Myocardial Ketone Metabolism in Heart Failure

by

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Abstract

Heart failure is a condition whereby the weakened cardiac muscle can no longer pump blood to the rest of the body, resulting in decreased quality of life. Despite the severity of this debilitating condition, there is no cure for heart failure. Modulation of cardiac energy metabolism has become an enticing therapeutic candidate to treat heart failure because the failing heart is energetically starved and metabolically inefficient. However, there is no definitive metabolic profile for the failing heart and recent evidence has emerged suggesting that the failing heart relies more on ketones, prompting the question of whether ketones are good or bad news for the failing heart. Therefore, the focus of my thesis is on the role of ketone metabolism in the setting of heart failure. The first objective was to define a metabolic profile for the failing heart. The second objective was to assess cardiac metabolism and efficiency in response to acute increases in ketone concentration in a healthy heart. The final objective was to assess cardiac function and metabolism following a chronic increase in ketones, via a ketogenic diet, to a failing heart. I hypothesized that if the failing heart has acute or chronic increases in ketone oxidation, this would be protective and mitigate further pathophysiological metabolic and structural remodeling of the failing heart.

To investigate the first objective, C57BL/6J mice underwent a sham or transverse aortic constriction surgery to induce pressure overload hypertrophy. Next, their hearts were excised and subjected to an isolated working heart perfusion to assess the failing heart's overall cardiac metabolism, function, and efficiency. To investigate the second objective, hearts from healthy mice were subjected to an isolated working heart perfusion and received increasing concentrations of ketones to assess the acute effect of increasing ketone delivery on cardiac metabolism of other substrates, cardiac function and efficiency. Finally, the third objective was

addressed by using mice that underwent a sham or left anterior descending coronary artery permanent ligation surgery to induce ischemic heart failure. Following the surgery, mice were randomized to receive a control or ketogenic diet for three weeks. Serial echocardiography was used to assess *in vivo* cardiac function and at the end of treatment, hearts were excised and subjected to an isolated working heart perfusion.

Hearts from mice with pressure overload hypertrophy exhibited decreased cardiac function alongside an abnormal metabolic profile compared to hearts from healthy mice. The observed failing heart's metabolic profile displayed decreases in absolute glucose oxidation rates, increases in normalized rates of fatty acid and ketone body oxidation. Furthermore, these metabolic changes contributed to the failing heart's decreased cardiac efficiency.

In healthy murine hearts perfused with increasing concentrations of ketones, myocardial ketone oxidation rates increased in a concentration-dependent manner with no effect on glucose or fatty acid oxidation rates. Furthermore, elevated ketone oxidation rates increased overall ATP production though this was not accompanied by changes in cardiac work, implying that an accumulation of reduced equivalents could be due to mitochondrial uncoupling. As a result, cardiac efficiency trended to decrease in hearts that were exposed to higher ketone concentrations.

In hearts from mice with ischemic heart failure, implementation of a ketogenic diet did not improve cardiac function compared to mice with heart failure fed a control diet. Furthermore, hearts from mice on the ketogenic diet displayed a notable shift to fatty acids for ATP production which was accompanied by blunted myocardial glucose oxidation rates (even in the presence of insulin). The increased reliance on fatty acids for energy contributed to the observed decrease in cardiac efficiency in heart failure mice fed a ketogenic diet.

My thesis has made several key advances for the field: firstly, the failing heart has blunted glucose oxidation and relies more on ketones for energy; secondly, ketones can increase cardiac ATP production without competing with glucose or fatty acids but do not improve cardiac efficiency; thirdly, the ketogenic diet is not a valid approach to increase myocardial ketone body oxidative rates and instead, shifts the heart's reliance on energy to fatty acids, causing a secondary decrease in myocardial glucose oxidation rates. Altogether, my thesis has revealed that ketones cannot acutely or chronically improve cardiac function and efficiency of the failing heart.

Preface

This thesis is an original work by Kim Ho. The research project, of which this thesis is a part of, received research ethics approval from the University of Alberta Research Ethics Board, Protection of the Ischemic Myocardium and Breeding Colony, AUP00000288, Monday, July 4, 2023.

A portion of the research conducted for this thesis forms part of an international research collaboration, led by Dr. Daniel Kelly at the University of Pennsylvania, with Dr. Gary Lopaschuk being the lead collaborator at the University of Alberta. The isolated working heart perfusions carried out in this thesis were performed by Dr. Gary Lopaschuk's lab technician, Mr. Cory Wagg. All data analysis throughout this thesis except for Chapter 3's echocardiographic data was carried out by me. Animal work in chapter 5 and biochemistry in chapter 3 through 5 were performed by me save for the ultra-performance liquid chromatography in chapter 4 was carried out by Mr. Ken Strynadka. Concluding analyses in chapters 3 through 5 are my original work, as well as the literature review in chapter 1. Methodological protocols presented in chapter 2 were all written up by myself with input from Dr. Liyan Zhang.

Chapter 3 of this thesis has been published as K.L. Ho, L. Zhang, C. Wagg, R.A. Batran, K. Gopal, J. Levasseur, T. Leone, J.R.B. Dyck, J.R. Ussher, D.M. Muoio, D.P. Kelly, and G.D. Lopaschuk, "Increased ketone body oxidation provides additional energy for the failing heart without improving cardiac efficiency" Cardiovascular Research, vol. 115, issue 11, 1606-1616. My role in this project included performing all experiments (except those noted below), data analysis, and manuscript composition. *Ex vivo* isolated working heart perfusions were performed by C. Wagg (Lab technician of G.D. Lopaschuk laboratory) and

echocardiography was performed by Ms. Donna Becker (former lab technician from the Cardiovascular Research Centre). G.D. Lopaschuk and J.R. Ussher were involved with concept formation and assisted with manuscript edits.

Chapter 4 of this thesis has been published as K.L. Ho*, Q.G. Karwi*, C. Wagg, L. Zhang, K. Vo, T. Altamimi, G.M. Uddin, J.R. Ussher, and G.D. Lopaschuk, "Ketones can become the major fuel source for the heart but do not increase cardiac efficiency" Cardiovascular Research, vol. 117, issue 4, 1178-1187. My role in this project included performing all the experiments (except those noted below) alongside the writing of the manuscript. Q.G. Karwi, a co-first author on this manuscript, assisted in writing a portion of the 'Discussion' section of the manuscript as well as the production of Figure 4.4 in the published manuscript. *Ex vivo* isolated working heart perfusions were performed by C. Wagg (Lab technician of G.D. Lopaschuk's laboratory). HPLC analysis of CoA and nucleotides was performed by Mr. Ken Strynadka. G.D. Lopaschuk and J.R. Ussher were involved with concept formation and assisted with manuscript edits.

