cardiac LCAD acetylation was observed in HFD obese mice (Figure 4-3B) accompanied by a significant increase in LCAD activity (Figure 4-3C). We also observed a strong positive correlation between LCAD acetylation and rate of fatty acid β-oxidation in the HFD obese and LFD mice (Figure 4-3D).

4.3.5 PGC-1α acetylation in hearts from HFD obese mice:

PGC-1α is an important transcriptional nuclear regulator of mitochondrial biogenesis and oxidative metabolism, and is also under acetylation control (173). We therefore determined if alterations in expression and acetylation of PGC-1α could contribute to the increase in fatty acid oxidation observed in the hearts of obese HFD obese mice. The amount of PCG-1α protein was significantly decreased in HFD obese mice compared to LFD controls (Figure 4-4A). Concomitantly, an increase in

overall acetylation of PGC-1α was observed, resulting in a marked increase in the Ac-PGC-1α/PGC-1α ratio (Figure 4-4B). Since acetylation of PGC-1α is believed to decrease PGC-1α transcriptional activity (173,209), the decrease in PGC-1α expression and increased acetylation are unlikely to explain the increase in fatty acid oxidation seen in the hearts from the HFD obese mice. Indeed, not only was overall expression of β-HAD or LCAD not altered (Figures 4-2A and 3B), neither was the expression of peroxisome proliferator-activated receptors alpha (PPARα) (Figure 4-4C), which is involved in the transcriptional control of fatty acid oxidative enzymes. Expression of the tricarboxylic acid (TCA cycle) enzyme, citrate synthase (CS) (Figure 4-4D) and the electron transport chain enzyme, Complex I (Figure 4-4E), were also unaffected. Combined, this suggests that the increase in fatty acid oxidation seen in hearts of HFD obese mice are not due to increases in mitochondrial biogenesis, but rather direct changes in acetylation control of fatty acid oxidation enzymes.

4.3.6 Role of acetylation in impaired cardiac insulin signaling in HFD obese mice:

As shown in Table 4-2, hearts from HFD obese mice showed a decrease in glucose oxidation that accompanied the increase in fatty acid oxidation. The ability of insulin to stimulate glucose oxidation in hearts from HFD obese mice was also markedly impaired compared to hearts from mice fed a LFD (Figure 4-5A). Pyruvate dehydrogenase (PDH), the

rate-limiting enzyme for glucose oxidation has recently been shown to be under acetylation control (59,191,192,201). We therefore determined if PDH expression and acetylation status were altered in hearts from obese HFD fed mice. Neither PDH expression (Figure 4-5B), nor the ratios of PDH acetylation/PDH expression were found to be different between hearts from obese HFD vs LFD mice (Figure 4-5C).

Akt is involved in insulin signaling, and can be modulated by phosphorylation and acetylation. SIRT1-mediated deacetylation of Akt, and its upstream activator, phosphoinositide dependent kinase 1 (PDK1) promotes Akt activation (164). Immunoblot analysis showed a decreased cardiac expression of Akt in obese HFD mice (Figure 4-6A). In contrast, the level of Akt acetylation was significantly increased in hearts from obese HFD mice (Figure 4-6B). This increase in acetylation was accompanied by a significant decrease in Akt phosphorylation (Figure 4-5C), suggesting that acetylation may contribute to the impaired insulin signaling seen in hearts from obese HFD mice (Figure 4-5A). GSK3β is a Ser/Thr protein kinase that phosphorylates and inactivates glycogen synthase (42). GSK3β itself is regulated by phosphorylation, with phosphorylation at Ser9 by Akt being inhibitory (250). GSK3β protein expression remained similar in hearts of HFD obese and LFD mice (Figure 4-6D). However, the level of GSK3β phosphorylation on Ser9 was significantly reduced, similar to what was seen with Akt phosphorylation in HFD obese mice (Figure 4-6D), and is consistent with the decrease in p-

Akt seen in hearts from HFD obese mice (Figure 4-6C). These findings are consistent with the insulin resistance observed in hearts from HFD obese mice.

4.3.7 Regulation of fatty acid β-oxidation enzymes by mitochondrial deacetylation:

Because SIRT3 protein levels were decreased in hearts from HFD-induced obese mice (Figure 4-1C), and SIRT3 has been implicated in deacetylation of fatty acid oxidative enzymes, we examined the effect of SIRT3 deletion on cardiac fatty acid β-oxidation and fatty acid oxidative enzyme acetylation status. Isolated working hearts from SIRT3 KO mice showed no differences in cardiac function compared to WT mice (Table 4-3). However, cardiac energy metabolism was significantly shifted, with a significant increase in fatty acid β-oxidation (Table 4-3, Figure 4-7B), and a decrease in glucose oxidation (Table 4-3). The increase in fatty acid oxidation and decrease in glucose oxidation remained when rates were normalized for cardiac work (Table 4-3). The increase in fatty acid β-oxidation in SIRT KO mouse hearts was accompanied by a significant increase in overall acetylation of cardiac proteins, when compared to WT mice (Figure 4-7A). We also measured the expression, acetylation and activity of fatty acid β-oxidation enzymes β-HAD and LCAD. While no changes in protein expression of β-HAD was observed, acetylation of β-HAD was significantly increased in hearts from SIRT3 KO mice in

comparison to WT mice (Figure 4-7C), which was accompanied by increase in β-HAD activity (Figure 4-7D). Similar to β-HAD, acetylation of LCAD was significantly increased in SIRT3 KO hearts without changes in protein expression (Figure 4-7E) and was associated with an increase in LCAD activity (Figure 4-7F).

4.3.8 Role of acetylation in regulation of cardiac glucose oxidation in SIRT3 KO mice:

Recent studies have implicated SIRT3 in deacetylation and inhibition of pyruvate dehydrogenase (PDH), the rate-limiting enzymes for glucose oxidation (201). In SIRT3 KO mouse hearts, insulin stimulated glucose oxidation was markedly impaired compared to hearts from WT mice (Figure 4-8A). We therefore determined if PDH acetylation was altered in hearts from SIRT3 KO mice. Neither PDH expression (Figure 4-8B), nor the ratios of PDH acetylation/PDH expression were different between hearts from SIRT3 KO versus WT mice (Figure 4-8C).

Table 4-1: Metabolic parameters of C57BL/6 mice fed a HFD for 18 weeks.

Heart weight (HW) normalized to tibia length (TL); Whole body fat mass as assessed by MRI; Oral GTT in overnight-fasted mice; Values represent mean±SEM. (n=5). *P<0.05, significantly different from LFD.

	LFD	HFD
Body weight (g)	31.2±1.2	48.3±1.51*
HW/TL (g)	8.4±0.4	10.4±0.5*
Fat mass (g)	5.8±0.32	18.3±0.61*
Percent Fat mass/ BW ratio	18±0.6	38.2±0.86*
GTT (A.U.C)	49.7±2.6	69.7±2.9*

Table 4-1

Figure 4-1: Overall cardiac protein acetylation and deacetylase expression in mice fed a high fat diet (HFD) for 16 wk.

A) Total protein acetylation in non-perfused hearts from LFD and HFD mice. **B)** Mitochondrial proteins acetylation in hearts from LFD and HFD mice. Mitochondria were isolated in the presence or absence of 500 nM TSA, and 10 mM nicotinamide. Protein expression of SIRT1 **(B)**, SIRT3 **(C)**, SIRT6 **(D)**, SIRT2 **(E)**, and GCN5L1 **(F)** in hearts from mice fed either a low fat diet (LFD) or HFD. Values represent mean ±SEM. (n=5/group). *P<0.05, significantly different from LFD.

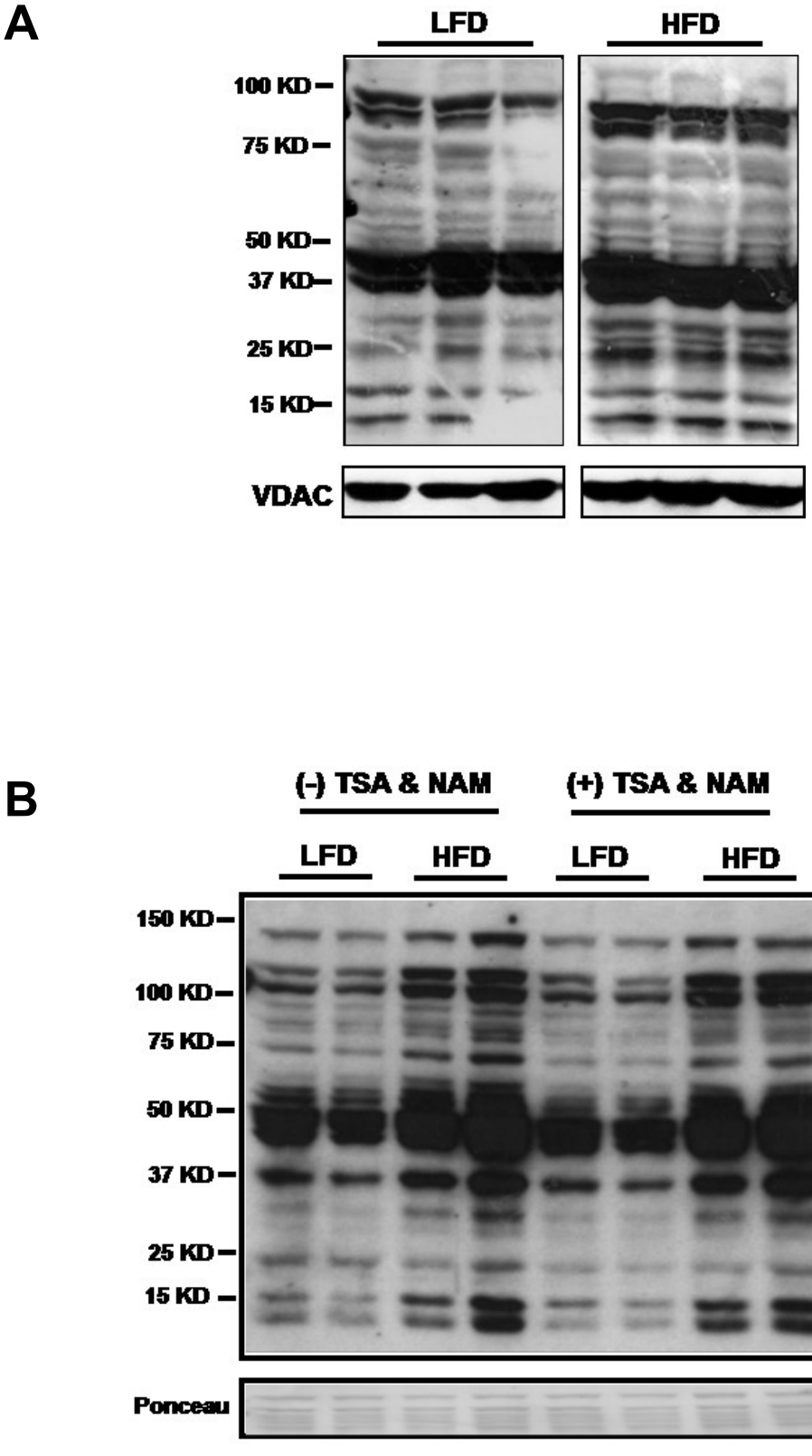
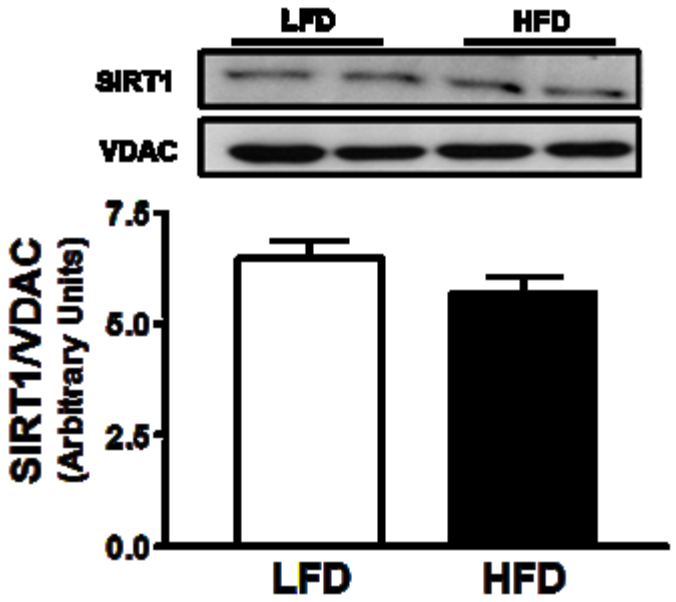

A
LFD
HFD
100 KD
75 KD
50 KD
37 KD
25 KD
15 KD
VDAC
B
(-) TSA & NAM
(+) TSA & NAM
LFD
HFD
LFD
HFD
150 KD
100 KD
75 KD
50 KD
37 KD
25 KD
15 KD
Ponceau

Figure 4-1

C


LFD
HFD
SIRT1
VDAC
SIRT1/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

D

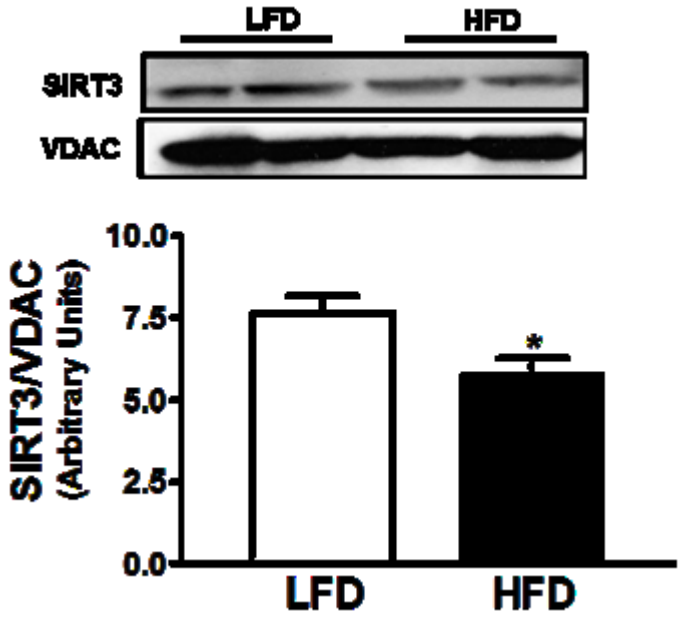
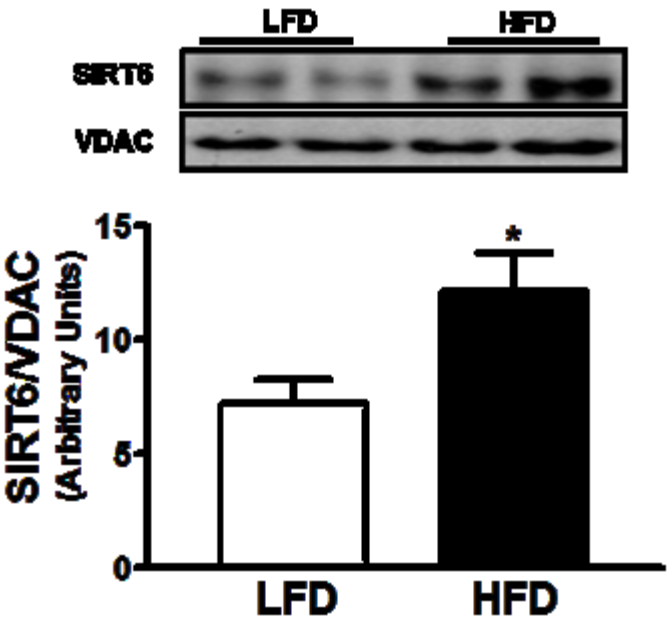

LFD
HFD
SIRT3
VDAC
SIRT3/VDAC
(Arbitrary Units)
10.0
7.5
5.0
2.5
0.0
*
LFD
HFD

Figure 4-1

E


LFD
HFD
SIRT6
VDAC
SIRT6/VDAC
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

F

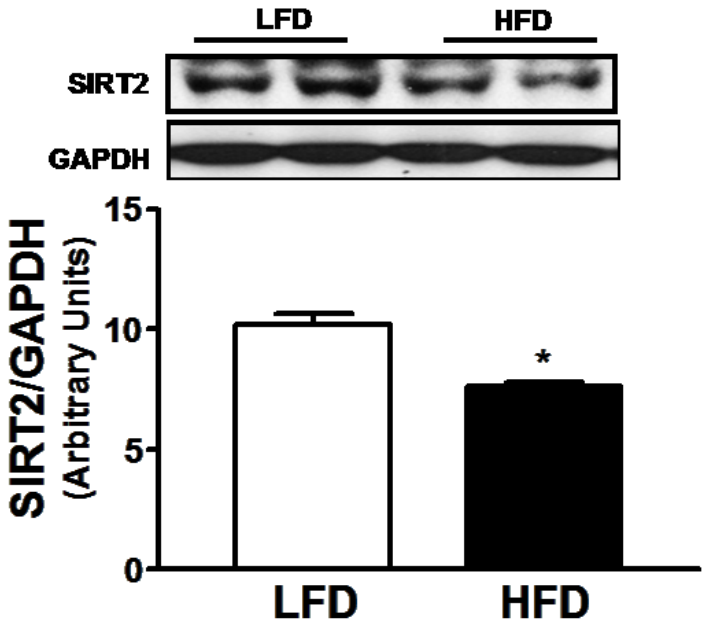

LFD
HFD
SIRT2
GAPDH
SIRT2/GAPDH
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

Figure 4-1

G

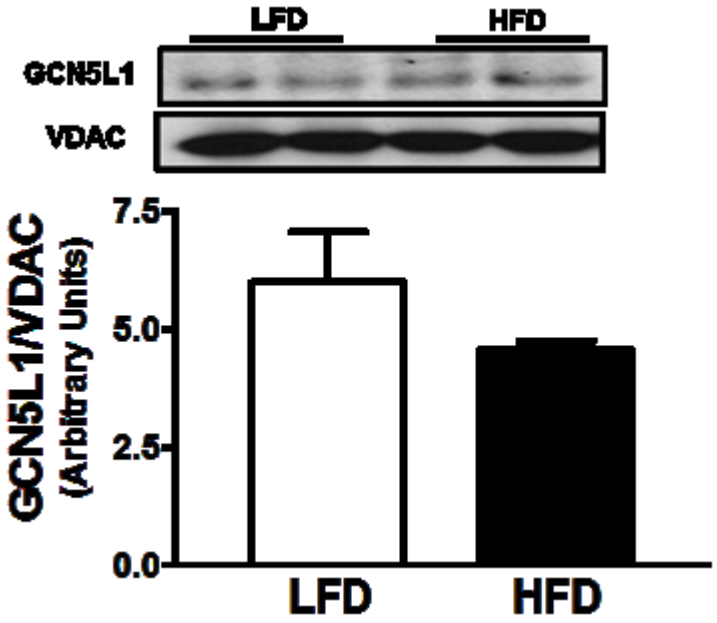

LFD
HFD
GCN5L1
VDAC
GCN5L1/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

Figure 4-1

Table 4-2: Effect of chronic HFD diet feeding on metabolic rates of palmitate and glucose oxidations in isolated working mice hearts

Isolated working hearts were perfused for a 60 min period in the presence of insulin. Rates of glucose and palmitate oxidation were determined as described in methods. Values represent mean±SEM; (n=8). *P<0.05, significantly different from LFD.

	LFD	HFD
	Absolute rates, nmol. g dry $wt^{-1}.min^{-1}$	
Palmitate Oxidation	551±87	845±76*
Glucose Oxidation	1235±160	635±146*
	Rates normalized for work differences, nmol. g dry wt^{-1}.mjoules	
Palmitate Oxidation	207±35	350±57*
Glucose Oxidation	463±55	263±65*

Table 4-2

Figure 4-2: Protein expression, acetylation, and activity of β-hydroxylacyl CoA dehydrogenase (β-HAD) in hearts from obese mice on a HFD.

