Maturation of Cardiac Energy Metabolism in the Normal and Hypertrophied Newborn Heart

by

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Abstract

Dramatic maturational changes occur in heart energy metabolism in the fetal to newborn transition, most predominantly marked by a decrease in glycolysis and an increase in fatty acid oxidation which then becomes the major energy providing substrate for the heart after birth. However, in the presence of hypertrophy, this maturation of fatty acid oxidation is delayed; wherein the dramatic increase of fatty acid oxidation after birth does not occur. Without the increase in fatty acid oxidation, the heart is at risk of having a decreased energy capacity since fatty acids are the major energy providing substrate for these hearts. The underlying mechanisms for the increase in fatty acid oxidation shortly after birth and the delay in fatty acid oxidation in hypertrophied newborn hearts are not yet fully elucidated.

The regulation of the maturation of newborn cardiac metabolism occurs at three levels: transcriptional control, allosteric control, and the most recently emerging is post-translational modifications (PTMs). PTMs can either activate or inhibit enzymatic activity of a protein. Previously, lysine acetylation was shown to activate cardiac fatty acid oxidative enzymes in obesity. Recently, metabolomics showed that the lysine PTM called succinylation is also important in regulating cardiac energy metabolism enzymes. However, the importance of acetylation and succinylation in the newborn setting of cardiac metabolism is unclear. We aim to investigate the role of acetylation and succinylation in the maturation of cardiac fatty acid metabolism and in hypertrophied newborn hearts. Also, in the case of hypoplastic left heart syndrome (HLHS), which is a severe congenital heart defect (CHD) consisting of underdevelopment of the left-sided cardiac structures, there may be cardiac metabolic abnormalities that differentiate this severe CHD from

other types of CHDs. We aim to investigate the differences in metabolism between HLHS and non-HLHS patients.

Hearts from rabbits aged 1-day, 7-days, and 21-days old underwent isolated heart perfusions and the frozen tissue was processed for Western blotting, immunoprecipitation, and enzymatic activity assays. Myocardial tissue obtained from infants aged 0-200 days old undergoing corrective heart surgery at the University of Alberta was used to analyze the succinylation status in these hearts and the status of metabolic proteins including protein abundance and acetylation of energy metabolic proteins in HLHS patients compared to non-HLHS patients. Also, 21-day old rabbit hearts with an aorto-caval shunt as a model for congenital heart defects and volume-overload hypertrophy were perfused in isolated biventricular working mode to measure flux through metabolic pathways and frozen tissue was used to investigate the role of acetylation and succinylation on fatty acid oxidation in hypertrophy.

We observed that total acetylation and succinylation abundance of mitochondrial proteins increased consistently from 1-day to 21 days post-birth and this was correlated with an increase in palmitate oxidation rates. In 21-day old hypertrophied rabbit hearts, palmitate oxidation and ATP production rates were significantly lower compared to the sham group. Acetylation, but not succinylation, of fatty acid oxidation enzymes long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) was also lower in the hypertrophied hearts compared to sham, and this acetylation was positively correlated with their enzymatic activity and palmitate oxidation rates. In human myocardial tissue, total lysine succinylation levels did not change with age, although there was a decrease in succinylation in the hypertrophied hearts compared to non-hypertrophied in the 101-200 day old group. In our study investigating HLHS infant hearts,

transcriptional, allosteric, and post-translational control of fatty acid oxidation was not compromised in HLHS patients compared to non-HLHS patients. In fact, increased cardiac PGC1α protein levels suggested an increased ability for mitochondrial biogenesis in HLHS patients.

Taken together we found that acetylation contributes to the normal increase of fatty acid oxidation in newborn hearts shortly after birth and to the delayed maturation of fatty acid oxidation in hypertrophied hearts. On the other hand, succinylation is another regulator of the maturation of fatty acid oxidation, having a stimulatory effect on LCAD, but the role of succinylation in hypertrophied hearts is less apparent although further studies in slightly older age groups is still required. HLHS infant hearts did not show compromised cardiac energetics and this may not be an important risk-factor in perioperative dysfunction of the right ventricle in newborns with HLHS.

Preface

In Chapter 2, modified versions of Figures 2.1-2.4 have been published in: Fukushima A, Alrob OA, Zhang L, Wagg CS, Altamimi T, Rawat S, Rebeyka IM, Kantor PF, and Lopaschuk GD. Acetylation and succinylation contribute to maturational alterations in energy metabolism in the newborn heart. *Am J Physiol Heart Circ Physiol.* 2016;311(2): H347-63. I was responsible for the conception and design of the research with Fukushima A and Lopaschuk GD. I performed the Western blots and immunoprecipitation, analyzed the data, and interpreted the results with Fukushima A. Zhang L performed the two enzymatic activity assays and provided intellectual contributions. Wagg CS performed the isolated heart perfusions. Lopaschuk GD also interpreted experimental results and provided editorial and intellectual contributions.

In Chapter 3, modified version of Figure 3.1 has been published in: Fukushima A, Zhang L, Huqi A, Lam VH, Rawat S, Altamimi T, Wagg CS, Dhaliwal KK, Hornberger LK, Kantor PF, Rebeyka IM and Lopaschuk GD. Cardiac hypertrophy in neonates delays acetylation control of cardiac energy metabolism. *JCI Insights*. 2018;3(10):e99281. Figure 3.2 and 3.3 are unpublished. I was responsible for the conception and design of the research with Fukushima A and Lopaschuk GD. I performed the Western blots and immunoprecipitation, analyzed the data, and interpreted the results with Fukushima A. Zhang L performed the two enzymatic activity assays and provided intellectual contributions. Lam VH performed the isolated bi-ventricular working heart perfusions. Lopaschuk GD also interpreted experimental results and provided editorial and intellectual contributions.

Chapter 4 of this thesis is a manuscript that is provisionally accepted at the *Journal of Thoracic and Cardiovascular Research*. I am the primary author of this manuscript. I was responsible for the conception and design of the research with Lopaschuk GD. I performed the Western blots and immunoprecipitation with Fukushima A. I analyzed the data and interpreted the results. Petinelli R assisted with the data analysis. Lopaschuk GD provided editorial and intellectual contributions.

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To my dear friend Tanzila Khawaja, thank you for continuous support and motivation all these years.

Dedication

This thesis is dedicated to my grandmothers, Mrs. Ram Pyari Devi and Mrs. Kusum Khandelwal, and my parents, Dr. Brijendra and Mrs. Nilima Rawat

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List of Abbreviations

AMPK: 5’ AMP-activated protein kinase

ACC: acetyl CoA carboxylase

Ac-β-HAD: acetylated β-hydroxyacyl CoA dehydrogenase

Ac-LCAD: acetylated long chain acyl CoA dehydrogenase

Acetyl-Lys: acetylated lysine

Ac-PGC1α: acetylated PPAR-γ coactivator 1α

ATP: adenosine triphosphate

ATGL: adipose triglyceride lipase

ANOVA: analysis of variance

AU: arbitrary units

β-HAD: β-hydroxyacyl CoA dehydrogenase

BSA: bovine serum albumin

CPT1: carnitine palmitoyl transferase 1

CS: citrate synthase

CHD: congenital heart defect

CDK4: cyclin-dependent kinase 4

DGAT2: diacylglycerol acyltransferase 2

GCN5L1: GCN5-like protein 1

HLHS: hypoplastic left heart syndrome

HIF1α: hypoxia-inducible factor 1α

IgG-HC: immunoglobulin heavy chain

IgG-LC: immunoglobulin light chain

LCAD: long chain acyl CoA dehydrogenase

MCD: malonyl CoA decarboxylase

NADH: nicotinamide adenine dinucleotide

PPARα: peroxisome proliferator activated receptor-α

p-AMPK: phosphorylated 5’ AMP-activated protein kinase

p-ACC: phosphorylated acetyl CoA carboxylase

p-Cyclin D1: phosphorylated cyclin D1

p-PDH: phosphorylated pyruvate dehydrogenase

p-Rb: phosphorylated retinoblastoma protein

PTM: post-translational modification

PGC1α: peroxisomal proliferator activated receptor-γ coactivator 1α

PDH: pyruvate dehydrogenase

PDK2: pyruvate dehydrogenase kinase 2

PDK4: pyruvate dehydrogenase kinase 4

SPT1: serine palmitoyltransferase 1

SPT2: serine palmitoyltransferase 2

SIRT: sirtuin

SE: standard error

SEM: standard error of the mean

Sc-LCAD: succinylated long chain acyl CoA dehydrogenase

Succinyl-Lys: succinylated lysine

Sc-PDH: succinylated pyruvate dehydrogrenase

t-AMPK: total 5’ AMP-activated protein kinase

t-ACC: total acetyl CoA decarboxylase

t-PGC1α: total PPAR-γ coactivator 1α

VDAC1: voltage-dependent anion channel

Chapter One

Literature Review

1.1 Importance of Energy Substrate Utilization by the Newborn Heart

The heart requires a large amount of energy in order to sustain continued contractile function to pump blood through the body. Due to this high energy requirement, the heart is able to use a variety of different carbon substrates for energy ranging from carbohydrates and fatty acids, to ketone bodies and some amino acids. However, there are marked differences in the energy substrate utilization in the adult versus developing heart. For example, the newborn heart has a reduced ability to oxidize fatty acids, which is in stark contrast to the adult heart, which gets 50-70% of its ATP requirements from oxidizing fatty acids.[1] For this reason, discoveries regarding regulation of energy metabolism in the adult heart cannot be extrapolated to the pediatric setting, as they are markedly different.

The type of energy substrate used by the heart has a profound effect on the heart's ability to manage ischemic stress. This is especially important for the 1% of newborns who are affected by congenital heart defects (CHDs). CHDs are the most prevalent type of congenital anomaly and result in the largest proportion of mortality based on birth defects (30-40%).[2] Many infants diagnosed with a CHD require corrective heart surgery during the newborn period.[3] Surgical repair techniques have benefited from major advances, while pediatric myocardial protective techniques are currently lacking in research.[4] This poses a problem because perioperative heart tissue damage and low cardiac output is a leading cause of death after corrective heart surgery.[4] The primary cardioprotective strategy used in open-heart surgery is hyperkalemic cardioplegia to delay the onset of an ischemic insult, which is tissue damage caused by decreased blood flow to the heart tissue.[4] Strategies that are being developed involve therapeutic additives to the cardioplegic solution in order to target metabolic derangements, calcium overload and accumulation of reactive

oxygen species, all of which can occur due to acute ischemia.[4] In order to target the metabolic derangements that occur from ischemic stress during pediatric corrective heart surgery, an understanding of the regulation of neonatal metabolism in health and disease is necessary.

1.2 Maturation of Cardiac Energy Metabolism in Newborn Hearts

1.2.1 Pathways Contributing to ATP Production

In the adult heart, almost all of the energy needed to sustain contractile function is derived from mitochondrial oxidative metabolism, primarily from using fatty acids and carbohydrates as energy substrates *via* the metabolic pathways of fatty acid oxidation and glucose oxidation (Figure 1.1).[5] In contrast, due to the low oxygen levels in the fetal environment, non-oxidative (anaerobic) glycolysis is a key energy-providing pathway. Prior to parturition, there are only minor contributions to ATP production from oxidizing fatty acids and carbohydrates.[6] In the rabbit heart, it was shown that between 1 to 7 days after birth there is a shift from obtaining ATP from glycolysis to predominantly fatty acids as an energy substrate.[6] Therefore, there is a dramatic shift in metabolism in the fetal to newborn transition as fatty acid oxidative metabolism begins to develop significantly.

The dramatic increase in oxidative metabolism shortly after birth is evident in the shift in proportion of ATP derived from oxidative versus non-oxidative energy-deriving pathways. Lopaschuk et al described that in fetal/immediate newborn rabbit hearts, 56% of cardiac ATP production comes from oxidative metabolism compared to 95% in neonatal 7-day-old hearts.[7] The most significant change in oxidation is evident in ATP production from fatty acid oxidation, which

supplies only 13% of ATP production in fetal/immediate newborn rabbit hearts, but jumps to 80% in 21-day-old hearts.[7] Concomitantly, glycolysis is high in fetal/immediate newborn hearts, accounting for 44% of ATP production, but drops substantially by 21 days following birth to 7%.[7] Glucose oxidation remains low throughout the neonatal period because it matures at a much slower rate; therefore, it does not increase to levels seen in the adult heart until later on in development.[8]

1.2.2 Regulation of the Maturation of Cardiac Energy Metabolism in Newborn Hearts

This dramatic increase in fatty acid oxidation post-birth is regulated at three levels: allosteric control, transcriptional control, and post-translational modifications. A key allosteric regulation of fatty acid oxidation involves levels of a metabolite called malonyl CoA (Figure 1.2). Malonyl CoA has a potent inhibitory effect on the mitochondrial fatty acid uptake gate-keeper, carnitine palmitoyltransferase 1 (CPT1). Lopaschuk et al showed a progressive decrease of malonyl CoA levels in 1-day, 1-week and 6-week old rabbit hearts.[9] This results in a decreased inhibition on CPT1 during maturation post-birth, allowing fatty acids to be taken up more readily by the mitochondria. With regards to regulation at the transcriptional level, the transcriptional factor peroxisome proliferator-activated receptor alpha (PPARα), which is responsible for regulating genes related to lipid metabolism, has an increased cardiac expression after birth,[10] thus contributing to the dramatic increase in fatty acid oxidation seen after birth. Regulation through post-translational modifications will be discussed in a later section.

1.2.3 Influence of Hypertrophy on the Maturation of Newborn Cardiac Energy Metabolism

Derangements in cardiac energy metabolism occur when the heart is under chronic stress.[11] Congenital heart defects cause atypical functioning and blood flow in the heart, which has the potential to cause hypertrophy – an initially adaptive, compensatory response that is meant to counteract myocardial fiber stress.[12] In the hypertrophied state, the heart muscle wall becomes enlarged due to a higher workload. Additionally, during surgical correction of congenital heart defects, the heart is subject to an obligatory period of ischemia in order to keep the heart immobile. This can deprive the heart tissue of the normal oxygen and nutrients that would normally be delivered to the beating heart. Metabolic differences have been observed in newborn hypertrophied hearts compared to non-hypertrophied hearts. Specifically, 3-week-old rabbits with cardiac hypertrophy have fatty acid oxidation rates that are 60% lower than non-hypertrophied controls.[13] Interestingly, rates of other oxidative metabolism pathways remain unchanged. Oka et al suggest that hypertrophy in the newborn heart leads to the re-emergence of the type of metabolic profile seen in the fetal heart.[13] Since fatty acid oxidation is a large provider of ATP after parturition, the fact that hypertrophy abrogates this ATP acquisition from fatty acids would lead to an energy deprived state in the hypertrophied heart,[13] leading to further cardiac stress. This leads to the idea that pharmacologically activating fatty acid oxidation in cardiac newborn hypertrophy could ease stress on the heart. In support of this, PPARα activation in neonatal hypertrophied rabbit hearts increases fatty acid oxidation, leading to an improvement in cardiac function post-ischemia.[14] Targeting other regulatory points to increase fatty acid oxidation in the newborn cardiac hypertrophy state may also be beneficial for congenital heart defect patients to ease cardiac stress and aid with recovery. This makes investigation of novel regulatory mechanisms of cardiac energy metabolism critical, such as post-translational modifications, in congenital heart defect patients. More specifically, it is also important to look at individual congenital heart diseases to elucidate

differences between them to obtain a more detailed understanding of cardiac metabolism in these patients.

1.3 Post-translational Modifications of Metabolic Enzymes

1.3.1 Post-translational Modifications of Proteins on Lysine Residues

Central dogma suggests that in order to express a protein, DNA gets transcribed to mRNA, which then gets translated to amino acids to make a protein.[15] Post-translational modifications (PTMs) occur on proteins after this translation from mRNA to protein. These modifications are covalent and occur on the side chains or back bones of amino acids.[16] Post-translational modifications allow for the diversification of the proteome because they are able to modify the function of proteins by altering the structure of those proteins.[16] Post-translational modifications have recently emerged as an important regulator of biological roles.[17]

Developments in mass spectrometry-based proteomic techniques have made it possible to detect and quantify a vast amount of post-translational modifications. About 260,000 PTM sites have been identified in the human proteome, but the biological roles of only a few have been deciphered.[17] Proteins that can undergo post-translational modification are located throughout the cell, including the nucleus, cytosol and mitochondrion. Recently, there has been a substantial focus on lysine PTMs in the mitochondrion.[18-22]

In recent years, PTMs have proved to hold a central role in regulating mitochondria function. The mitochondrion is an important organelle to focus investigative efforts on because it is central to cellular changes in health and disease. The regulation of various essential cellular pathways such as energy metabolism, autophagy, and apoptosis are regulated by PTMs in mitochondria.[18-22]

Dysfunction in mitochondrial regulation is linked with many diseases such as heart disease, diabetes, metabolic syndrome, cancer and neurodegenerative diseases.[21] PTMs are a useful method of regulation in energy metabolism since it allows for a quick response to metabolic cues.[21] It is important to note that PTMs can occur on various amino acids, but the most common residue to be modified is lysine.[23] Lysine acetylation refers to when a two-carbon chain with a carbonyl is added to the amino group of lysine (Figure 1.3). Lysine succinylation is the addition of a four-carbon chain with a carbonyl onto the amino group of lysine (Figure 1.3). Figure 1.3 shows double-headed arrows because these post-translational modifications are reversible.

Acetyl CoA is the substrate for acetylation on lysine residues of proteins. This acetyl CoA is produced by the oxidation of fatty acids, glucose, ketones, and branched-chain amino acids in mitochondria or from malonyl CoA via the malonyl CoA decarboxylase enzyme.[24] Acetyl CoA can become completely oxidized by going into the tricarboxylic (TCA) cycle to produce energy, used in the synthesis of hormones or neurotransmitters, or it can be used in protein lysine acetylation. The substrate of lysine succinylation is succinyl CoA. Succinyl CoA is an intermediate in the TCA cycle in which it is produced from α-ketoglutarate or it can be synthesized from the odd chain fatty acid propionyl CoA.

Oxidative metabolism occurs in mitochondria and is largely regulated by PTMs on lysine residues of proteins. Among the most commonly researched PTMs are phosphorylation, ubiquitination, and acetylation, while recently succinylation, SUMOylation, and citrullination have emerged.[17] There are numerous reviews, which discuss various PTMs in the mitochondrion such as acetylation, succinylation, malonylation, and phosphorylation.[18, 20, 21]

1.3.2 Lysine Acetylation of Metabolic Enzymes and its Regulatory Proteins

Acetylation on lysine residues of metabolic enzymes is the most extensively studied PTM. In adult hearts, lysine acetylation of non-histone proteins is known to regulate metabolic proteins such as the transcription factor FOXO1[25] and fatty acid translocase CD36.[26] Other important acetylation targets are fatty acid oxidation enzymes, which lead to derangements in the oxidation of fatty acids in diseases such as obesity, diabetes, and heart failure.[27] Enzymes that are involved in the process of protein acetylation and other post-translational modifications have been researched in order to gain an understanding about how acetylation occurs and how it can be modified in health and disease. These enzymes include deacetylases, such as the sirtuins, and acetyltransferases, such as GCN5L1, which are discussed below.

When investigating lysine acetylation, it is important to consider the role of sirtuins (SIRTs), which are NAD^+-dependent deacetylases. The removal of acetyl groups from proteins is not a spontaneous process, therefore requiring the catalytic activity of deacetylases. There are seven SIRTs numbered 1 to 7 and are either localized in the nucleus, cytosol, or mitochondrion.[21] SIRT1, 6, and 7 are nuclear, while SIRT2 is cytosolic.[28] SIRT3, 4, and 5 are localized in mitochondria. Although SIRTs as a family are referred to as deacetylases, SIRT5 is in fact a desuccinylase and demalonylase with low activity as a deacetylase.[28, 29] The main mitochondrial SIRT that has been focused on in metabolic research is SIRT3 because it has widespread deacetylase activity.[30] The specific roles of SIRT4 and 5 are newly emerging.

Whether there is an opposing enzyme to SIRT3 to acetylate proteins had been a topic of investigation and in 2012, the Sack laboratory published on a mitochondrial acetyltransferase called general control of amino acid synthesis 5-like 1, abbreviated as GCN5L1.[31] Part of this study was to knockdown GCN5L1 in HepG2 cells, where a decrease in mitochondrial protein

acetylation was observed compared to controls, as well as an increased mitochondrial respiration in cells with GCN5L1 knocked down. With regard to cardiac energy metabolism, GCN5L1 has been quantified in at least three different situations: 1) in high-fat diet fed mice,[32] which had no change in GCN5L1 expression, 2) in newborn rabbits,[27] which showed an increase in GCN5L1 expression with development, which occurs concomitant with an increase of both overall acetylation and mitochondrial acetylation, and 3) in hypertrophied human newborn hearts,[33] where a blunted expression of GCN5L1 is seen in the hypertrophied state associated with a decreased acetylation.