Chapter 5 of this thesis is in preparation for submission for publication. My role in this project included performing all experiments (except those noted below), data analysis alongside manuscript composition. *Ex vivo* isolated working heart perfusions were performed by C. Wagg (Lab technician of G.D. Lopaschuk's laboratory). G.D. Lopaschuk and J.R. Ussher were involved with concept formation and assisted with manuscript edits.

Dedication

This thesis is dedicated to my parents, Cam and Thanh Ho, my brother, Huan Ho, and the most emotionally intelligent person I know, Ryan Hunt.

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First and foremost, I would like to sincerely thank my supervisors, Dr. Gary Lopaschuk and Dr. John Ussher, for giving me the opportunity to study under their tutelage and supervision during my graduate studies. Without their constant support, patience and help, I would never have made it this far into my graduate career. I am truly lucky to have them both as my mentors.

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Lastly, I would like to thank my best friend and talented author, Ryan Hunt, for proofreading all of my work throughout my graduate studies and always believing in me amidst my mental health struggles. There would be no thesis without your unconditional love and support.

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Abbreviations

Abbreviation	Definition
-/-	Knock Out
a'	Late Diastolic Mitral Annulus Velocity
AC-	Acetylated
ACC	Acetyl CoA Carboxylase
ADP	Adenosine Diphosphate
AKT (PKB)	Protein Kinase B
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate Activated Protein Kinase
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
AU	Arbitrary Units
BDH1	Beta-Hydroxybutyrate Dehydrogenase 1

BSA	Bovine Serum Albumin
CA^{2+}	Calcium Ion
CO_2	Carbon Dioxide
COA	Coenzyme A
CPT1	Carnitine Palmitoyltransferase 1
db/db	Leptin Receptor Deficient
e'	Mitral Annulus Early Diastolic Velocity
ECL	Enhance Chemiluminescence
EF	Ejection Fraction
ETC	Electron Transport Chain
FABP	Fatty Acid Binding Protein
$FADH_2$	Flavin Adenine Dinucleotide
FAO	Fatty Acid Oxidation
FAT/CD36	Fatty Acid Translocase
FATP	Fatty Acid Transporter Protein

FFA	Free Fatty Acids
FS	Fractional Shortening
GLUT4	Glucose Transporter Type 4
GO	Glucose Oxidation
GTP	Guanosine Triphosphate
HF	Heart Failure
HFD	High Fat Diet
HK	Hexokinase
HOMA	Homeostatic Model Assessment
HW	Heart Weight
IGG	Immunoglobulin
IM	Intramuscular
IP	Intraperitoneal
LAD	Left Anterior Descending Coronary Artery
LCAD	Long Chain Acyl CoA Dehydrogenase

LDL	Low Density Lipoprotein
LV	Left Ventricle
MCD	Malonyl CoA Decarboxylase
MI	Myocardial Infarction
min	minute
MPC	Mitochondrial Pyruvate Carrier
MTOR	Mammalian Target of Rapamycin
NADH	Nicotinamide Adenine Dinucleotide
PCR	Phosphocreatine
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PFK1	Phosphofructokinase 1
PH	Potential Hydrogen
PPAR	Peroxisome Proliferator Activated Receptors
PTM	Post Translational Modification

ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RT	Room Temperature
SC	Subcutaneous
SCOT	Succinyl Coa:3-Ketoacid CoA Transferase
SDS	Sodium Dodecylsulfate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
sec	Second
SEM	Standard Error of Mean
TAC	Transverse Aortic Constriction
TCA	Tricarboxylic Acid Cycle
TG/TAG	Triacylglycerols
TL	Tibia Length
UCP	Uncoupling Protein
UPLC	Ultra Performance Liquid Chromatography

wk	week
βHAD	Beta-Hydroxy Acyl CoA Dehydrogenase
βOHB	beta-hydroxybutyrate

Chapter I
Introduction

Chapter 1 Introduction

1.1 Introduction

Before embarking on this big journey of reading my thesis, I thought I would cushion the overwhelming nature of this body of text by starting off with a short and sweet explanation of my research, one that helped me win first place at my university's 3-Minute-Thesis competition in 2022. Figure 1.1 accompanies the following speech.

"Have you ever run out of fuel while driving on the road? If you have, you probably know that it's quite scary. But what's even scarier is not your car running out of fuel one day, it's your heart running out of fuel. Just like a car engine uses fuel from gas stations, your heart also uses fuels obtained from blood. But instead of gasoline, if I bring your attention to (Figure 1), the healthy heart uses fats, carbohydrates and ketones for its fuels.

All these fuels are constantly being metabolized by the heart to produce energy that it uses to pump blood throughout the body. However, this is not the case in people who suffer from heart failure. In heart failure, a chronic condition where the cardiac muscle is weakened, the heart can no longer effectively pump blood to the rest of the body and you have a situation where a once efficient engine is now not producing enough energy and ultimately, is like an engine out of fuel. And what are the consequences? Well, approximately 50% of patients die after 5 years following diagnosis, and unfortunately there is no cure for heart failure.

My research therefore is focused on improving our understanding of the failing heart's energy profile and more specifically, the heart's reliance on ketones for energy. Why ketones though? Well, several years ago, work from our lab and others found that

the failing heart relies more on ketones for energy compared to a healthy heart. The question then surfaced – are ketones good or bad news for a failing heart? Could they be a super fuel for a heart already running low on fuels?