A) β-HAD expression. **B)** Level of β-HAD acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for β-HAD. **C)** β-HAD enzyme activity. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

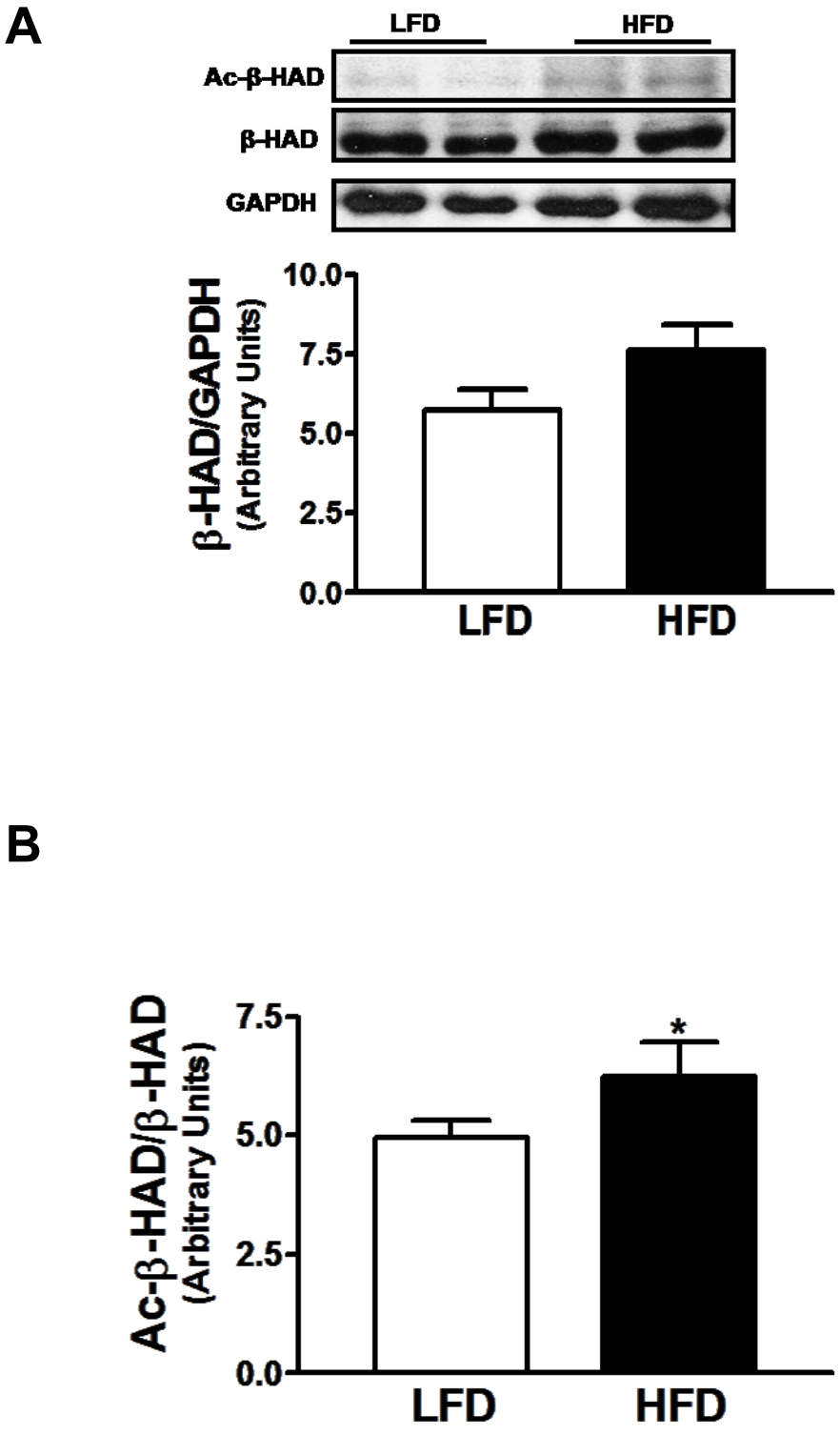

A
LFD
HFD
Ac-β-HAD
β-HAD
GAPDH
β-HAD/GAPDH
(Arbitrary Units)
10.0
7.5
5.0
2.5
0.0
LFD
HFD
B
Ac-β-HAD/β-HAD
(Arbitrary Units)
7.5
5.0
2.5
0.0
*
LFD
HFD

Figure 4-2

C

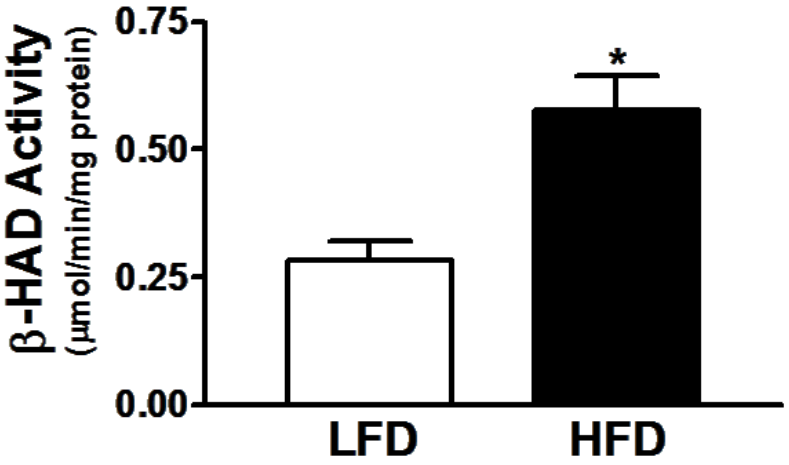
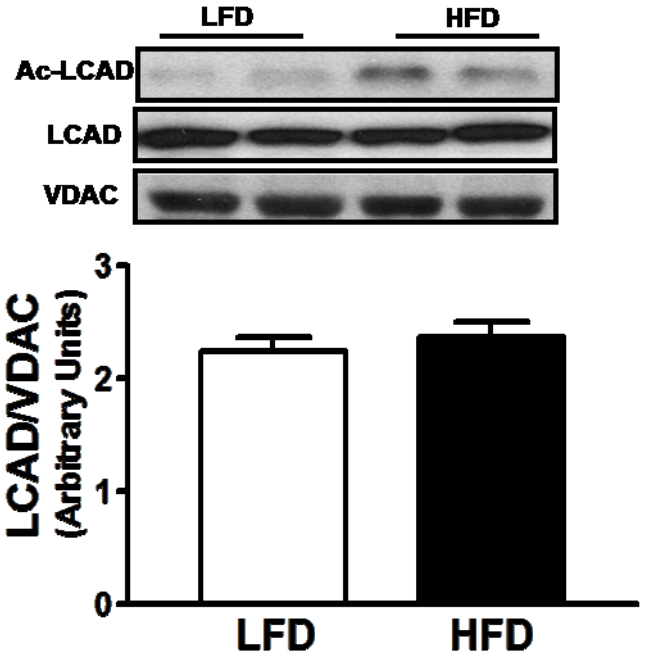


Figure 4-2

Figure 4-3: Level of long chain acyl CoA dehydrogenase (LCAD) acetylation and its correlation to rates of palmitate oxidation.

A) LCAD expression. **B)** Level of LCAD acetylation. Total lysate from isolated working hearts were immunoprecipitated with anti-acetyl-lysine antibodies and then immunoblotted with antibodies specific for LCAD. **C)** LCAD activity. **D)** Correlation curve between palmitate oxidation and LCAD acetylation. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
Ac-LCAD
LCAD
VDAC
LCAD/VDAC
(Arbitrary Units)
3
2
1
0
LFD
HFD

B

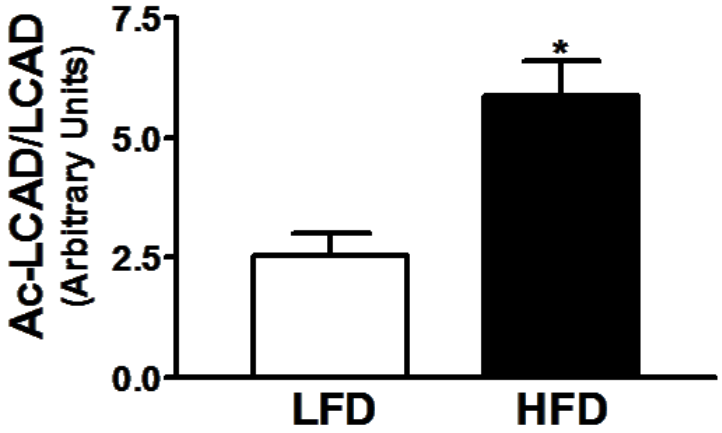
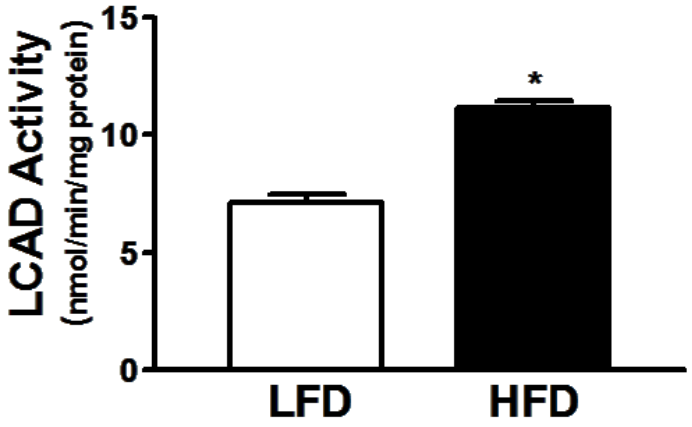

Ac-LCAD/LCAD
(Arbitrary Units)
7.5
5.0
2.5
0.0
*
LFD
HFD

Figure 4-3

C


LCAD Activity
(nmol/min/mg protein)
15
10
5
0
*
LFD
HFD

D

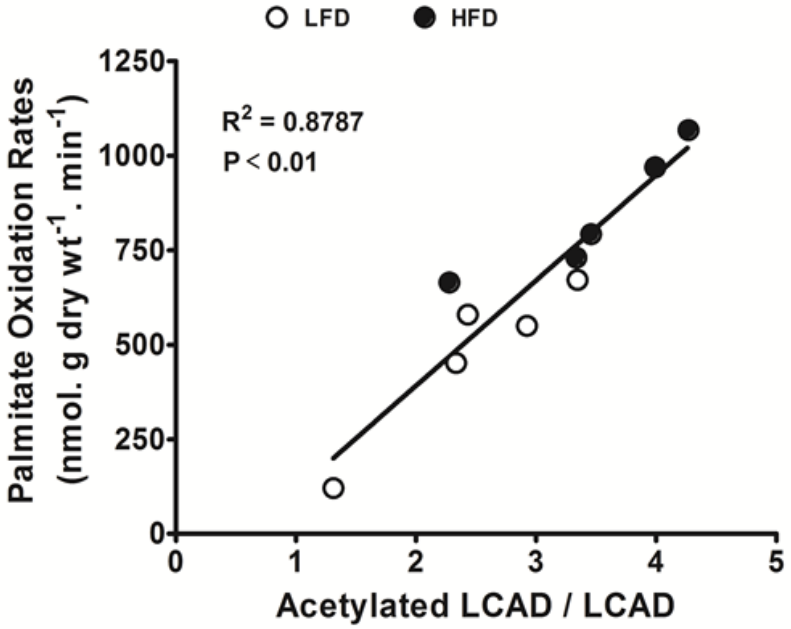
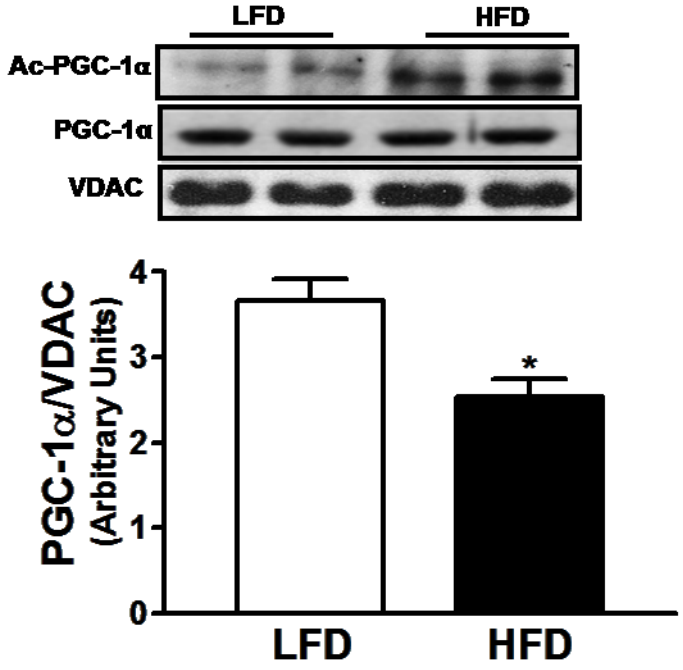

LFD
HFD
Palmitate Oxidation Rates
(nmol. g dry wt-1. min-1)
1250
1000
750
500
250
0
R2 = 0.8787
P < 0.01
0
1
2
3
4
5
Acetylated LCAD / LCAD

Figure 4-3

Figure 4-4: Level of PGC-1α acetylation and its downstream target protein expression in non-perfused hearts from obese mice on a HFD.

A) PGC-1α expression. **B)** Level of PGC-1α acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGC-1α. Protein expression of PPARα **(C)**, CS **(D)**, and complex I **(E)** in hearts from mice fed either a LFD or HFD. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
Ac-PGC-1α
PGC-1α
VDAC
PGC-1α/VDAC
(Arbitrary Units)
0
1
2
3
4
*
LFD
HFD

B

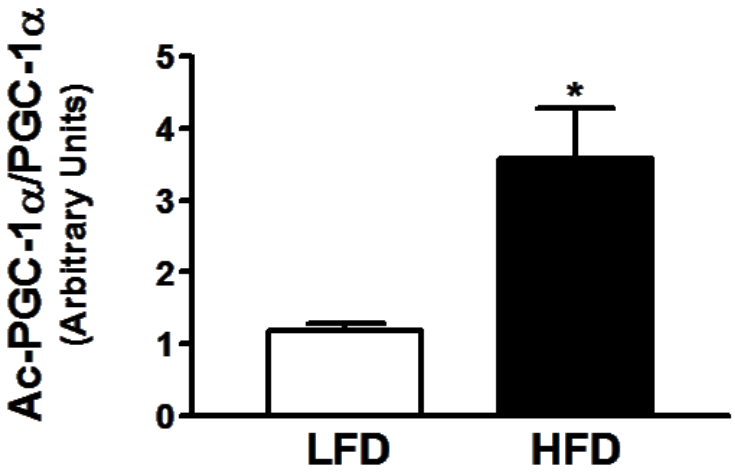
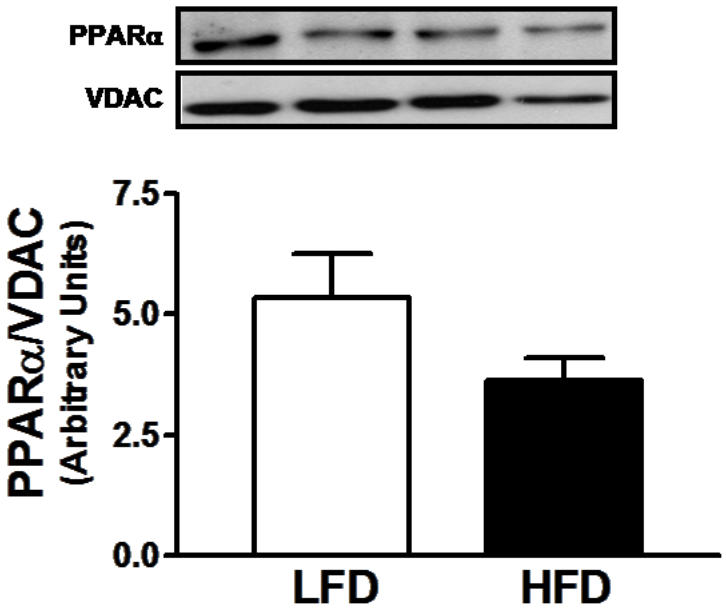

Ac-PGC-1α/PGC-1α
(Arbitrary Units)
0
1
2
3
4
5
*
LFD
HFD

Figure 4-4

C


PPARα
VDAC
PPARα/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

D

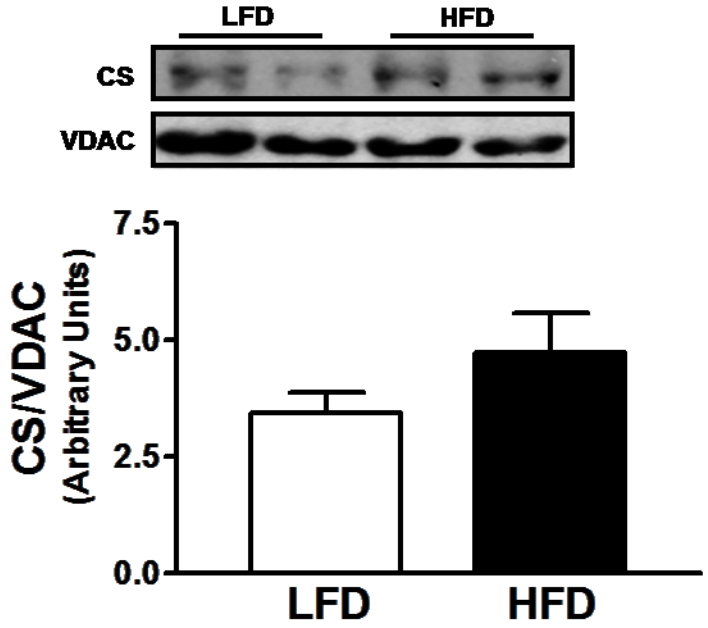

LFD
HFD
CS
VDAC
CS/VDAC
(Arbitrary Units)
7.5
5.0
2.5
0.0
LFD
HFD

Figure 4-4

E

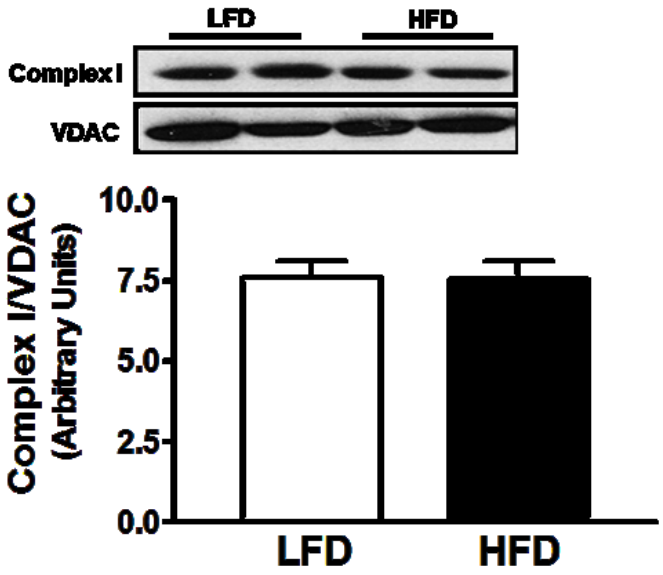

LFD
HFD
Complex I
VDAC
Complex I/VDAC (Arbitrary Units)
10.0
7.5
5.0
2.5
0.0
LFD
HFD

Figure 4-4

Figure 4-5: Glucose oxidation rates and PDH acetylation levels in hearts from obese mice on a HFD.

A) Glucose oxidation rates in hearts from mice fed either a LFD or HFD, in the presence (+) or absence (-) of insulin. **B)** PDH expression. **C)** Level of PDH acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-lysine antibodies and then immunoblotted with antibodies specific for PDH. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

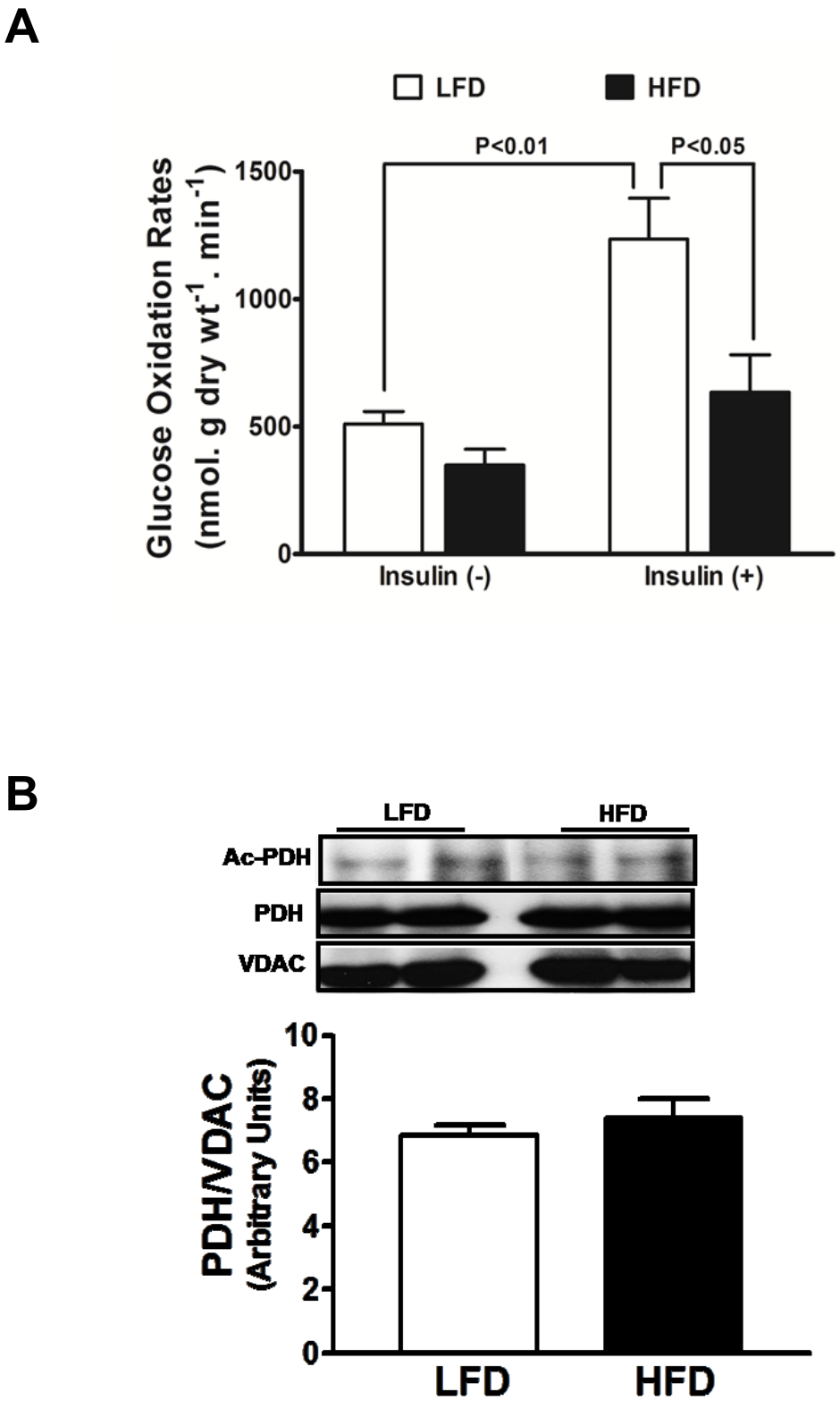

A
LFD
HFD
P<0.01
P<0.05
Glucose Oxidation Rates
(nmol. g dry wt-1. min-1)
1500
1000
500
0
Insulin (-)
Insulin (+)
B
LFD
HFD
Ac-PDH
PDH
VDAC
PDH/VDAC
(Arbitrary Units)
10
8
6
4
2
0
LFD
HFD

Figure 4-5

C

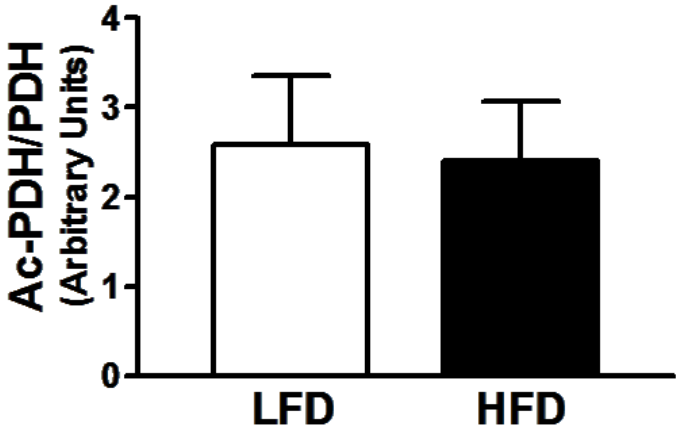
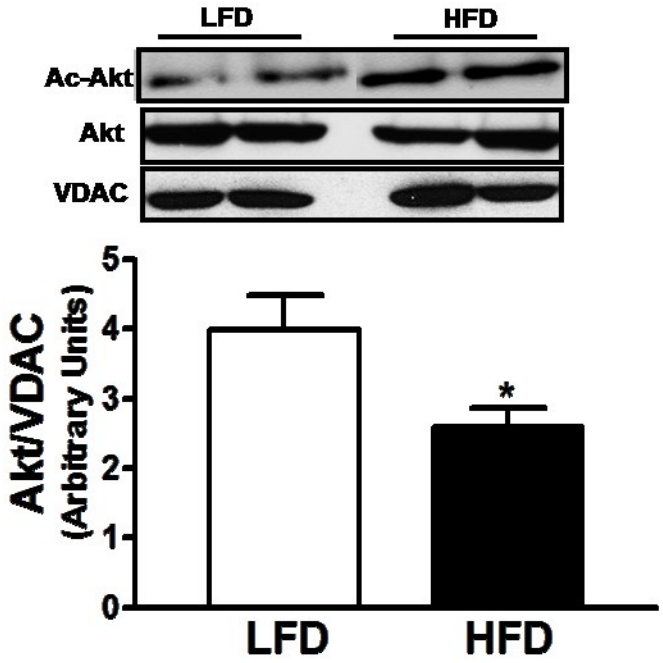


Figure 4-5

Figure 4-6: Effect of chronic HFD feeding on Akt acetylation and activity.