1.3.3 Lysine Succinylation of Metabolism Enzymes

Zhang et al showed lysine succinylation to be a new and highly abundant post-translational modification using mass spectrometry.[34] Its abundance had led researchers to hypothesize that succinylation has important cellular functions. Du et al published in *Science* that all of the succinylated proteins that they detected in bovine liver mitochondria were energy metabolic enzymes.[29] Sadhukan et al performed a metabolomics-assisted proteomics study in various types of mouse tissue of SIRT5 knockout mice.[35] In these mice, protein lysine succinylation predominantly occurred in the heart compared to liver, kidney, brain and muscle. The protein identified in this study to have the most succinylation sites was an enzyme involved in fatty acid oxidation. A more in-depth investigation is necessary in both the healthy and diseased neonatal heart.

1.3.4 Post-translational Modifications in Newborn Energy Metabolism

Large changes in cardiac mitochondrial enzyme activity occur during the fetal to newborn transition. Changes in energy substrate preference in the neonatal heart are regulated at the allosteric,[9, 36] transcriptional,[5] and post-translational levels. PTMs are currently the least studied among these, despite their potential importance in regulating cardiac energy metabolism.

The affect acetylation has on fatty acid oxidation enzyme activity has been a point of controversy because there are some groups that have found acetylation of fatty acid oxidation enzymes in fact decreases their activity. Hirschey et al published a paper in a 2010 issue of *Nature* using SIRT3 knockout mice and showed that there was an accumulation of long-chain acylcarnitine species in the liver, which they claim is because fatty acid oxidation is impaired.[37] Also, Bharathi et al suggest that acetylation of the fatty acid oxidation enzyme long chain acyl-CoA dehydrogenase (LCAD) at Lys-318 and Lys-322 inhibited its enzymatic activity.[38] On the other hand, a recent study suggests that the hyperacetylation present in SIRT3 knockout mice has minimal effect on cardiac bioenergetics.[39] However, in a previous study in obese mice by our group, we have shown acetylation to have a stimulatory effect on fatty acid oxidation.[32] In support of our results, Vasquez et al have also seen an increase in fatty acid oxidation with increase acetylation in mitochondria of diabetic hearts.[40] Thapa et al recently observed that increased cardiac acetylation has a stimulatory effect on fatty acid oxidation enzymes in high-fat diet fed mice.[41] Jing et al in *Diabetes* showed that the mostly oxidative hemidiaphragm of SIRT3 knockout mice, which have increased acetylation compared to wild type, also had high palmitate oxidation rates.[42] This controversy has yet to be fully elucidated.

In summary, although much research has been done to investigate the role of allosteric and transcriptional control in the newborn heart, the post-translational modifications of acetylation and

succinylation still require a considerable research effort. Acetylation has been shown to be an important regulator of metabolism in the adult heart. Many studies using mass spectrometry have shown the importance of succinylation in cardiac metabolism. By gaining an understanding of the role of post-translational modifications in the newborn heart, possible therapeutic approaches can be derived in order to increase ATP production in congenital heart defect patients to assist with recovery after corrective heart surgery.

1.4 Background: Infants with Hypoplastic Left Heart Syndrome

Hypoplastic left heart syndrome (HLHS) is a severe CHD consisting of underdevelopment of the left side of the heart. HLHS occurs in 0.21 out of 1000 live births and has one of the worst prognoses of all CHDs.[43] It is one of the most complex CHDs to manage[44] and if left untreated, it is lethal with a 98% mortality in the first 6 weeks of life.[45-47] Children diagnosed with HLHS have also been shown to have lower quality of life compared to healthy children at 2-10 years of age.[48] HLHS infants are more susceptible to delayed neurodevelopment due to decreased oxygen or glucose supply to the brain.[49-51] It has also been shown that the myocytes of HLHS patients are inherently different in terms of their myofibrillar pattern and a persistent fetal gene expression pattern and have differing gene expression of alpha myosin heavy chain and β-adrenergic signaling.[52, 53] Additionally, impairment in coronary circulation, low oxygen saturation, high cardiac troponin I, low pH, increased markers for brain injury, high serum cortisol, and decreased gestational age, to name a few, lead to a greater morbidity or mortality post-surgery in infants with HLHS.[54-60]

The main intervention for HLHS patients is staged palliation, consisting of 3 surgeries: the Norwood in the first weeks of life, followed by the Glenn and Fontan later during development. In the 1990s, the surgical technique of the Norwood procedure greatly improved, leading to fewer post-operative mortalities.[61-63] However, this, much like other corrective heart surgeries, subjects the heart to ischemic stress and can cause perioperative heart tissue damage and low cardiac output, leading to poor outcomes after the surgery.[4] In a study determining early and midterm outcomes of the Norwood procedure, the mortality rate after the surgery was 19% from the year 1998-2000.[61]

1.5 Cardiac Energy Metabolism in Infants with Hypoplastic Left Heart Syndrome

In some CHD patients, particularly those presenting with cardiac hypertrophy, there is a delay in the maturation of cardiac fatty acid β-oxidation,[64] leading to a decreased energy capacity in these hearts.[13, 14, 33] This can have an adverse effect on infants with HLHS in whom systemic circulation is provided by the right ventricle that is pumping under stressed conditions. These patients also require multiple complex corrective heart surgeries to lessen cardiac load and improve function, which subjects the heart to obligatory periods of ischemia during surgery. Although there are some studies that have examined cardiac energy metabolism in CHD patients, little is known about HLHS specifically. In order to target the metabolic derangements that may occur in HLHS patient hearts, an understanding of the regulation of metabolism in the hearts of these infants is crucial.

1.6 Hypothesis and Aims

In this thesis, we aim to investigate changes in acetylation and succinylation during the maturation of cardiac fatty acid metabolism in the newborn heart. We also aim to determine whether there are

changes in succinylation in the presence of hypertrophy or a CHD. We will use rabbit hearts from 1-day, 7-day, and 21-day old rabbit hearts. Also, tissue from rabbit hearts with an aortocaval shunt as a model for CHDs and volume-overload hypertrophy will be used. Lastly, we will also use human myocardial tissue obtained from infants undergoing corrective heart surgery at the University of Alberta. We hypothesize that acetylation and succinylation will increase with maturation and will stimulate the activity of fatty acid oxidation enzymes. In hypertrophied hearts, acetylation and succinylation will be blunted, correlating with a decreased fatty acid oxidation compared to non-hypertrophied or sham hearts.

Another aim of this thesis is to determine if infants diagnosed with HLHS have delayed mitochondrial biogenesis and cardiac metabolism compared to infants diagnosed with other CHDs. Changes in cardiac fatty acid oxidation enzyme expression and acetylation, as well as alterations in transcriptional and allosteric regulation of cardiac metabolism may suggest altered energetics in this severe form of congenital defect. We hypothesize that there will be an alteration in cardiac metabolic regulators in HLHS hearts suggesting a delayed maturation of fatty acid oxidation in these hearts.

1.7 Figures

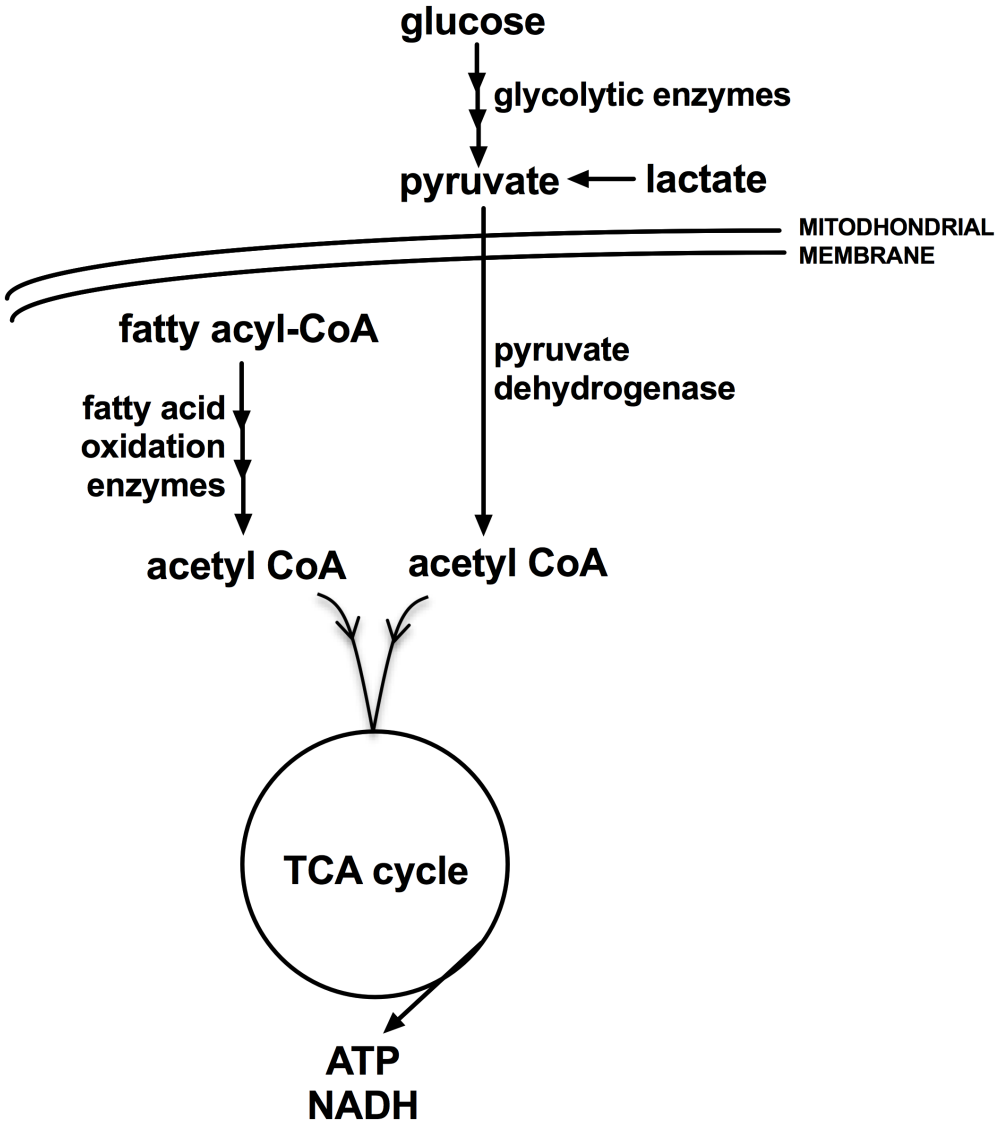

Figure 1.1 Fatty acid and carbohydrate metabolism pathways. Fatty acyl-CoA is oxidized by fatty acid oxidation enzymes to produce acetyl CoA. Glucose is converted to pyruvate via glycolysis. Pyruvate transported into the mitochondria undergoes glucose oxidation by pyruvate dehydrogenase producing acetyl CoA. Lactate can undergo lactate oxidation by being converted to pyruvate and then further oxidized in the mitochondria. Acetyl CoA produced by these pathways goes into the TCA cycle.

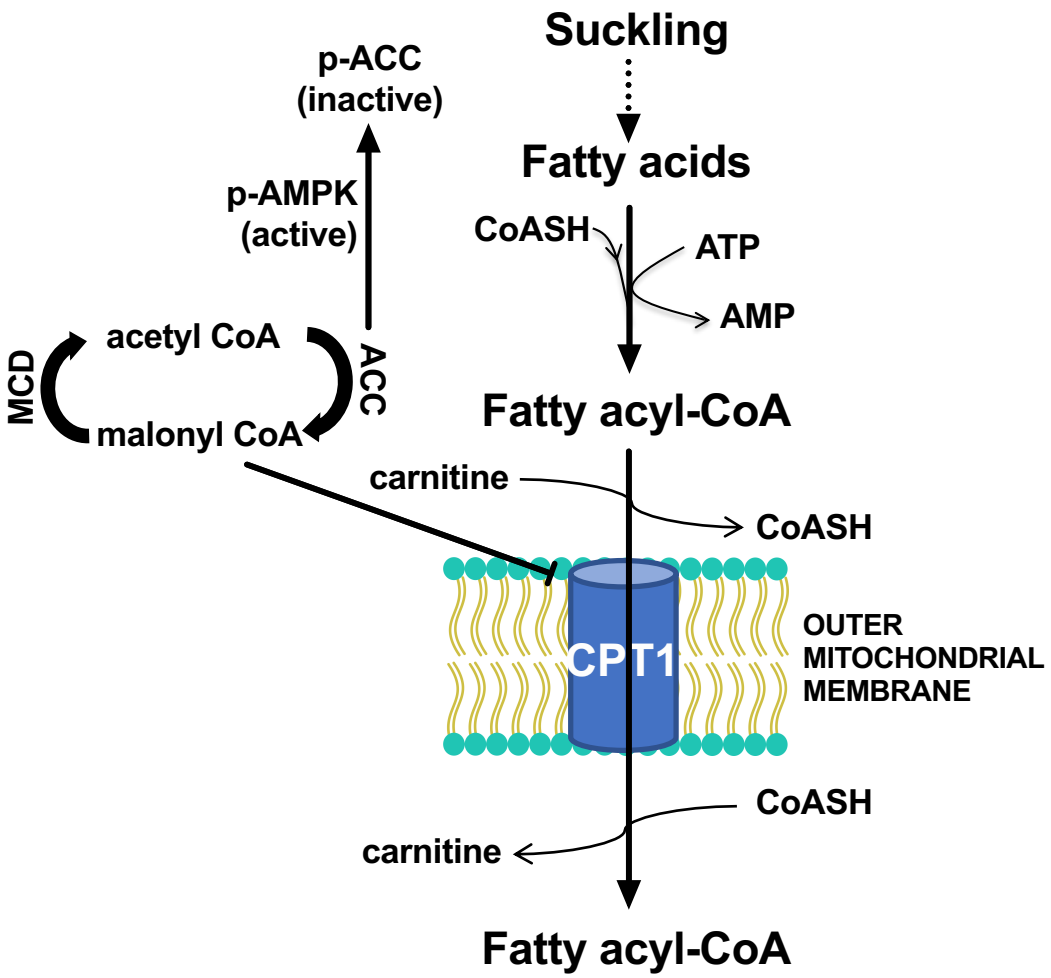

Figure 1.2 Malonyl CoA inhibition of fatty acid uptake protein carnitine palmitoyl transferase (CPT1) in the outer mitochondrial membrane. Suckling provides increased fatty acids in infants which are then converted into fatty acyl-CoA. For transport through CPT1, fatty acyl-CoA is converted to fatty acyl-carnitine and then back into fatty acyl-CoA. Malonyl CoA, which is produced by acetyl CoA carboxylase (ACC) and degraded by malonyl CoA decarbosylase (MCD), is a potent inhibitor of CPT1. Malonyl CoA levels are low when ACC is inactivated via phosphorylation by phosphorylated 5' AMP-activated protein kinase (p-AMPK). Adapted from Kantor et al.[65]

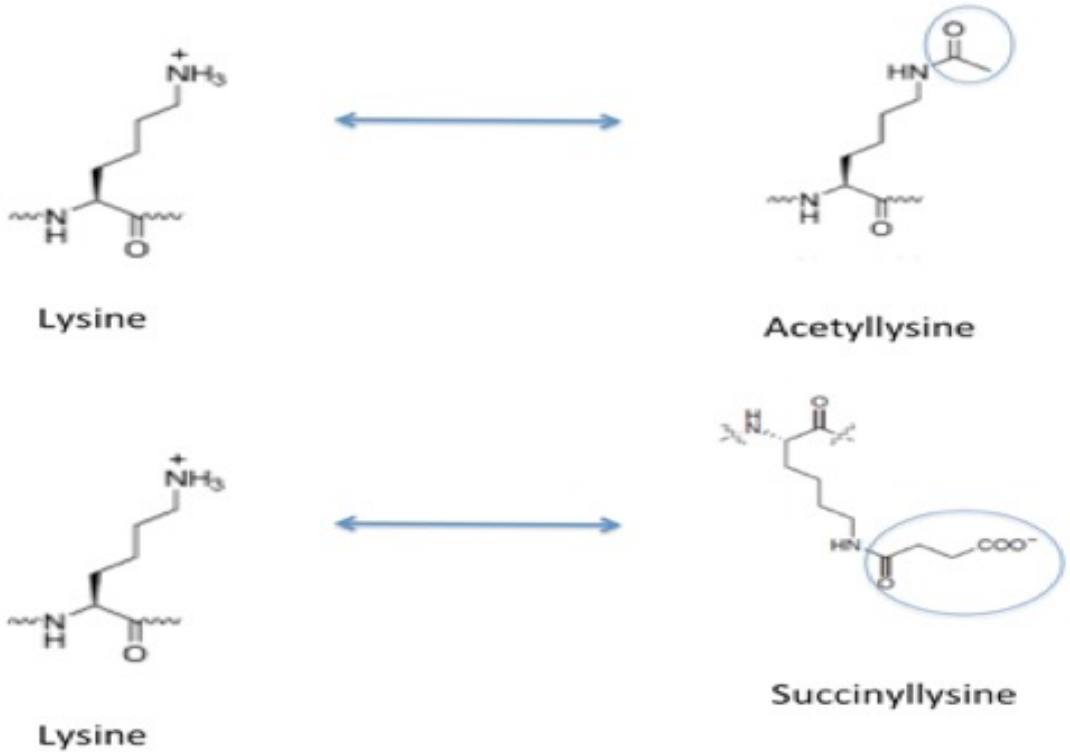

Figure 1.3 Acetylation and succinylation on lysine residues of proteins. Lysine residues on proteins can be acetylated or succinylated in a reversible reaction. The blue circles indicate the two-carbon acetyl or four-carbon succinyl group attached to lysine. Adapted from Walsh et al.[16]

Chapter Two

Acetylation and Succinylation in the Maturation of Cardiac Energy Metabolism in Newborn Rabbit and Human Hearts

Contribution:

Sonia Rawat: conception and design of research, Western blots, immunoprecipitation, data analysis, interpretation of experiment results
Arata Fukushima: conception and design of research, Western blots, immunoprecipitation, data analysis, interpretation of experiment results
Liyan Zhang: enzymatic activity assay
Cory S. Wagg: isolated heart perfusions
Gary D. Lopaschuk: conception and design of research, interpretation of experiment results, editorial and intellectual contribution

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2.1 Introduction

Congenital heart defects (CHD) affect more than 1% of newborns, one-third of which need surgery within the first month of life.[3] This palliative surgery is associated with a 4% mortality rate or a late onset heart failure, a significant cause of which is an obligatory period of ischemia and inadequate cardioprotection during surgery.[66, 67] The type of energy substrate used by the heart has a profound impact on its ability to manage ischemic stress. A decreased energy pool or reduced cardiac efficiency can further exacerbate poor cardiac function and post-surgical outcomes. Shifts in myocardial energy substrate use during maturation will influence the outcome of CHD patients receiving surgical repairs and has the potential to aggravate cardiac dysfunction.

Dramatic maturational changes occur in newborn energy metabolism in the fetal to newborn transition, most predominantly marked by a decrease in glycolysis and increase in fatty acid oxidation after birth.[6] Fatty acids quickly become the major energy providing substrate for the heart, while the oxidation of glucose remains low until weaning. An understanding of the mechanisms responsible for the maturation of fatty acid oxidation in the newborn heart will further our understanding of the newborn cardiac metabolic milieu and may provide avenues to modulate energy production under stressed conditions when the cardiac energy pool is reduced. The maturation of fatty acid β-oxidation is partly due to decreased allosteric inhibition of mitochondrial fatty acid uptake, increases in transcriptional factors controlling fatty acid β-oxidation, and changes in post-translational modifications that control key enzymes of fatty acid β-oxidation.

Post-translational modifications (PTMs) on proteins modify their structure, function, and/or enzymatic activity, usually by either activating or inhibiting the protein. Lysine acetylation is a

PTM that has recently been shown to be important in regulating energy metabolism.[37, 68] Also, the lysine PTM called succinylation has recently been shown through metabolomics to be important in regulating energy metabolism enzymes.[35] However, the importance of acetylation and succinylation in the newborn setting of cardiac metabolism is unclear. A more in-depth investigation is required to elucidate the role of acetylation and succinylation in the maturation of metabolism, specifically in fatty acid metabolism.

Results from this study can potentially be used to modulate the PTMs acetylation and succinylation in newborn CHD patients to prevent the energy-deprived state seen in many congenital heart defect patients and mitigate the negative effects of surgery on these hearts.

2.2 Methods

2.2.1 Experimental Animals

All animals were treated according to the guidelines of the Canadian Council on Animal Care and all procedures on animals were approved by the University of Alberta Health Sciences Animal Welfare Committee. One-, 7-, 21-, and 42-day-old New Zealand White rabbits of either sex were obtained from Charles River Laboratories (Charles River, Quebec, Canada) and were used in this study. Rabbit hearts are of an appropriate size to performed isolated heart perfusions unlike mouse or rat hearts at these age groups which are smaller and therefore unsuitable for isolated heart perfusion experiments.