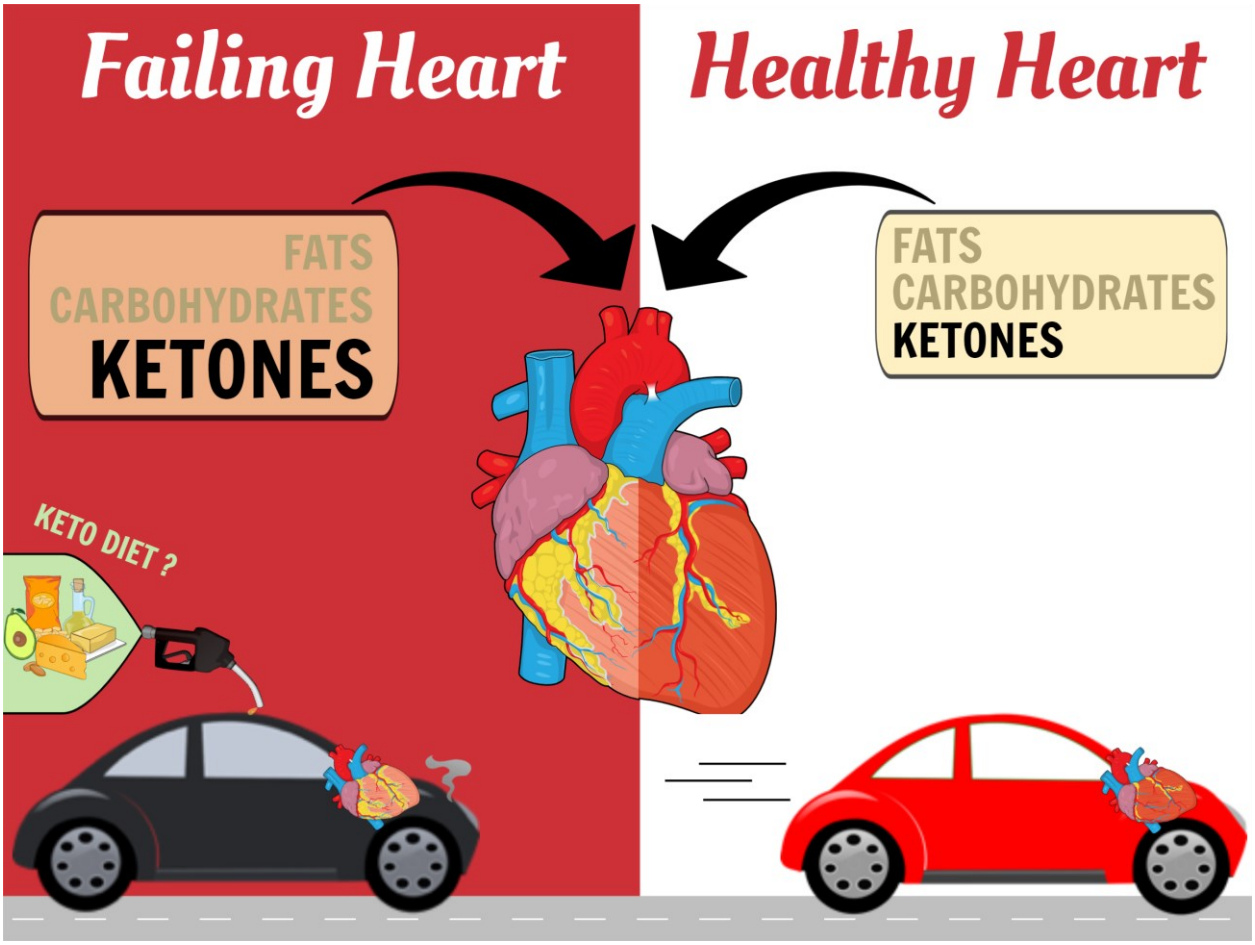
To investigate this, I use mice. This allows me to ethically carry out the following experiments that would otherwise not be possible with humans. Using mice with heart failure, their hearts are removed and perfused with a special solution that you can think of as simulated blood. Now even though the heart has been removed from the mouse's body, it can beat in isolation and the heart's function and metabolism can be measured. What I found was that giving ketones to the heart increased energy production. This, of course, is great news for a fuel-deficient failing heart.

The neat thing is, I am now feeding mice with heart failure a ketogenic diet to increase ketone levels and measure their heart function to see if this popular diet can be used as a therapy for heart failure.

So, if we take a step back to the car engine analogy, my research will help determine if ketones can be used like the AMA's emergency fuel delivery service for an engine out of fuel."

I hope that with the inclusion of my 3-Minute-Thesis speech, I have provided enough enticement to delve deeper into my thesis and explore cardiac ketone body metabolism with me. In the following sections, I will be delving deeper into cardiac energy metabolism.

Figure 1.1 Fueling the failing heart with ketones. A simplistic illustration of my research to accompany my 3-Minute-Thesis (3MT) speech.



According to the Heart and Stroke Foundation of Canada, 600 000 Canadians are living with heart failure.[1] Diabetes also increases the risk for heart failure 2.7 fold.[2] However, recent evidence from the EMPA-REG OUTCOME trial[3] suggests that treatment with the glucose-lowering agent, empagliflozin, improves cardiovascular outcomes in people with type 2 diabetes. Interestingly, empagliflozin's cardioprotection has been postulated to be due to increases in myocardial ketone body oxidation ketone body oxidation rates.[4,5] However, evidence of a beneficial effect of increased ketone body oxidation in heart failure has not been shown, and recent studies have demonstrated increased myocardial ketone body oxidation in animal models and humans with heart failure[6,7]. We have also shown that ketone body oxidation is increased in mice with heart failure secondary to pressure-overload[8]. Whether or not this increase in myocardial ketone body oxidation is adaptive or maladaptive during heart failure [9-12], and its implications in the context of empagliflozin-induced cardioprotection remains controversial.[13] In fact, a recent study proposed that part of the compromised cardiac energetics of heart failure may be due to increased ketone utilization during heart failure which increases the mitochondrial acetyl CoA pool and consequently, contributes to mitochondrial protein hyperacetylation and impaired mitochondrial bioenergetics[14]. In contrast, blunting myocardial ketone body oxidation in succinyl-CoA:3-ketoacid CoA transferase (rate-limiting enzyme in ketone body oxidation) knockout mice accelerates pathological remodelling during experimental heart failure [15]. In support of this, cardiac-specific overexpression of β-hydroxybutyrate-dehydrogenase-1 (a key enzyme in ketone body oxidation) protects mice against pressure-overload hypertrophy.[16] Despite the sparse amount of evidence available, it seems plausible that increasing myocardial ketone metabolism may improve heart failure outcomes. However, no reported studies have directly measured myocardial ketone

metabolism or assessed the effects of increasing cardiac ketone oxidation on the failing heart's metabolism, ATP production and cardiac efficiency.