A) Akt expression. **B)** Level of Akt acetylation. Total lysate from non-perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for Akt. **C)** Level of Akt phosphorylation at Ser407 residue. **D)** Level of GSK3β phosphorylation at Ser9 residue. Values represent mean ± SEM. (n=5/group). *P<0.05, significantly different from LFD.

A


LFD
HFD
Ac-Akt
Akt
VDAC
Akt/VDAC
(Arbitrary Units)
5
4
3
2
1
0
*
LFD
HFD

B

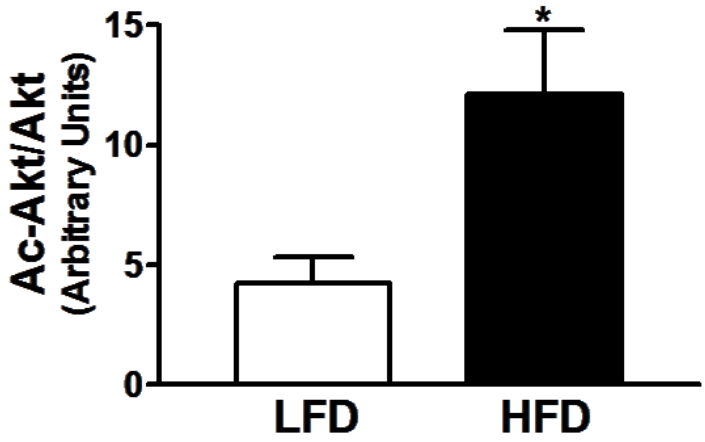
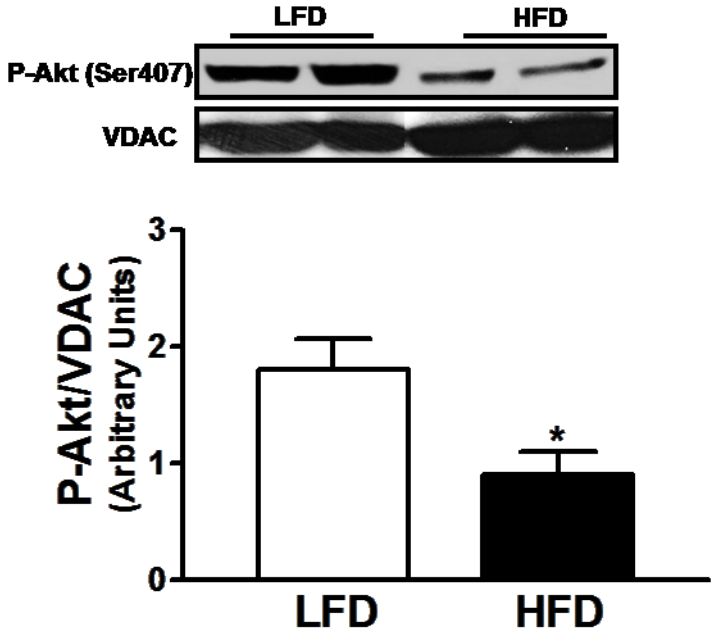

Ac-Akt/Akt
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

Figure 4-6

C


LFD
HFD
P-Akt (Ser407)
VDAC
P-Akt/VDAC
(Arbitrary Units)
3
2
1
0
*
LFD
HFD

D

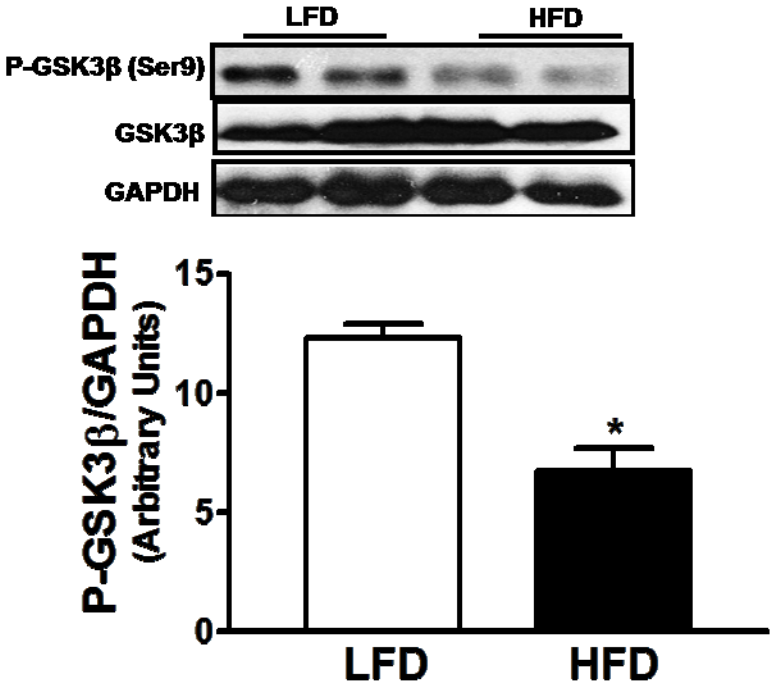

LFD
HFD
P-GSK3β (Ser9)
GSK3β
GAPDH
P-GSK3β/GAPDH
(Arbitrary Units)
15
10
5
0
*
LFD
HFD

Figure 4-6

Table 4-3: Effects of SIRT3 deletion on cardiac function and cardiac metabolic rates

Isolated working hearts from wild type (WT) and SIRT3 KO mice were perfused for a 60 min period for measurement of cardiac function, palmitate oxidation, and glucose oxidation. Rates of glucose and palmitate oxidation were determined as described in experimental methods. Values represent mean±SEM. *P<0.05, significantly different from WT.

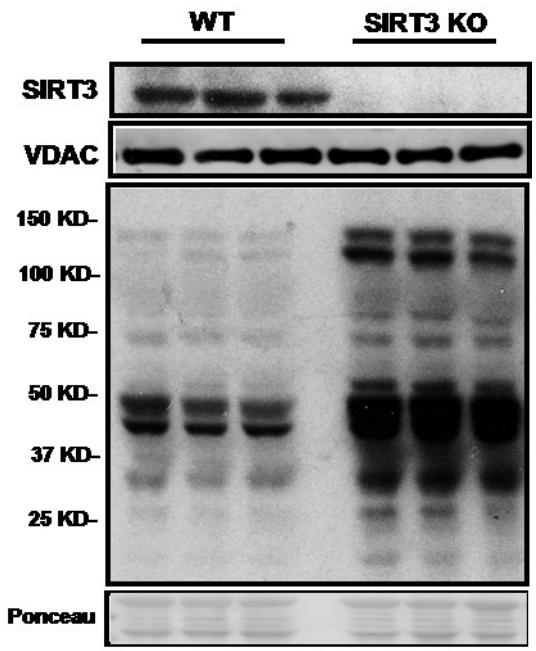
	WT n=8	SIRT3 KO n=8
	Cardiac function	
Heart rate (beat/min)	313±4	272±15
Cardiac output (ml/min)	9.6±0.4	8.7±0.5
Cardiac work (joules/min/g dry wt)	1.8±0.1	1.8±0.1
	Oxidative rates (nmol. g dry wt^{-1}.min^{-1})	
Palmitate Oxidation	291±17	422±29*
Glucose Oxidation	1983±174	1262±121*
	Oxidative rates normalized for work (nmol. g dry wt^{-1}.mjoules)	
Palmitate Oxidation	158±16	240±32*
Glucose Oxidation	1100±99	712±62*

Table 4-3

Figure 4-7: Effects of SIRT3 deletion on cardiac fatty acid β-oxidation.

A) Total protein acetylation in isolated working hearts from WT and SIRT3 KO mice. **B)** Palmitate oxidation rates. **C)** Acetylated β-HAD (Ac-β-HAD) normalized to its level of expression. **D)** β-HAD enzyme activity. **E)** Acetylated LCAD (Ac-LCAD) normalized to its level of expression. **F)** LCAD activity. Values represent mean ± SEM. (n=7/group). *P<0.05, significantly different from WT.

A


WT
SIRT3 KO
SIRT3
VDAC
150 KD-
100 KD-
75 KD-
50 KD-
37 KD-
25 KD-
Ponceau

B

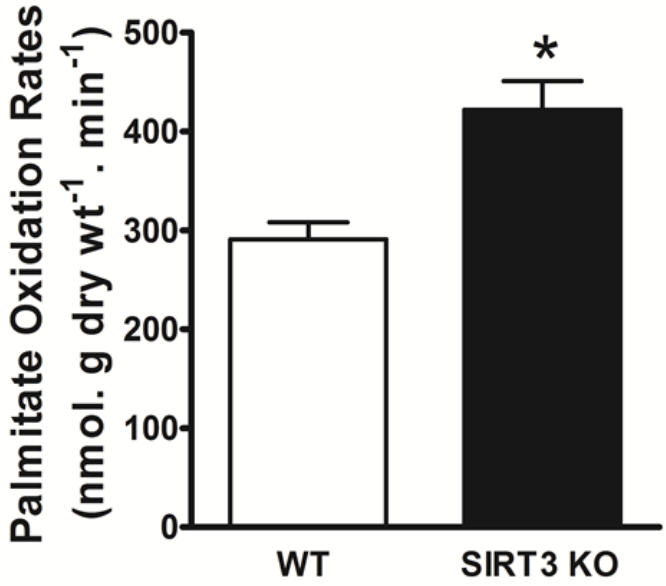
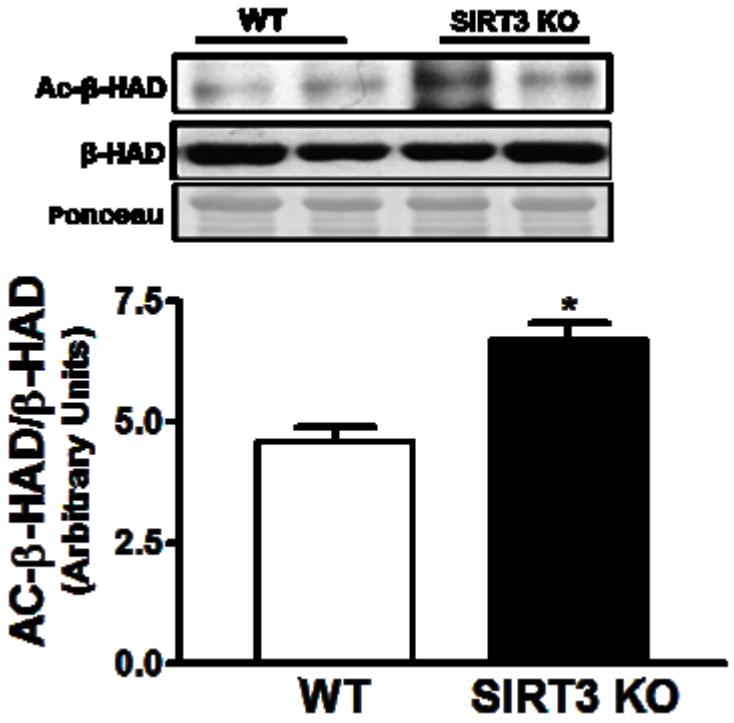

Palmitate Oxidation Rates
(nmol. g dry wt-1. min-1)
500
400
300
200
100
0
*
WT
SIRT3 KO

Figure 4-7

C


WT
SIRT3 KO
Ac-β-HAD
β-HAD
Ponceau
AC-β-HAD/β-HAD
(Arbitrary Units)
7.5
5.0
2.5
0.0
*
WT
SIRT3 KO

D

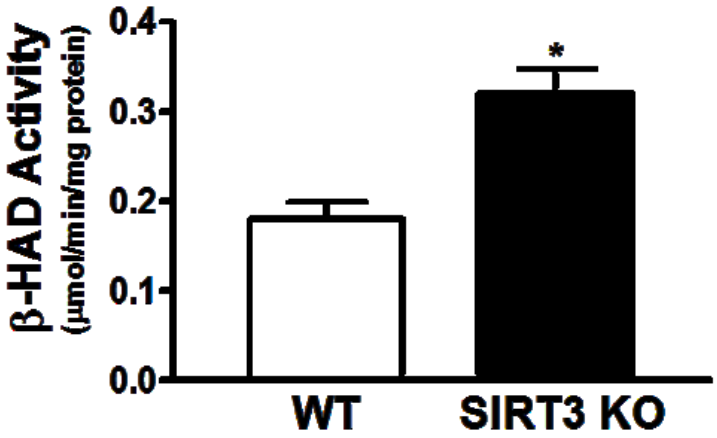
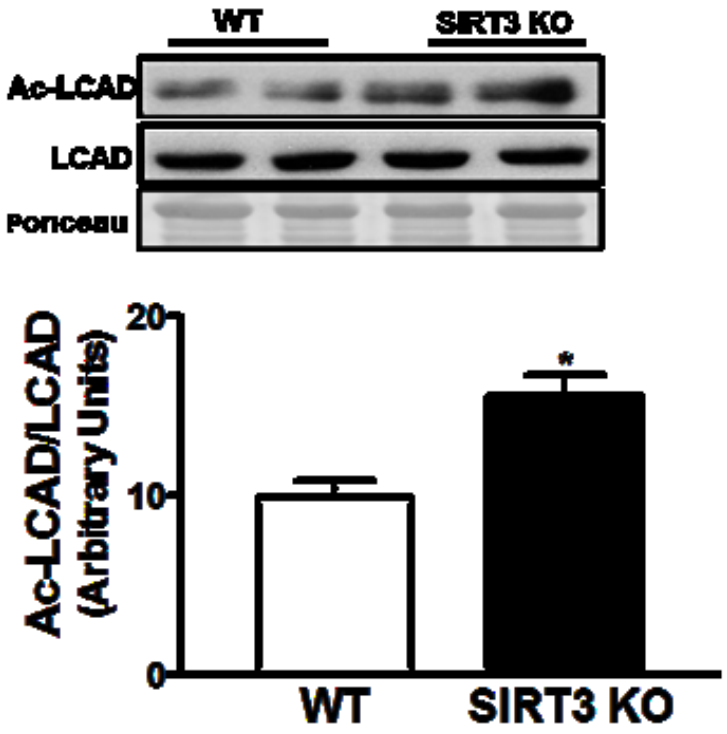

β-HAD Activity
(μmol/min/mg protein)
0.4
0.3
0.2
0.1
0.0
*
WT
SIRT3 KO

Figure 4-7

E


WT
SIRT3 KO
Ac-LCAD
LCAD
Ponceau
Ac-LCAD/LCAD (Arbitrary Units)
20
10
0
*
WT
SIRT3 KO

F

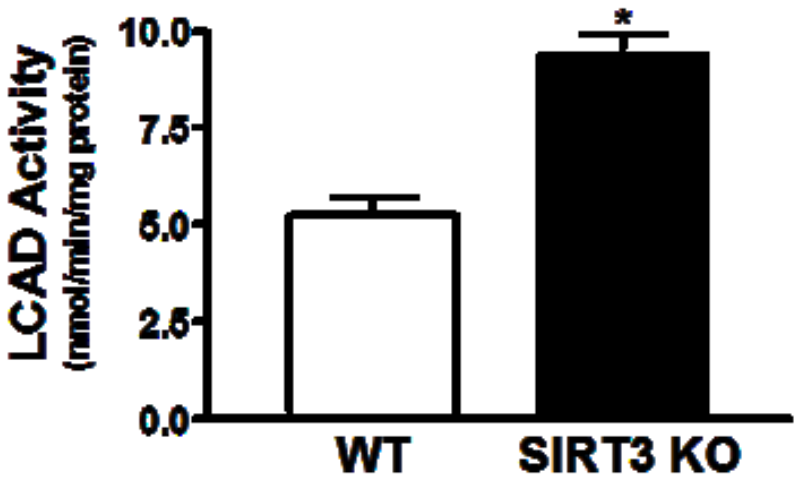

LCAD Activity (nmol/min/mg protein)
10.0
7.5
5.0
2.5
0.0
*
WT
SIRT3 KO

Figure 4-7

Figure 4-8: Glucose oxidation rates and pyruvate dehydrogenase (PDH) acetylation levels in hearts from SIRT3 deficient mice.

A) Glucose oxidation rates in isolated working hearts from WT or SIRT3 KO mice, in the presence (+) or absence (-) of insulin. **B)** PDH expression. **C)** Level of PDH acetylation. Total lysate from perfused hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PDH. Values represent mean ± SEM. (n=7/group). *P<0.05, significantly different from same group in response to inulin.

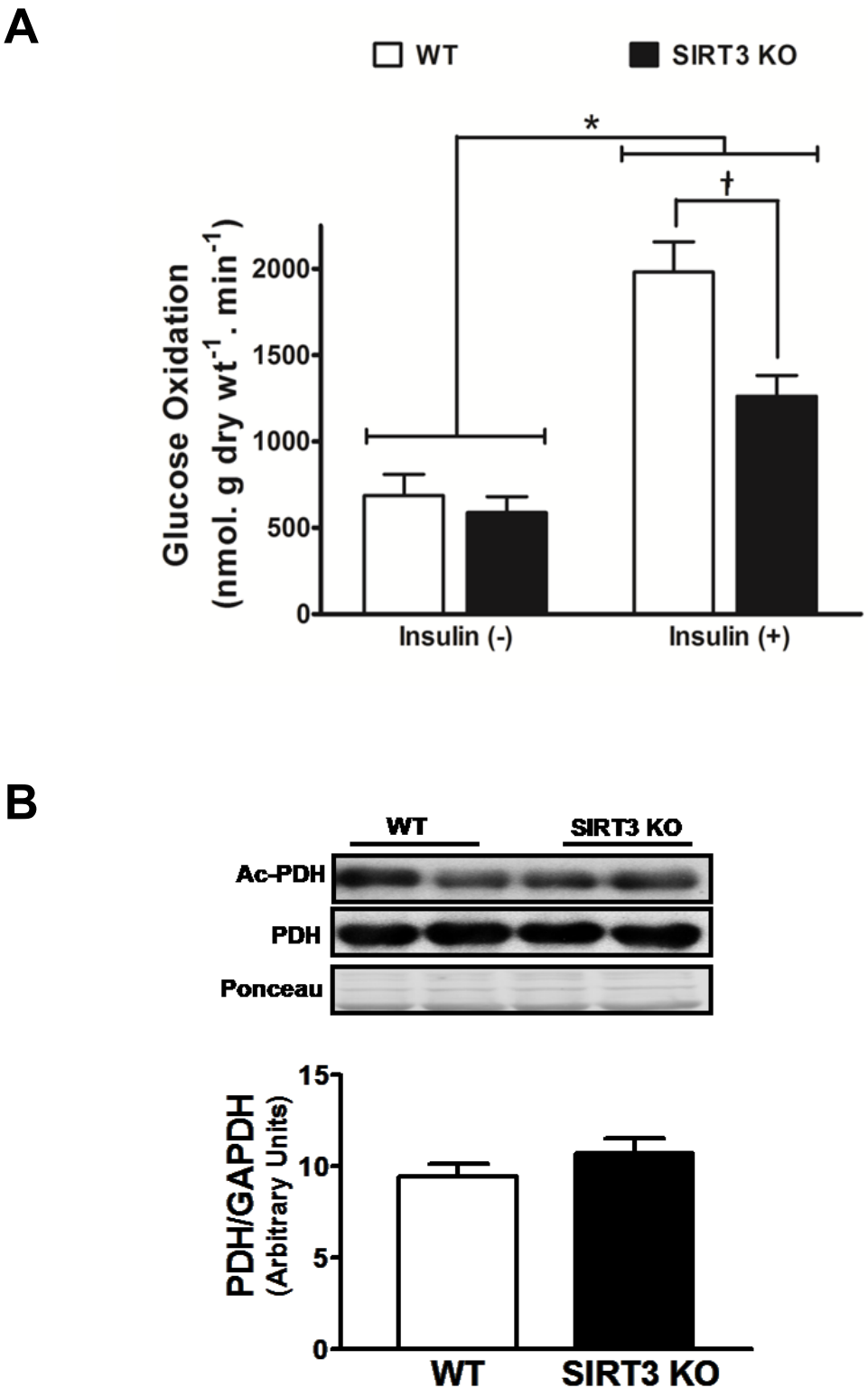

A
WT
SIRT3 KO
*
†
Glucose Oxidation
(nmol. g dry wt-1. min-1)
2000
1500
1000
500
0
Insulin (-)
Insulin (+)
B
WT
SIRT3 KO
Ac-PDH
PDH
Ponceau
PDH/GAPDH
(Arbitrary Units)
15
10
5
0
WT
SIRT3 KO

Figure 4-8

C

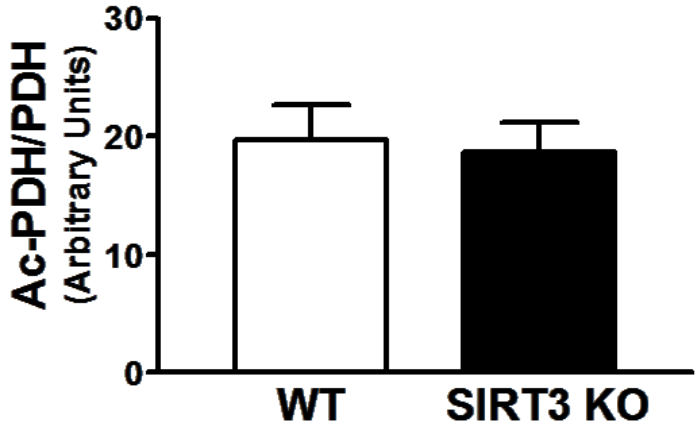


Figure 4-8

Figure 4-9: Model for the role of SIRT3 in regulation of cardiac energy metabolism.