2.2.2 Isolated heart perfusions

One-, 7-, and 21-day-old rabbits were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and hearts were extracted and retrogradely perfused with Krebs-Henseleit solution (2.5 mM Ca^{2+}, 5 mM glucose, 0.4 mM palmitate prebound to 3% bovine serum albumin (BSA), and 1 mM lactate), as described previously.[6] The hearts were initially perfused for 30 minutes without insulin followed by a 30-minute perfusion with 100 μU/mL of insulin. A 2.5 mM concentration of Ca^{2+} was used based on the large requirement of Ca^{2+} in the immature hearts, as previously described.[8] Palmitate oxidation, glycolysis, lactate oxidation, and glucose oxidation rates were measured using radiolabeled [9,10-^{3}H]palmitate, [5-^{3}H]glucose, [U-^{14}C]lactate, and [U-^{14}C]glucose, respectively, as described previously.[8] Steady-state rates of ATP production from exogenous substrates was calculated with the values of 2 and 31 mol ATP/mol of glucose passing through glycolysis and glucose oxidation, respectively, 15 mol ATP/mol of lactate oxidized through lactate oxidation, and 105 mol ATP/mol of palmitate oxidized from palmitate oxidation.[13]

2.2.3 Immunoblotting

Thirty milligrams of frozen right ventricular tissue was homogenized for 30 seconds with a Polytron homogenizer in buffer of pH 7.5 containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% NP-40, 1% Triton-X 100, 5 mmol/L EDTA, and 0.1% SDS in the presence of phosphatase and protease inhibitors (MilliporeSigma) as well as deacetylase inhibitors (10 mmol/L nicotinamide, 10 μmol/L trichostatin A, and 10 mmol/L sodium butyrate). Mitochondria were also isolated and homogenized from the neonatal heart by the standard differential centrifugation method, as previously described.[14] Thirty micrograms of the denatured proteins was subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes as previously described.[69] After blocking in 5% fat-free milk for 1 hour, membranes were probed with one of the following primary

antibodies at a 1 in 1000 dilution: β-HAD (catalogue ab37673), LCAD (catalogue ab129711), SIRT3 (catalogue ab86671), and SIRT5 (catalogue ab13697) from Abcam; succinyllysine (catalogue PTM-401) from PTM Biolabs; acetyllysine (catalogue #9441), PDH (catalogue #2784), histone H3 (catalogue #9715) and VDAC1 (catalogue #4866) from Cell Signaling Technologies; and GCN5L1 (provided by M.N. Sack, NIH, Bethesda, Maryland, USA). Membranes were incubated with the appropriate secondary antibodies (goat anti-rabbit, catalogue sc-2054; goat anti-mouse, catalogue sc-2055; goat anti-chicken, catalogue sc-2428) at a 1 in 5000 dilution for 1 hour. These bands were visualized with enhanced chemiluminescence and quantified with ImageJ software (NIH). Tubulin (catalogue #2144; Cell Signaling Technology) was used as an internal control to normalize for any variation in protein loading between samples.

2.2.4 Immunoprecipitation

A total of 300 micrograms of lysates was precleared with 20μL protein A/G-agarose beads. The lysates were incubated with succinyllysine antibodies (catalogue PTM-401; PTM Biolabs) or acetyllysine antibodies (catalogue #9441; Cell Signaling Technologies) (3μg/300μg lysate) overnight at 4°C overnight at 4°C, and 40 μL protein A/G-agarose beads was added to each sample and incubated on a rotator for 6 hours at 4°C, as previously described.[27, 32] After 6 hours, samples were washed 3 times and centrifuged at 16,000g for 5 minutes. The immune complexes were then subjected to immunoblot analysis as described above with an increase in membrane incubation time in primary antibody to 3 hours. As a negative control, heart lysates were immunoprecipitated with normal rabbit IgG (sc-2027; Santa Cruz) coupled to agarose A/G beads or agarose A/G beads alone. Heart lysates without rabbit IgG were used as a positive control. The heavy chain of IgG was used as the loading control.

2.2.5 Enzymatic activity of LCAD and β-HAD

LCAD activity was assayed in total heart lysates prepared from frozen heart tissues as previously described.[32] In brief, 20 micrograms of homogenate was pipetted in a 96-well plate containing potassium phosphate assay buffer. The reaction was initiated by the addition of 50 μM palmitoyl-CoA. To determine the LCAD enzymatic activity, a spectrophotometer measuring absorbance at 300 nm wavelength was used to follow the initial decrease in absorbance upon reduction of the ferricenium ion over 2 minutes. The ferricenium ion is the electron acceptor that gets reduced upon the oxidation of palmitoyl-CoA with the catalyzing action of the LCAD enzyme.

β-HAD activity was assayed as previously described.[69] In brief, heart lysates were pipetted in a 96-well plate containing assay buffer with 50 mmol/L imidazole and 150 mmol/L NADH. The reaction was initiated by the addition of acetoacetyl-CoA and the disappearance of NADH was measured at the 340 nm wavelength absorbance for 5 minutes.

2.2.6 Statistical Analysis

Data are represented as mean ± SEM. Comparisons were performed using a one-way ANOVA followed by Tukey's multiple-comparison test. The correlation was examined by linear regression analysis using the least-squares method. A p value < 0.05 was considered statistically significant.

2.3 Results

In isolated heart perfusion experiments, palmitate oxidation rates were found to be significantly higher in the 21-day old hearts compared to 1-day and 7-day old hearts (Figure 2.1A). Glycolysis

was lower in 7- and 21-day old hearts compared to the 1-day old hearts, and glucose oxidation was also lower in 21-day old hearts (Figures 2.1B and 2.1C). Lactate oxidation was lower in the 21-ay old hearts compared to the 7-day old hearts (Figure 2.1D). Overall ATP production was significantly higher in 21-day old hearts compared to 1- and 7-day old hearts (Figure 2.1E).

Total cardiac lysine acetylation was significantly higher in 21-day old rabbit hearts compared to 1-day old hearts in whole lysate and in purified mitochondria and purified nucleus (Figures 2.2A and 2.2B). To explain this change in acetylation, we analyzed protein levels of two acetylation regulators. Although NAD^+-dependent deacetylase sirtuin 3 (SIRT3) was not different across groups (Figure 2.2C), the mitochondrial acetyltransferase GCN5L1 was significantly higher in 21-day old hearts (Figure 2.2D). This increase in GCN5L1 coincides with the increase in acetylation observed in 21-day old hearts.

The proteins levels of fatty acid β-oxidation long chain acyl-CoA dehydrogenase (LCAD) was increased in 7- and 21-day old hearts compared to 1-day old hearts (Figure 2.3A). Another key fatty acid β-oxidation enzyme, β-hydroxyacyl CoA dehydrogenase (β-HAD), was increased in 21-day old hearts compared to 1-day old hearts (Figure 2.3B). Lysine acetylation levels of both LCAD and β-HAD were also increased with maturation (Figures 2.3C and 2.3D). This was accompanied by an increase in the enzymatic activity of LCAD and β-HAD in these hearts (Figures 2.3E and 2.3F). This suggests a maturational increase in fatty acid β-oxidation enzyme expression, acetylation, and activity in newborn rabbit hearts.

We next investigated lysine succinylation of proteins in the newborn hearts. Total cardiac lysine succinylation was significantly increased in 21-day old hearts compared to 1-day old hearts (Figure

2.4A and 2.4B). SIRT5, a known desuccinylase in mitochondria, decreased in 7-day and 21-day old hearts compared to 1-day old hearts (Figure 2.4C), being a possible contributor to the increase in total cardiac lysine succinylation observed. Succinylation of LCAD was increased in 21-day old hearts compared to 1-day old hearts and this was positively correlated with the enzymatic activity of LCAD (R^2=0.50, p<0.01) (Figures 2.4D and 2.4E). This did not, however, correlate with the changes in the rates of palmitate oxidation (R^2=0.19, p=0.15) (Figure 2.4F). We also investigated lysine succinylation of the enzyme that is the rate limiting step of glucose oxidation, pyruvate dehydrogenase (PDH) and observed a significantly higher level of PDH succinylation in 21-day old hearts compared to 1-day old hearts (Figure 2.4G).

2.4 Discussion

Acetylation is a potentially important regulator of the maturation of cardiac fatty acid oxidation by stimulating fatty acid oxidation enzymes in the newborn period. We observed an increase in cardiac lysine acetylation with maturation in whole lysate, mitochondria, nuclei, and the cytosol from 1-day to 21-days in newborn rabbits. Although we did not see a parallel decrease in the mitochondrial sirtuin, SIRT3, the mitochondrial acetyltransferase that promotes acetylation increased. When investigating the maturation of fatty acid metabolism in these hearts, we observed an increase in protein levels of LCAD and β-HAD with age. A novel finding in this study is that there was an increase in the acetylation levels of LCAD and β-HAD with age in newborn rabbit hearts. Interestingly, this acetylation was associated with an increase in enzymatic activity of LCAD and β-HAD. In our investigation of succinylation in newborn cardiac metabolism maturation, we obtained a novel finding that cardiac lysine succinylation increased from 1-day to 21-days in newborn rabbits. Furthermore, we have noted an increase in total lysine succinylation

levels in 42-day old hearts compared to 21-day old hearts, suggesting a role of succinylation throughout the newborn period. This increase in lysine succinylation was parallel to a decrease in SIRT5 protein levels. The fatty acid oxidation enzyme LCAD showed increased succinylation from 1-day to 21-days and this was positively correlated with an increase in LCAD enzymatic activity. There was also an increase in succinylation of glucose oxidation enzyme PDH with maturation from 1-day to 21-day old hearts.

Post-translational modifications on lysine residues of proteins including acetylation and succinylation are recently recognized regulators of mitochondrial energy metabolism, particularly in studies using SIRT3 or SIRT5 knockout mice.[22, 35, 37, 70] In the present study, we observed dramatic increases in total cardiac acetylation and succinylation during maturation that coincide with the dramatic increase in cardiac fatty acid oxidation that normally occurs post-birth. The level of acetylation is balanced by the deacetylase and acetyltransferase enzymes SIRT3 and GCN5L1, respectively. Although we did not observe changes in SIRT3 protein levels to explain the changes in total cardiac acetylation, we did observe increases in GCN5L1, a newly discovered mitochondrial acetyltransferase.[71] GCN5L1 has been shown to acetylate fatty acid oxidation enzyme β-HAD in the liver.[72] This led to subsequent investigation of acetylation of fatty acid oxidation enzymes.

Fatty acid oxidation rates in the heart dramatically increase post-birth. Allosteric regulators and transcription factors have been established to be key in regulating the maturation of fatty acid β-oxidation in newborns.[9, 73-75] In the present study, along with the increase in total lysine acetylation there is an age-dependent increase in specific acetylation and enzymatic activity of mitochondrial enzymes responsible for fatty acid β-oxidation, LCAD and β-HAD, with age. Acetylation and

activity of these enzymes showed a significant positive correlation with each other and was accompanied with an increase in cardiac fatty acid oxidation rates seen in maturation. The effect of acetylation on the activity of fatty acid oxidation enzymes has been a matter that is not yet agreed upon. Some groups have found that SIRT3 knockout mice with high acetylation levels have decreased levels of fatty acid oxidation in the liver.[37, 76] However, some specific residues for acetylation change the enzymatic activity by altering protein conformation of the LCAD active site, suggesting differences in the specific acetylation site may be resulting in varied activity.[38] In this study and in a previous study in obese mice, we have shown acetylation to have a stimulatory effect on fatty acid oxidation.[32] In support of our results, Vasquez et al have also seen an increase in fatty acid oxidation with increase acetylation in mitochondria of diabetic hearts.[40] Zhao et al investigated acetylation in human liver tissue and observed that acetylation activated fatty acid oxidation enzymes.[22] Thapa et al recently observed that increased cardiac acetylation has a stimulatory effect on fatty acid oxidation enzymes in high-fat diet fed mice.[41] Jing et al in *Diabetes* showed that the mostly oxidative hemidiaphragm of SIRT3 knockout mice, which have increased acetylation compared to wild type, also had high palmitate oxidation rates.[42] Taken together, these findings suggest that acetylation has a stimulatory effect on the activity of fatty acid oxidation enzymes, contributing to increased fatty acid oxidation rates in the maturation of newborn hearts.

Upon further investigation, we found that the PTM succinylation also changes with maturation in the newborn heart. We show for the first time that there is an increase in total succinylation both in whole lysates and isolated mitochondria parallel to a drop in mitochondrial desuccinylase SIRT5 expression with age. Interestingly, this increase in succinylation continues on past the 21-day old age group, as there was an approximately 1.5-fold increase at 42 days of age compared to 21-day old hearts. This considerable increase indicates that lysine succinylation may have a role in more

mature hearts. When investigating specific protein succinylation, an increase in lysine succinylation of LCAD seen with maturation was positively correlated with LCAD activity. However, the lack of correlation of LCAD succinylation with fatty acid oxidation rates suggests that succinylation may not be a major contributor to the changes associated with newborn fatty acid metabolism maturation in this age range. Nonetheless, proteomics studies have highlighted the importance of succinylation in mitochondrial metabolism in the heart, liver, and other organs.[23, 35] An investigation of fatty acid oxidation enzyme succinylation in more mature newborn hearts will help further elucidate whether this PTM has a role in fatty acid oxidation enzyme regulation.

It has been previously observed that acetylation of PDH inhibits its enzymatic activity in mouse skeletal muscle of SIRT3 knockout mice.[77] However, the effect of succinylation on PDH is not known. We observed an increase in PDH succinylation in 21-day old hearts compared to 7-day old hearts. PDH has been observed to be suppressed when succinylation levels are low.[78] However, we observed decreased glucose oxidation rates parallel to increased PDH succinylation levels in maturation, suggesting an inhibitory role of succinylation on PDH activity. Further work to determine the effect of succinylation on PDH activity is necessary.

2.4.1 Limitations

One limitation of our study is that in our investigation of acetylation and succinylation of specific proteins, our techniques do not provide us with the proportion of the proteins that are acetylated compared to non-acetylated. Secondly, our experiments show protein levels of SIRT3 and SIRT5, but not the enzymatic activity of these proteins. Knowing the activity of these proteins would provide a more accurate understanding of their effect on the maturation of newborn metabolism. Lastly, due to the difficulty of determining the sex of the rabbits in the immediate newborn period,

both male and female mice were used in this study, contributing to heterogeneity in the experimental animals.

In summary, our findings show that the post-translational modification acetylation is important in the regulation of the dramatic increase in fatty acid oxidation in the newborn period. Increased fatty acid oxidation enzyme acetylation contributes to an increase in their activity. Succinylation also has a stimulatory effect on fatty acid oxidation enzyme LCAD but does not correlate with fatty acid oxidation rates in newborn rabbit hearts. These results identify a pathway that can potentially be used to pharmacologically stimulate cardiac fatty acid oxidation, which can be beneficial for newborns with congenital heart defects that have a decreased energy pool.

2.5 Figures

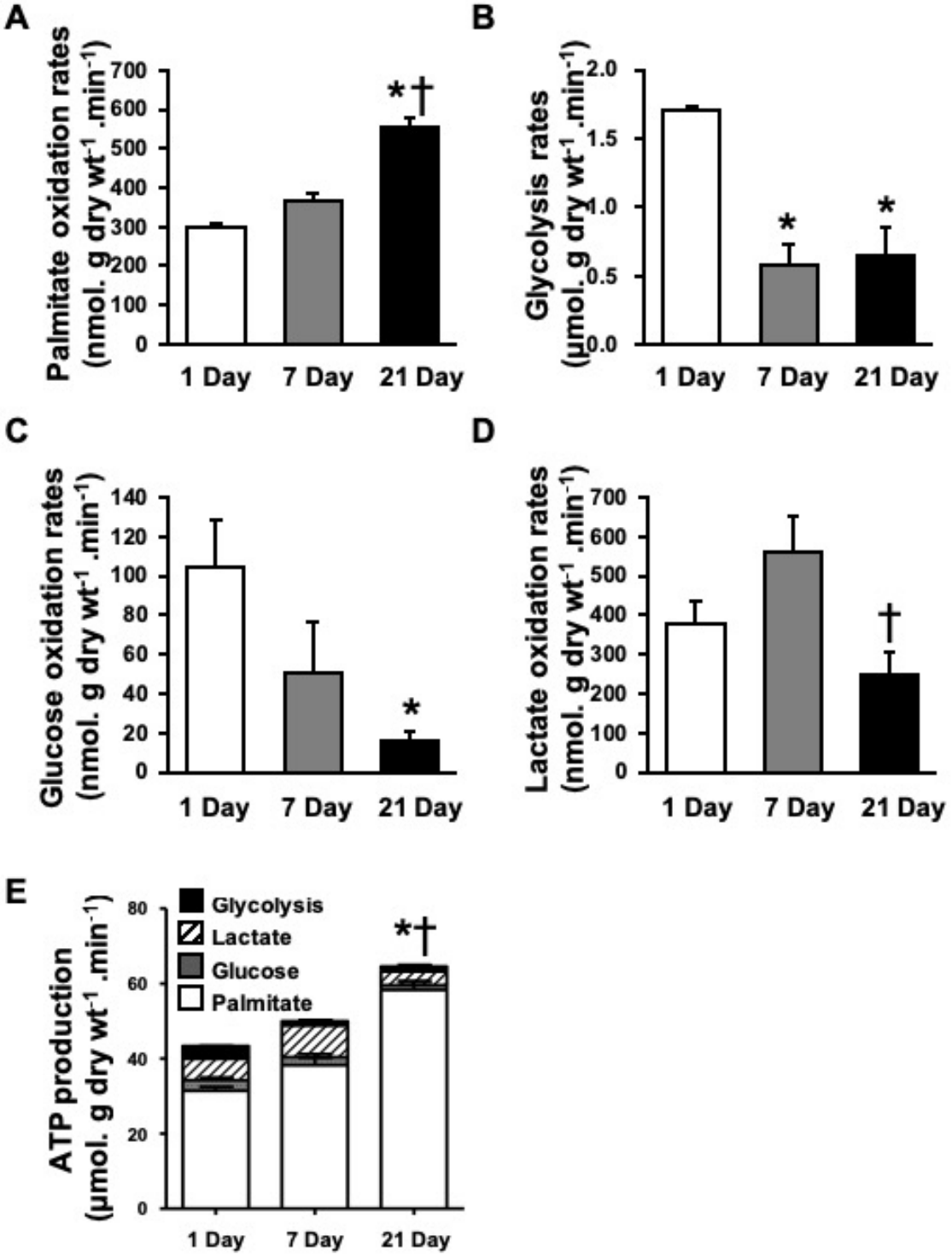

Figure 2.1 Absolute metabolic rates in isolated Langendorff newborn rabbit hearts. Metabolic rates are shown for palmitate oxidation (A), glycolysis (B), glucose oxidation (C), lactate oxidation (D), and total ATP production (E). n=4-6/group. Data are presented as mean ±

SE. Data was analyzed by One-Way ANOVA followed by Tukey's multiple-comparison test. *$p<0.05$ is considered as significantly different compared to 1-day old group and †$p<0.05$ is considered as statistically different compared to 7-day old group.