1.2 An overview of cardiac energy metabolism

Intermediary metabolism, or the sum total of both anabolic and catabolic chemical events occurring, is based on the fact that energy cannot be synthesized or destroyed. Energy can only be transformed or transferred between cells. Much of the potential energy cells get is from energy that is 'trapped' within a chemical bond of a molecule. Notably, the molecule known as adenosine triphosphate (ATP). Made up of a five-carbon sugar, adenine base and 3 phosphates, energy can be released when the phosphoanhydride bonds (P-O) bonds are broken. Specifically, 7 kcal/mole of energy is released per mole of ATP. Therefore, ATP is like a reservoir of high energy for a cell to tap into for its cellular processes[17]. In the particular case of a cardiac cell (cardiomyocyte), the myosin head can bind to ATP and use its energy to pull actin filaments resulting in muscle contraction[18]. So, with a better understanding of the primary currency used in energy metabolism, ATP, the next section will explore all the different ways in which we can procure ATP in the healthy heart.

1.3 Energy metabolism in a healthy heart

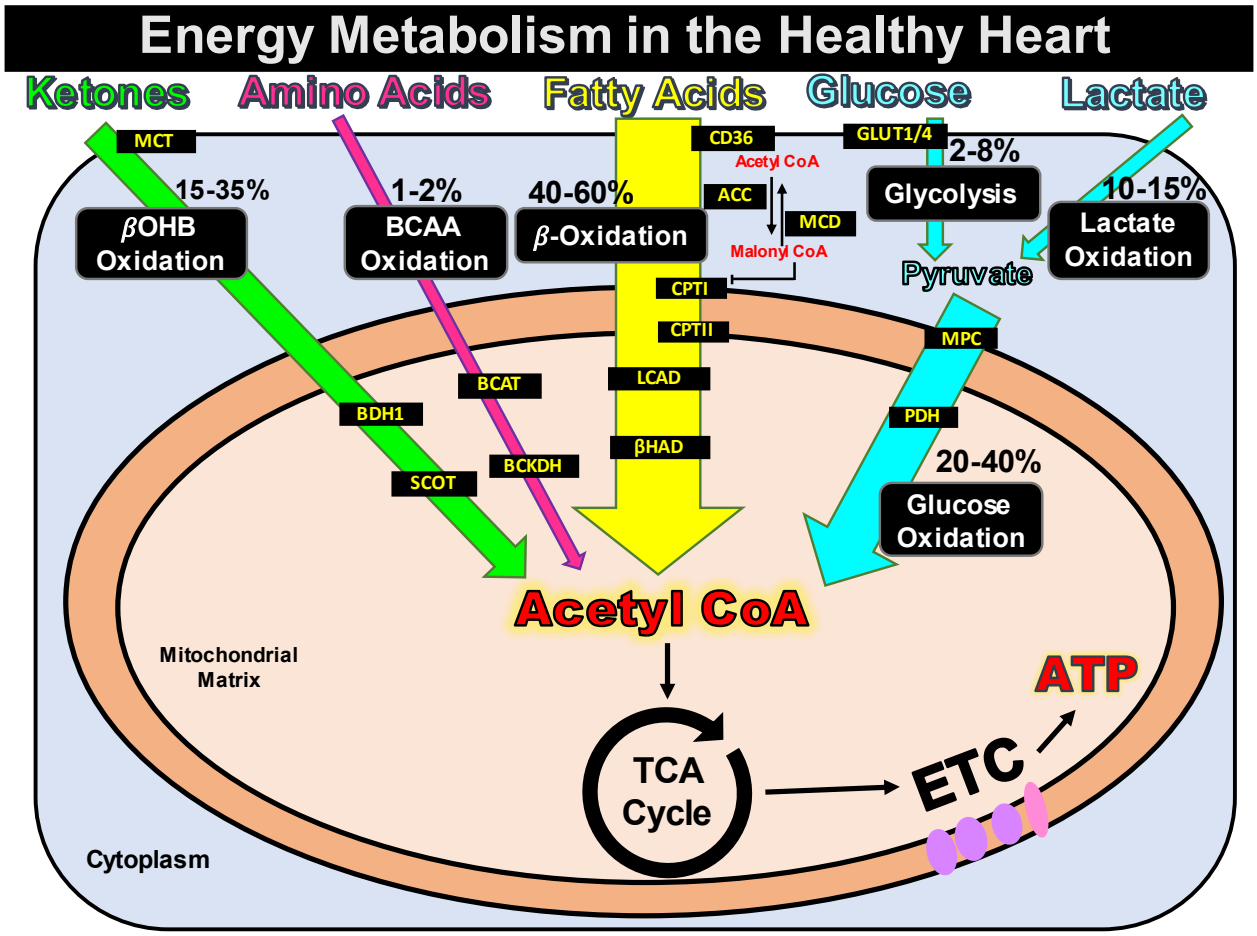
In a healthy heart, most of the ATP is derived from mitochondrial oxidative phosphorylation (approximately 95%), while the remaining 5% of ATP comes from substrate level phosphorylation during glycolysis[19]. More specifically, 40-60% of ATP comes from the oxidation of fatty acids, 20-40% from glucose and the remaining ATP comes from the metabolism of ketones, amino acids, and lactate[20]. As you can see in Figure **1.2**, the heart is very much like an omnivore of different fuel types. Depending on whether we have just eaten

a big meal or have been fasting, the healthy heart is flexible with what types of fuels it can consume.

For example, in a fed state, circulating insulin levels will be high in our blood and thus, our heart will preferentially use glucose for energy. Conversely, in a fasted state when insulin levels are lower, the heart will prefer to use fatty acids for energy. I like to think of the heart as a very 'hungry' organ that constantly requires high amounts of energy (ATP) to power its mechanical processes. In addition to sustaining contractile function, the heart must also use energy to maintain ionic homeostasis and keep basal metabolic processes running. In fact, without a constant supply of ATP, the heart would only have enough internal ATP stores to sustain 5 beats (our hearts beat around 60-80 beats per minute)[21]. Thus, it is crucial that the heart continually synthesizes ATP via intermediary metabolism of fatty acids, glucose, lactate, amino acids, and ketones.

The following sub-sections will detail how the healthy heart gets its constant supply of ATP via the intermediary metabolism of fatty acids, glucose, and ketones.

Figure 1.2 Overview of cardiac energy metabolism in a healthy heart. This figure is published in the textbook chapter 'Cardiac energy Metabolism' by Lopaschuk and Ho (in press).