A) In normal condition, cardiac SIRT3 expression is abundant and leads to deacetylation of the fatty acid β-oxidation enzymes, β-HAD and LCAD. This is associated with phosphorylation of Akt and GSK3β resulting in enhanced insulin sensitivity. **B)** In contrast, decreased cardiac SIRT3 expression by high fat feeding or genetic deletion leads to hyperacetylation and activation of β-HAD and LCAD which results in increased fatty acid β-oxidation. Increased acetylation of Akt inhibits its ability to phosphorylate GSK3β and impairs insulin sensitivity.

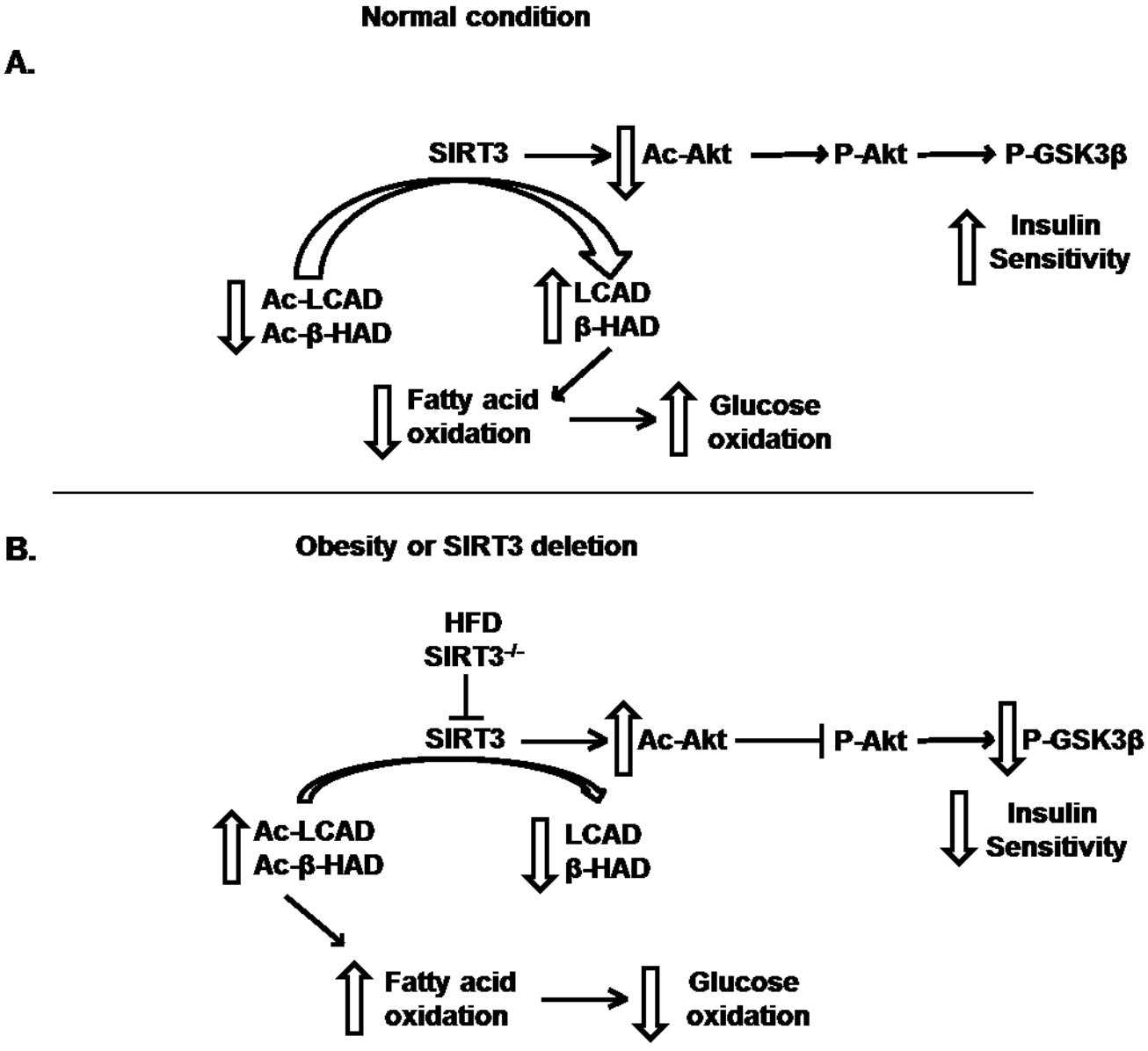

A.
Normal condition
SIRT3
Ac-Akt
P-Akt
P-GSK3β
Insulin Sensitivity
Ac-LCAD
Ac-β-HAD
LCAD
β-HAD
Fatty acid oxidation
Glucose oxidation
B.
Obesity or SIRT3 deletion
HFD
SIRT3-/-
SIRT3
Ac-Akt
P-Akt
P-GSK3β
Insulin Sensitivity
Ac-LCAD
Ac-β-HAD
LCAD
β-HAD
Fatty acid oxidation
Glucose oxidation

Figure 4-9

4.4 DISCUSSION

This study identified a number of key roles for lysine acetylation in the control of cardiac energy metabolism and insulin signaling. First, obesity is associated with an overall increase in acetylation of cardiac proteins, with a concomitant decrease in SIRT3 expression. Second, the increase in fatty acid oxidation rates observed in hearts from obese mice is accompanied by an increased acetylation and activity of the fatty acid oxidation enzymes LCAD and β-HAD, demonstrating that the acetylation of these enzymes increases rather than decreases flux through the fatty acid oxidative pathway. Third, the marked cardiac insulin resistance observed in hearts from obese mice is associated with an increased acetylation and decreased phosphorylation/activity of Akt. Fourth, SIRT3 deletion increases cardiac fatty acid oxidation, which is accompanied by a significant decrease in glucose oxidation. Finally, SIRT3 deletion induces hyperacetylation and activation of the fatty acid β-oxidation enzymes, β-HAD and LCAD. Collectively, these data demonstrate that lysine acetylation has an important role in regulating fatty acid β-oxidation in the heart and that increased acetylation of fatty acid oxidative enzymes contributes to the excessive reliance of the heart on fatty acid β-oxidation in obesity.

Post-translational lysine acetylation has emerged as a potentially important mechanism for controlling many energetic pathways (165,200).

Lysine acetylation occurs on a large number of enzymes involved in mitochondrial metabolism and glycolysis (191,192). Mitochondrial metabolism, is regulated by lysine acetylation, with SIRT3 being an important enzyme involved in the mitochondrial deacetylation process (194). A key role for SIRT3 in mitochondrial electron transport chain activity and TCA cycle activity is evident. For instance, complex I and complex II in the respiratory chain are hyperacetylated and inactivated in $SIRT3^{-/-}$ mice (204,205). In addition, SIRT3 deacetylates and activates TCA cycle enzymes, such as isocitrate dehydrogenase, during caloric restriction (199). Another important target of SIRT3 is the fatty acid β-oxidation pathway, with both LCAD and β-HAD being activated by acetylation. However, conflicting results have been reported that this post translational modification inhibits enzyme activity (196,200,206). The increase in overall acetylation and decrease in SIRT3 we observed in obese mice hearts is also consistent with other studies. Chronic HFD feeding and diabetes, both of which are associated with high fatty acid oxidation rates (61,88,207,243), lead to a decrease in SIRT3 protein expression (11, 24, 41) a result also seen in our study. Overall protein acetylation is also increased in livers from mice fed a HFD (196,197). Recently, lysines 318/322 located near the active site of LCAD were identified as a SIRT3 target (206). Interestingly, we show that LCAD is hyperacetylated and activated in hearts from HFD fed mice. Because fatty acid β-oxidation is enhanced in hearts from mice subjected to HFD, our

result suggest that acetylation of LCAD correlates positively with an increase, rather than a decrease, in fatty acid oxidation. In addition, the acetylation and activity of β-HAD, another enzyme of the fatty acid β-oxidation pathway is also enhanced in HFD obese mice. Collectively, these results suggest a stimulatory effect of lysine acetylation on enzymes involved in fatty acid β-oxidation.

Elevation in circulating levels of free fatty acids and triacylglycerol seen in obesity contributes to an increase in myocardial fatty acid uptake and fatty acid β-oxidation, which can potentially lead to the accumulation of lipid metabolites that perturbs insulin signaling (89). Furthermore, high fatty acid β-oxidation rates can decrease cardiac efficiency, and therefore could have important consequences in insulin-resistant hearts, as well as in the setting of obesity where the overall rates of fatty acid utilization are markedly increased (58,98,243). Our data implicates post-translational regulation of fatty acid oxidative enzymes as a contributing factor to the high fatty acid β-oxidation rates in obesity. As a result, decreasing acetylation status of fatty acid oxidative enzymes has potential therapeutic approach to decreasing fatty acid oxidation in obesity and diabetes. In support of this, we demonstrate that increasing acetylation via deletion of SIRT3 does increase fatty acid β-oxidation in the heart at the expense of glucose oxidation. While this was not associated with any functional deficit in the heart, it is possible that in times of stress, this increase in fatty acid oxidation may contribute to cardiac pathology. It is clear that

excessive fatty acid β-oxidation, which inhibits glucose oxidation, can contribute to cardiovascular disease and induce cardiac insulin resistance (1,2,59,88,89,207). In support of this, previous studies have shown that increasing SIRT3 can decrease cardiac hypertrophy and protect hearts from oxidative stress injury (198,225).

GCN5L1 as a component of the mitochondrial acetyltransferase machinery plays an important role in modulating the acetylation levels of various mitochondrial enzymes. In our study, GCN5L1 protein expression was unaltered by the type of diet. This result suggests that decreased activity of the mitochondrial deacetylase, SIRT3, is primarily responsible for the increased mitochondrial enzyme acetylation under HFD feeding conditions, including LCAD and β-HAD acetylation (159,194,200). Our data provides evidence that HFD feeding decreases SIRT3 expression and enhances acetylation and activity of enzymes involved in fatty acid oxidation.

Previous studies have shown that acetylation of PGC-1α represses its ability to function as a transcriptional coactivator (140,171,178). We found that PGC-1α was robustly acetylated in obese HFD fed mice. This enhanced acetylation of PGC-1α could have potentially contributed to impaired mitochondrial biogenesis and dysregulation of mitochondrial function seen in obesity (173,251). While SIRT1 is a key activator of PGC-1α (171), we did not find any changes in SIRT1 expression between HFD and LFD mice experimental groups. Previous study has reported that

SIRT1 is not required for PGC-1α deacetylation and importantly that GCN5 acetyltransferase activity is the key regulator of PGC-1α acetylation (252). Interestingly, we found a significant increase in SIRT6 expression in obese HFD mice. Although not detected in the current study, recent results show that SIRT6 can deacetylate and activate GCN5 thereby increasing PGC-1α acetylation (179). Thus; this raises the possibility that SIRT6 can deacetylate and activate the nuclear acetyltransferase GCN5 and hence increase PGC-1α acetylation in hearts from HFD fed mice. Interestingly, SIRT6 is able to remove long chain fatty acyl groups from lysine residues (253). Thus, SIRT6-mediated deacylation of enzymes involved in metabolism may represent a potential post-translational regulation of enzyme activities and this process warrants further investigation.

As shown in this study, and as reported previously (1,61,207,88,254), glucose oxidation rates are decreased in hearts from obese mice. Recent studies have shown that the rate-limiting enzyme for glucose oxidation, PDH, is also under acetylation control. We therefore determined whether PDH acetylation was altered in hearts of obese HFD fed mice. We found that the acetylation level of PDH was not altered by a HFD diet. Despite this finding, glucose oxidation rates were markedly decreased in mice fed a HFD compared to LFD fed mice. These results suggest that mechanisms other than PDH acetylation primarily regulate PDH activity under HFD feeding conditions. The inverse relation between

fatty acid and glucose oxidation as described by Randle *et al* at 1963, could be the main determinant for the low glucose oxidation seen in hearts from HFD fed mice. Increases in fatty acid β-oxidation will directly inhibit glucose oxidation in hearts from HFD mice. Moreover, up-regulation of pyruvate dehydrogenase kinase 4 results in hyper-phosphorylation and inactivation of PDH in HFD fed mice (207).

HFD induced obesity contributes to the development of myocardial insulin resistance and dysfunction (61,88). Previous studies by us and others suggest that accumulation of lipid intermediates, such as diacylglycerol and ceramide, enhance the activity of protein kinases that can phosphorylate and inactivate Akt (61,249,255). In this study, we also provide data potentially linking alterations in acetylation to Akt inactivation in hearts from HFD fed mice. The level of Akt acetylation was increased in hearts from HFD obese mice. Recent finding showed that deacetylation of Akt is a prerequisite for its activation, and that activation of SIRT1 will enhance insulin signaling in obesity and diabetes (164-166,244). In addition, SIRT2 overexpression leads to deacetylation and activation of Akt in hepatic cells (256). Hyperacetylation blocked Akt phosphorylation in hearts from HFD mice. To our knowledge, this is the first report demonstrating that HFD induces Akt acetylation and inactivation, which can subsequently lead in part to cardiac insulin resistance.

Cardiac fatty acid β-oxidation rates were significantly increased in SIRT3 KO compared to WT. These results are consistent with a recent

study that reported an increase in palmitate oxidation in skeletal muscles of SIRT3 KO mice compared to WT mice (201). This study suggested that hyperacetylation and inactivation of PDH induces a switch in skeletal muscle energy substrate utilization from glucose to fatty acids (201). However, we did not observe changes in acetylation of PDH. On the other hand, our study suggests that deletion of SIRT3 leads to hyperacetylation and activation of β-HAD and LCAD, which increases cardiac fatty acid oxidation and that occurred primarily at the expense of glucose oxidation (Randle cycle). In support of this, deletion of SIRT4 (which is another mitochondrial deacetylase) was recently shown to increase lipid oxidation in liver and skeletal muscles (216,217). These results challenged the notion that acetylation is always a signal of inhibition.

Our study implicates alterations in enzyme acetylation as having important effects on cardiac energy metabolism. A limitation of our study is that we did not use isolated heart mitochondria to assess acetylation level of enzymes localized in mitochondria, such as β-HAD, LCAD, and PDH. However, mitochondrial isolation takes several hours to be completed. During this time, changes in acetylation/deacetylation of proteins may occur. Unlike phosphatase and kinase inhibitors that can be added during mitochondrial isolation, acetylase inhibitors are not yet available. Therefore, we chose to perform our experiments on whole heart lysates, to limit isolation time. It is important to emphasize, however, that our results

from frozen heart tissue mimicked what was previously found in mitochondrial preparations of liver tissue (196,197,200).

In summary, a decrease in SIRT3 seen following chronic HFD feeding or SIRT3 deletion enhances acetylation of the fatty acid oxidation enzymes, β-HAD and LCAD (Figure 9). This induces a switch in cardiac energy substrate utilization from glucose to fatty acid oxidation. Our results offer new insights into the role of acetylation in regulating cardiac energy metabolism.

CHAPTER 5.

The Role of Lysine Acetylation in Regulating Cardiac Fatty Acid β-Oxidation and Hypertrophy

My role in this work involved performing all experiments (except those noted below), as well as the writing of the manuscript. Cory Wagg perfused the mouse heart, and Sowndramalingam Sankaralingam performed perfusion data analysis. Donna Becker performed the echocardiography for in vivo cardiac functions.

Manuscript Status: This manuscript is in preparation for submission as an original article.

CHAPTER 5.

The Role of Lysine Acetylation in Regulating Cardiac Fatty Acid β-Oxidation and Hypertrophy

Abstract

Aims: Lysine acetylation has emerged as a novel post-translational pathway that regulates the activities of enzymes involved in fatty acid β-oxidation and hypertrophy. We examined whether lysine acetylation controls heart fatty acid β-oxidation and mediators of hypertrophy in pressure overload-induced heart failure.

Methods and results: C57Bl/6J mice (4-wk of age) were subjected to a high fat diet (HFD, 60% kcal from fat) to induce obesity. Heart failure was induced by abdominal aortic constriction (AAC). At 4 wk post-AAC, mice were either switched to a low-fat diet (LFD, 4% kcal from fat; HF AAC LF) or maintained on a HFD (HF AAC HF) for a further 10 wk period. After 18 weeks, HF AAC HF mice weighed significantly more than HF AAC LF mice. Cardiac hypertrophy evident in HF AAC HF mice was associated with SIRT1 mediated inhibition of FoxO1 while activation of FoxO1 and AMPK blunted this effect in HF AAC LF mice. In isolated working hearts, insulin stimulated glucose oxidation rates were also significantly increased in HF AAC LF hearts, compared to HF AAC HF hearts. Activity of the fatty acid β-oxidation enzyme, long chain acyl CoA dehydrogenase (LCAD) was

significantly increased in hearts from HF AAC HF vs HF AAC LF mice, and was associated with LCAD hyperacetylation. Heart failure observed *in vivo* by echocardiography in HF AAC HF was significantly improved in HF AAC LF mice.

Conclusions: We conclude that increased LCAD and FoxO1 acetylation enhances cardiac fatty acid β-oxidation and reduces hypertrophy, respectively.

5.1 INTRODUCTION

Obesity is widely recognized as a risk factor for heart failure (257,258). Obesity is associated with left ventricular hypertrophy and dilatation, features that are known to precede the development of overt heart failure (259,260). For every increase in body mass index (BMI) by 1 the risk of heart failure increases by 5% in men and 7% in women (261). Compared with lean subjects, overweight and obese individuals had a 49% and 180% increased risk of heart failure respectively (262).

One major pathway that is altered in both obesity and heart failure is cardiac energy metabolism (1,89). Insulin resistance states such as obesity and diabetes are associated with dramatic changes in cardiac energy metabolism, which include an increase in fatty acid β-oxidation, and a decrease in glucose oxidation (58,61,89). We previously showed that mice fed a high fat diet developed cardiac insulin resistance that was accompanied by increased cardiac fatty acid β-oxidation and decreased glucose oxidation (61). Similarly *ob/ob* and *db/db* mice that exhibit insulin resistance and obesity have increased cardiac fatty acid β-oxidation and reduced cardiac efficiency (58). Obesity itself markedly decreases glucose oxidation. In obese women, both myocardial fatty acid uptake and oxidation are increased and directly correlate with the degree of insulin resistance (91). Thus obesity and the associated insulin resistance

adversely affect cardiac metabolism and function. Heart failure even in the absence of risk factors such as obesity can also lead to dramatic alterations in cardiac energy metabolism (1). A decrease in energy production and or a decrease in energy efficiency can result in a state of "energetic deficit" in the heart (129,137,263). Using various experimental models of heart failure, we have shown that a decrease in insulin-stimulated glucose oxidation precedes heart failure and that stimulating glucose oxidation can improve both cardiac efficiency and function (59,137,138,264).

Non-histone lysine acetylation is suggested as an important post-translational modification which can regulate cardiac energy metabolism (18-21). Key enzymes of glycolysis, glucose oxidation, fatty acid β-oxidation, and tricarboxylic cycle are acetylated at least on one lysine residue (159,194,191,200). Furthermore, proteins involved in cardiac hypertrophy such as FoxO1 have been shown to be under acetylation control (187,188). However, the impact of this post-translational modification on the activity of key enzymes involved in fatty acid metabolism and hypertrophy is poorly defined. In mitochondria, general control of amino acid synthesis 5 like 1(GCN5L1) is recently characterized as an acetyltransferase which plays important role metabolism (211). Protein deacetylation is mediated by a family of seven enzymes called sirtuins (SIRT1-7) (159,191,194). SIRT3 is suggested as a major mitochondrial deacetylase which controls the activity of key enzymes

involved in glucose and fatty acid β-oxidation (191,194,200). The impact of lysine acetylation on fatty acid β-oxidation in the setting of heart failure has never been addressed before. In addition, SIRT1 plays an important role in cardiac hypertrophy (228,236). The transcriptional factor FoxO1 which play a key role in regulation of cardiomyocyte proliferation and cardiac hypertrophy is a target for SIRT1 deacetylation (187,188). SIRT1 can deacetylate and repress FoxO1 activity (187,188). The purpose of this study is to determine the role of lysine acetylation in regulation of cardiac energy metabolism and the contribution of this post-translational modification to the development of cardiac hypertrophy.