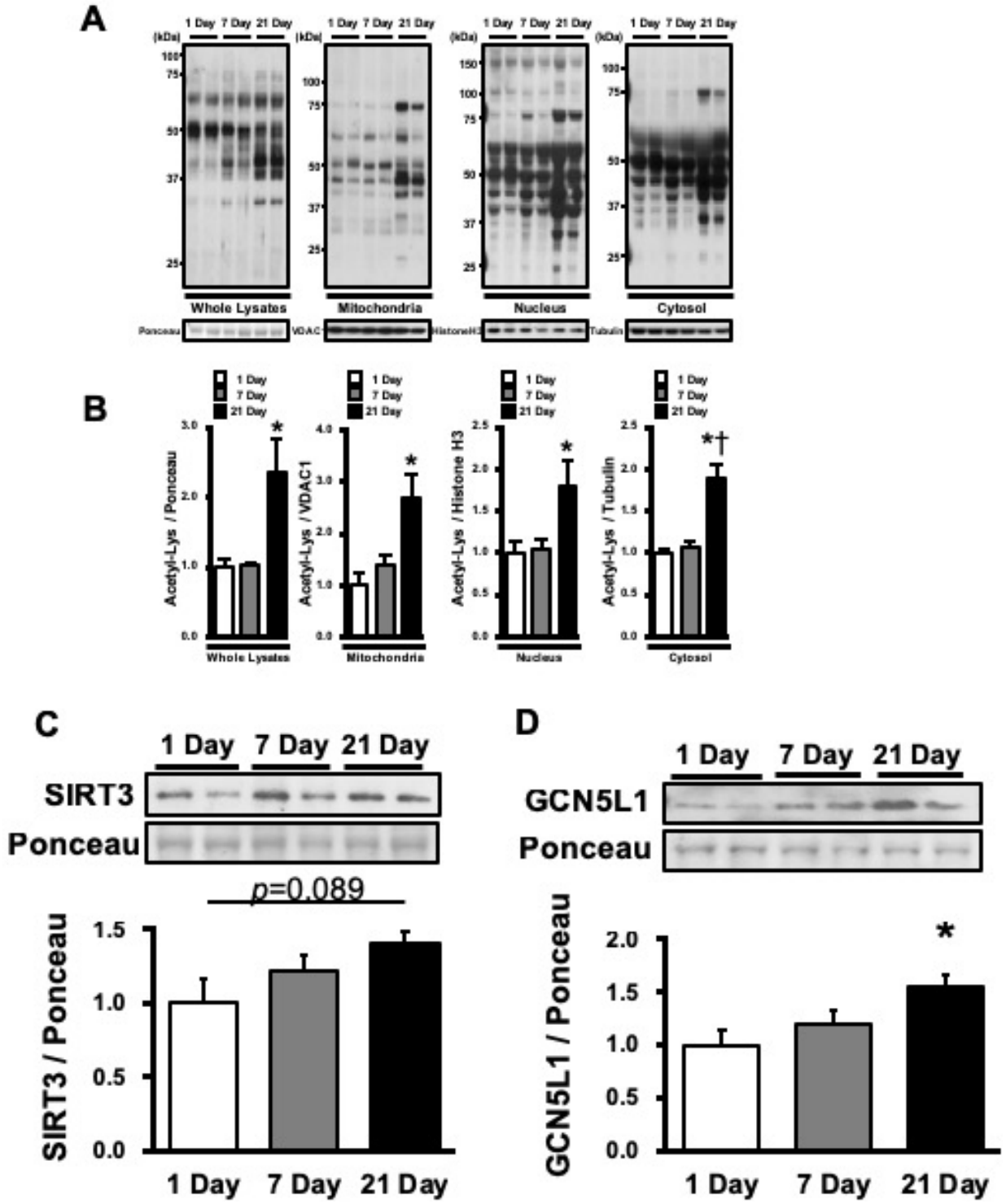

Figure 2.2 Total cardiac lysine acetylation in the maturation of newborn rabbits. Total cardiac lysine acetylation blots in whole lysate, isolated mitochondria, isolated nuclei, and cytosol (A) with quantification (B) along with protein levels of mitochondrial deacetylase sirtuin 3 (SIRT3)

(C) and mitochondrial acetyltransferase GCN5L1 (D) are shown. n=6-8/group. Data are presented as mean ± SE and analyzed by One-Way ANOVA followed by Tukey's multiple-comparison test. *$p<0.05$ is considered as significantly different compared to 1-day old group and †$p<0.05$ is considered as statistically different compared to 7-day old group. Panels A and B are work done by Fukushima A. Panels C and D are work done in conjunction by Rawat S and Fukushima A.

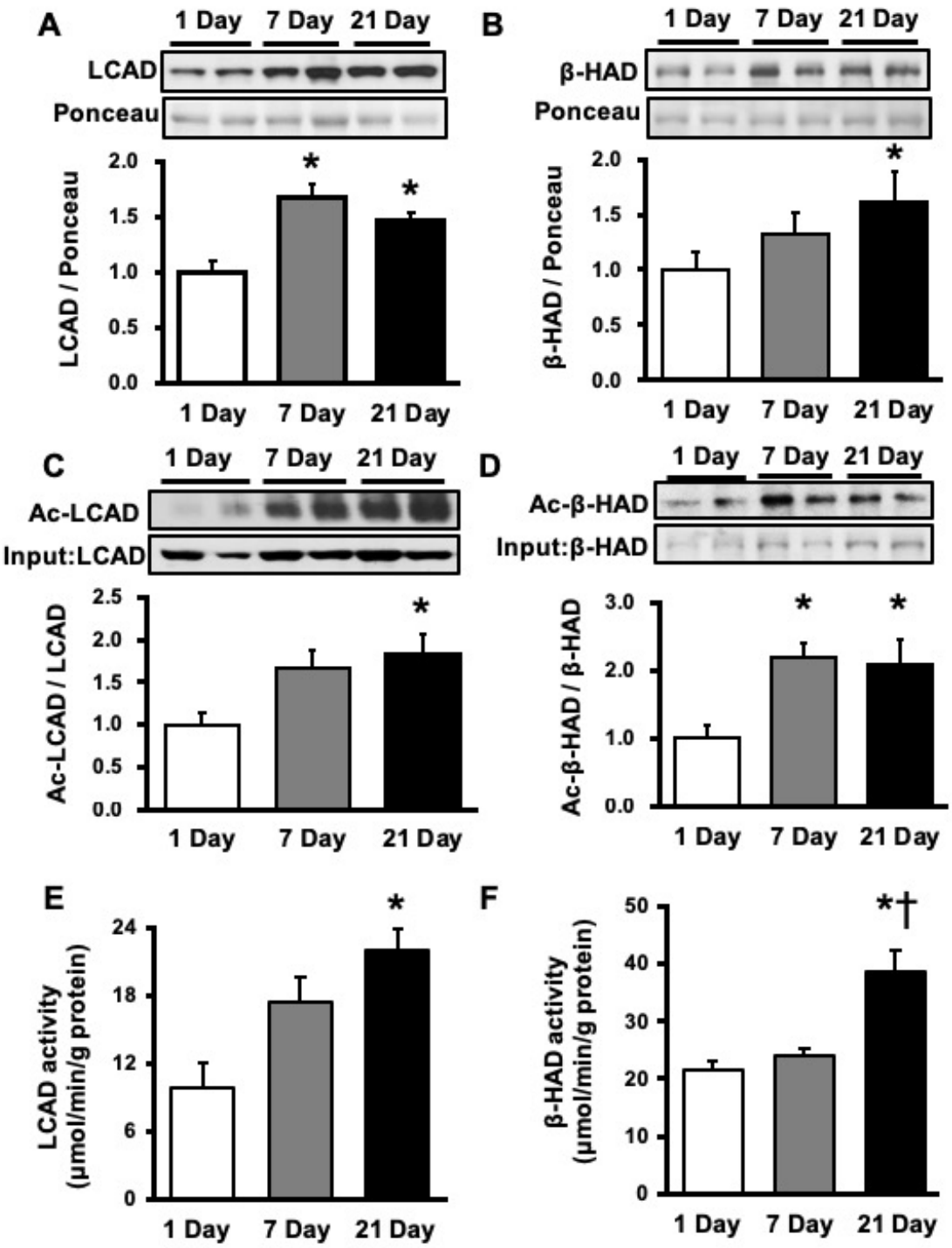

Figure 2.3 Fatty acid oxidation enzymes protein levels and acetylation. Protein levels of fatty acid oxidation enzymes long chain acyl CoA dehydrogenase (LCAD) and β–hydroxyacyl CoA dehydrogenase (β-HAD) (B), followed by the acetylation of LCAD (C) and β-HAD (D) normalized for total LCAD or β-HAD levels in the same hearts, respectively, and the enzymatic

activities of LCAD (E) and β-HAD (F) are shown. n=6-8/group. Data are presented as mean ± SE and analyzed by One-Way ANOVA followed by Tukey's multiple-comparison test. *$p<0.05$ is considered as significantly different compared to 1-day old group and †$p<0.05$ is considered as statistically different compared to 7-day old group. Panels A-D are work done in conjunction by Rawat S and Fukushima A.

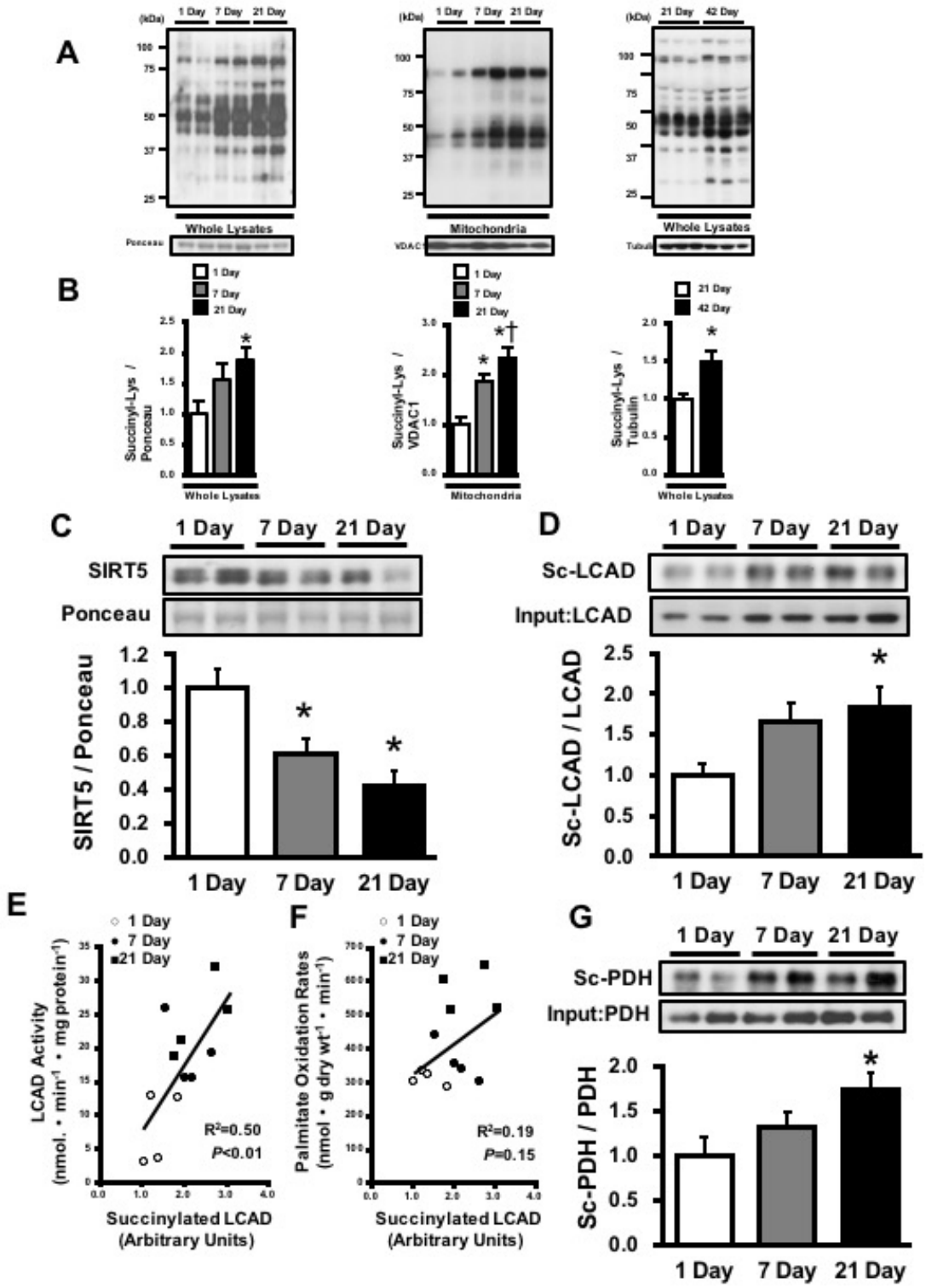

Figure 2.4 Cardiac lysine succinylation in the maturation of newborn rabbits. Total cardiac lysine succinylation blot in whole lysates and in isolated mitochondria in age groups ranging from 1-42 days old (A) with quantification (B) protein levels of mitochondrial desuccinylase sirtuin 5

(SIRT5) (C) along with succinylation levels of LCAD normalized for total LCAD levels in the same hearts (D), correlation of levels of succinylated LCAD to its enzymatic activity (E), correlation of levels of succinylated LCAD to rates of cardiac palmitate oxidation (F), and succinylated levels of PDH normalized for total PDH levels in the same hearts (G) are shown. n=6-8/group for each except panel G has n=4/group. Data are presented as mean ± SE and analyzed by One-Way ANOVA followed by Tukey's multiple-comparison test or linear regression analysis using the least squares method. *$p<0.05$ is considered as significantly different compared to 1-day old group and †$p<0.05$ is considered as statistically different compared to 7-day old group. Panels A and B are work done by Fukushima A. Panels C, D, and G are work done by Rawat S.

Chapter Three

Acetylation and Succinylation as Regulators of Cardiac Energy Metabolism in Hypertrophied Rabbit and Human Newborn Hearts

Contribution:

Sonia Rawat: conception and design of research, Western blots, immunoprecipitation, data analysis, interpretation of experiment results
Arata Fukushima: conception and design of research, Western blots immunoprecipitation, data analysis, interpretation of experiment results
Liyan Zhang: enzymatic activity assays, intellectual and editorial contribution
Victoria H. Lam: isolated biventricular working heart perfusions
Gary D. Lopaschuk: conception and design of research, interpretation of experiment results, intellectual and editorial contribution

A modified version of Figure 3.1 has been published in:

Fukushima A, Zhang L, Huqi A, Lam VH, Rawat S, Altamimi T, Wagg CS, Dhaliwal KK, Hornberger LK, Kantor PF, Rebeyka IM and Lopaschuk GD. Cardiac hypertrophy in neonates delays acetylation control of cardiac energy metabolism. *JCI Insights*. 2018;3(10):e99281.

Figure 3.2 and 3.3 are unpublished.

3.1 Introduction

1% of newborns are affected by a CHD, many of whom require heart surgery during the newborn period.[79] Despite recent advancements in cardiac surgical techniques for CHD patients, chronic and acute stress on these heats is still a concern, making heart failure a major determinant of outcomes in these patients.[80] The presence of cardiac hypertrophy, abnormal blood flow through the heart, and an obligatory period of global ischemia during surgery are all factors contributing to risk of heart failure in CHD patients.[81, 82] Derangements in cardiac energy metabolism during maturation occur in hearts subjected to volume and/or pressure overload and may also be a key factor in the development of heart failure associated with CHDs.[13, 14, 65] Therefore, the type of energy substrate used by the heart may have a profound impact on the heart's ability to manage volume and/or pressure overload conditions.

Dramatic maturational changes occur in newborn energy metabolism in the fetal to newborn transition, most predominantly marked by a decrease in glycolysis and increase in fatty acid oxidation after birth, making fatty acids the major energy-providing substrate for the heart.[1, 6] However, the presence of hypertrophy delays the maturation of fatty acid oxidation; wherein the dramatic increase of fatty acid oxidation after birth does not occur.[13, 14, 65] Metabolic differences have been observed in newborn hypertrophied hearts compared to non-hypertrophied hearts. Specifically, 3-week-old rabbits with cardiac hypertrophy have fatty acid oxidation rates that are 60% lower than non-hypertrophied controls.[13] Without the dramatic increase in fatty acid oxidation, the heart is at risk of having a decreased energy capacity. The regulation of the maturation of fatty acid oxidation is complex and occurs at three levels: allosteric control,

transcriptional control, and the most recently emerging one being post-translational modifications (PTMs).

The PTM lysine acetylation has recently been shown to be an important regulator of cardiac energy metabolism.[32, 83] We and others have observed lysine acetylation to have a stimulatory effect on fatty acid oxidation enzyme activity.[27, 32, 40] We investigated whether lysine acetylation has a role in the delayed maturation of fatty acid oxidation in hypertrophied hearts compared to non-hypertrophied hearts in newborn rabbit hearts. Also, the lysine PTM succinylation has recently been shown through metabolomics to be important in regulating energy metabolism enzymes.[35] However, their importance in the newborn setting of cardiac metabolism is unclear. Previously, we observed that total succinylation levels of proteins increases consistently from 1-day post-birth to 7-days, 21-days and 42-days of age, suggesting an association with maturation.[27] Similarly, mitochondrial succinylation levels also show an increase. We therefore investigated the role of succinylation in the cardiac metabolism of rabbit and human newborn hearts.

3.2 Methods

3.2.1 Newborn subjects

Right ventricular samples were collected from patients undergoing corrective heart surgery for a congenital heart defect at the University of Alberta Hospital from 2006 to 2015. Infants were stratified in two age groups and further stratified based on the presence or absence of hypertrophy. Collection of specimens from newborn patients were approved by the University of Alberta Health Research Ethics Board protocol (ID no. Pro0001112). All participants or their guardians provided

written informed consent for the sample collection, following analysis and prior to inclusion in the study.

3.2.2 Animal Study

New Zealand White newborn rabbits of either sex (Charles River Co.) were used for this study. All animals were treated according to the guidelines of the Canadian Council on Animal Care. All animal protocols were approved by the University of Alberta Health Sciences Animal Welfare Committee. All rabbits were anaesthetized using inhaled isofluorane (2%) at 7-days of age for placement of an aortocaval shunt to induce volume-overloaded cardiac hypertrophy or sham surgery. 14-days after the surgery, the rabbits were anaesthetized using sodium pentobarbital (60 mg/kg body weight), and the hearts were extracted for isolated biventricular working heart perfusions, snap-frozen in liquid nitrogen, and crushed for biochemical analysis.

3.2.3 Isolated Bi-Ventricular Working Heart Perfusions

Rabbit hearts were excised and retrogradely perfused using a modified Krebs-Henseleit solution that contains 2.5 mmol/L Ca^{2+}, 5.5 mmol/L glucose, 1.2 mmol/L palmitate prebound to 3% BSA, 0.5 mmol/L lactate, and 100 μU/mL insulin, as described previously.[13, 14] In brief, during 15 minutes of retrograde perfusion, the superior vena cava, left atria, and pulmonary artery were cannulated and inferior vena cava was ligated. At the end of the 15 minutes, flow into the left atria, opening of the aortic afterload line, and termination of retrograde perfusion initiated LV work. RV load was added by opening SVC flow, producing a biventricular working heart. Rates of palmitate oxidation, glycolysis, and glucose oxidation were measured during the aerobic periods using radiolabeled [9,10-^{3}H]palmitate, [5-^{3}H]glucose, and [U-^{14}C]glucose, respectively. Heart rates and

peak systolic pressures of aortas and pulmonary veins were measured using a Gould P21 pressure transducer attached to the aortic and pulmonary outflow line. Normalized cardiac function was calculated as [heart rates x (aortic peak systolic pressure + pulmonary vein peak systolic pressure)], as previously described.[13, 14] At the end of the aerobic perfusion protocol, hearts were immediately frozen in liquid N_2 and stored at –80 degrees Celcius. Steady-state rates of ATP production from exogenous substrates was calculated with the values of 2 and 31 mol ATP/mol of glucose passing through glycolysis and glucose oxidation, respectively and 105 mol ATP/mol of palmitate oxidized from palmitate oxidation.[13]

3.2.4 Immunoblotting

Immunoblotting was performed as described in section 2.2.3. Membranes were probed with one of the following primary antibodies at a 1 in 1000 dilution: β-HAD (catalogue ab37673), LCAD (catalogue ab129711), SIRT3 (catalogue ab86671), and SIRT5 (catalogue ab13697) (both from Abcam); succinyllysine (catalogue PTM-401) from PTM Biolabs; PDH (catalogue #2784) and acetyllysine (catalogue #9441) from Cell Signaling Technologies; and GCN5L1 provided by M.N. Sack, NIH, Bethesda, Maryland, USA.

3.2.5 Immunoprecipitation

Please refer to section 2.2.4 for the immunoprecipitation methods.

3.2.6 Enzymatic activity of LCAD and β-HAD

Please refer to section 2.2.5 for the enzymatic activity assay methods.

3.2.7 Statistical Analysis

All data are presented as mean ± SEM. A Student's t-test for the experiments using the rabbit heart model or a one-way ANOVA for the human heart tissue experiments was used for the analysis. A p value < 0.05 was considered statistically significant.

3.3 Results

We started by investigating the absolute metabolic rates in rabbit newborn hearts using biventricular working heart perfusion. Palmitate oxidation rates were significantly lower in hypertrophied hearts compared to the sham group (Figure 3.1A). Glycolysis rates were higher in hypertrophied hearts without an accompanied increase in glucose oxidation (Figures 3.1B and 3.1C). Overall cardiac ATP levels were significantly lower in the hypertrophied group (Figure 3.1D). While the protein levels of fatty acid oxidation enzymes long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were unchanged between groups, the acetylation of these enzymes was lower in hypertrophied hearts (Figure 3.1E-3.1H). This was not parallel to changes in SIRT3 protein levels, but the changes seen in fatty acid oxidation enzyme acetylation did occur in parallel to a decrease in mitochondrial acetyltransferase GCN5L1 levels (Figures 3.1I and 3.1J). There was an accompanying decrease in enzymatic activity of LCAD and β-HAD and we found a positive correlation between the acetylation and activity of these enzymes. Our analysis showed a significant positive correlation between the level of acetylated LCAD or β-HAD and the rates of palmitate oxidation in these hearts (Figures 3.1K-3.1P).