1.3.1 Fatty acid metabolism

Much of section 1.3.1 pays homage to the excellent review of 'Myocardial Fatty Acid Metabolism in Health and Disease' by Lopaschuk and colleagues[22].

1.3.1.1 Source of fatty acids that can be used by the heart

Fatty acids can come from various sources throughout our body. In a post-prandial state, fatty acids can be synthesized from glucose via *de novo* lipogenesis in the liver to form very low-density lipoproteins (VLDL). Fatty acids can also come from our diet itself and be exported directly from enterocytes (intestines) in the form of chylomicrons.

Three fatty acids can then be esterified to glycerol 3-phosphate to produce triacylglycerol (TAG). TAGs are stored in white adipose tissue and can be mobilized when we are in a fasted state. Mobilization of fat stores from adipose tissue begins with the hydrolysis of triacylglycerols by various lipases. Circulating free fatty acids (FFAs) either bound to albumin or released by the cell surface enzyme lipoprotein lipase (LPL) from triacylglycerol (TAG) contained in very density lipoproteins (VLDL) or chylomicrons can then enter the cardiomyocyte either by a carrier mediated pathway or facilitated diffusion[23].

The mobilization of fatty acids from adipose tissue is termed lipolysis and involves the breakdown of triacylglycerol into three fatty acids and one glycerol through several steps. Firstly, triacylglycerol is hydrolyzed to diacylglycerol via adipose triacylglycerol lipase (ATGL), releasing one acyl molecule. Diacylglycerol is then hydrolyzed to monoacylglycerol via hormone-sensitive lipase (HSL), releasing a second fatty acid molecule. And finally, monoacylglycerol is hydrolyzed to glycerol via monoacylglycerol lipase (MAGL), releasing a

third fatty acid molecule. In total, three fatty acid molecules are obtained from the lipolysis of one triacylglycerol[24,25].

In addition to free fatty acids bound to albumin, the heart can also receive fatty acids that are broken down from triacylglycerol that is derived from lipoproteins, specifically, very-low-density-lipoproteins (VLDL) or chylomicrons.

1.3.1.2 Uptake of fatty acids into the cell

Fatty acids enter the cell through passive diffusion or through the scavenger receptor known as 'cluster of differentiation 36' (CD36) also known as fatty acid translocase (FAT). Other modes of entry into the cell include the use of fatty-acid-binding proteins (FABPs) and fatty acid transport proteins (FATP). Studies have shown that CD36/FAT-mediated translocation of fatty acids across the cardiomyocyte's sarcolemmal membrane contributes about 50-60% to the overall fatty acid uptake in the heart[26,27].

Once fatty acids have entered the cell, they are converted/activated into long-chain acyl CoA esters in the cytoplasm via fatty acyl CoA synthetase (FACS). Long-chain acyl CoAs can then transfer the fatty acid moiety to carnitine to facilitate fatty acid uptake into the mitochondria (Section 1.3.1.3). Alternatively, long-chain acyl CoAs can be used to synthesize intracellular lipid intermediates.

1.3.1.3 Uptake of fatty acids into the mitochondria

In order for the activated acyl CoA to cross the inner mitochondrial membrane, a transport system is required. This transport system is called the carnitine shuttle and consists of carnitine/acylcarnitine translocase alongside two carnitine-palmitoyl transferases (CPTs). The first CPT, named CPT-1 is located on the outer mitochondrial membrane and catalyzes

the conversion of long-chain acyl CoA into long-chain acylcarnitine. Long-chain acylcarnitine is then shuffled into the mitochondrial matrix through the inner mitochondrial membrane-bound carnitine/acylcarnitine translocase. A second CPT, named CPT-2 is located on the inner mitochondrial membrane and subsequently catalyzes the conversion of long-chain acylcarnitine into carnitine and long-chain acyl CoA (now located inside of the mitochondria!).

1.3.1.4 β-oxidation of fatty acids

Now that the fatty acid has finally arrived within the mitochondrial matrix, after having been derived from triacylglycerol from the intestine, liver, adipose tissue or from within the heart itself (endogenous triacylglycerol stores), it can now be oxidized through a metabolic process known as β-oxidation. With each cycle of β-oxidation, two carbons are cleaved from the fatty acyl moiety, producing acetyl CoA, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$).

The first step in β-oxidation is the dehydrogenation of the fatty acyl-CoA into Δ^2-enoyl-CoA via an acyl CoA dehydrogenase and concurrent reduction of FAD to $FADH_2$. Different isoforms of acyl CoA dehydrogenase exist to cater to specific lengths of fatty acids (ex. LCAD, long-chain acyl CoA dehydrogenase; MCAD, medium-chain acyl CoA dehydrogenase; SCAD, short-chain acyl CoA dehydrogenase).

The second step in β-oxidation is the hydration of *trans*-Δ^2-enoyl-CoA into 3-hydroxyacyl-CoA via enoyl-CoA hydratase and concurrent consumption of one water (H_2O) molecule.

The third step in β-oxidation is the dehydrogenation of 3-hydroxyacyl-CoA into 3-ketoacyl-CoA via *L*-hydroxyacyl-CoA dehydrogenase and concurrent reduction of NAD^+ to $NADH + H^+$.

The fourth and final step in β-oxidation is the thiolysis of 3-ketoacyl-CoA into a fatty acyl-CoA that has now been shortened by two carbons. During this step, 3-oxoacyl CoA thiolase utilizes coenzyme A to break the bond at the third carbon and release one unit of acetyl CoA alongside the shortened fatty acyl CoA.

Finally, acetyl CoA can then enter the tricarboxylic acid (TCA) cycle where additional NADH and $FADH_2$ are produced. NADH and $FADH_2$ can then enter the electron transport chain to produce ATP. In total, the oxidation of a single palmitate molecule produces 104 ATP (Table 1.1).

Table 1.1 The amount of ATP, NADH/$FADH_2$ produced by various metabolic pathways.

This is based on the more recently corrected ATP values for NADH (2.5) and $FADH_2$ (1.5).

Substrate	ATP	Acetyl CoA	NADH/ FADH2	O2 consumed	P/O Ratio
Glycolysis	2	0	2	0	
Glucose Oxidation	29	2	10	6	2.58
Palmitate oxidation	104	8	46	23	2.33
Ketone body oxidation	21.25	2	9	4.5	2.50

1.3.1.5 Regulation of fatty acid metabolism in the heart

The rate of fatty acid uptake into the cardiomyocyte is dependent on the concentration of fatty acids in the blood and thus supply of fatty acid to the heart. As such, post-prandial insulin secretion and subsequent inhibition of lipolysis will result in decreased fatty acid levels in the blood and thus, decreased fatty acid supply and metabolism in the heart.