5.2 MATERIALS AND METHODS

Mice received care and were treated according to the guidelines of the Canadian Council on Animal Care, and all procedures performed on animals were approved by the University of Alberta Health Sciences Animal Welfare Committee. GTT and echocardiography were conducted to determine levels of blood glucose and *in vivo* cardiac function, respectively. Hearts isolated from pentobarbital euthanized mice were perfused as isolated working hearts with Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 5 mM [U-^{14}C]glucose, 0.8 mM [9,10-^{3}H]palmitate prebound to 3% albumin, in the presence or absence of 100 μU/mL insulin, as described in details in **chapter 2**. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. Protocols for assessment of LCAD activity, immunoprecipitation, immunoblotting, and statistical analysis are described in details in **chapter 2** of this thesis.

5.3 RESULTS

5.3.1 Body weight and glucose tolerance:

After 8 weeks mice (4-wk of age) fed the standard chow (Low Fat Diet, LFD) weighed 26.1±1.2 g (Figure 5-1A). HFD fed mice weighed significantly more than LFD fed mice (34.8±2.0 g, $P<0.05$). HF AAC HF and HF AAC LF mice had similar body weights after 8 weeks of HFD (35.7±1.4 vs. 36.0±2.2 g). Although mice were randomly assigned to the HF AAC LF sub-group after 8 weeks from the HF AAC group, data from this subgroup of mice are presented separately at 8 weeks to demonstrate that the parameters are similar at 8 weeks before being switched to LFD. In our study this process of randomization has removed any selection bias. After 18 weeks HFD fed mice had a significantly higher body weight compared to LFD fed mice (48.3±1.5 vs. 31.2±1.2 g, $P<0.001$). Interestingly, HF AAC mice switched to LFD (HF AAC LF) after 8 weeks, did not lose but maintained their body weight and was significantly lower compared to HF AAC HF mice (43.5±1.9 vs. 35.1±1.6 g, $P<0.01$).

To analyze the response of mice to a glucose challenge, we performed oral GTT after 8 weeks to confirm the development of impaired glucose tolerance in these mice before switching the diet. After 8 weeks of feeding protocol, the area under the curve (AUC) was significantly higher in all groups of HFD compared to LFD fed mice (Figure 5-1B). The AUC for HFD (56.9±2.6 mmol/l.min), HF AAC HF (58.6±2.1), HF AAC LF

(60.1±2.7 mmol/l.min) was higher compared to LFD fed mice (46.1±1.2 mmol/l.min, P<0.05).

After 18 weeks, HFD fed mice had sustained impaired glucose tolerance (Figure 5-1B). The AUC for HFD fed and HF AAC HF mice (68.7±2.6 and 69.1±3.2 mmol/l.min) was significantly higher (P<0.01) than that of LFD fed mice (44.5±1.4 mmol/l.min). There was a dramatic improvement in glucose tolerance in HF AAC mice switched to LFD. The AUC in the HF AAC LF group was 49.1±1.0 mmol/l.min and was not different from LFD fed mice.

5.3.2 In vivo (echocardiography) analyses of cardiac function:

LFD fed sham mice had normal diastolic and systolic function. AAC induced a diastolic dysfunction in HFD fed mice as early as 2 weeks after the procedure as assessed by an increased E/A ratio (HF AAC; 1.9±0.1vs. 1.5±0.04, p<0.05) compared to baseline (Figure 5-2A). Switching to LFD improves diastolic function as shown by a decrease in E/A ratio 1.6±0.05, P<0.05 that was evident after 18 weeks. Mitral tissue doppler E'/A' ratio and E/E' ratio (marker of left atrial filling pressure) were also measured as markers of diastolic function (Figure 5-2B). Decrease in E'/A' ratio and an increase in E/E' ratio represent diastolic dysfunction. Significant changes in these parameters are noted as early as 2 weeks post-AAC compared to baseline suggesting diastolic dysfunction in HF AAC HF mice. Being more sensitive markers of diastolic function, improvements in E'/A' ratio and

E/E' ratio were observed as early as 6 weeks after switching the HF AAC mice to a LFD even before significant changes in E/A ratio was observed (Figure 5-2C). Systolic function measured as percent ejection fraction was normal in LFD fed sham and HFD fed sham mice (54.8±4.4 and 57.7±3.1%) (Figure 5-2D). AAC induced a small but significant reduction in ejection fraction (45.3±2.3%) compared to baseline (55.3±2.5%, $P<0.05$) in HFD fed mice (Figure 5-2D). Switching to LFD also improved systolic function (51.2±1.6%).

5.3.3 Mechanisms of cardiac hypertrophy in obese heart failure mice:

AAC induced a modest level of cardiac hypertrophy in HFD fed mice compared to LFD and HFD fed sham mice (LV mass, 149.2±5.7 vs. 93.7±2.0 and 117.7±5.5 mg, $P<0.01$) (Figure 5-3A). Switching HF AAC mice to LFD resulted in significantly lower LV mass (122.0±2.9, $P<0.01$). It is important to note that both HF AAC HF and HF AAC LF mice had similar LV mass after 8 weeks. While hearts from both groups of mice continued to hypertrophy, the rate of hypertrophy was blunted in the HF AAC LF mice, and was significant as early as 14 weeks. Hypertrophy assessed by echocardiography was confirmed by measuring heart weights after 18 weeks (Figure 5-3B). We wanted to understand the mechanisms involved in cardiac hypertrophy in HF AAC HF hearts. When compared to HFD fed sham mice, a 2.5-fold increase in SIRT1 expression (Figure 5-3C) was observed that was accompanied by a 50% reduction in

abundance of acetylated FoxO1 (Ac-FoxO1) (inhibition) in hearts from HF AAC HF mice (Figure 5-3D). Inhibition of FoxO1 was accompanied by decrease in downstream atrogin-1, a mediator of muscle atrophy (Figure 5-3E). On the other hand, a decrease in SIRT1 and an increase in acetylation (activation) of FoxO1 were associated with increased atrogin-1 expression which played a role in decreased LV mass and a blunted rate of cardiac hypertrophy observed in HF AAC LF hearts. Activation (phosphorylation) of P38MAPK accompanied the activation of AMPK and has been suggested to increase atrogin-1 expression observed in our study (Figure 5-3F).

In addition to SIRT1, a dramatic increase in PKC-α also contributes to cardiac hypertrophy in HF AAC HF hearts (Figure 5-4A). This could subsequently result in activation (increased phosphorylation) of mTOR and P70S6K ultimately resulting in increased protein synthesis and cardiac hypertrophy in HF AAC HF hearts (Figure 5-4B&C). The decrease in LV mass observed in HF AAC LF was also associated with activation (increased phosphorylation) of AMPK (Figure 5-4D).

5.3.4 Cardiac energy metabolism and insulin resistance:

Insulin stimulated a 2-fold increase in cardiac glucose oxidation in the LFD fed mice (Figure 5-5A). This response was blunted in both HFD fed sham and HF AAC HF mice suggesting insulin resistance. Interestingly, switching to LFD completely reversed insulin resistance that

resulted in a dramatic 3-fold increase in glucose oxidation in the HF AAC LF hearts. In HF AAC LF hearts, the insulin stimulated glucose oxidation was significantly higher than HF AAC HF hearts even after normalizing to cardiac work. Further, in response to insulin, LFD fed sham mice had a significant decrease in cardiac palmitate oxidation (Figure 5-5B) an effect that was blunted in HF AAC HF hearts but reversed in HF AAC LF hearts. In summary, in HF AAC HF hearts with diastolic dysfunction insulin resistance and greater reliance on palmitate oxidation are observed. In contrast, HF AAC LF hearts with improved diastolic function are insulin sensitive and rely heavily on glucose oxidation.

5.3.5 Acetylation control of cardiac metabolic enzymes:

Acetylation as a post-translational modification has gained considerable interest in the regulation of energy metabolism. HFD stimulated a 2-fold increase in GCN5L1 expression compared to LFD fed sham mice and was further increased in the HF AAC HF hearts (Figure 5-6A). HF AAC LF hearts had significantly lower GCN5L1 expression compared to HF AAC HF hearts. However, we did not find changes in SIRT3 expression (Figure 5-6B). Parallel to increased abundance of GCN5L1, acetylation of LCAD was increased in HFD fed sham and HF AAC HF mice (Figure 5-6C). Interestingly, HF AAC LF mice had decreased acetylation of LCAD. LCAD activity increased in parallel to LCAD acetylation (Figure 5-6D). We further show a positive correlation

between abundance of acetylated LCAD and palmitate oxidation rates suggesting that acetylation of LCAD could activate LCAD and therefore stimulate fatty acid β-oxidation in these hearts (Figure 5-6E).

5.3.6 Insulin signaling in failing hearts:

Phosphorylation of Akt was markedly enhanced in obese-heart failure mice switched to LFD (Figure 5-7A). Hearts from HFD fed sham and HF AAC HF mice are insulin resistant as shown by decreased abundance of phosphorylation of GSK-3β (Figure 5-7B). Switching to LFD resulted in enhanced insulin signaling as shown by increased P-Akt and P-GSK3β expression in HF AAC LF group.

Figure 5-1: Total body weight and glucose tolerance for LFD and HFD fed mice.

A) Feeding high fat diet for 18 weeks increases body weight in both HFD fed sham mice and HF AAC HF groups compared to mice fed low fat diet (LFD). Switching HF AAC mice to LFD lowers body weight. **B)** High fat feeding for 18 weeks impairs oral glucose tolerance in mice fed a high fat diet (HFD fed sham and HF AAC HF) in comparison to LFD fed mice. Switching HF AAC mice to LFD dramatically improves glucose tolerance. ** $P<0.01$, *** $P<0.001$ vs. LF Sham; † $P<0.05$ vs. HF AAC HF, †† $P<0.01$ vs. HF AAC HF. Values represent mean ± SEM (n=6).

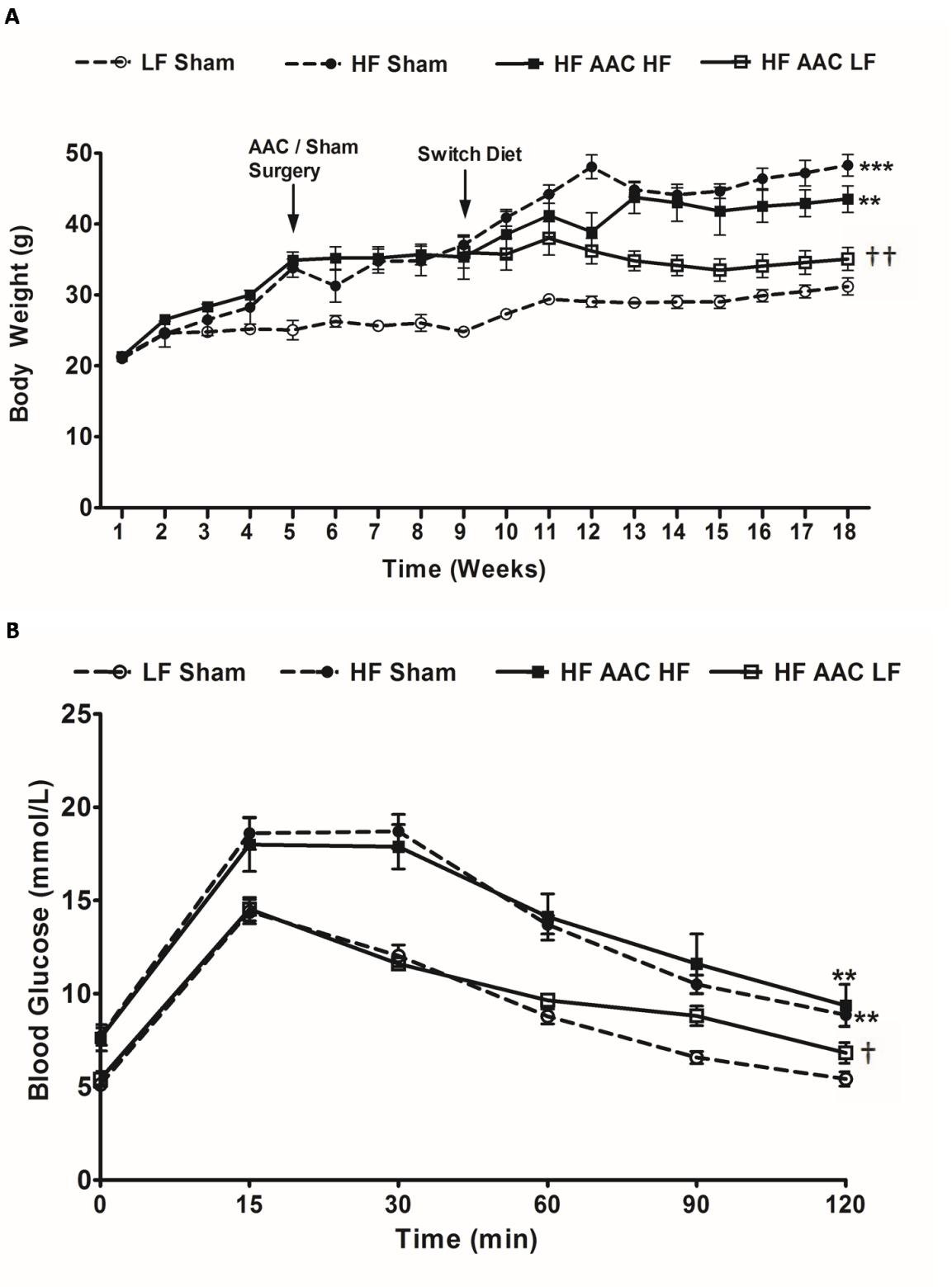
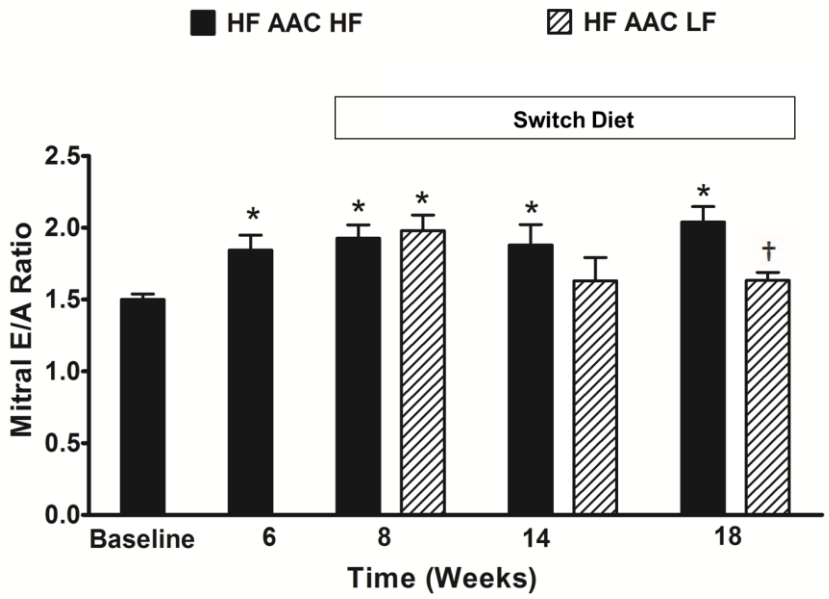

A
LF Sham
HF Sham
HF AAC HF
HF AAC LF
AAC / Sham Surgery
Switch Diet
Body Weight (g)
Time (Weeks)
***
**
††
B
LF Sham
HF Sham
HF AAC HF
HF AAC LF
Blood Glucose (mmol/L)
Time (min)
**
**
†

Figure 5-1

Figure 5-2: In vivo cardiac function as assessed by echocardiography.

A) HF AAC HF mice developed diastolic dysfunction as measured by an increase in E/A ratio that improved when switched to LFD. **B)** HF AAC mice developed diastolic dysfunction as seen by a decrease in E'/A' ratio and the improvement in function in HF AAC LF mice is seen as early as 14 weeks. **C)** HF AAC HF mice developed diastolic dysfunction as seen by an increase in E/E' ratio and the improvement in function in HF AAC LF mice are seen as early as 14 weeks. **D)** Systolic dysfunction in HF AAC HF mice was observed after 14 weeks and improved upon switching to LFD.
† $P<0.05$ vs. HF AAC HF at the same time point. * $P<0.05$, ** $P<0.01$ vs. baseline; Values represent mean ± SEM (n=6).

A


HF AAC HF
HF AAC LF
Switch Diet
Mitral E/A Ratio
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
6
8
14
18
Time (Weeks)

B

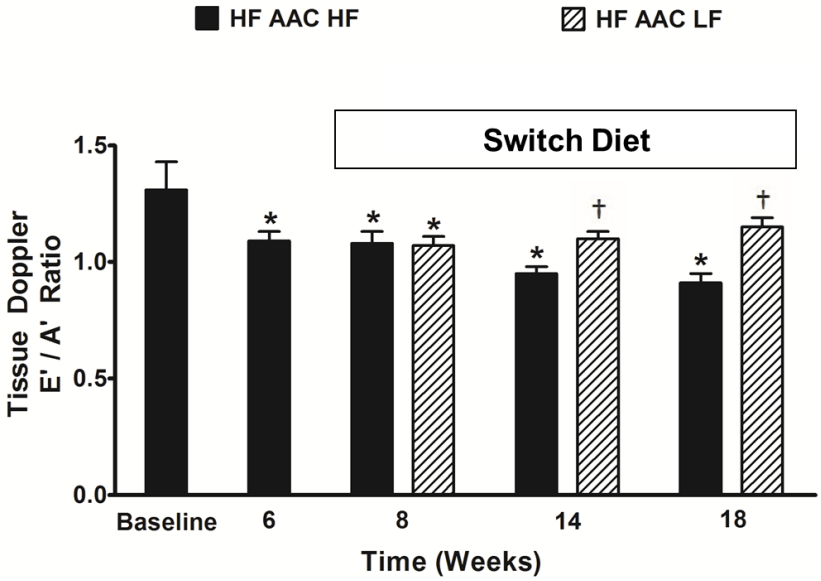
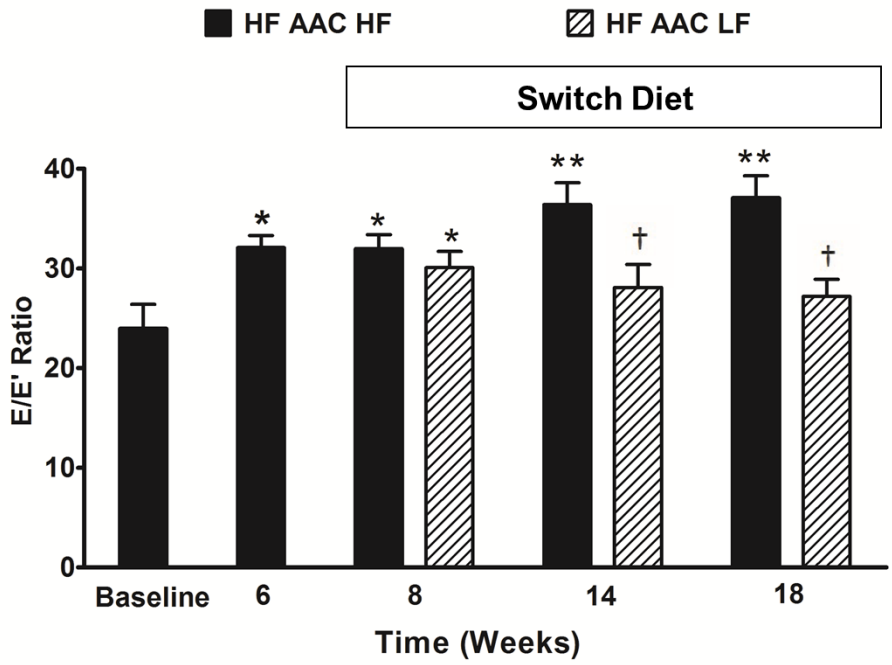

HF AAC HF
HF AAC LF
Switch Diet
Tissue Doppler
E' / A' Ratio
1.5
1.0
0.5
0.0
Baseline
6
8
14
18
Time (Weeks)

Figure 5-2

C


HF AAC HF
HF AAC LF
Switch Diet
E/E' Ratio
40
30
20
10
0
*
*
*
**
†
**
†
Baseline
6
8
14
18
Time (Weeks)

D

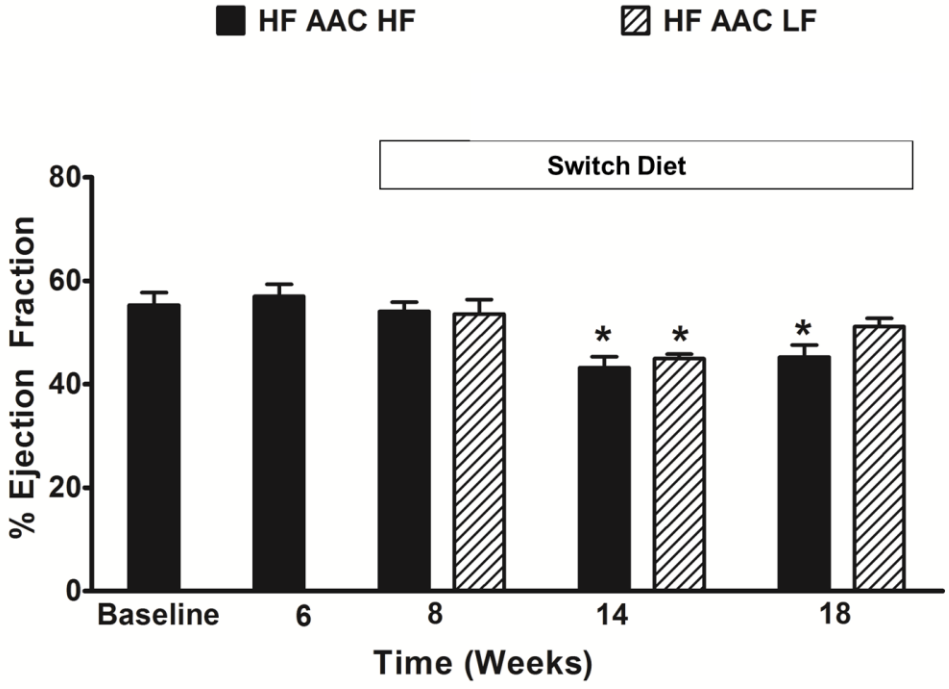

HF AAC HF
HF AAC LF
Switch Diet
% Ejection Fraction
80
60
40
20
0
*
*
*
Baseline
6
8
14
18
Time (Weeks)

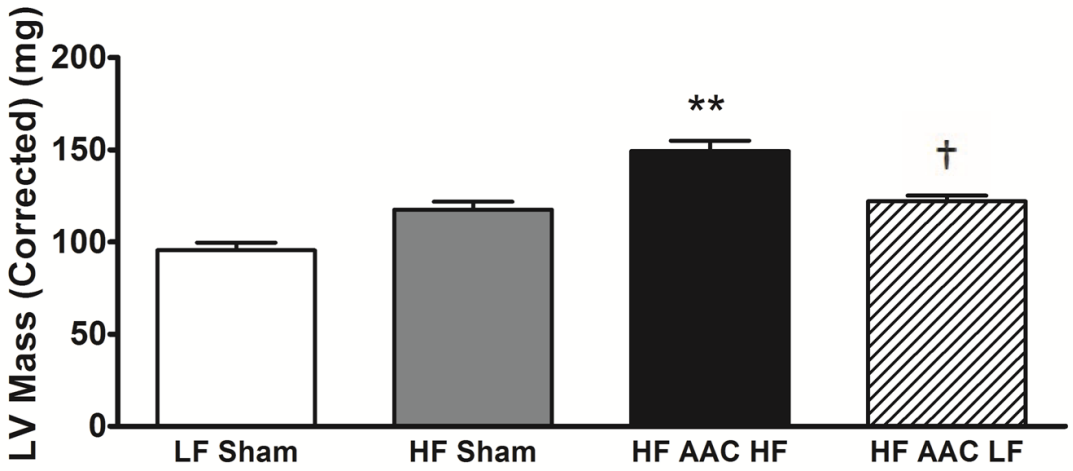
Figure 5-2

Figure 5-3: Mechanisms of cardiac hypertrophy in mice fed a low or high fat diet.