Following our investigation into examining the role of acetylation in newborn cardiac fatty acid oxidation, we examined the role of lysine succinylation in regulating cardiac energy metabolism. Total cardiac lysine succinylation levels were not different between sham and hypertrophied rabbits (Figures 3.2A and 3.2B). The sirtuin SIRT5, which has been shown to have desuccinylase and demalonylase activity also did not show a difference between groups (Figure 3.2C). Lysine succinylation levels of pyruvate dehydrogenase and long chain acyl CoA dehydrogenase normalized for the heavy chain of the antibody used in the pulldown step of immunoprecipitation were also not different between groups, suggesting succinylation may not play a significant role in the metabolic delay of fatty acid oxidation observed in hypertrophied hearts during the newborn period (Figures 3.2D and 3.2E).

3.4 Discussion

Alterations in PTMs are a potentially key mechanism in the delayed metabolic maturation, particularly of fatty acid oxidation, observed in newborn hypertrophied hearts. In this study, we investigated the role of acetylation and succinylation in regulating the metabolic changes that occur in hypertrophied newborn hearts that contribute to a decreased energy pool. We found that in a rabbit model of CHD, there is a decrease in cardiac fatty acid β-oxidation and overall ATP production in hypertrophied hearts compared to sham. A novel finding is that lysine acetylation of fatty acid β-oxidation enzymes was also decreased in hypertrophied hearts and this decreased acetylation had a positive correlation with fatty acid oxidation rates. The decrease in lysine acetylation of these enzymes in hypertrophied hearts occurred in parallel with a decrease in mitochondrial acetyltransferase GCN5L1 protein levels. Another novel finding was in our investigation of lysine succinylation, which showed total lysine succinylation was not changed,

nor was succinylation of the fatty acid and glucose oxidation enzymes LCAD and PDH, respectively, altered. However, in human cardiac newborn tissue, there was a decrease in total lysine succinylation in the hypertrophied group compared to the non-hypertrophied group in hypertrophied hearts of infants 101-200 days of age, suggesting a role of succinylation in hypertrophied human hearts but not hypertrophied rabbit hearts.

It has been shown in various disease states that changes in lysine acetylation are linked to metabolic derangements in obesity, diabetes, and heart failure.[32, 84, 85] Previously, we have shown that acetylation of fatty acid oxidation enzymes activates these enzymes.[27, 32, 85] In this study, we observed that in the newborn heart, acetylation acts to stimulate the activity of fatty acid oxidation enzymes LCAD and β-HAD, potentially helping to ameliorate the decreased energy pool that we observed in hypertrophied rabbit newborn hearts. The fatty acid oxidation enzymes are a major target for SIRT3.[37] However, SIRT3 did not show any changes in protein levels in our study. The opposing enzyme to SIRT3 is the acetyltransferase GCN5L1, which we observed to be decreased in the hypertrophied rabbit hearts, leading to decreased fatty acid oxidation enzyme acetylation, lower ATP production, and further exacerbation of cardiac hypertrophy.

Our main aim was to elucidate whether there are changes in succinylation in hypertrophied rabbit and human newborn hearts compared to the non-hypertrophied hearts from rabbits and humans. We did not observe a change in total lysine succinylation between sham and hypertrophied rabbit hearts, although there was a significant change in hypertrophied human newborn hearts in the 101-200 days old age group. Since dramatic changes in cardiac succinylation have been seen later in the maturation process (i.e., 42-day old rabbits compared to 21-day old rabbits), perhaps investigating older hypertrophied rabbit hearts will provide clarity on the effect of hypertrophy in

the heart on succinylation levels. Additionally, the possibility exists that there may be differential maturation of lysine succinylation levels between rabbits and humans at this age. Lastly, the opportunity to further experiment on human newborn hearts to elucidate whether changes in succinylation levels of specific cardiac metabolic enzymes such as β-HAD and LCAD remains to be elucidated as well.

Results from this study can potentially be used to pharmacologically increase lysine acetylation of cardiac metabolic proteins to enhance energy production in CHD patients and mitigate the negative effects of surgery on these hearts. Further research is necessary to elucidate the role of succinylation on fatty acid oxidation in the human hypertrophied newborn heart during later maturation. Specific succinylation of fatty acid oxidation enzymes at the 101-200 age range and older is necessary to determine whether succinylation, in addition to acetylation, contributes to the delayed maturation of fatty acid oxidation in the hypertrophied state.

3.4.1 Limitations

Firstly, the clinical population used for control group labeled “non-hypertrophied” group are not representative of the normal population. Also, the techniques we have used do not allow us to determine the proportion of succinylated proteins to non-succinylated proteins. However, Zhao et al have shown, using mass spectrometry, that there is a large absolute change in acetylation of fatty acid oxidation enzyme upon pharmacological inhibition of β-HAD, strengthening the idea that acetylation of fatty acid oxidation enzymes can have a considerable impact on fatty acid oxidation rates. Further studies will be required to elucidate the absolute levels of succinylation on these proteins. Next, due to ethical concerns, we were only able to obtain right ventricular tissue normally resected during heart surgery. Studies have suggested that metabolic processes may be

different in each ventricle.[82] This affects the generalizability of our results, but our data is important nonetheless, since the right ventricle is understudied compared to the left ventricle. Lastly, due to limited tissue availability from CHD patients undergoing heart surgery, we were unable to do succinylation immunoprecipitation analysis on human tissue. Due to decreases seen in total succinylation in the hypertrophied group compared to non-hypertrophied hearts in the 101-200-day age group, future experiments on specific succinylation of cardiac metabolic enzymes is warranted upon availability of human tissue around this age group.

In conclusions, our results show that acetylation, but not succinylation, may regulate the metabolic shift in hypertrophied newborn hearts. Results from this study can potentially be used to pharmacologically increase lysine acetylation of cardiac metabolic proteins to enhance energy production in CHD patients and mitigate the negative effects of surgery on these hearts. Further research is necessary to elucidate the role of succinylation on fatty acid oxidation in the human hypertrophied newborn heart later in maturation. Specific succinylation of fatty acid oxidation enzymes at the 101-200 age range and older is necessary to determine whether succinylation, in addition to acetylation, contributes to the delayed maturation of fatty acid oxidation in the hypertrophied state.

3.5 Figures

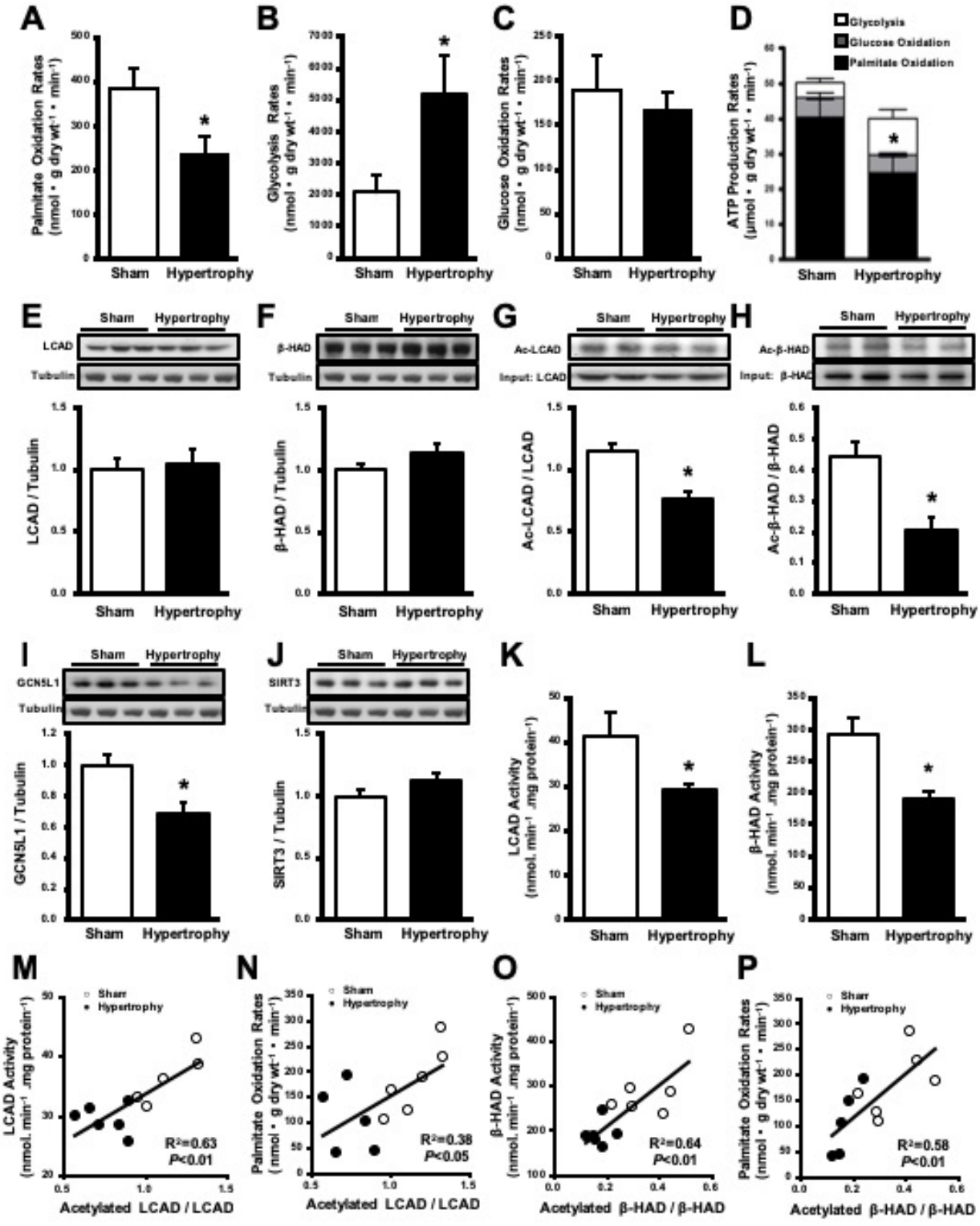

Figure 3.1 Absolute metabolic rates and acetylation of fatty acid oxidation enzymes in 21-day old rabbits with volume-overload hypertrophy. Rates of cardiac palmitate oxidation (A),

glycolysis (B), glucose oxidation (C), and ATP production (D) are shown. Protein levels of LCAD (E) and β-HAD (F), followed by the acetylation of LCAD (G) and β-HAD (H) normalized for total LCAD or β-HAD levels in the same hearts, respectively, are shown. Also, protein levels of mitochondrial acetyltransferase GCN5L1 (I) and deacetylase SIRT3 (J), the enzymatic activities of LCAD (K) and β-HAD (L), and correlations of acetylated LCAD levels to enzymatic activity (M) or to palmitate oxidation rates (N) and the same for β-HAD (O-P). n=4-7/group. Data are presented as mean ± SEM and analyzed by Students' t-test or linear regression analysis using the least squares method. *$p<0.05$ is considered as significantly different. Panels E, F, I, and J are work done by Fukushima A. Panels G and H are done in conjunction by Rawat A and Fukushima A.

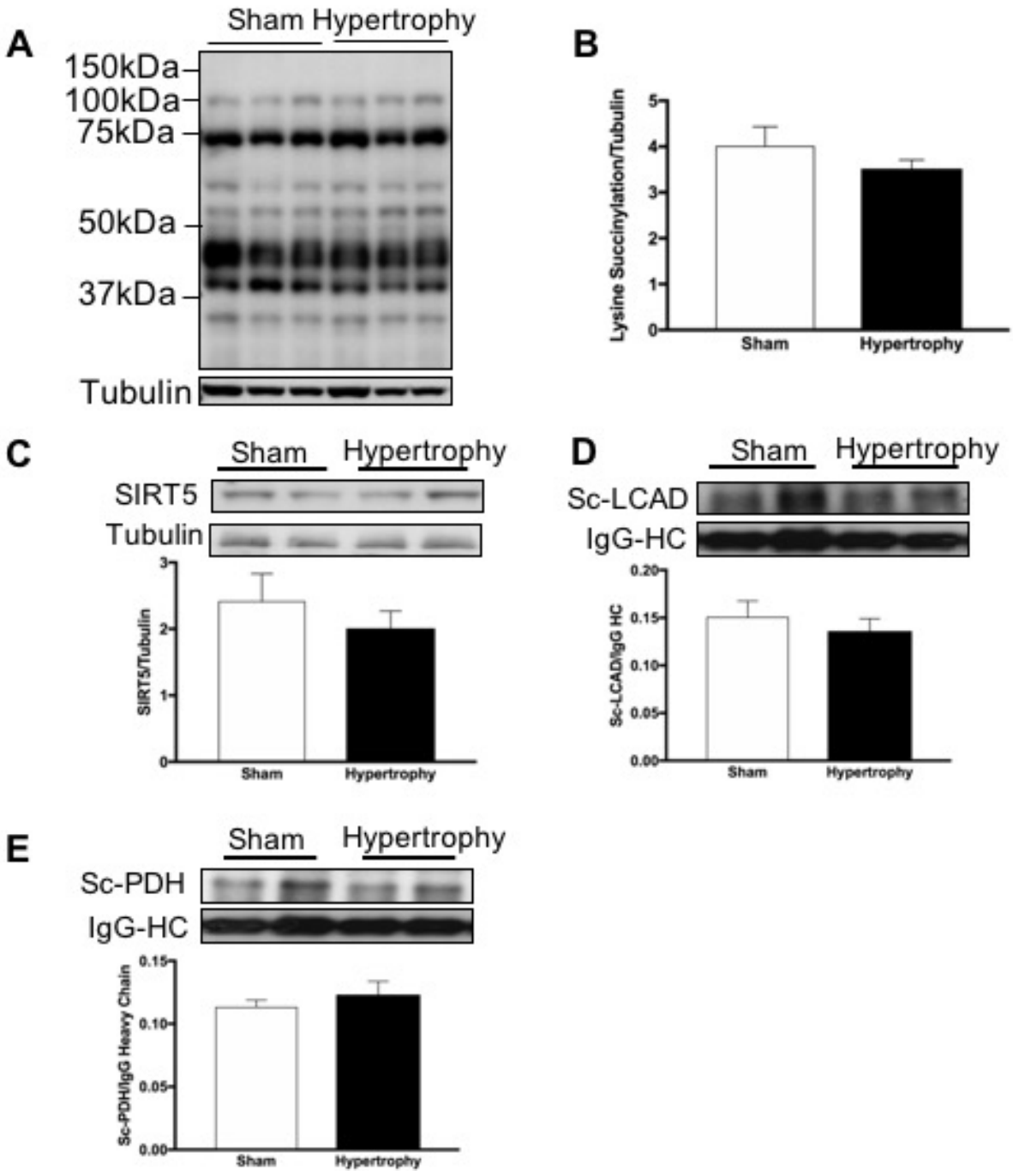

Figure 3.2 Cardiac lysine succinylation in 21-day old rabbits with volume-overload hypertrophy. Total cardiac lysine acetylation blot (A) with quantification (B) along with expression of mitochondrial desuccinylase sirtuin 5 (SIRT5) (C) are shown followed by succinylation of both LCAD (D) and PDH (E) normalized for total LCAD or PDH levels

respectively. n=6/group. Data are presented as mean ± SEM and analyzed by Student's t-test. Panels A-E are work done by Rawat S.

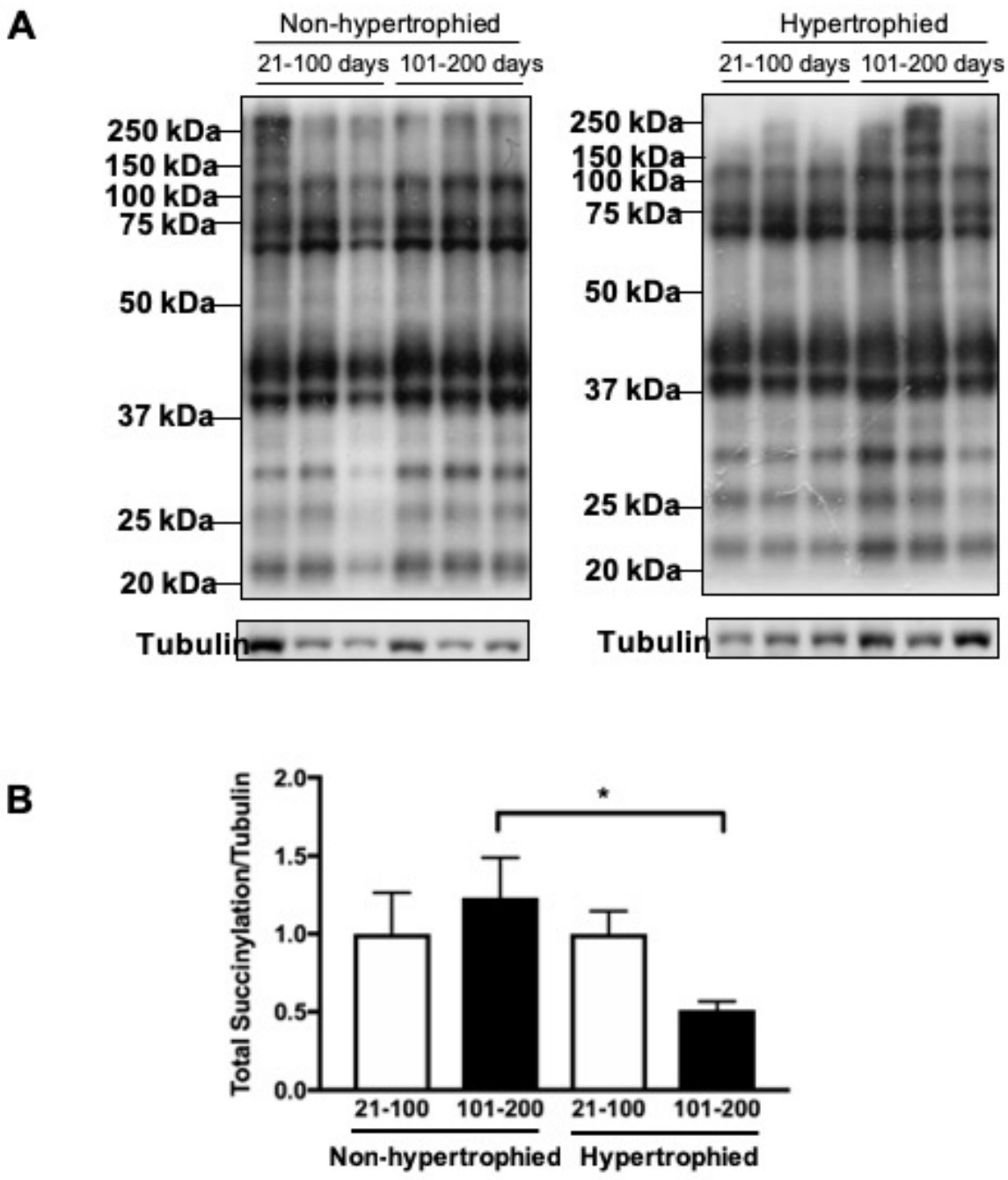

Figure 3.3 Total cardiac lysine succinylation during the development of newborn congenital heart defect patients. Total cardiac lysine succinylation blots (A) with quantification (B) are shown. n=3/group. Data are presented as mean ± SEM and analyzed by One-Way ANOVA followed by Fisher's Least Significant Difference Test. *$p<0.05$ was considered as statistically significant. Panels A and B are work done by Rawat S.