Malonyl CoA is a potent inhibitor of CPT-1 and is a key mechanism by which myocardial fatty acid oxidation rates are regulated. Malonyl CoA is synthesized from acetyl CoA via acetyl CoA carboxylase (ACC) and degraded by malonyl CoA decarboxylase (MCD)[28].

A key regulator of ACC activity is AMPK, a 'fuel sensor' that promotes fatty acid oxidation when we need more energy and lowers fatty acid oxidation when we don't need energy. Specifically, ACC activity is inhibited by AMPK phosphorylation[29,30]. Thus, decreases in energy demand result in decreased AMPK activity, decreased phosphorylation of ACC, increased ACC activity, increased malonyl CoA levels, decreased mitochondrial uptake of fatty acids and decreased fatty acid oxidation[31,32].

Another regulatory mechanism utilized to control rates of fatty acid oxidation includes the post-translational modification (i.e. acetylation, succinylation and malonylation) of enzymes in the β-oxidation pathway. Previous work from our lab has shown that hyperacetylation of long-chain acyl CoA dehydrogenase as well as 3-hydroxyacyl-CoA-dehyrogenase increases its enzymatic activity[33]. Certain diseased states promote hyperacetylation of proteins, one of which is heart failure[34]. Additionally, the availability of coenzyme A and coenzymes NAD^+ and FAD also allosterically regulates the rate at which fatty acids are oxidized.

Transcriptional changes in fatty acid oxidative enzymes are largely mediated by peroxisomal proliferator-activated receptor γ coactivator-1 (PGC-1α) as well as peroxisomal proliferator activated receptor (PPAR)α [35]. For example, PPARα's target genes include, but are not limited to, CD36/FAT, FABP, FACS, MCD, CPT 1, and MCAD. Studies have shown that overexpression of PPARα increases cardiac fatty acid uptake and oxidation while knock-out of PPARα decreases cardiac fatty acid uptake and oxidation[36].

Through these various regulatory points for fatty acid metabolism, the heart can metabolically adapt to changes in our nutritional, energetic and hormonal state.

1.3.2 Glucose metabolism

1.3.2.1 Source of glucose that can be used by the heart

The heart can obtain glucose from either the bloodstream or from endogenous glycogen stores.

1.3.2.2 Uptake of glucose into the cell

Glucose can be taken up in an insulin-dependent manner via the GLUT4 transporter or in an insulin-independent manner via the GLUT1 transporter. Once glucose enters the cell, it can be used in glycogen synthesis, the hexosamine biosynthetic pathway, the pentose phosphate pathway, the polyol pathway or enter glycolysis. In the heart, glycolysis is the predominant pathway that glucose is subjected to.

1.3.2.3 Glycolysis

Once glucose is inside of the cell and specifically the cytoplasm, glucose is phosphorylated to glucose-6-phosphate via an enzyme called glucokinase/hexokinase, consuming one molecule of ATP in the process. Another ATP molecule is then used to convert

glucose-6-phosphate to fructose 1,6-bisphosphate via the enzyme phosphofructokinase-1. Phosphofructokinase is considered the rate-limiting step in glycolysis.

Fructose 1,6-bisphophate is then split into two triose phosphates via fructose 1,6-bisphosphate, namely: dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, which are interconvertible. All subsequent reactions in glycolysis are hence doubled due to the two triose phosphate molecules.

Glyceraldehyde 3-phosphate dehydrogenase then oxidizes glyceraldehyde 3-phosphate using an inorganic phosphate to produce 1,3-bisphophoglycerate. In this step as well, NADH is formed and can then enter the electron transport chain to produce three molecules of ATP.

1,3-bisphophoglycerate has a phosphate removed by phosphoglycerate kinase to produce 3-phosphoglycerate and one molecule of ATP (two in total due to the double triose phosphate molecules previously mentioned). A mutase enzyme, phosphoglycerate mutase, then converts 3-phosphoglycerate into 2-phosphoglycerate. 2-phosphoglycerate is then converted into phosphoenolpyruvate via a hydro-lyase known as phosphopyruvate hydratase or enolase. Finally, pyruvate kinase converts phosphoenopyruvate alongside ADP into ATP and pyruvate.

At this point, anaerobic glycolysis has finished in the cytosol and yields a total of 4 molecules of ATP though recall that 2 molecules of ATP were invested during the beginning of glycolysis. Thus, a net total of 2 ATP molecules are produced from each cycle of glycolysis from glucose.

1.3.2.4 Glucose oxidation

Glucose oxidation involves the oxidation of pyruvate through the Krebs cycle, producing NADH and $FADH_2$ which can subsequently go through the electron transport chain to produce ATP.

The first step in glucose oxidation is the uptake of pyruvate into the mitochondria from the cytosol. This is facilitated by the mitochondrial pyruvate carrier.

Once inside the mitochondria, pyruvate dehydrogenase (PDH), considered the rate-limiting enzyme for glucose oxidation, converts of two pyruvate molecules into 2 acetyl CoA molecules. Notably, pyruvate dehydrogenase consists of three units, termed E1 through E3, and thus is referred to as the pyruvate dehydrogenase complex (PDC). Together, the complex takes pyruvate, coenzyme A and NAD^+ and converts them to acetyl-CoA, CO_2 and NADH. The two molecules of acetyl CoA can then enter the citric acid cycle to produce a total of 10 NADH, 2 GTP (metabolized to 2 ATP), and 2 $FADH_2$, totaling 29 ATP produced from the oxidation of pyruvate.

Together with glycolysis and glucose oxidation, a total of 31 molecules of ATP are produced from a single glucose molecule (Table 1.1).

1.3.2.5 Regulation of glucose metabolism in the heart

Glucose metabolism is primarily dependent on circulating insulin levels since insulin stimulates translocation of GLUT4 to the plasma membrane to increase glucose uptake (and metabolism) in the cell. Work from our lab has also shown that insulin can directly stimulate glucose oxidation in the heart[37]. When insulin levels increase after feeding, increased GLUT4-mediated glucose uptake will result in increased intracellular glucose levels and ultimately, increased glucose metabolism in the heart[38].