A) Cardiac hypertrophy as measured by an increase in LV mass developed in the HF AAC HF mice that was blunted in the HF AAC LF mice. **B)** The increase in left ventricular mass in HF AAC HF compared to LFD fed sham mice partially resolved on switching to LFD. **C)** HFD decreased while HF AAC HF increased SIRT1 expression in comparison to LFD fed sham mice, an effect that was reversed upon switching obese-heart failure mice to LFD. **D)** In parallel to SIRT1 expression, acetylated (Ac) FoxO1 increased in HFD fed sham mice while the abundance decreased in HF AAC HF. Upon switching to LFD, HF AAC LF mice had increased acetylated (activated) FoxO1. **E)** Atrogin-1 expression was significantly decreased in the HF AAC HF mice, while the expression was increased in HF AAC LF mice. **F)** An increase in P-P38MAPK was observed in HF AAC LF mice in comparison to HF AAC HF mice.

* $P<0.05$ vs. baseline or LF Sham; † $P<0.05$ vs. HF AAC HF; †† $P<0.01$ vs. HF Sham or HF AAC HF (as appropriate); ‡ $P<0.05$ vs. HF AAC HF. Values represent mean ± SEM (n=6).

A


LV Mass (Corrected) (mg)
200
150
100
50
0
**
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

B

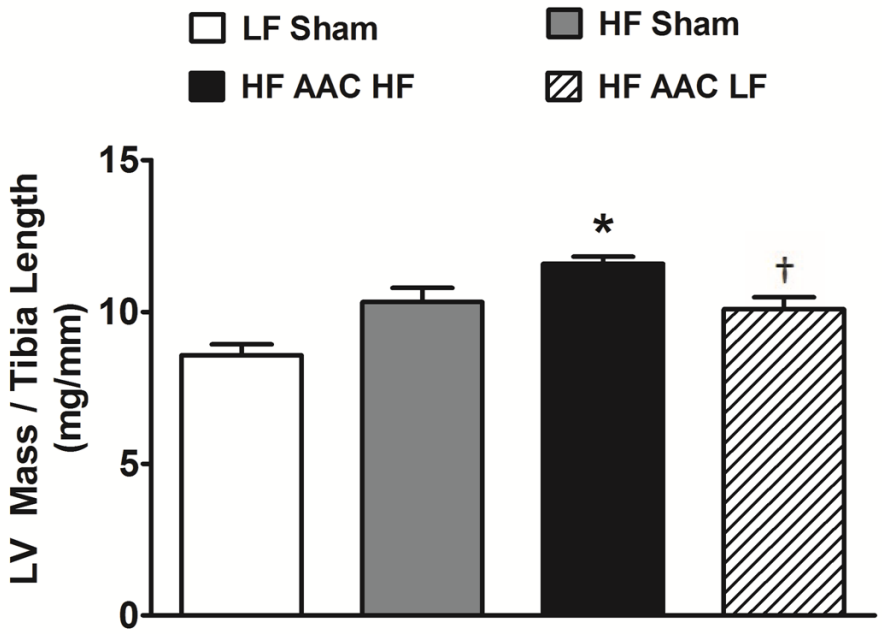
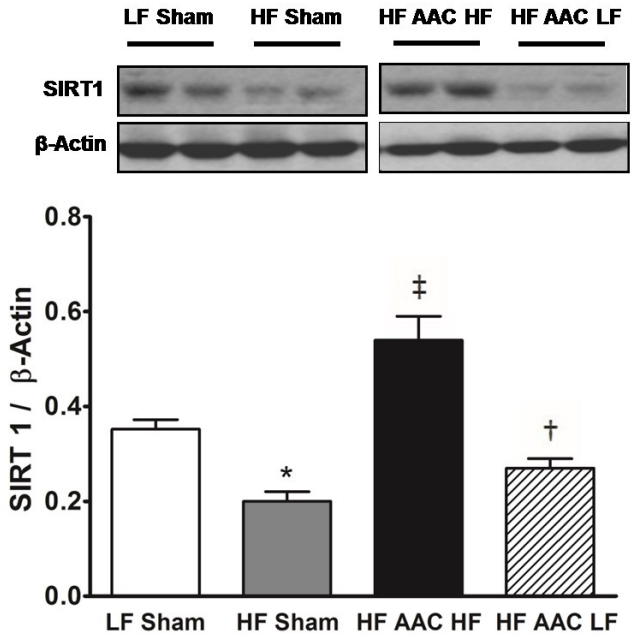

LF Sham
HF Sham
HF AAC HF
HF AAC LF
LV Mass / Tibia Length (mg/mm)
15
10
5
0
*
†

Figure 5-3

C


LF Sham
HF Sham
HF AAC HF
HF AAC LF
SIRT1
β-Actin
SIRT 1 / β-Actin
0.8
0.6
0.4
0.2
0.0
*
‡
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

D

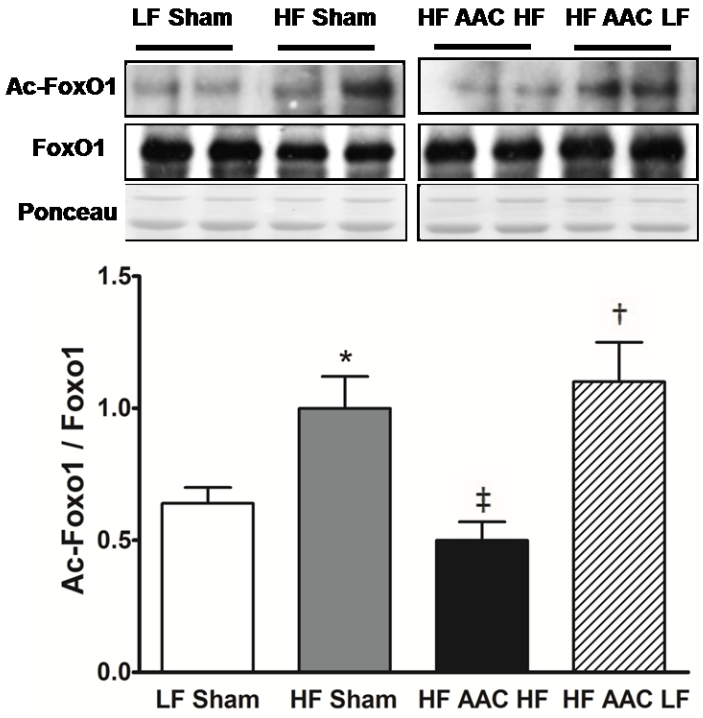
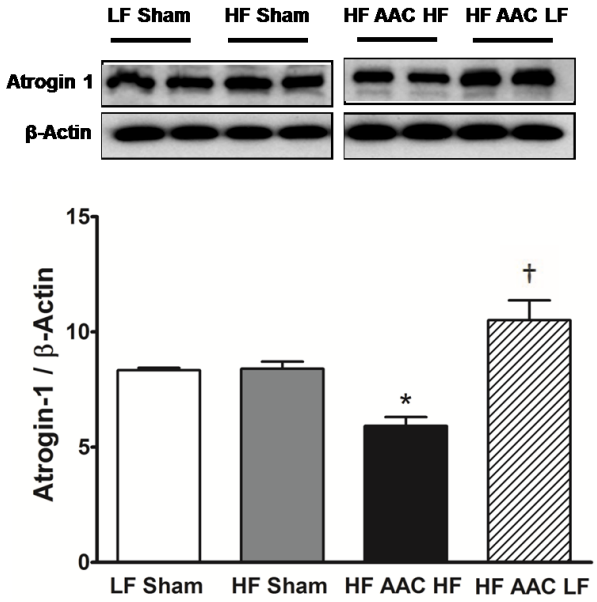

LF Sham
HF Sham
HF AAC HF
HF AAC LF
Ac-FoxO1
FoxO1
Ponceau
Ac-Foxo1 / Foxo1
1.5
1.0
0.5
0.0
*
‡
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

Figure 5-3

E


LF Sham
HF Sham
HF AAC HF
HF AAC LF
Atrogin 1
β-Actin
Atrogin-1 / β-Actin
15
10
5
0
*
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

F

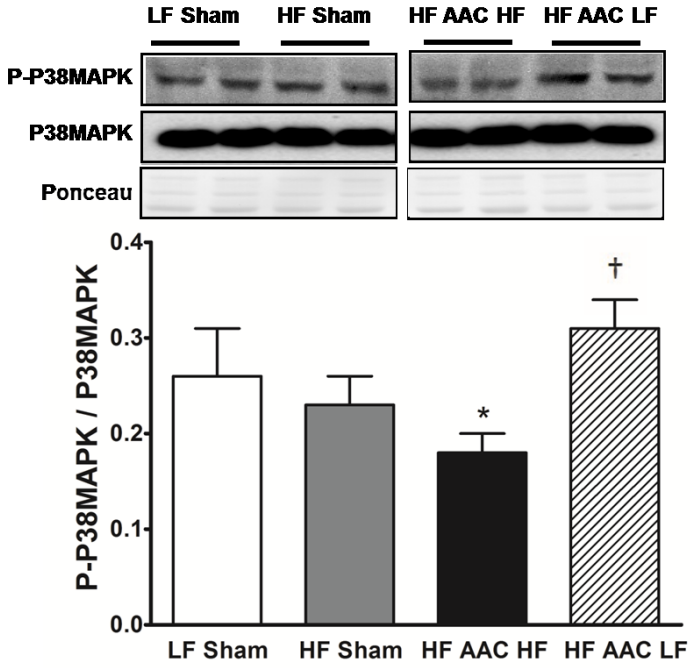

LF Sham
HF Sham
HF AAC HF
HF AAC LF
P-P38MAPK
P38MAPK
Ponceau
P-P38MAPK / P38MAPK
0.4
0.3
0.2
0.1
0.0
*
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

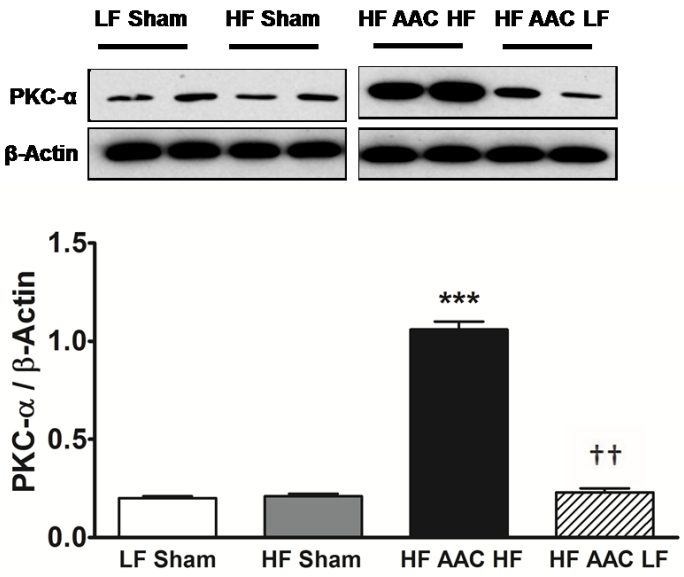
Figure 5-3

Figure 5-4: Contribution of PKC-α-mTOR pathway to cardiac hypertrophy in LFD and HFD fed mice.

A) Dramatic increase in PKC-α is observed in HF AAC HF that is markedly blunted in HF AAC LF. **B)** P-mTOR is increased in HF AAC HF mice. Switching to low fat diet reverses this effect. **C)** The increased P-P70S6K expression in HF AAC HF mice was reversed by switching to low fat diet. **D)** High fat feeding did not affect AMPK. However, phosphorylation of AMPK is increased in HF AAC HF mice switched to low fat diet.

* $P<0.05$, *** $P<0.001$ vs. LF Sham; † $P<0.05$, †† $P<0.01$ vs. HF AAC HF. Values represent mean ± SEM (n=5-6).

A


LF Sham
HF Sham
HF AAC HF
HF AAC LF
PKC-α
β-Actin
PKC-α / β-Actin
1.5
1.0
0.5
0.0
***
††
LF Sham
HF Sham
HF AAC HF
HF AAC LF

B

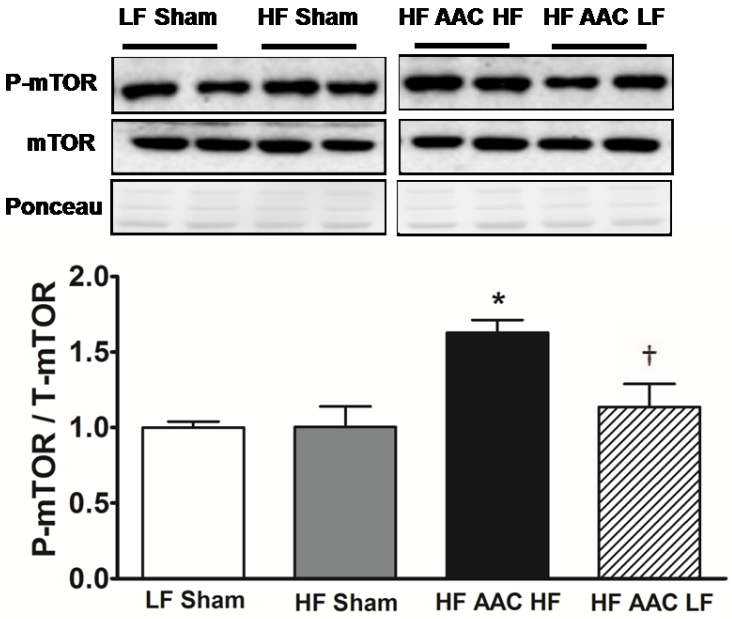
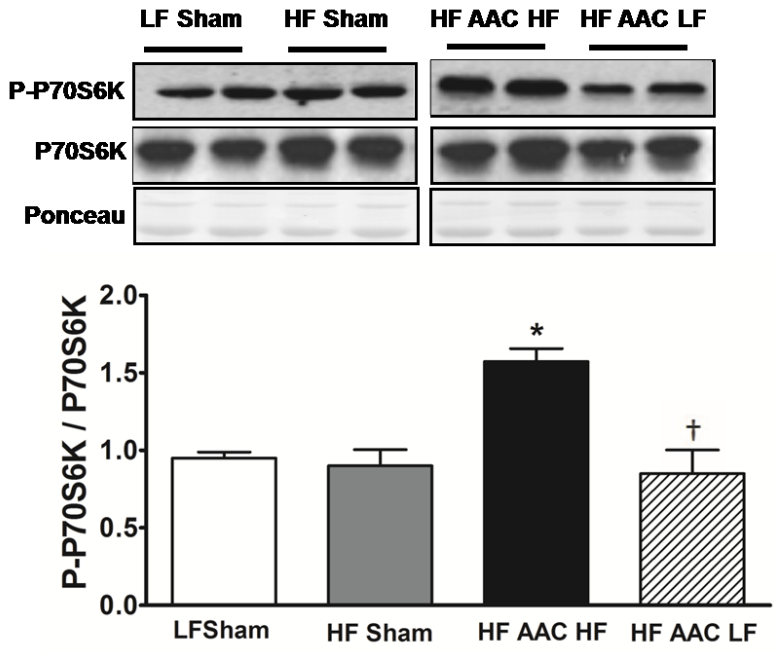

LF Sham
HF Sham
HF AAC HF
HF AAC LF
P-mTOR
mTOR
Ponceau
P-mTOR / T-mTOR
2.0
1.5
1.0
0.5
0.0
*
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

Figure 5-4

C


LF Sham
HF Sham
HF AAC HF
HF AAC LF
P-P70S6K
P70S6K
Ponceau
P-P70S6K / P70S6K
2.0
1.5
1.0
0.5
0.0
*
†
LFSham
HF Sham
HF AAC HF
HF AAC LF

D

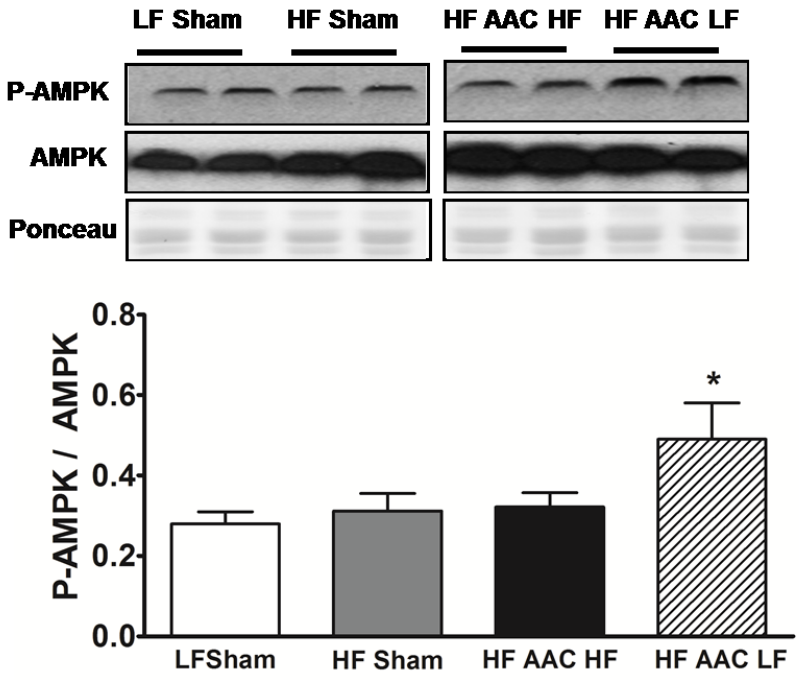

LF Sham
HF Sham
HF AAC HF
HF AAC LF
P-AMPK
AMPK
Ponceau
P-AMPK / AMPK
0.8
0.6
0.4
0.2
0.0
*
LFSham
HF Sham
HF AAC HF
HF AAC LF

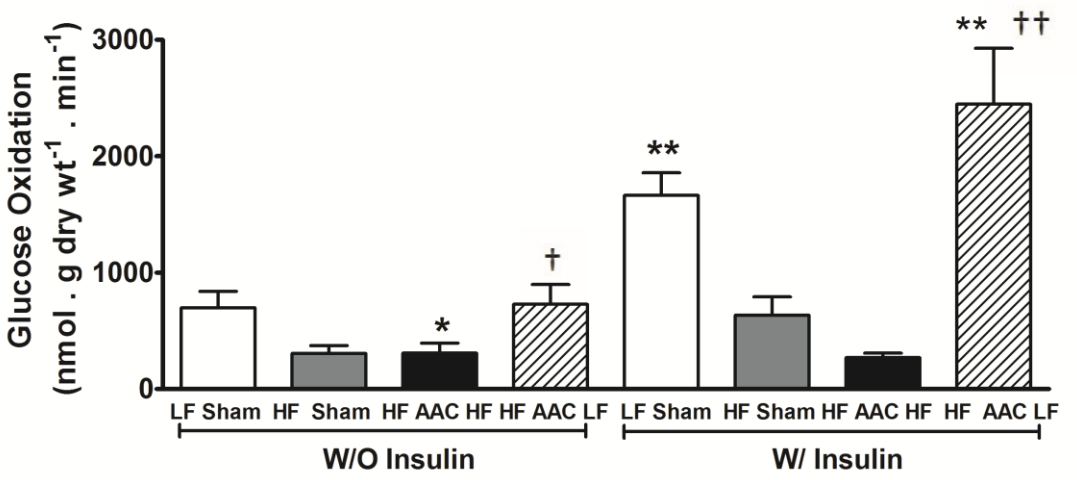
Figure 5-4

Figure 5-5: Cardiac energy metabolism in LFD and HFD fed mice.

A) Insulin stimulated glucose oxidation is increased in the LFD fed sham mice. This response is markedly blunted in the HF AAC HF mice. Switching obese-heart failure mice to LFD results in a dramatic improvement in glucose oxidation. **B)** Insulin induced decrease in palmitate oxidation is blunted in the HF AAC HF mice. Switching to LFD normalizes this effect.

* P<0.05, ** P<0.01 vs. respective group W/O insulin; †† P<0.01 vs. HF AAC HF W/ Insulin. Values represent mean ± SEM (n=6).