Chapter Four

Maturation of Cardiac Fatty Acid Metabolism in Hypoplastic Left Heart Syndrome Patients

Contribution:

Sonia Rawat: conception and design of research, Western blots, immunoprecipitation, data analysis, primary author
Arata Fukushima: Western blots, immunoprecipitation
Liyan Zhang: intellectual contribution
Renan Petinelli: data analysis
Gary Lopaschuk: conception and design of research, intellectual and editorial contribution

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4.1 Introduction

Hypoplastic left heart syndrome (HLHS) is a severe congenital heart defect (CHD) characterized by underdevelopment of the left side of the heart. HLHS occurs in 0.21 out of 1000 live births and has one of the worst prognoses of all CHDs.[43] HLHS is one of the most difficult CHDs to manage[44] and, if left untreated, is lethal with a 98% mortality in the first 6 weeks of life.[45, 46] Children diagnosed with HLHS have also been shown to have lower quality of life compared to healthy controls at 2-10 years of age.[48]

The main intervention for HLHS patients is staged palliation consisting of 3 surgeries: the Norwood procedure in the first weeks of life, followed by the bidirectional Glenn and Fontan procedures later in infancy and early childhood, respectively. In the 1990s, the surgical technique of the Norwood procedure greatly improved, leading to fewer post-operative mortalities.[61, 62] However, this, much like other corrective heart surgeries, subjects the heart to ischemic stress and can cause perioperative heart tissue damage and low cardiac output leading to poor post-surgical outcomes.[4] In one study, the mortality rate after the Norwood procedure from 1998-2000 was 19%.[61]

A critical consideration for hearts under stress due to ischemia during surgery or abnormal cardiac demands, as occurs in HLHS patients, is the energy substrate preference of the heart. Following birth there is a rapid increase in cardiac fatty acid β-oxidation, which becomes the main source of energy for the newborn heart.[6, 86, 87] This increase in fatty acid oxidation occurs as a result of: 1) increased transcriptional activation of mitochondrial biogenesis and fatty acid oxidation enzymes,[14] decreased allosteric control of fatty acid oxidation by malonyl CoA,[9, 88] and increased

post-translational modification of fatty acid oxidation enzyme phosphorylation and acetylation.[9, 27, 33, 40] However, in the presence of hypertrophy in CHD patients, there is a delay in this maturation of fatty acid β-oxidation,[13, 14, 64] leading to a decreased energy capacity in these hearts.[33] This delay occurs as a result of impaired transcriptional, allosteric, and post-translational modification of fatty acid oxidation in the hypertrophied newborn heart.[33] However, it is not known if a similar delay in the maturation of fatty acid oxidation occurs in the hearts of HLHS patients. If such a delay occurs, it may impact the maturation of metabolism in the systemic ventricle of patients with HLHS, resulting in increased susceptibility to ischemia, and impaired cardiac function. It is therefore important to understand whether the normal maturation of fatty acid oxidation that occurs post-birth is altered in the hearts of HLHS-diagnosed patients.

In this study, we determined if infants diagnosed with HLHS have delayed mitochondrial biogenesis and cardiac metabolism compared to infants diagnosed with other CHDs. Changes in cardiac fatty acid oxidation enzyme expression and acetylation, as well as alterations in transcriptional and allosteric regulation of cardiac metabolism, if present, may suggest altered energetics in this severe form of CHD. Such information could contribute to the development of novel strategies to optimize cardiac metabolism in an effort to improve outcomes.

4.2 Methods

4.2.1 Newborn subjects

From 2006 to 2015, a total of 22 right ventricular samples were collected from patients undergoing corrective heart surgery for a CHD at the University of Alberta Hospital. Infants were grouped based on whether they were diagnosed with HLHS (the "HLHS" group, n=13) or other CHDs (the

"non-HLHS" group, n=9). All infants were age-matched and were between the ages of 4 days and 16 days old. Biopsies were taken following cardioplegic arrest. In the HLHS group, biopsies were obtained from the Sano conduit insertion site and this was uniform in almost every patient. In the non-HLHS group, biopsies were obtained from the right ventricular (RV) free wall in tetralogy of Fallot patients and from the RV outflow tract in isolated aortic stenosis and double outlet right ventricle patients. For the patients with transposition of the great arteries and tricuspid atresia in whom RV outflow tract surgery is not performed, these patients required a Sano or VSD closure which provided access to RV trabeculi. All biopsies were immediately immersed in liquid nitrogen in the operating room and transported to the laboratory to be stored at -80 degrees Celsius. At a later date, the tissue was powdered and processed for biochemical analysis altogether. Collection of specimens from newborn patients were approved by the University of Alberta Health Research Ethics Board protocol (ID no. Pro0001112). All participants or their guardians provided written informed consent for the sample collection following determination of eligibility for the study and prior to the surgical procedure.

4.2.2 Immunoblotting

Immunoblotting was performed as described in section 2.2.3. Membranes were probed with one of the following primary antibodies at a 1 in 1000 dilution: ACC (catalogue 3662), acetylated lysine (catalogue 9441), AMPK (catalogue 2532), p-AMPK (Thr172) (catalogue 2531), ATGL (catalogue 2439), CDK4 (catalogue 12790), cyclin D1 (catalogue 2922), p-cyclin D1 (Thr286) (catalogue 3300), PGAM1 (catalogue 7534), and p-Rb (Ser780) (catalogue 9307) (all from Cell Signaling Technology); β-HAD (catalogue ab37673), E2F1 (catalogue ab86431), LCAD (catalogue ab129711), PDK4 (catalogue ab71240), PGC1α (catalogue ab77210), PPARα (catalogue ab8934), SIRT1 (catalogue ab7343), SIRT2 (catalogue ab75436), SIRT3 (catalogue

ab86671), SIRT4 (catalogue ab124521), and SIRT5 (catalogue ab13697) (all from Abcam); p-ACC (Ser79) (07-303), p-PDH (Ser293) (catalogue ABS204), and SIRT6 (catalogue S4322) (all from MilliporeSigma); HIF1α (catalogue NB100-449) and DGAT2 (catalogue NB100-57851) (both from Novus Biologicals); CS (catalogue sc-242444), GCN5 (catalogue sc-20698), PDK2 (catalogue sc-100534), SPT1 (catalogue sc-32916), and SPT2 (catalogue sc-27500), (all from Santa Cruz); GCN5L1 (provided by M.N. Sack, NIH, Bethesda, Maryland, USA); and MCD (produced at the University of Alberta).

4.2.3 Immunoprecipitation

Please refer to section 2.2.4 for the immunoprecipitation methods.

4.2.4 Statistical Analysis

All data are presented as mean ± SEM. A Student's t-test was used for the analysis. A p value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Characteristics of the study subjects

Table 4.1 shows the patient characteristics. Among the collected right ventricular tissue used for this study, thirteen were from infants diagnosed with HLHS and nine were from infants diagnosed with other congenital heart defects including transposition of the great arteries (n=2), tetralogy of Fallot (n=2), tricuspid atresia (n=2), isolated aortic stenosis (n=1), double outlet right ventricle

(n=1, unbalanced atrial septal defect with dominant RV), and pulmonary atresia (n=1). The average age of infants the day they underwent corrective heart surgery and subsequent tissue collection was not statistically different between the two groups (Table 1). In the non-HLHS group, 6 out of the 9 infants (67%) were male, while only 3 out of 13 (23%) were males in the HLHS group (Table 4.1). Body surface area and weight were not statistically different between the two groups (Table 4.1).

4.3.2 Fatty acid oxidation is not impaired in HLHS patient hearts compared to non-HLHS patient hearts

Peroxisome proliferator activated receptor (PPAR)-γ coactivator 1α (PGC1α) is a regulator of mitochondrial biogenesis and PPAR-mediated transcription, which normally increases post-birth.[5] Interestingly, the expression of PGC1α was significantly higher in HLHS hearts (Figure 4.1A), suggesting that transcriptional control of cardiac mitochondrial biogenesis was not impaired, but was actually increased in HLHS patients. Expression of cardiac PPARα, a transcription factor important for fatty acid storage and oxidation, along with citrate synthase, a marker of mitochondrial biogenesis, were not different in HLHS infants (Figures 4.1B and 4.1C). Expression of the enzymes responsible for the breakdown (i.e., oxidation) of fatty acids to generate ATP, namely long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were also not significantly different between groups (Figure 4.1D and 4.1E). This data suggests that post-birth maturation of fatty acid oxidation is not impaired in the myocardium of HLHS patients.

4.3.3 Total lysine acetylation and acetylation of proteins in the fatty acid oxidation pathway is not blunted in HLHS patient hearts

The attachment of a two-carbon entity onto enzymes, referred to as acetylation, can change the activity of an enzyme. Post-translational acetylation of lysine residues increases the activity of cardiac fatty acid oxidation enzymes in the newborn heart,[27, 32, 33, 41] and is reduced in the presence of hypertrophy in congenital heart defect patients.[33] We therefore assessed total cardiac and fatty acid oxidative enzyme acetylation in HLHS patients. Both total lysine acetylation of proteins and acetylation of the fatty acid oxidation enzymes LCAD and β-HAD were not different between the hearts of non-HLHS and HLHS patients (Figures 4.2A-4.2D). Acetylation of PGC1α, which inhibits its activity,[89] was also not different between the groups (Figure 4.2E and 4.2F). This suggests that the normal increase in acetylation of the cardiac fatty acid oxidative enzymes, which increases fatty acid oxidation, was not impaired in HLHS patients. It also suggests that acetylation of PGC1α, which decreases its transcriptional activity, was not impairing mitochondrial biogenesis in the HLHS patients.

In a recent study we found GCN5-like protein 1 (GCN5L1, a key mitochondrial acetylase in the heart) to be decreased in the hypertrophied newborn human heart, leading to decreased acetylation and fatty acid oxidation.[33] However, as shown in Figure 4.3A and 4.3B, we did not observe any changes in expression of mitochondrial and nuclear acetyltransferases GCN5L1 and GCN5, respectively, in HLHS infants. This is consistent with the lack of change in cardiac acetylation observed in the HLHS infants (Figure 4.2).

Deacetylation of mitochondrial proteins primarily occurs via the actions of Sirtuins (SIRTs). SIRT 1-6 are localized in various cell compartments and have different enzymatic targets. SIRT1 and

SIRT6 are primarily in the nucleus, SIRT2 in the cytoplasm, and SIRT3, 4, and 5 in the mitochondrial matrix.[90, 91] As shown in Figures 4.3C-4.3H, the only cardiac SIRT that was significantly different in HLHS infants was SIRT1, which plays a role in protecting the heart against hypertrophic response, and it was decreased in HLHS patients. No change in the expression of SIRT3 and SIRT4 were observed, which are the primary deacetylases found in the mitochondria.[91] This lack of change in mitochondrial SIRT3 and 4 is again consistent with the lack of change in acetylation of mitochondrial fatty acid oxidation enzymes observed in HLHS patients (Figure 4.2C and 4.2D).

4.3.4 Allosteric control of cardiac fatty acid oxidation by malonyl CoA is not altered in HLHS patients

The increase in fatty acid oxidation observed post-birth in normal newborn hearts is due in part to a decrease in malonyl CoA, which is a potent inhibitor of mitochondrial fatty acid uptake.[88, 92] This decrease in malonyl CoA is due to an increased phosphorylation and inhibition of acetyl CoA carboxylase (which synthesizes malonyl CoA) by 5' AMP-activated protein kinase (AMPK), and an increased expression and activity of malonyl CoA decarboxylase (MCD) which degrades malonyl CoA (Figure 4.4A).[64, 93] In the hypertrophied newborn heart, acetyl CoA carboxylase (ACC) phosphorylation by AMPK is impaired, while MCD expression is decreased, leading to an increase in malonyl CoA levels and a decrease in the maturation of fatty acid oxidation.[94] As shown in Figure 4.4B, cardiac MCD expression was not altered in HLHS patients. In addition, neither ACC expression or ACC phosphorylation was different in the HLHS infants compared to non-HLHS patients (Figures 4.4C-4.4E). There was also no difference in cardiac AMPK expression (Figure 4.4F). This suggests that malonyl CoA control of fatty acid oxidation was not compromised in hearts of HLHS patients. Of note, however, is that phosphorylation of cardiac

AMPK, which is activated in low energy states, was increased in HLHS patients versus non-HLHS patients (Figure 4.4G and 4.4H). This variation in phosphorylation levels of AMPK suggests a different cardiac energetic environment in HLHS patients, possibly due to increased demands on the heart in HLHS patients since AMPK activation is seen in context of stress.[95] We also correlated p-AMPK levels with the age of the infants at the time of surgery and did not observe a significant correlation (Figure 4.4I).

4.3.5 Unaltered glucose metabolism regulation and triacylglycerol storage/breakdown in the hearts of HLHS infants

In newborn hearts, oxidative glucose metabolism is low and does not mature until weaning.[7, 87] However, we did examine whether enzymes involved in cardiac glucose oxidation were altered in HLHS infants. We found no changes in cardiac expression of glucose oxidation regulators pyruvate dehydrogenase kinases PDK4 and PDK2, along with no change in the phosphorylation and deactivation of the rate-limiting step of glucose oxidation, pyruvate dehydrogenase (Figures 4.5A-4.5D). Additionally, the transcription factor E2F1 has been shown to control PDK4 expression.[96] This pathway leads to activation of E2F1-mediated PDK4 transcription and consists of cyclin D1 translocation to the nucleus, its association with cyclin-dependent kinase 4 resulting in phosphorylation of retinoblastoma protein, and subsequent E2F1-induced transcription of PDK4 (Figure 4.5I). Expression of the proteins involved in the E2F1 pathway did not differ in HLHS hearts compared to controls (Figures 4.5E-4.5H). Taken together, these results suggest that HLHS patients do not demonstrate differences in the regulation of glucose oxidation metabolic pathways compared to the non-HLHS group.

We also examined whether the presence of HLHS altered the expression of enzymes involved in triacylglycerol synthesis/breakdown, namely diacylglycerol acyltransferase 2 (DGAT2) and adipose triglyceride lipase (ATGL), as well as enzymes for ceramide synthesis serine palmitoyltransferase (SPT) 1 and 2. We found no significant differences between non-HLHS and HLHS patients in any of these enzymes (Figures 4.6A-4.6E). Also, no difference was seen between groups in the transcription factor hypoxia-inducible factor 1α that is upregulated in low-oxygen environments or in the glycolytic enzyme phosphoglycerate mutase 1 (Figure 4.6F and 4.6G).

4.4 Discussion

We sought to determine if metabolic differences occur in the newborn hearts of HLHS patients. Our focus was on mitochondrial fatty acid β-oxidation, which rapidly becomes the key ATP-producing pathway in the heart after birth and prior to weaning.[6] We recently demonstrated in both humans and rabbits that some types of CHDs, namely those with hypertrophy, have a delay in the normal post-natal maturation of fatty acid oxidation, which can lead to an "energy starved" heart.[33] This delayed maturation of fatty acid oxidation that was shown in the newborn hypertrophied hearts occurs due to a combination of decreased transcriptional control of mitochondrial biogenesis and fatty acid oxidative enzymes,[33, 75] an increased malonyl CoA inhibition of fatty acid oxidation,[94] and a decreased post-translational acetylation of fatty acid oxidation enzymes.[27] Since it is not known if a similar delay occurs in HLHS patients specifically, we investigated whether the maturation of cardiac fatty acid oxidation may be impaired in the right ventricular myocardium of these patients. However, our data demonstrates that this was not the case, and that at a transcriptional, allosteric and post-translational level, the control of fatty acid oxidation was not compromised in HLHS patients compared to non-HLHS patients. In fact, evidence of an increased

ability for mitochondrial biogenesis was observed in the HLHS patients (Figure 4.7). This suggests that compromised cardiac energetics may not be an important risk-factor in perioperative dysfunction of the right ventricle in newborns with HLHS. Therefore, surgeons performing a Norwood procedure need not be concerned about metabolic perturbations in the right systemic ventricle of HLHS patients.

A number of changes occur in the fetal to newborn transition, resulting in a "maturation" of cardiac metabolism.[1, 86] Changes in fatty acid β-oxidation regulation after birth occur in at least three levels: allosteric regulation, transcriptional control, and post-translational modifications. Allosteric regulation of fatty acid oxidation is mainly mediated by malonyl CoA inhibition of mitochondrial fatty acid uptake.[64, 88] Also, various transcription factors involved in fatty acid and glucose oxidation and glycolysis have been shown to change in expression and acetylation levels in the newborn period.[27] Finally, non-histone protein acetylation has recently been shown to be an important post-translational modification in newborn heart maturation and is associated with the metabolic changes that accompany hypertrophy in congenital heart defect patients.[33] Our data suggests that these regulatory pathways are intact in the hearts of newborn HLHS patients, suggesting that the normal maturation of myocardial fatty acid oxidation is intact at this developmental stage. Whether these pathways become altered as the demands of the single right ventricle and right ventricular hypertrophy evolve is not certain.

High AMP levels, which are present in energy-deprived states, are a stimulus that activate AMPK by favouring its phosphorylated form. In the present study, a higher level of p-AMPK was observed in HLHS hearts compared to non-HLHS hearts, suggestive of a lower energy pool and a possible altered metabolic milieu in the hearts of HLHS patients. Although, further studies are

required to determine the energy status in HLHS hearts, ATP and other energy currencies are easily varied by the condition of the tissue upon collection, which is difficult to control in human studies. Unfortunately, the tools necessary to accurately determine the absolute energy levels in human tissue are currently limited.

PGC1α is a co-activator of PPARα-mediated transcription. Previously, in a neonatal hypertrophied rabbit heart model we showed that activating PPARα, and thereby stimulating fatty acid oxidation, can improve heart function and decrease ischemic injury.[14] In our study, an increase in PGC1α in HLHS hearts suggests increased mitochondrial biogenesis. This increase may be compensatory, increasing PPAR-mediated transcription due to the lower energy status exhibited by increased AMPK phosphorylation.

The lack of observed change in total acetylation and fatty acid oxidative enzyme acetylation in HLHS patients is supported by the absence of differences in GCN5L1, a key mitochondrial acetylase, and SIRT 3 and 4, two key mitochondrial deacetylases. SIRTs are NAD^+-dependent deacetylases that are localized in various cellular compartments. SIRT1, SIRT3, and SIRT6 have been implicated in the pathogenesis of cardiac hypertrophy and heart failure.[27] SIRT1 deficiency displays a lethal phenotype with severe developmental cardiac defects,[97] suggesting that SIRT1 is essential to protect the heart against hypertrophic response. Interestingly, we saw a lower level of this protective SIRT in HLHS hearts, possibly indicative of a mechanism which enables altered right ventricular myocardial development in HLHS. Currently, the mechanism responsible for the increase in cardiac SIRT1 in HLHS patients is unclear. SIRT1 is known to interact with AMPK to activate PGC1α; however, we observed a negative relationship between the levels of both AMPK and PGC1α compared to SIRT1 in HLHS patients. In future studies, it would be valuable to

determine what other changes are associated with increased SIRT1 expression in HLHS to further understand mechanistic differences in this complex congenital defect.

In the adult heart, glucose oxidation and fatty acid oxidation together produce ~95% of the ATP used to support contractile function. However, in the newborn heart, glucose oxidation does not mature until weaning, making fatty acids the major contributor to total ATP production from oxidative metabolism.[7, 87] Therefore, since the age range of participants used in this study was 4-16 days old, it is expected that glucose oxidation should be a minor contributor to ATP production. Regardless of the contribution of glucose oxidation to ATP production during this period, we saw no differences in the cardiac glucose oxidation enzymes and its regulators in the HLHS patients compared to the non-HLHS patients.

4.4.1 Limitations

One of the limitations in this study was that we had performed our assessments at the time of surgery, whenever it occurred. All of the tissue was harvested within the first several days after delivery. Generally, dramatic maturational changes occur rapidly during the initial 200 days after birth.[33] Whether more dramatic cardiac metabolic changes occur in these HLHS patients later on in infancy throughout the progression of myocardial metabolic maturation is not certain and remains to be investigated. Another limitation was that the non-HLHS group constituted of a spectrum of CHD pathology with varying levels of hypertrophy and cyanosis, and volume and/or pressure overload contributing to the heterogeneity of the non-HLHS control group.

In summary, we have demonstrated that fatty acid β-oxidation is not impaired in the hearts of newborns with HLHS compared to non-HLHS hearts. In addition, the maturation of fatty acid β-oxidation in HLHS hearts may be facilitated by an increase in AMPK and PGC1α activity. Therefore, compromised cardiac energetics may not be an important risk-factor for right ventricular dysfunction in newborns with HLHS undergoing the Norwood procedure. Further work is necessary to elucidate the evolution of cardiac metabolism through later developmental and pathophysiological stages.