Another important mode of regulation for glucose metabolism is the reciprocal relationship that glucose and fatty acid metabolism have, known as the 'Randle Cycle'[39]. Simply put, when fatty acid oxidation increases, glucose oxidation decreases. Conversely, when glucose oxidation increases, fatty acid oxidation decreases. The tightly regulated reciprocal nature is conferred through the inhibition of PDH (rate limiting enzyme of glucose oxidation) by fatty acid oxidation. Specifically, increases in the [acetyl-CoA]/[CoA] ratio feedback inhibit PDH as well as contribute to increases in cytosolic citrate levels. Increases in citrate inhibit PFK and causes a 'traffic jam', increasing glucose-6-phosphate levels which inhibit hexokinase. Furthermore, increases in glucose oxidation result in increases in malonyl-CoA which, as previously discussed in section 1.3.1.5, serve as a potent inhibitor of CPT-1 (mitochondrial fatty acid uptake).

1.3.3 Ketone metabolism

Since 1938 when Barnes and Waters, alongside their colleagues, first established that the heart could use ketones as an energy substrate[40,41], ketones have remained an elusive substrate of the heart. While an impressive amount of literature exists to describe the role and regulation of fatty acid and glucose metabolism in the heart, myocardial ketone metabolism remains understudied and poorly understood especially in a pathological condition such as heart failure (See review by Karwi and colleagues [11]).

There are three ketone bodies and they are 3-hydroxybutyrate (βOHB), acetoacetate and acetone. Unlike glucose and fat, ketone bodies cannot be conveniently consumed and instead, are produced from our liver using fatty acids. Evolutionarily, ketones were made to be a fuel for the brain during fasting periods as long chain fatty acids cannot be metabolized by the brain for energy[42].

1.3.3.1 Source of ketones that can be used by the heart

The heart can also use ketones as a fuel and the primary source of ketones for the heart comes from the ketones produced by the liver and released into the blood. Hepatic ketogenesis is the process whereby ketone bodies are produced in the liver during periods of starvation, prolonged exercise or uncontrolled diabetes. Ketone bodies are produced from triacylglycerols as well as several amino acids. Specifically, in a fasted state, mobilization of lipid stores will release fatty acids into the blood where they are bound to albumin and can travel to the liver. Once in the liver, the fatty acids will undergo β-oxidation and subsequently ketogenesis.

The first step in ketogenesis utilizes the acetoacetyl CoA thiolase enzyme to convert two molecules of acetyl CoA (from β-oxidation) into acetoacetyl CoA. Acetoacetyl CoA is then condensed with a third acetyl CoA to produce 3-hydroxy 3-methylglutaryl CoA (HMG CoA) via HMG CoA synthase. HMG CoA is then cleaved into acetoacetate and acetyl CoA by HMG CoA lyase. Acetoacetate can then be interconverted into βOHB, the main circulating ketone body in our blood, via 3-hydroxybutyrate dehydrogenase (BDH1).

1.3.3.2 Uptake of ketones into the cell

Ketone bodies are thought to be taken up by the cell through a transporter known as SLC16A1 after which it can enter the mitochondria for oxidation[43].

1.3.3.3 Ketone body oxidation

Once inside the mitochondria, ketone body oxidation can occur and begins with βOHB being converted into acetoacetate via BDH1, releasing a single molecule of NADH in the process. Next, 3-ketoacyl-CoA transferase (SCOT) utilizes succinyl CoA to convert acetoacetate into acetoacetyl CoA, releasing succinate in the process. SCOT is considered the

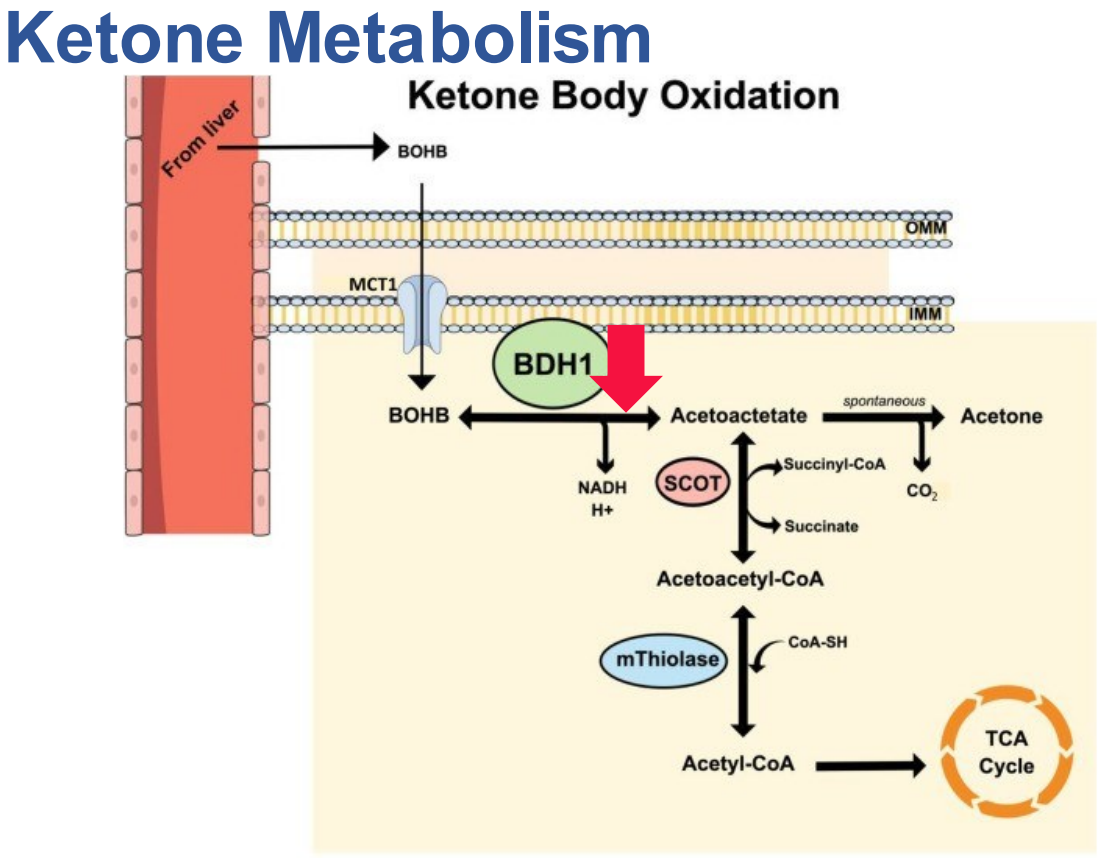
rate limiting enzyme for ketone body oxidation. Lastly, acetoacetyl CoA is converted into two acetyl CoA molecules via the acetoacetyl CoA thiolase enzyme. This is illustrated in Figure 1.3.