A


Glucose Oxidation
(nmol . g dry wt-1 . min-1)
3000
2000
1000
0
*
†
**
** ††
LF Sham HF Sham HF AAC HF HF AAC LF
LF Sham HF Sham HF AAC HF HF AAC LF
W/O Insulin
W/ Insulin

B

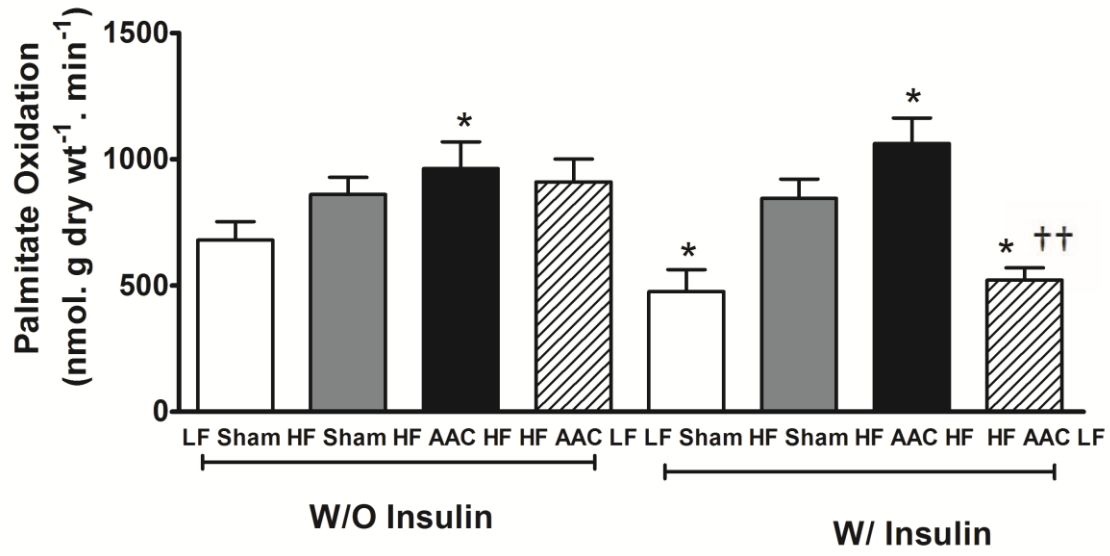

Palmitate Oxidation
(nmol. g dry wt-1 . min-1)
1500
1000
500
0
*
*
*
*
* ††
LF Sham HF Sham HF AAC HF HF AAC LF
LF Sham HF Sham HF AAC HF HF AAC LF
W/O Insulin
W/ Insulin

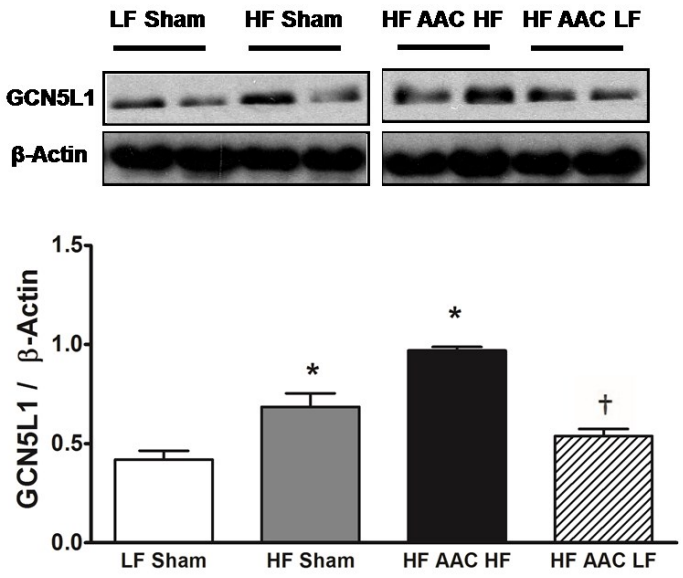
Figure 5-5

Figure 5-6: Level of LCAD acetylation and activity in hearts from LFD and HFD fed mice.

A) HFD increases expression of the acetyl transferase GCN5L1. Switching to LFD reverses this effect. **B)** No change in expression of the deacetylase SIRT3 was observed. **C)** Acetylation of LCAD is increased in response to HFD. A further increase is noted in HF AAC HF mice. Switching to LFD completely reverses this effect. **D)** LCAD activity parallels acetylation status of LCAD. **E)** LFD fed sham and HF AAC LF mice that have a lower abundance of acetylated LCAD have lower palmitate oxidation rates. The HFD fed sham and HF AAC HF mice with higher palmitate oxidation rates have higher abundance of acetylated LCAD.

*P<0.05, **P<0.01 vs. LF Sham; † P<0.05 vs. HF AAC HF; ‡ P<0.05 vs. HF Sham; Values represent mean ± SEM (n=6).

A


LF Sham
HF Sham
HF AAC HF
HF AAC LF
GCN5L1
β-Actin
GCN5L1 / β-Actin
1.5
1.0
0.5
0.0
*
*
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

B

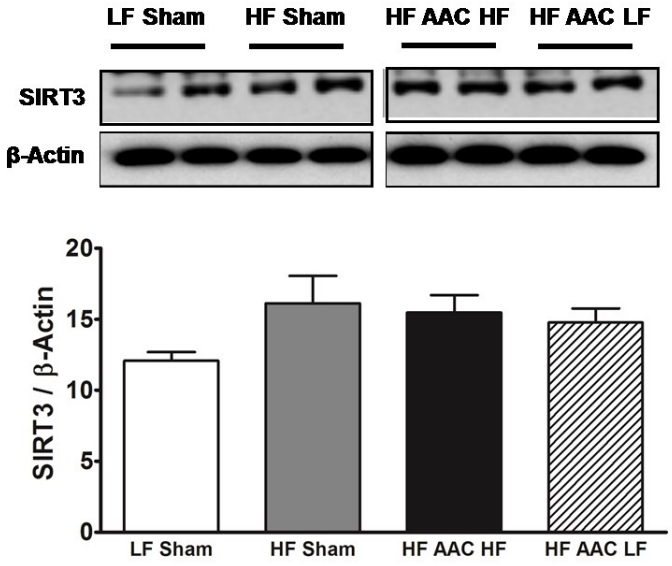

LF Sham
HF Sham
HF AAC HF
HF AAC LF
SIRT3
β-Actin
SIRT3 / β-Actin
20
15
10
5
0
LF Sham
HF Sham
HF AAC HF
HF AAC LF

Figure 5-6

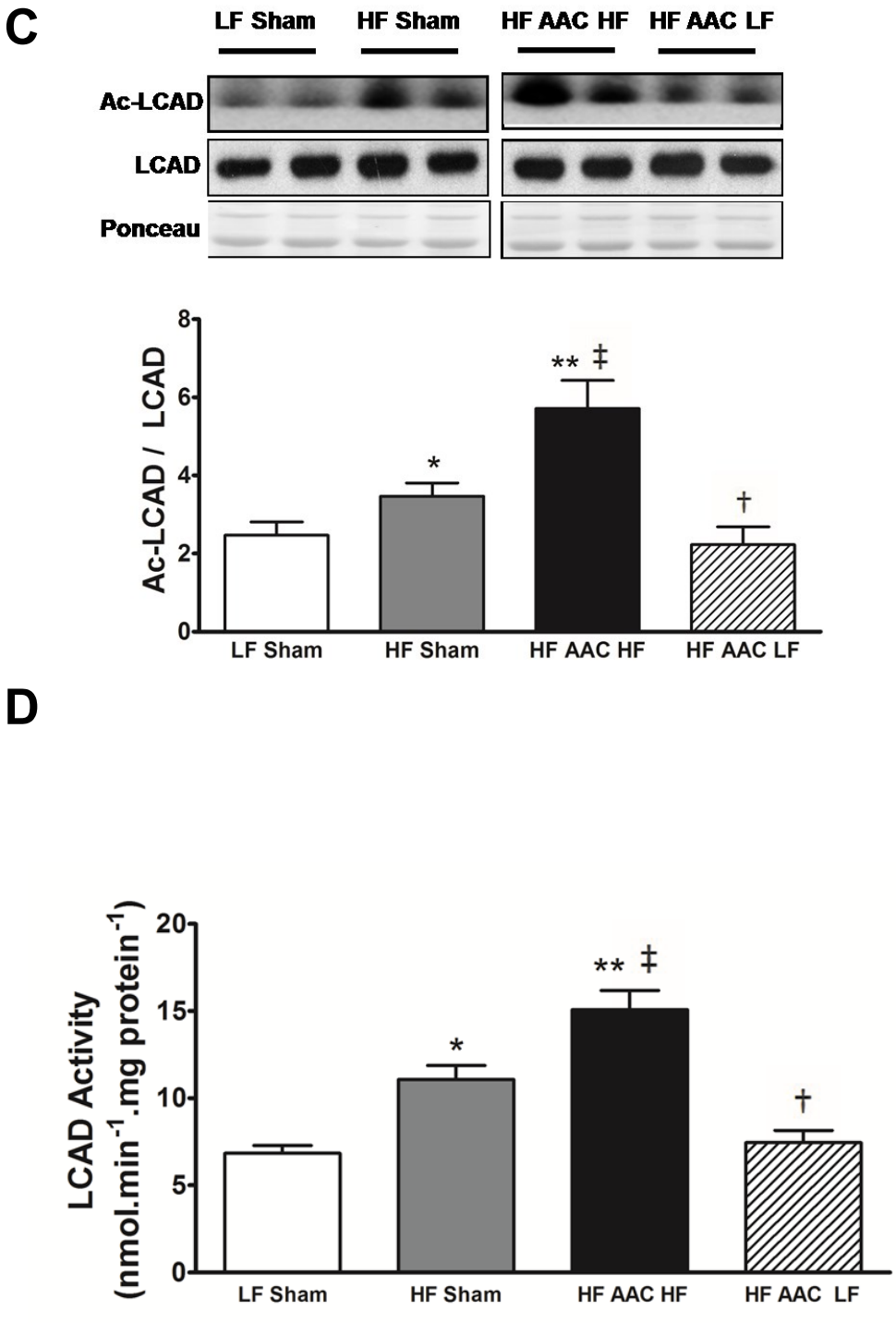

C
LF Sham
HF Sham
HF AAC HF
HF AAC LF
Ac-LCAD
LCAD
Ponceau
Ac-LCAD / LCAD
0
2
4
6
8
*
** ‡
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF
D
LCAD Activity
(nmol.min-1.mg protein-1)
0
5
10
15
20
*
** ‡
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

Figure 5-6

E

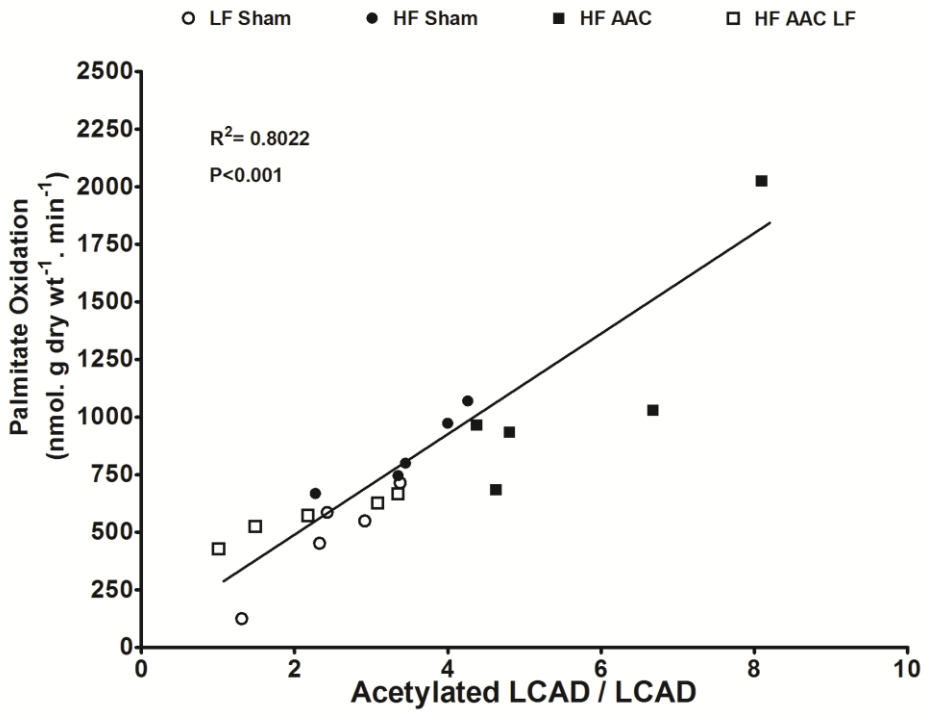

LF Sham
HF Sham
HF AAC
HF AAC LF
R^2= 0.8022
P<0.001
Palmitate Oxidation (nmol. g dry wt^{-1}. min^{-1})
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
0
2
4
6
8
10
Acetylated LCAD / LCAD

Figure 5-6

Figure 5-7: Insulin signaling pathway in mice fed a low or high fat diet.

A) Phosphorylation of Akt (P-Akt) is significantly increased in HF AAC LF. **B)** Phosphorylation of GSK3β is decreased by high fat feeding (HF Sham and HF AAC HF). Switching to low fat diet rescues this effect.

*P<0.05; ‡P<0.05 vs. HF AAC HF. Values represent mean ± SEM (n=6).

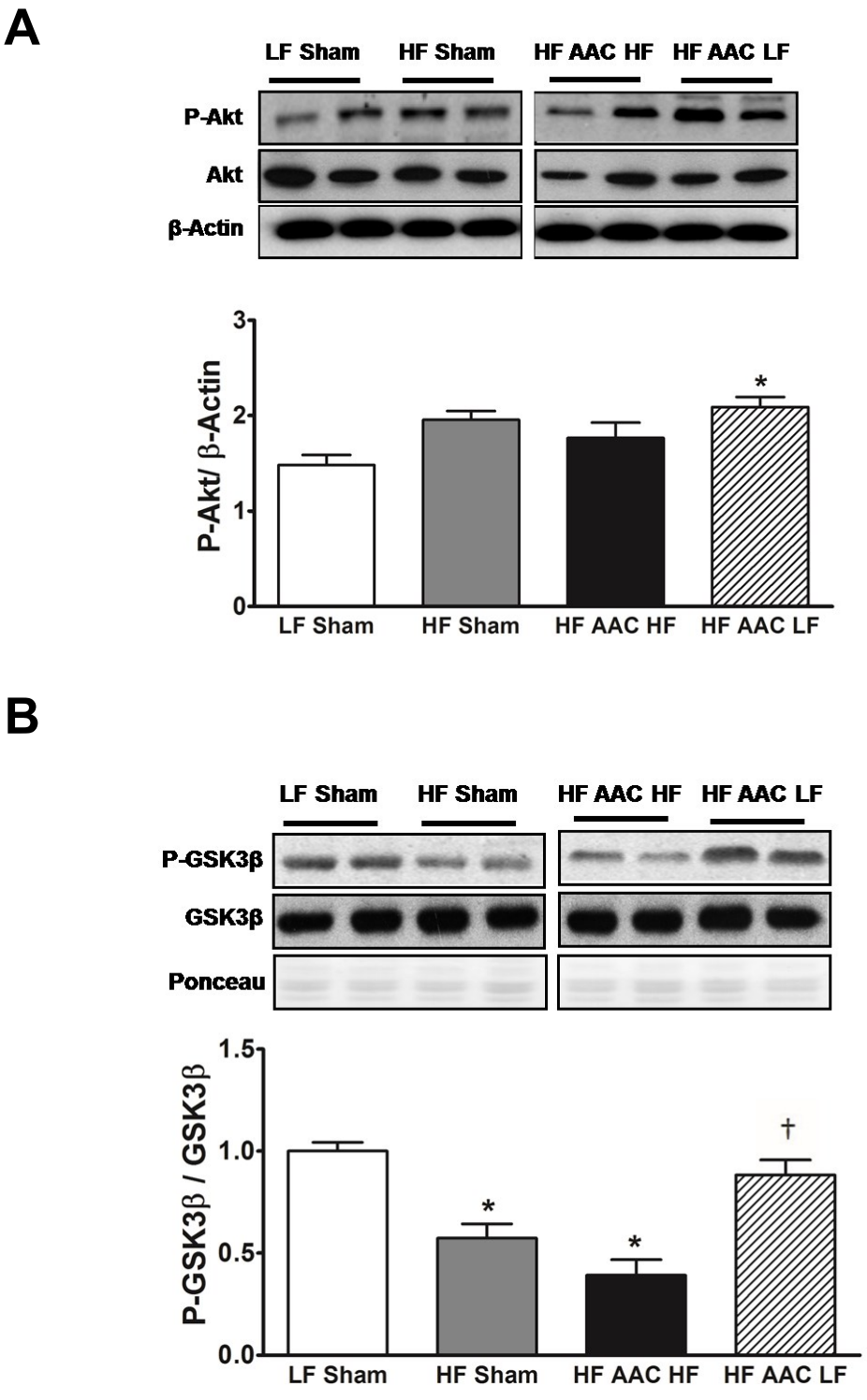

A
LF Sham
HF Sham
HF AAC HF
HF AAC LF
P-Akt
Akt
β-Actin
P-Akt/ β-Actin
3
2
1
0
*
LF Sham
HF Sham
HF AAC HF
HF AAC LF
B
LF Sham
HF Sham
HF AAC HF
HF AAC LF
P-GSK3β
GSK3β
Ponceau
P-GSK3β / GSK3β
1.5
1.0
0.5
0.0
*
*
†
LF Sham
HF Sham
HF AAC HF
HF AAC LF

Figure 5-7

5.4 DISCUSSION

In this study we showed that obese mice with heart failure that were switched to LFD had improved cardiac function. This was accompanied by a dramatic improvement in insulin sensitivity that led to an increase in insulin-stimulated glucose oxidation and a decrease in cardiac hypertrophy. In our study, we show that by switching the obese heart failure mice to LFD, we completely reversed cardiac insulin resistance. Hearts from HF AAC HF mice developed insulin resistance that resulted in low glucose oxidation rates. On the other hand, switching these mice to LFD resulted in improved insulin sensitivity and increased glucose oxidation. Thus insulin resistance observed in HF AAC HF hearts was associated with impaired insulin signaling (Akt and GSK3b phosphorylation) and therefore decreased glucose oxidation rates.

Recent studies from our group have shown a decrease in glucose oxidation in TAC (138) and angiotensin – II (59,264) induced mouse models of heart failure. We also showed that in mice lacking malonyl CoA decarboxylase *MCD*$^{-/-}$, the enzyme that inhibits fatty acid β-oxidation through modulating malonyl CoA levels, a robust increase in cardiac insulin stimulated glucose oxidation was seen in mice on a HFD (88). Therefore, a decrease in glucose oxidation and an increase in fatty acid β-oxidation could have contributed to cardiac dysfunction in the HF AAC HF mice. In contrast, *CPT-1b*$^{+/-}$ mice that partially lack CPT-1 have decreased

cardiac fatty acid β-oxidation and they develop cardiac dysfunction and hypertrophy under mild pressure overload conditions (16). Furthermore, preserved cardiac function and attenuated cardiac hypertrophy was observed in *ACC2*$^{-/-}$ subjected to TAC when compared to WT mice subjected to TAC (136). These studies underscore the role of fatty acid β-oxidation in maintaining cardiac function in heart failure.

One effect of insulin resistance is increased fatty acid utilization by hearts. We observed increased fatty acid β-oxidation rates in hearts from obese mice with diastolic dysfunction. Switching these mice to low fat diet resulted in marked increase in glucose oxidation and a reciprocal decrease in fatty acid β-oxidation. Consistent with our results, hearts from mice fed high fat diet (61) or from ob/ob and db/db mice (58) have increased fatty acid β-oxidation. We therefore wanted to understand mechanisms that underlie dramatic changes in substrate utilization.

Several studies have shown that acetylation of lysine residues regulate activity of enzymes involved in energy metabolism (159,191,194,200). We therefore wanted to assess the acetylation status of a key enzyme in fatty acid β-oxidation, LCAD. Acetylation of LCAD was increased in obese mice with heart failure. This was associated with an increase in fatty acid β-oxidation rates in aerobically perfused hearts. In hearts from HF AAC LF mice acetylation of LCAD was decreased and so was fatty acid β-oxidation rate. Hyperacetylation of LCAD was also associated with increased LCAD activity. There is a controversy in the

literature whether acetylation of LCAD or other fatty acid β-oxidation enzymes is activating or inhibitory. Hirschey *et al* demonstrated that in hepatocytes, deacetylation of the fatty acid β-oxidative enzyme LCAD increases fatty acid β-oxidation rates (200). On the otherhand, Zhao *et al* showed that in cardiomyocytes, hyperacetylation of β-HAD, another enzyme involved in fatty acid β-oxidation resulted in its activation (159). A recent study reported increased fatty acid β-oxidation in skeletal muscle of SIRT3 KO mice (201). Consistent with the latter studies, we observed a positive correlation between acetylation of LCAD and fatty acid β-oxidation rates supporting evidence in the literature that acetylation of LCAD activates fatty acid β-oxidation.

Cardiac hypertrophy in HF AAC HF mice was estimated by increased LV mass, and increased posterior wall thickness, findings that were confirmed by *ex vivo* measurement of LV mass / tibia length. HF AAC HF mice had cardiac hypertrophy that progressed to heart failure. Mice switched to LFD continued to hypertrophy albeit to a lesser extent ultimately resulting in a lower LV mass. We therefore wanted to understand mechanisms of cardiac hypertrophy in HF AAC HF hearts. Inhibition of FoxO1 and the decrease in downstream atrogin expression have been implicated in high fat diet induced cardiac hypertrophy (265). On the otherhand, activation of FoxO1 has been shown to promote cardiac (266) and skeletal muscle atrophy (267). In several tissues, FoxO1 and 3 are known to be regulated by SIRT1 (187,188). De-acetylation of

FoxO1 by SIRT1 has been shown to repress its activity (187,188). Because FoxO1 that regulates atrophy are in turn regulated by SIRT1, we wanted to understand if SIRT1 would be involved in regulating cardiac hypertrophy in diastolic dysfunction. We observed significantly increased SIRT1 expression in HF AAC HF mice that was associated with decreased acetylation and inhibition of FoxO1 which resulted in decreased atrogin-1 expression. Atrogin-1 is associated with skeletal muscle atrophy and therefore decreases in atrogin-1 possibly contributed to cardiac hypertrophy as well. Consistent with our study, SIRT1 has been shown to be involved in the inhibition of FoxO1 (187,188) and cardiac hypertrophy (164). In HF AAC HF mice switched to low fat diet, a decrease in SIRT1 was associated with increased acetylation and activation of FoxO1 and the subsequent increase in atrogin-1 expression. In contrast, activation of FoxO1 has been shown to be involved in high fat diet induced cardiac hypertrophy and metabolic alterations. We find that a decrease in SIRT1 in HFD fed sham mice resulted in increased acetylation and therefore activation of FoxO1 leading to increase in LV mass/tibia length. Thus, cardiac hypertrophy in sham and AAC mice could involve divergent pathways.