4.5 Figures

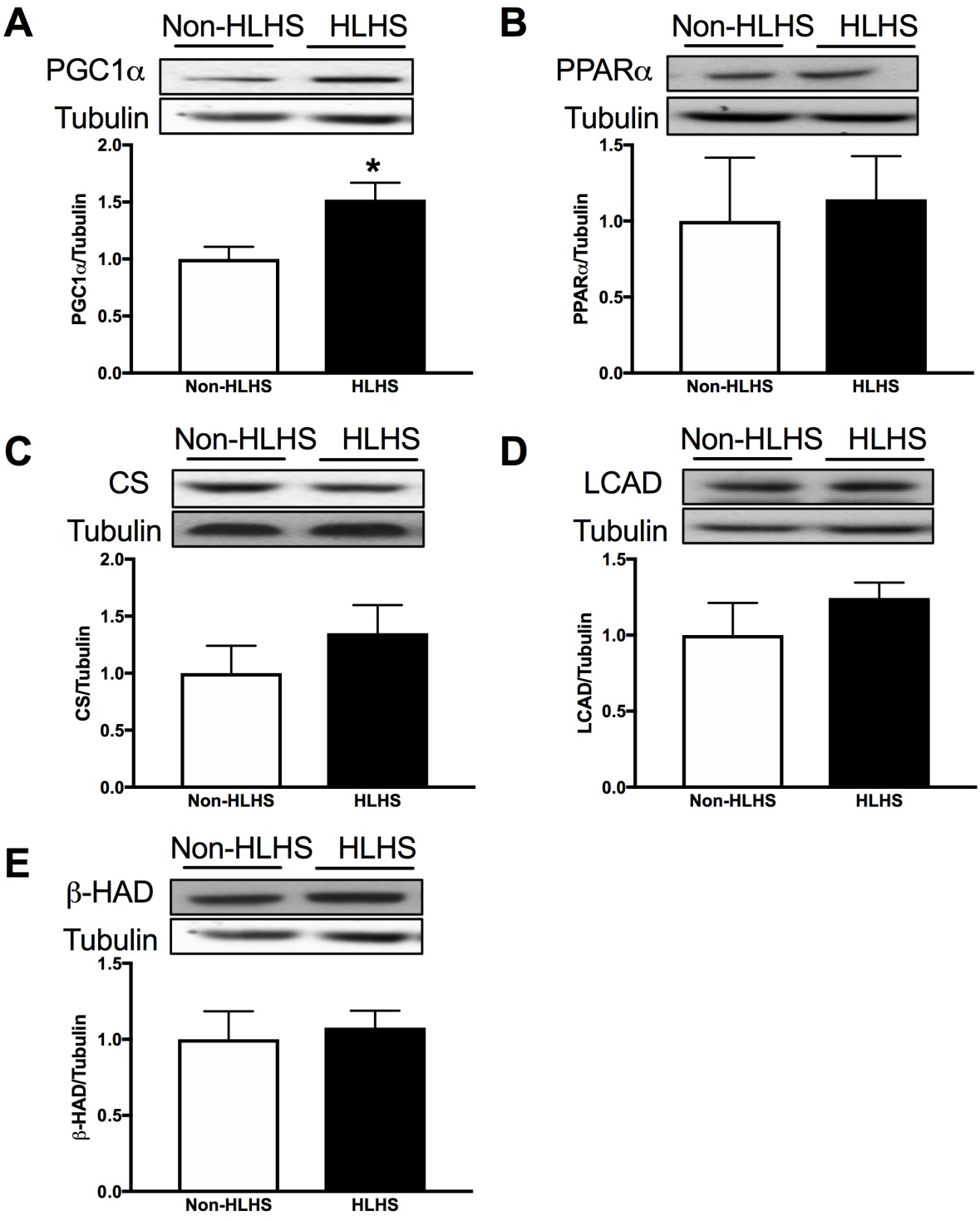

Figure 4.1 Level of proteins involved in cardiac mitochondrial biogenesis and fatty acid oxidation in HLHS patients was unchanged compared to non-HLHS except mitochondrial biogenesis master regulator PGC1α. Relative protein levels of transcriptional coactivator

PPAR-ɣ coactivator 1α (PGC1α) involved in mitochondrial biogenesis (A) shows an increase in expression in HLHS patient hearts. Protein levels of transcription factor peroxisome proliferator-activated receptor alpha (PPARα) responsible for lipid metabolism (B), mitochondrial biogenesis marker citrate synthase (CS) (C), fatty acid β-oxidation enzymes long chain acyl CoA dehydrogenase (LCAD) (D) and β-hydroxyacyl CoA dehydrogenase (β-HAD) (E) were not different between groups. Right ventricular biopsies were utilized to produce lysate samples for the above Western blots. n=5 for non-HLHS group, n=7 for HLHS group. Data are presented as box-and-whisker plots in which the upper and lower borders of the box represent the upper and lower quartiles, the middle horizontal line represents the median, and the upper and lower whiskers represent the maximum and minimum values of non-outliers. Black circles and squares represent values from individual hearts in the non-HLHS and HLHS group, respectively. Data were analyzed by Student's t-test. *$p<0.05$ was considered as statistically significant.

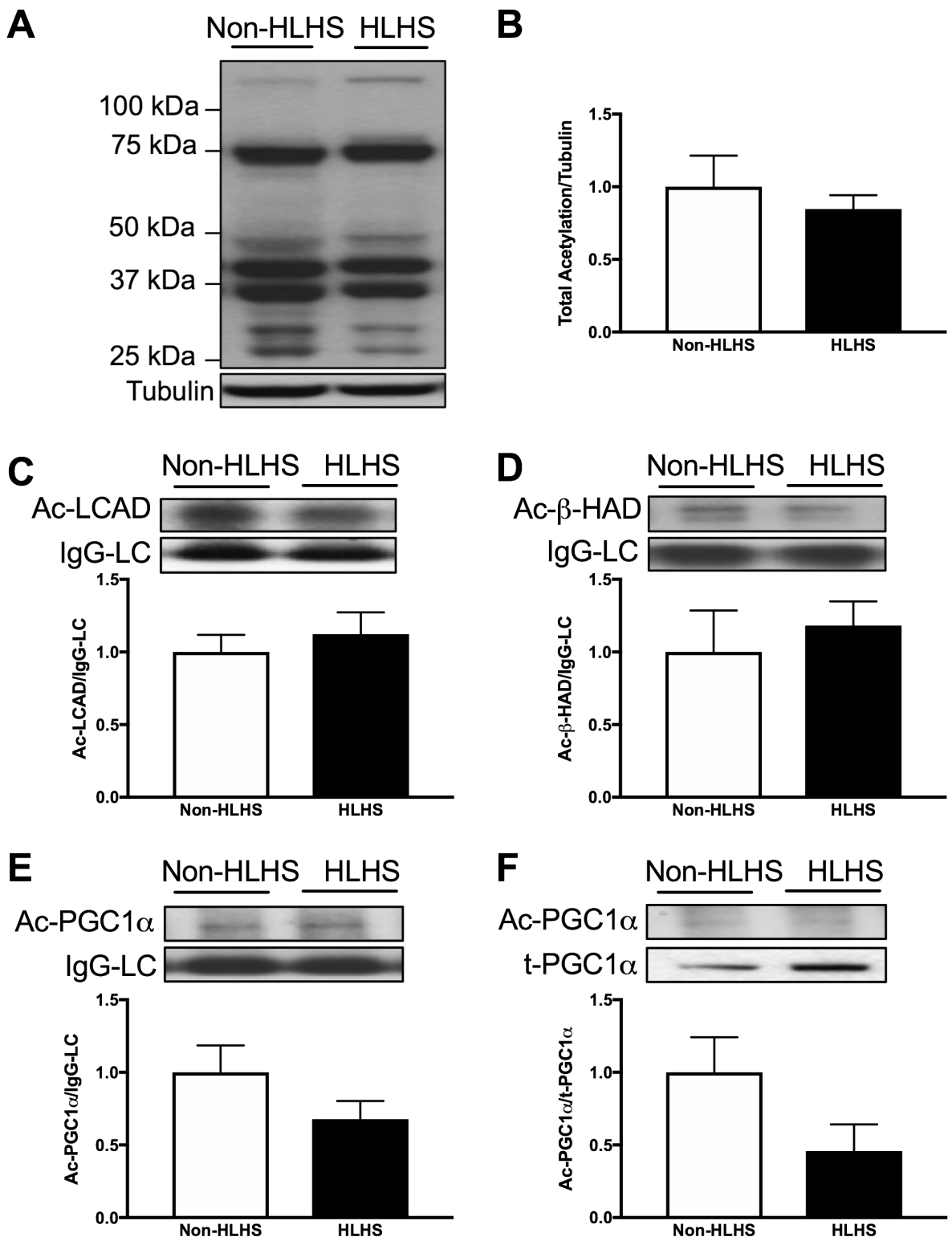

Figure 4.2 Total lysine acetylation and acetylation of proteins was unchanged in cardiac fatty acid oxidation in HLHS patients was unchanged. Total cardiac lysine acetylation blot (A) with

quantification (n=6 for non-HLHS group, n=10 for HLHS group) (B) shows no different between groups. Acetylation of fatty acid β-oxidation enzymes long chain acyl CoA dehydrogenase (Ac-LCAD) (C) and β-hydroxyacyl CoA dehydrogenase (Ac-β-HAD) (D) which has a stimulatory effect on enzymatic activity and acetylation of transcription factor PPAR-ɣ coactivator 1α (Ac-PGC1α) responsible for mitochondrial biogenesis (E) normalized with long chain of immunoglobulin G (IgG-LC) from antibody used for pull-down in immunoprecipitation also did not show changes between groups. Acetylation of PGC1α was also normalized for total PGC1α (t-PGC1α) expression (n=2 for non-HLHS group, n=6 for HLHS group) (F). Right ventricular biopsies were utilized to produce lysate samples for the above protein assessment. n=5 for non-HLHS group, n=10 for HLHS group unless stated otherwise. Data are presented as box-and-whisker plots in which the upper and lower borders of the box represent the upper and lower quartiles, the middle horizontal line represents the median, and the upper and lower whiskers represent the maximum and minimum values of non-outliers. Black circles and squares represent values from individual hearts in the non-HLHS and HLHS group, respectively. Data were analyzed by Student's t-test. *$p<0.05$ was considered as statistically significant.

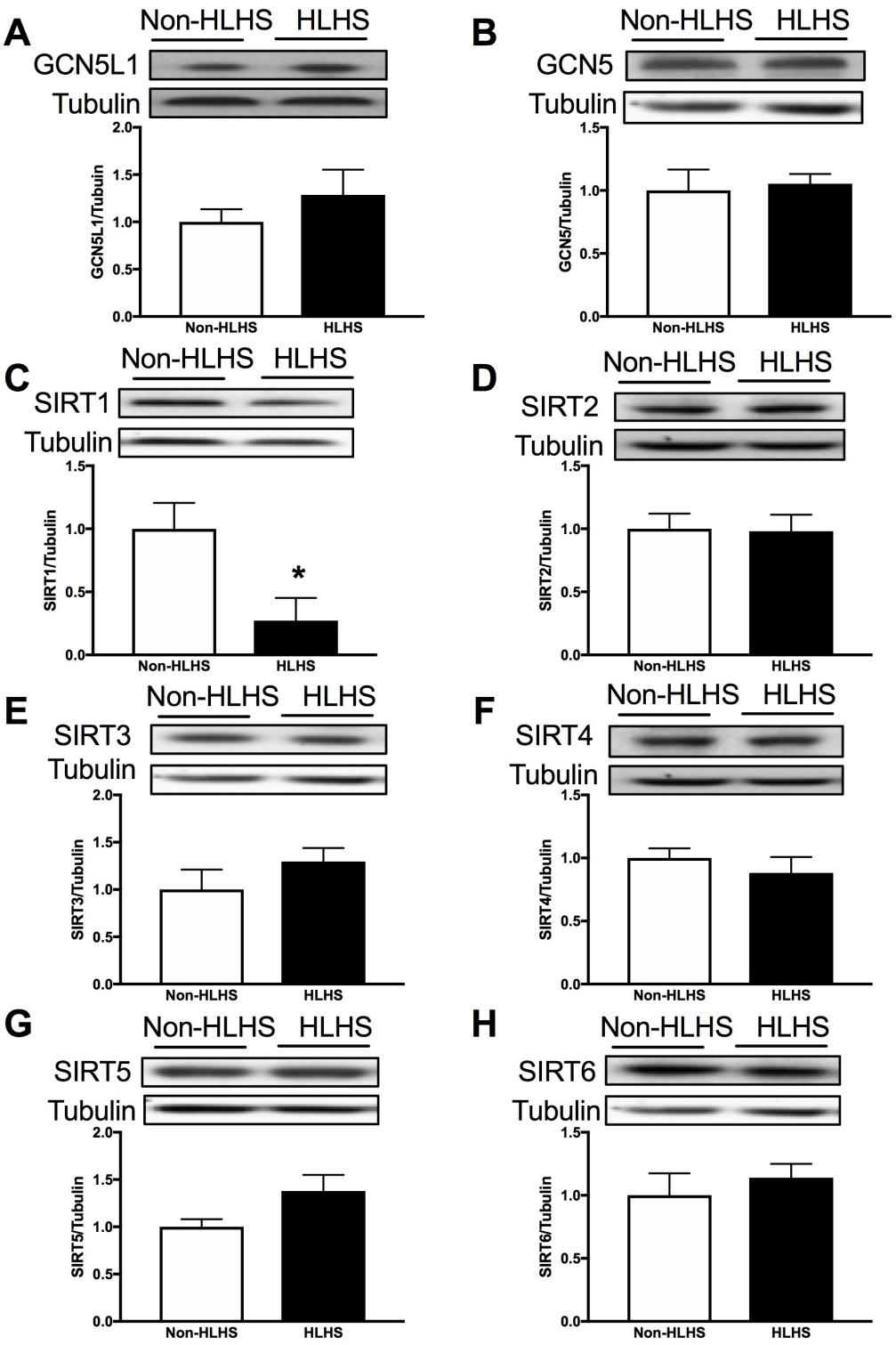

Figure 4.3 Level of the cardiac NAD^+-dependent deacetylases, sirtuins, and the acetyltransferases in HLHS patients was unchanged except SIRT1 compared to non-HLHS. Relative protein levels of mitochondrial acetyltransferase GCN5-like protein 1

(GCN5L1) (A), nuclear acetyltransferase GCN5 (B) and sirtuin (SIRT) 1-6 that regulate deacetylation of proteins (C-H) are shown as no significant change except in SIRT1 which was decreased in HLHS hearts compared to non-HLHS hearts. Right ventricular biopsies were utilized to produce lysate samples for the above protein assessment. n=5 for non-HLHS group, n=7 for HLHS group. Data are presented as box-and-whisker plots in which the upper and lower borders of the box represent the upper and lower quartiles, the middle horizontal line represents the median, and the upper and lower whiskers represent the maximum and minimum values of non-outliers. Black circles and squares represent values from individual hearts in the non-HLHS and HLHS group, respectively. Data were analyzed by Student's t-test. *$p<0.05$ was considered as statistically significant.

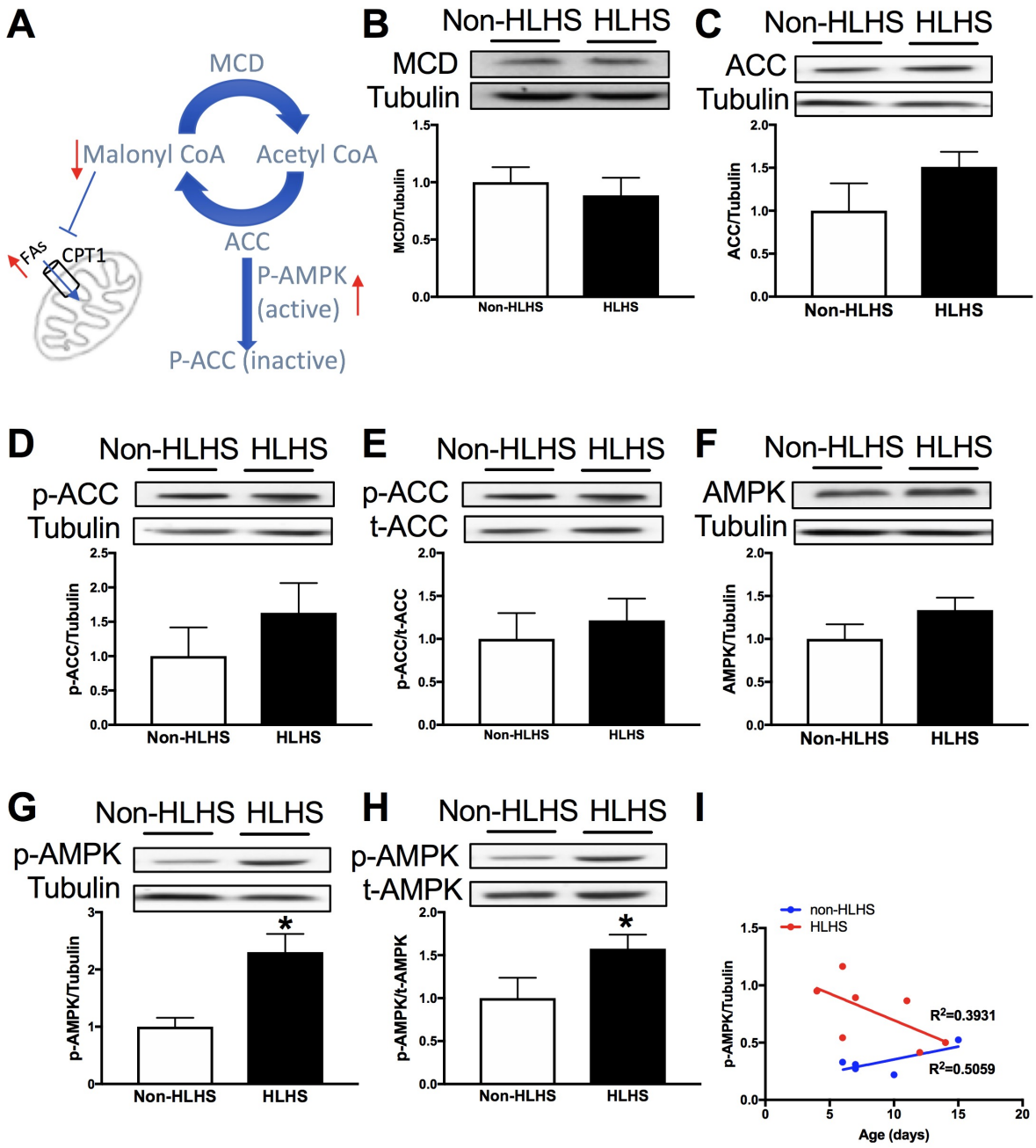

Figure 4.4 Allosteric regulation of cardiac mitochondrial fatty acid uptake in HLHS patients was unchanged compared to non-HLHS. A schematic view of mitochondrial fatty acid uptake regulation by phosphorylated 5' AMP-activated protein kinase (p-AMPK) and malonyl CoA inhibition on carnitine palmitoyl transferase 1 (CPT1) is shown (A). There was no change in protein levels of malonyl CoA decarboxylase (MCD) which breaks down malonyl CoA (B), acetyl CoA carboxylase (ACC) which produces malonyl CoA (C), and phosphorylation of ACC (p-ACC,

inactive state) normalized with tubulin (D) or total ACC expression (t-ACC) (E) and the kinase for ACC called AMPK (F) between groups. The phosphorylated Thr172 form (p-AMPK, active state) normalized by tubulin (G) or normalized by total AMPK expression (t-AMPK) (H) are significantly increased in the HLHS group. Total AMPK expression correlated with age of patients (I) is also shown as not significant. Right ventricular biopsies were utilized to produce lysate samples for the above Western blots. n=5 for non-HLHS group, n=7 for HLHS group. Data are presented as box-and-whisker plots in which the upper and lower borders of the box represent the upper and lower quartiles, the middle horizontal line represents the median, and the upper and lower whiskers represent the maximum and minimum values of non-outliers. Black circles and squares represent values from individual hearts in the non-HLHS and HLHS group, respectively. Data were analyzed by Student's t-test. *$p<0.05$ was considered as statistically significant.