Another pathway involved in cardiac hypertrophy is protein kinase C (PKC). PKC activity is increased during the development of heart failure (268). In a pressure overload induced model of heart failure in guinea pigs, constriction of the descending thoracic aorta was accompanied by

increase in PKC α expression during decompensated cardiac hypertrophy (269). Moreover, PKC is also involved in cardiac hypertrophy. PKC is hypothesized to modulate cardiac hypertrophy by phosphorylation of transcription factors controlling expression of hypertrophic genes. Among these transcription factors found to be modulated by PKC in agonist stimulated cardiomyocytes are c-jun and fos (270). PKC also promotes phosphorylation of mTOR and P70S6K resulting in activation of the cardiac hypertrophic pathways (271). Cardiac hypertrophy was associated with activation of mTOR and P70S6K in HF AAC HF hearts. On the other hand, in HF AAC LF hearts activation of AMPK resulted in inhibition of mTOR and P70S6K pathway that led to lesser degree of cardiac hypertrophy. In a transverse aortic constriction induced rat model of cardiac hypertrophy, AICAR, an AMPK activator reduced cardiac hypertrophy (272). Resveratrol, an AMPK activator also reduced cardiac hypertrophy in the spontaneously hypertensive rats (272). We observed an increased activation of AMPK in obese heart failure mice switched to LFD. Activation of AMPK can further activate P38MAPK (272) that can increase atrogin-1 independent of FoxO (273,275). Thus, SIRT1 mediated hypertrophy in HF AAC HF mice, while activation of AMPK and FoxO1 blunted the hypertrophic response in HF AAC LF mice. However, further studies are needed to ascertain the mechanism of cardiac hypertrophy to a lesser extent in HF AAC LF mice.

Switching HF AAC mice to low fat diet resulted in an improvement in diastolic function that was associated with a lesser degree of hypertrophy and an increase in insulin-stimulated glucose oxidation. At 8 weeks (4 weeks post-AAC) when the HF AAC mice were switched to a LFD, decompensation had already set in despite cardiac hypertrophy. Our data shows that in the HF AAC LF mice, despite continued hypertrophy (possibly due to constant AAC mediated pressure overload) an increase in insulin-stimulated glucose oxidation is able to improve both diastolic and systolic function. This study clearly demonstrates the importance of targeting cardiac energy metabolism in the treatment of hypertrophy associated heart failure.

CHAPTER 6.

DISCUSSION AND FINAL CONCLUSIONS

CHAPTER 6

DISCUSSION AND CONCLUSIONS

6.1 Summary

It is becoming more readily obvious that alterations in lysine acetylation contribute significantly to changes in energy metabolism (6,152,176,192,201,204,206). In addition, major cardiovascular diseases such as heart failure, and myocardial infarction as well as metabolic syndrome are associated with changes in lysine acetylation and sirtuins activity (59,151,166,196,199,227). One of the major pathways of energy production namely fatty acid β-oxidation was shown to be markedly enhanced in the aforementioned diseases (58,89,94,121,243). Previous studies showed that increased lysine acetylation of fatty acid oxidation enzymes inhibit their activity (196,197,200,206,246). Furthermore, increases in lysine acetylation of insulin signaling proteins are suggested to contribute to the development of insulin resistance in various tissues (164-166,196,208,244). Finally, in HF, a selective reduction in glucose oxidation as a source of cardiac energy as well as increased reliance on fatty acid β-oxidation believed to impair cardiac efficiency and function (58,59,129,136-138,261,264).

The specific aim of this thesis was to understand whether alterations in lysine acetylation could contribute to changes in cardiac fatty acid β-oxidation seen during maturation and in obesity and HF. In the liver,

it is suggested that lysine acetylation is inhibitory signal for fatty acid oxidative enzymes (196,200,206,246). This concept is in direct contrast to other studies that suggested lysine acetylation as a stimulatory signal for fatty acid oxidative enzymes in cardiac myocytes, and skeletal muscles (159,201).

In the first part of this thesis we addressed the controversy whether lysine acetylation is a stimulatory or inhibitory signal for fatty acid β-oxidation in the heart, and demonstrated that increased lysine acetylation was associated with increased cardiac fatty acid β-oxidation during maturation. Fatty acid oxidation enzymes; β-HAD and LCAD both were hyperacetylated and activated during maturation. Our results are highly suggestive that lysine acetylation is a stimulatory signal for cardiac fatty acid β-oxidation, and argues against the notion that increasing lysine acetylation lead to inhibition of fatty acid β-oxidation. Following on these findings, we next investigated in this thesis contrary to popular theory, whether obesity increases lysine acetylation and accelerates fatty acid β-oxidation. We achieved this by utilizing HFD-induced obese mouse model. Previous studies from our lab and others showed that obesity induced by HFD feeding or genetic manipulation, such as leptin deletion leads to increases in cardiac fatty acid β-oxidation (61,88,98,243). Interestingly, we showed that HFD feeding increases cardiac fatty β-oxidation and led to increased lysine acetylation and activity of β-HAD and LCAD. Furthermore, using SIRT3 KO mice, we showed that increases in fatty

acid β-oxidation rates were accompanied with hyperacetylation and activation of both fatty acid oxidation enzymes β-HAD and LCAD, suggesting that lysine acetylation increases fatty acid β-oxidation in the heart.

The last part of this thesis focused on the role of lysine acetylation in regulating fatty acid β-oxidation and hypertrophy in pressure overload-induced HF mouse model. We showed that rates of fatty acid β-oxidation are increased in obese-HF and were associated with hyperacetylation and activation of LCAD. Furthermore, cardiac hypertrophy evident in obese-HF mice were associated with decreased lysine acetylation of FoxO1.

Overall, the novel findings of this thesis have clearly demonstrated that lysine acetylation enhances fatty acid β-oxidation in the heart. Furthermore, we have evidently demonstrated that inhibition of SIRT3 in the heart by HFD feeding or genetic deletion, leads to increases in fatty acid β-oxidation. The rest of this chapter will focus in more details about the implications of the findings, justification of the methods used in this thesis, and future directions.

6.2 Enzymatic versus Spontaneous Acetylation

Lysine acetylation is abundant both in mitochondria and cytosol. Acetyl CoA generated from the oxidation of fatty acids and glucose is the substrate for proteins acetylation. As the primary site for acetyl CoA

utilization, mitochondrial total CoA pool is about 2mM, which is 150 times higher than that of the cytosol in the heart (276). It was demonstrated previously that the high pH and acetyl CoA concentrations in the mitochondria are sufficient to drive enzyme-independent acetylation of mitochondrial proteins *in vitro* (277). Furthermore, incubating recombinant LCAD with acetic anhydride *in vitro* was able to acetylate LCAD on 13 lysine residues (206). However, the basis of this concept would be feeble if mitochondrial acetyltransferases are identified in mitochondria. Indeed, a mitochondrial acetyltransferase was recently identified in the heart, skeletal muscles, and liver (211). Interestingly, genetic knockdown of GCN5L1 without changing acetyl CoA concentrations results in a marked decrease in mitochondrial proteins acetylation (211). However, further characterizations of GCN5L1 kinetics and molecular components are needed. In addition, of the 13 chemically acetylated lysines (206), only 8 lysine residues in LCAD were identified as a target for lysine acetylation in the liver (200). These differences in the number of acetylated lysines further support that enzymatic acetylation is key for regulation of mitochondrial proteins acetylation. Furthermore, the abundance of lysine acetylation in the cytoplasm although concentrations of acetyl CoA are low and the non-alkaline pH argues against the notion of non-enzymatic acetylation. Moreover, in conditions that are accompanied by increased overall acetylation such as obesity, diabetes, and ischemia-reperfusion (142,191,201,210,246,278), the acetylation levels of specific proteins in

the mitochondria are not equal and could significantly differ from each other. For example, in this thesis I showed that mitochondrial enzymes LCAD and β-HAD were hyperacetylated in hearts isolated form obese mice. However, acetylation of another mitochondrial enzyme PDH was not changed, suggesting that lysine acetylation is a highly specific post-translational modification. In addition, Affinity of some acetyltransferases to acetyl CoA is similar to physiological acetyl CoA concentrations (279); this may suggest that fluctuations in acetyl CoA concentrations may regulate proteins acetylation. Finally, enzymatic site-specific acetylation may occur in parallel with spontaneous non-enzymatic acetylation, which adds more sophisticated level of how post-translational modifications might be controlled.

6.3 Differential Regulation of Sirtuins

An interesting aspect in sirtuins physiology is the differential regulation of sirtuins activity and expression in different tissues. Calorie restriction increases SIRT1 protein expressions in various tissues including brain, kidney, cardiomyocytes, skeletal muscles, and liver (148,158,280,281). Furthermore, protein expressions of SIRT3 were reported to be enhanced in liver, adipose tissue, cardiomyocytes, and skeletal muscles by calorie restriction (140,152,158,192,209,212,280) . However, it was reported by Schwer *et al* that mitochondrial protein lysine

acetylation is altered by calorie restriction in a tissue-specific manner (282). Their data show that calorie restriction markedly increases mitochondrial proteins acetylation in liver. However, mitochondrial proteins acetylation was significantly decreased by calorie restriction in adipose tissue. Interestingly, acetylation was unchanged in mitochondria isolated from heart, brain, and kidney (282). In fasting, SIRT3 protein levels were decreased in skeletal muscles but increased in liver (200,201,208). Furthermore, SIRT3 is active in the fed state in skeletal muscles, whereas in the liver SIRT3 is more active in the fasting state (201). Interestingly, metabolic effects of SIRT3 seem to be tissue-specific as well. For example, SIRT3 deletion results in hyperacetylation of LCAD and inhibition of liver lipid oxidation (200), whereas deletion of SIRT3 in skeletal muscles increases fatty acid β-oxidation (201). In addition, specific lysine residues could be targeted in different conditions. Four lysine residues in the enzyme superoxide dismutase are targets for SIRT3 deacetylation. Lysines 53 and 89 are deacetylated during calorie restriction (283), whereas lysines 122 and 68 are deacetylated by SIRT3 in response to ionizing radiation stress and increased reactive oxygen species levels, respectively (284,285). This dichotomous role of sirtuins in different tissues may explain the differences in metabolic effects we noticed in the heart compared to what others reported in the liver (196,200).

6.4 Mitochondrial Versus Total Heart Lysates

We used total heart lysates to determine the acetylation levels of nuclear, cytoplasmic, and mitochondrial enzymes. However, in previous studies mitochondria were isolated and then acetylation levels of mitochondrial proteins were evaluated (191,192,200,201,226). Our concern is that mitochondrial isolation takes several hours to be completed. During this time changes in acetylation/deacetylation of proteins may occur. Unlike phosphatase and kinase inhibitors that can be added during mitochondrial isolation, acetylase inhibitors are not yet available. It is important to emphasize, however, that our results from frozen heart tissue mimicked what was previously found in mitochondrial preparations of liver and skeletal muscles tissue (196,197,200,201). Furthermore, trichostatin A (TSA) which is a selective class I and II histone deacetylase inhibitor, and nicotinamide (NAM) which is a noncompetitive sirtuins inhibitor were added to mitochondrial isolation and immunoprecipitation experiments. The purpose of such intervention is to preserve acetylation status of proteins during the time of experiment. However, we did not use these inhibitors in our experiments. We believe that this intervention could be futile, since acetylation can be regulated by both acetyltransferases and deacetylases. Therefore, inhibiting only one arm of the reaction using deacetylases inhibitors such as TSA and NAM, although could inhibit deacetylases, acetylation catalyzed by

acetyltransferases could proceed uninterrupted. Nonetheless, we isolated mitochondria from non-perfused hearts in the presence and absence of TSA and NAM, and we found that overall proteins acetylation is not affected by inhibitors treatment.

6.5 Final Conclusions

Overall, the findings in this thesis have addressed very important questions regarding the contributions of lysine acetylation towards the regulation of cardiac energy metabolism. The novel findings in this thesis should open the doorway for a new avenue to explore in the treatment of obesity and heart failure. This avenue will have a central focus on the optimization of cardiac energy metabolism by targeting sirtuins based on the following conclusions:

1) Overall proteins acetylation is increased during maturation, accompanied by hyperacetylation and activation of fatty acid oxidative enzymes; β-HAD and LCAD.
2) Increased expression of the nuclear deacetylase SIRT1 and the decreases in SIRT6 cardiac protein levels during maturation were accompanied by decreased acetylation of PGC-1α and increased ATP production.

3) Hyperacetylation of the glycolytic enzymes; HK and PGM were associated with decreased glycolytic rates during maturation.
4) Decreased cardiac SIRT3 expression in obesity or by genetic deletion increases fatty acid β-oxidation, acetylation and activities of β-HAD and LCAD.
5) Last, cardiac hypertrophy evident in pressure overload-induced heart failure was associated with SIRT1 mediated inhibition of FoxO1. Such observations suggest that SIRT1 may induce cardiac hypertrophy.

6.6 Future Directions

Although exciting and novel data has been presented in this thesis, there remains a significant amount of experiments that need to be performed to validate the role of sirtuins and acetylation in regulation of cardiac energy metabolism. One of the most important aspects that need to be addressed is whether pharmacological activation of SIRT3 will improve cardiac metabolism and function in a disease states such as obesity, diabetes, and heart failure. Although cardiac-specific overexpression of SIRT3 protects the heart from hypertrophy and improves cardiac function, there are no specific activators or inhibitors for

SIRT3 and the data regarding its role in regulating cardiac energy metabolism is still scarce.

Another important area for future investigation lies on the fact that many lysine residues are acetylated in the same enzyme. Further investigation of the acetylated lysines and determination of SIRT3 target sites are key for improving our understanding about the mechanisms by which acetylation/deacetylation modulates enzymatic activities. Furthermore, acetylation, succinylation, and malonylation counterbalance each other because these post-translational modifications may occur in the same lysine residue. This suggests the existence of intricate interplay between acetylation, succinylation, and malonylation in the activation or inhibition of various metabolic enzymes. It will be fascinating to study their relationship in conditions such as fasting and cardiovascular diseases associated with obesity and diabetes.

The role of acetylases in controlling cardiac energy metabolism is never investigated. Compared to our understanding of deacetylation, the processes involved in acetylation of lysine residues of metabolic enzymes are less well characterized. A role for GCN5L1 as a mitochondrial acetylase and GCN5 as a nuclear acetylase has recently been described (178,211). Recently, MCD has also been proposed to be an acetyltransferase whereby it channels acetyl CoA that is produced via MCD into acetylation reactions (286). It will be very interesting to determine the role of MCD in cardiac acetylation. Furthermore, SIRT4

deacetylates and represses MCD activity. SIRT4 knockdown in liver and muscle results in a significant upregulation of MCD activity, as well as increased fatty acid β-oxidation (216). It will be just as interesting to investigate whether such mechanisms may also apply in the heart, as SIRT4 has received very little attention as a possible regulator of cardiac fatty acid β-oxidation. It is worth mention that MCD is also localized in the peroxisomes where it regulates the level of malonyl CoA and peroxisomal fatty acid β-oxidation (287,288). The finding that protein acetylation is abundant in human liver peroxisomes (210), could be a prelude to the existence of a similar mechanism in cardiac peroxisomes.

The last area that is going to require extensive investigation is to delineate the functions of each sirtuin in each subcellular compartment, and the impact of acetylation on TCA and ETC enzymes in the heart. In addition, several post-translational modifications include phosphorylation and ubiquitination have been identified to regulate SIRT1 activity (148,289). However, so far no post-translational modifications of SIRT3 have been identified (198). It is very important for future research to determine if post-translation modifications regulate SIRT3 activity, which will greatly help us to develop new therapeutic agents to target SIRT3 and treat obesity associated cardiovascular diseases, where SIRT3 seems to play a pivotal role in pathogenesis.

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List of Publications:

Original Research Articles:

O Abo Alrob, S Sankaralingam, JS Jaswal, MN Sack, R Lehner, RS Padwal, DE Johnstone, AM Sharma, N Fillmore, GD Lopaschuk. Obesity-Induced Lysine Acetylation Increases Cardiac Fatty Acid Oxidation and Impairs Insulin Signaling. Cardiovascular Res. (Accepted) 2014

Jun Mori, Vaibhav B. Patel, Tharmarajan Ramprasath, **Osama Abo Alrob**, Jessica DesAulniers, James W. Scholey, Gary D. Lopaschuk and Gavin Y. Oudit. Angiotensin 1-7 mediates renoprotection against diabetic nephropathy by reducing oxidative stress, inflammation and lipotoxicity. Am J Physiol Renal Physiol. 2014

Mori J, Patel VB, **Abo Alrob O**, Basu R, Desaulniers J, Wagg CS, Kassiri Z, Lopaschuk GD, Oudit GY. Angiotensin 1-7 Ameliorates Diabetic Cardiomyopathy and Diastolic Dysfunction in db/db Mice by Reducing Lipotoxicity and Inflammation. Circ Heart Fail. 2014

Jun Mori, **Osama Abo Alrob**, Cory S Wagg, Robert A Harris, Gary D. Lopaschuk, Gavin Y Oudit. Ang II causes insulin resistance and induces cardiac metabolic switch and inefficiency: a critical role of PDK4. Am J Physiol Heart Circ Physiol. 2013

Review Articles:

Osama Abo Alrob and Gary D. Lopaschuk. Role of CoA and Acetyl CoA in Regulating Cardiac Fatty Acid and Glucose Oxidation. Biochemical Society Transactions. 2014

Filmore N*, **Abo alrob O***, Lopaschuk G. Fatty Acid Beta Oxidation. AOCS Lipid Library. 2011, Online review. * Equal contribution

Manuscripts in preparation:

Osama Abo Alrob, Sowndramalingam Sankaralingam, Cary Ma, Cory S. Wagg, Waleed GT. Masoud, Jagdip S. Jaswal, and Gary D. Lopaschuk. Acetylation of Cardiac Regulatory Proteins Increases Fatty Acid β-Oxidation and Represses Glycolysis During Maturation. For submission to Cardiovascular Research journal

Waleed GT. Masoud, **Osama Abo Alrob**, Gary D. Lopaschuk, and Alexander S Clanachan. Tolerance to ischemic injury in remodeled mouse hearts: less ischemic glycogenolysis and preserved metabolic efficiency. Under revisions of Cardiovascular Research journal

S Sankaralingam, **O Abo Alrob**, JS Jaswal, C Wagg, RS Padwal, DE Johnstone, AM Sharma, MN Sack, GD Lopaschuk. Lowering body weight in obese mice with diastolic dysfunction improves cardiac insulin sensitivity and function. For Submission to Diabetes journal

S Sankaralingam, **O Abo Alrob**, JS Jaswal, C Wagg, RS Padwal, DE Johnstone, AM Sharma, MN Sack, GD Lopaschuk. High fat diet exacerbates cardiac hypertrophy. For submission to Circulation Research journal

Selected conference presentations and peer reviewed abstracts:

Osama Abo Alrob, Sownd Sankaralingam, Cory S. Wagg, and Gary D. Lopaschuk. Role of Lysine Acetylation in the Maturation of Energy Metabolism in the Newborn Heart. Pediatrics research day, Edmonton, Canada, 2014

Osama Abo Alrob, Sownd Sankaralingam, Cory S. Wagg, and Gary D. Lopaschuk. Mitochondrial Acetylation Increases Fatty Acid Oxidation in the Heart. Keystone symposia, Vancouver, Canada, 2014

Abo Alrob O, Sankaralingam S, Masoud WGT, Sack MN, Clanachan AS , and Lopaschuk GD. Lysine Acetylation Enhances Cardiac Fatty Acid β-Oxidation. American Heart Association, Dallas, USA, 2013

Cary Ma, **Osama Abo Alrob**, Richard Lehner, Cory Wagg, and Gary Lopaschuk. Acetylation Enhances Cardiac Fatty Acid Oxidation in SIRT3 KO mice. Summer student research day, Edmonton, Canada

Abo Alrob O, Sankaralingam S, Jaswal JS, Sack MN, Lehner R, Padwal RS, Johnstone DE, Sharma AM, Fillmore N, and Gary D. Lopaschuk. Acetylation Enhances Cardiac Fatty Acid Oxidation in High Fat Diet Induced-Obese Mice. Keystone, USA, 2013

Sowndramalingam Sankaralingam, **Osama Abo Alrob**, Jagdip S. Jaswal, Raj S. Padwal, David E. Johnstone, Arya M. Sharma, Cory S. Wagg, Gary D. Lopaschuk. Lowering body weight in obese mice with heart failure improves cardiac insulin sensitivity and cardiac function. ADI research day, Edmonton, Canada, 2012

Osama Abo Alrob, Jagdip S. Jaswal, Natasha Fillmore, Victoria Lam, and Gary D. Lopaschuk. Role of Lysine Acetylation in the Maturation of Energy Metabolism in the Newborn Heart. ISHR, Banff, Canada, 2012

G.D. Lopaschuk, **O. Abo Al-Rob**, V.H. Lam, J.S. Jaswal, I. M. Rebeyka. Delayed maturation of fatty acid oxidation in the newborn hypertrophied heart. SHVM, Oxford, UK, 2012