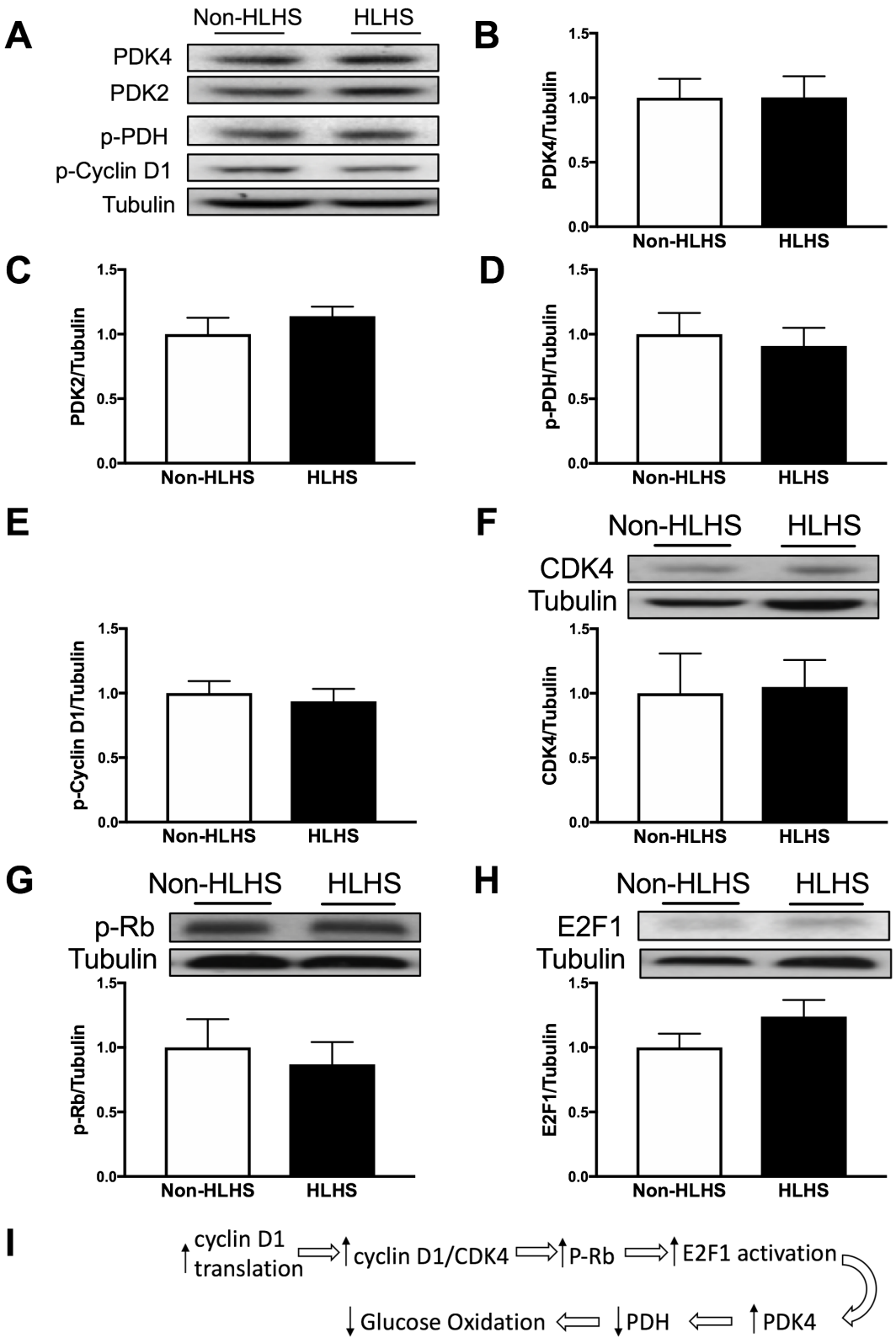

Figure 4.5 Level of proteins responsible for glucose metabolism regulation in the hearts of HLHS patients compared to non-HLHS. Western blots for protein levels of the glucose

oxidation regulators pyruvate dehydrogenase kinase 4 (PDK4) and 2 (PDK2), as well as Ser293 phosphorylation of pyruvate dehydrogenase (p-PDH, inactive state) and cyclin-D1 (Thr286) (A) with their quantifications (B-E) are shown as no statistical significance. Protein levels of cyclin-dependent kinase 4 (CDK4) (F), Ser780 phosphorylation of retinoblastoma protein (p-Rb) (G), and protein levels of E2F transcription factor 1 (E2F1) (H) that controls PDK4 expression are also shown as not significant. The scheme of the E2F1 pathway leading to suppression of pyruvate dehydrogenase (PDH) activity and subsequently decreased glucose oxidation (I) is also shown. Right ventricular biopsies were utilized to produce lysate samples for the above Western blots. n=5 for non-HLHS group, n=7 for HLHS group. Data are presented as box-and-whisker plots in which the upper and lower borders of the box represent the upper and lower quartiles, the middle horizontal line represents the median, and the upper and lower whiskers represent the maximum and minimum values of non-outliers. Black circles and squares represent values from individual hearts in the non-HLHS and HLHS group, respectively. Data were analyzed by Student's t-test. *$p<0.05$ was considered as statistically significant.

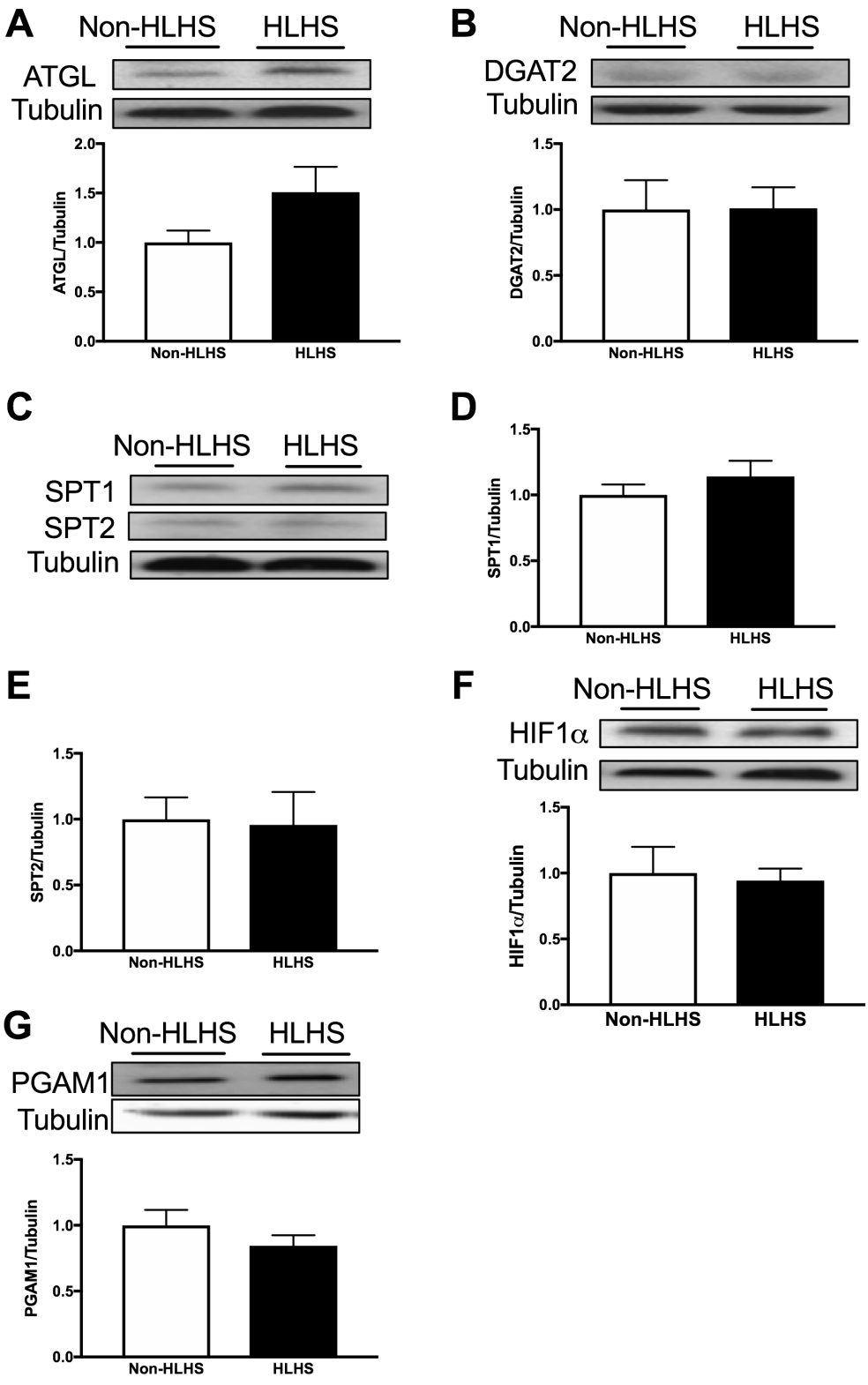

Figure 4.6 Level of enzymes for cardiac triacylglycerol/ceramide synthesis and regulation of glycolytic enzymes in HLHS patients was not different compared to non-HLHS. Protein levels

of triacylglycerol synthesis enzyme adipose triglyceride lipase (ATGL) (A), triacylglycerol breakdown enzyme diacylglycerol acyltransferase 2 (DGAT2) (B), blots for ceramide synthesis enzymes serine palmitoyltransferase 1 (SPT1) and 2 (SPT2) (C), and quantification for SPT1 and SPT2 (D and E) are shown as not significant. The transcription factor activated in hypoxic conditions, hypoxia-inducible factor 1α (HIF-1α) expression (F) and glycolytic enzyme phosphoglycerate mutase 1 (PGAM1) are also shown to be unchanged between groups. Right ventricular biopsies were utilized to produce lysate samples for the above protein assessment. n=5 for non-HLHS group, n=7 for HLHS group. Data are presented as box-and-whisker plots in which the upper and lower borders of the box represent the upper and lower quartiles, the middle horizontal line represents the median, and the upper and lower whiskers represent the maximum and minimum values of non-outliers. Black circles and squares represent values from individual hearts in the non-HLHS and HLHS group, respectively. Data were analyzed by Student's t-test. *$p<0.05$ was considered as statistically significant.

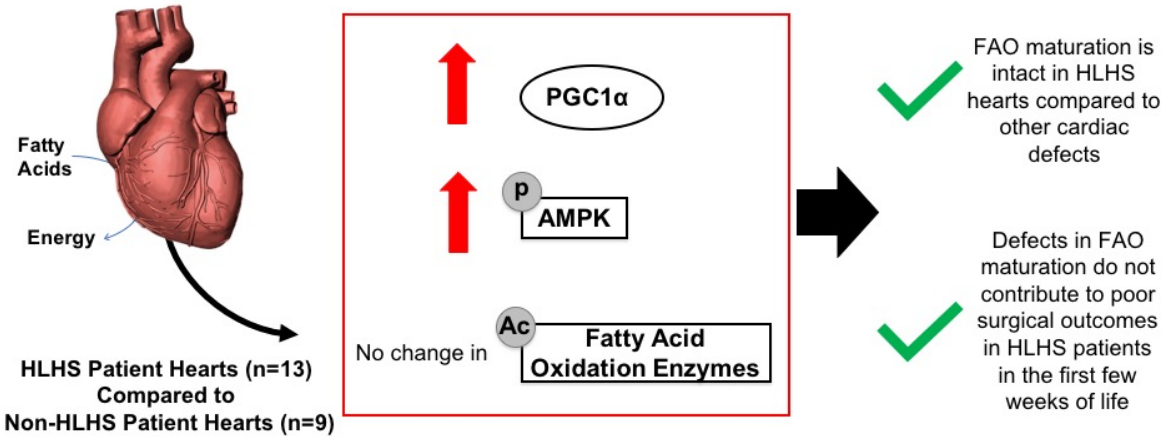

Figure 4.7 Fatty acid oxidation in HLHS patients 4-16 days old is not compromised compared to non-HLHS patients RV tissue from patients diagnosed with hypoplastic left heart syndrome (HLHS) or another CHD ("non-HLHS") was used to look at differences in metabolic enzymes and regulators during the first few weeks of life. Mitochondrial biogenesis master regulator PGC1α (PPAR-ɣ coactivator 1α) and fatty acid oxidation stimulator p-AMPK (phosphorylated 5' AMP-activated protein kinase) were increased in HLHS hearts, while total lysine acetylation and acetylation of fatty acid oxidation enzymes long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were unchanged. Our results show that fatty acid β-oxidation was not compromised in HLHS patient hearts compared to non-HLHS patients. Therefore, surgeons performing a Norwood procedure need not be concerned about metabolic perturbations in the right systemic ventricle of HLHS patients.

Table 4.1 Characteristics of hypoplastic left heart syndrome (HLHS) and non-HLHS patients

Demographic Factors	Non-HLHS Group (n=9)	HLHS Group (n=13)	p value
Age, days	11.3 ± 1.5	9.5 ± 1.1	0.34
Males, n (%)	6 (67%)	3 (23%)	N/A
Body surface area, m^2	0.200 ± 0.011	0.205 ± 0.007	0.67
Body weight, kilograms	3.24 ± 0.25	3.35 ± 0.17	0.71

Data are presented as mean ± SEM and analyzed by Students' t-test.

Chapter Five

General Discussion

In this group of studies we investigated the role of acetylation and succinylation on maturation of fatty acid oxidation in the newborn heart during the first few weeks of life and in hearts of newborns with CHD, particularly HLHS. Firstly, our experiments show that total cardiac lysine acetylation in whole lysate and isolated mitochondria from rabbit hearts significantly increases at 21-days of age compared to younger hearts. This occurs in parallel with an increase in palmitate oxidation, and an increase in mitochondrial acetyltransferase GCN5L1. Acetylation of mitochondrial fatty acid oxidation enzymes LCAD and β-HAD in newborn rabbit hearts increases with maturation from 1-day old to 7-days and 21-days, and this was positively correlated with an increase in its enzymatic activity. Secondly, lysine succinylation significantly increases with maturation and this is parallel to a decrease in desuccinylase SIRT5 protein levels. Succinylation of LCAD also significantly increases with maturation, and this positively correlates with enzymatic activity but is not significant when correlated with palmitate oxidation rates. Thirdly, rabbit hypertrophied hearts have lower fatty acid oxidation and lower acetylation and activity of LCAD and β-HAD. Total lysine succinylation is not changed in hypertrophied rabbit newborn hearts, nor was succinylation of fatty acid and glucose oxidation enzymes LCAD and PDH, respectively. In human heart tissue, total succinylation is unchanged except when comparing the non-hypertrophied and hypertrophied human hearts at 101-200 days of age. Fourthly, patients with HLHS do not have changes in fatty acid oxidation regulation on the allosteric, transcriptional, or post-translational levels, although they do show enhancement of mitochondrial biogenesis transcription factor PGC1α. Taken together we have found that acetylation contributes to the normal increase of fatty acid oxidation in newborn hearts shortly after birth and contributes to the delayed maturation of fatty acid oxidation in hypertrophied hearts. On the other hand, succinylation is another regulator of the maturation of fatty acid oxidation, having a stimulatory

effect on fatty acid oxidation enzymes, but the role of succinylation in hypertrophied hearts is less apparent although further studies in slightly older age groups is still required.

In the recent years, there has been a lack of consensus about the effect of acetylation on the activity of fatty acid oxidation enzyme activity. Some studies that have modulated the expression of mitochondrial deacetylase SIRT3 or acetyltransferase GCN5L1 have noted that increased acetylation is associated with a decreased fatty acid oxidation in the liver or heart.[37, 72] One study by Fisher-Wellman et al observed no changes in cardiac bioenergetics in SIRT3 knockout mice.[39] However, Zhao et al showed in the liver that acetylation activates the fatty acid oxidation enzyme β-HAD.[22] We and others have investigated in various disease states including diabetes and obesity that increased acetylation leads to activation of fatty acid oxidation enzymes LCAD and β-HAD in the heart.[32, 40, 41] In the present studies, we have extended this to the newborn heart and observed acetylation to be a stimulator of fatty acid oxidation that aids fatty acids to become the primary energy-producing substrate during the newborn period. We also observed that in the hypertrophied state, newborn rabbit hearts have lower acetylation and fatty acid oxidation rates compared to controls.

Succinylation is a more recently appreciated, and currently less studied, post-translational modification. Its importance in cardiac metabolism has been highlighted in metabolomic studies that have shown that in mice the most abundant acyl CoA is succinyl CoA and that the heart has the largest amount of succinylation that particularly affects metabolism.[35] Looking at the cardiac tissue of newborn rabbits, we saw an elevation of lysine succinylation levels with maturation, interestingly even when comparing more mature hearts at 42-days of age with 21-day old rabbit hearts. When furthering our investigation by looking into lysine succinylation of specific metabolic

enzymes, our finding that succinylation of LCAD increases with maturation suggests that succinylation plays a role in the maturation of cardiac metabolism in newborn hearts. However, our data does not support succinylation as a regulator of the changes in metabolism seen in hypertrophied newborn hearts compared to sham hearts.

For a more in-depth look at the pathological state of newborn cardiac health, we analyzed the cardiac metabolic enzymes in HLHS patients, a population that has high post-surgery mortality rates relative to other congenital heart defects. We investigated whether the maturation of cardiac fatty acid oxidation may be impaired in the right ventricular myocardium of these patients compared to non-HLHS patients since enlargement of the right ventricle is an adaptive requirement in these patients. However, our data demonstrates that this was not the case, and that at a transcriptional, allosteric and post-translational level, the control of fatty acid oxidation was not compromised in HLHS patients compared to non-HLHS patients. In fact, evidence of an increased ability for mitochondrial biogenesis was observed in the HLHS patients. This suggests that compromised cardiac energetics may not be an important risk-factor in perioperative dysfunction of the right ventricle in newborns with HLHS for surgeons to consider.

The data observed in the hypertrophied state in both rabbits and humans coincide with the theory that neonatal hypertrophied hearts revert to a more fetal metabolic phenotype consisting of higher glycolytic rates and with a less mature fatty acid oxidation. The perfusion data in the rabbit newborn hearts with an aortocaval shunt to induce hypertrophy show that glycolysis increases and palmitate oxidation decreases to rates similar to that seen in the fetal heart.[13, 98, 99] GCN5L1 contributes to this shift by decreasing acetylation and subsequently decreasing the acetylation and activity of LCAD and β-HAD in the hypertrophied state. Further work is required to elucidate

whether succinylation also contributes to this change because the role of succinylation in the hypertrophied heart is unclear with our data from rabbit and human hypertrophied hearts.

The decrease in energy production in hypertrophied newborn hearts is characterized not only by a decrease in the oxidation of fatty acids, but also by an increase in glycolysis, an inefficient generator of ATP that uses glucose. Typically, glycolysis rates are high in oxygen poor environments and is regulated by transcription factor HIF1α.[100] Interestingly, high rates of glycolysis even in the presence of oxygen can be found, particularly in tumor cells - a phenomenon known as the Warburg effect. The Warburg effect promotes cell growth and helps to incorporate nutrients into the biomass of proliferating cells.[101, 102] Based on our results, this preference for glucose metabolism by increasing glycolysis is also present in newborn hearts that consist of cells undergoing proliferation. The Warburg effect is considered to be increased in proliferating cells, which includes fetal and early newborn cardiac tissues. However, in later newborn hearts that have hypertrophy, the Warburg effect perhaps re-emerges as the heart reverts to a fetal phenotype. In fact, the Warburg effect has been shown to take place in cardiac hypertrophic states in adult mice.[103] This insight of the Warburg effect occurring in newborn hearts helps provide a deeper understanding of the metabolic characterizations of the heart in this age group.

Limitations: There are some limitations to the projects presented here to discuss. Firstly, in terms of the tissue acetylation and succinylation status, we were only able to obtain the proportion of acetylation or succinylation levels in the tissue, and not the absolute levels, with the techniques that we used. We also were able to obtain proteins levels of acetylation and succinylation regulators, namely sirtuins and GCN5L1, rather than their enzymatic activities, which would have provided a more accurate representation of the regulatory role of sirtuins and acetyltransferases.

Secondly, when considering the newborn rabbit studies, rabbits of both sexes were used because of the difficulty to determine the sex of the rabbits in the first few days after birth, contributing to the heterogeneity of these groups. With regards to the human studies, due to ethical constraints, it was not feasible to obtain tissue that is representative of the normal population for the control group, resulting in us using caution when making comparisons between the non-hypertrophied and hypertrophied heart groups. For the hypoplastic left heart syndrome study, the assessments were performed on tissue that was resected at the time of surgery, whenever that may have occurred, contributing to the heterogeneity of these samples also. Lastly, the assays are performed on the right ventricle which is known to have different metabolism compared to the left ventricle, affecting the generalizability of these results.

In conclusion, it has been shown that high levels of fatty acids in the plasma of newborn rabbits can increase contractile function following ischemia.[73] This combined with our results suggests that stimulating fatty acid oxidation by increasing acetylation, and potentially succinylation, can improve the contractile function of newborn hearts that have undergone ischemic stress. Pharmacologically activating the mitochondrial acetyltranferase GCN5L1 may be a promising approach to improve the functioning of these hearts. Although HLHS is a high mortality congenital heart defect that is relatively complicated to treat, there need not be an added concern about the cardiac metabolism of HLHS patients compared to other congenital heart defects.

Future Directions: When looking at the maturation of cardiac energy metabolism in newborn hearts, we observed an increase in PDH succinylation in 21-day old hearts compared to 7-day old hearts. However, the effect of succinylation on PDH is not known. PDH has been observed to be suppressed when succinylation levels are low.[78] However, we observed decreased glucose

oxidation rates parallel to increased PDH succinylation levels in maturation, suggesting an inhibitory role of succinylation on PDH activity. Further work to determine the effect of succinylation on PDH activity is necessary. Also, since we observed dramatic changes in total cardiac succinylation levels later in the maturation process (i.e., 42-day old rabbits compared to 21-day old rabbits), perhaps investigating hypertrophied rabbit hearts at an age group older than the 21-day old rabbits will provide clarity on the effect of hypertrophy in the heart on succinylation levels. Also, the opportunity to further experiment on human newborn hearts to elucidate whether changes in succinylation levels of specific cardiac metabolic enzymes such as β-HAD and LCAD remains to be elucidated as well.

In our study investigating metabolism in HLHS infant hearts, whether the pathways we investigated become altered as the demands of the single right ventricle and right ventricular hypertrophy evolve is not certain. This can be addressed in future studies that can elucidate the metabolic milieu in older HLHS infant hearts. Whether more dramatic cardiac metabolic changes occur in these HLHS patients later on in infancy throughout the progression of myocardial metabolic maturation is not certain. Also, further studies are required to determine the energy status in HLHS hearts and what other changes are associated with increased SIRT1 expression in HLHS to further understand mechanistic differences in this complex congenital defect.

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