Together, 1 NADH (from the BDH1 reaction), 6 NADH + 2 $FADH_2$ (from the 2 acetyl CoA), alongside the succinate entering into the Krebs cycle totals 21.25 ATP produced from a single ketone molecule (0.25 ATP is loss due to 1 hydrogen being used by a phosphate carrier) (Table 1.1).

1.3.3.4 Regulation of ketone metabolism in the heart

Cardiac ketone metabolism is dependent on the concentration of ketones in the blood and hence, the amount of ketones the heart is 'seeing'. Furthermore, transcriptional regulation of ketolytic enzymes plays an important role in regulating the rate at which the heart oxidizes ketones. Work from Dr. Peter Crawford's lab has shown that exposure to ketones in a chronic manner causes a compensatory decrease in the transcriptional machinery used in ketone oxidation[44].

Figure 1.3 Ketone body oxidative pathway. This figure was previously published in the paper 'Lopaschuk GD, Karwi QG, Ho KL, Pherwani S, Ketema EB. Ketone metabolism in the failing heart. Biochim Biophys Acta Mol Cell Biol Lipids. 2020 Dec;1865(12):158813. doi: 10.1016/j.bbalip.2020.158813. Epub 2020 Sep 10. PMID: 32920139.

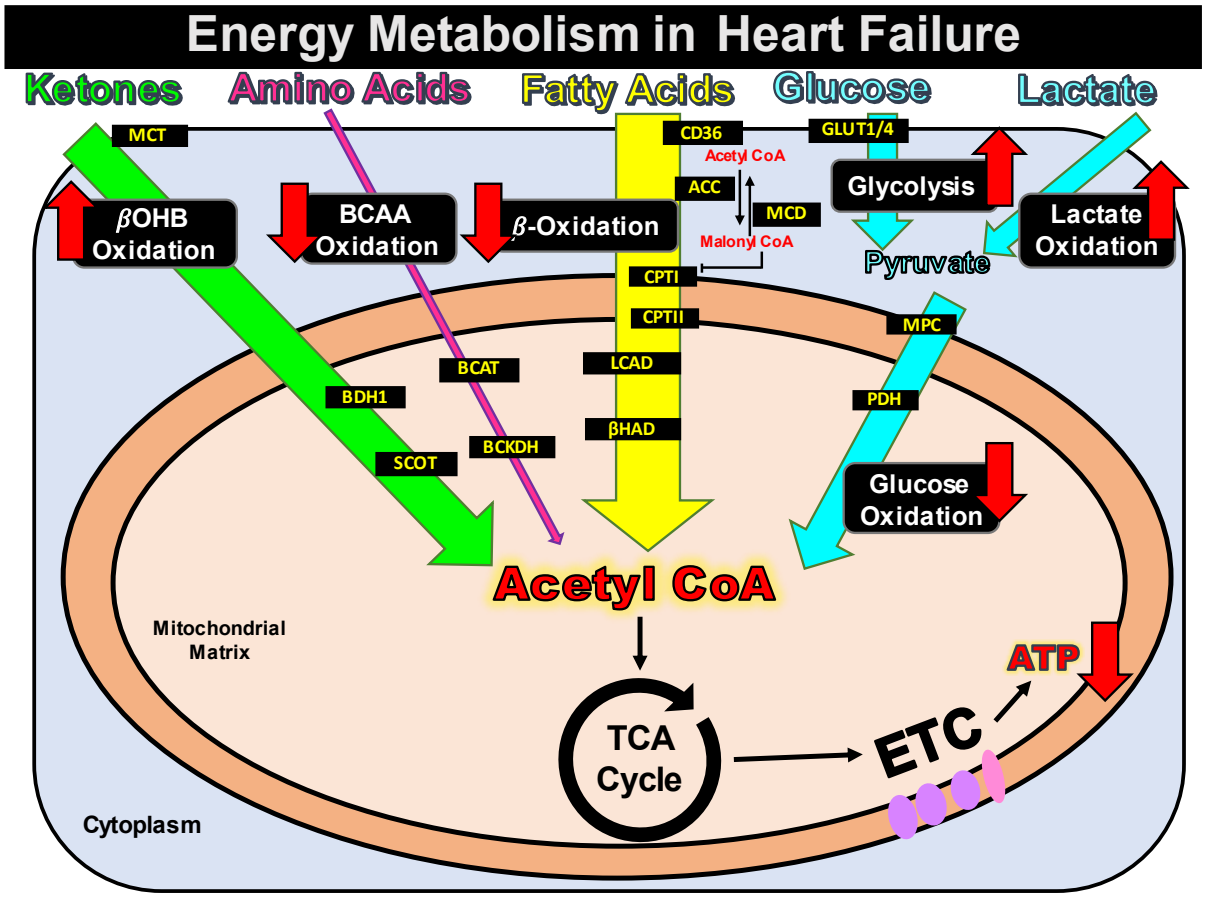


1.4 Energy metabolism in a failing heart

In the late 1950s, Dr. Richard Bing started suggesting that the root cause of myocardial failure is metabolic failure [45]. Much of this occurs due to the fact that 95% of the energy the heart gets is from oxidative metabolism but in a failing heart, mitochondrial oxidative capacity is impaired. Mitochondrial function is compromised in the failing heart due to several reasons which include decreased mitochondrial biogenesis, impaired mitofusion, sustained mitophagy, increased reactive oxygen species production as well as increased autophagic cell death of cardiomyocytes (see [46] for review). Altogether, the metabolic dysfunction leads to lack of metabolic flexibility that one would see with a healthy heart (as previously discussed) as well as an 'energy-starved' state. In fact, heart failure can decrease ATP content in the human heart by 30-40%[47].

While the healthy heart derives most of its energy from fatty acids followed by glucose then ketones, the failing heart experiences changes in its ability to utilize these fuels properly, leading to impaired cardiac ATP production and cardiac efficiency (Figure 1.4). The following sub-sections will detail the way in which fatty acid, glucose and ketone metabolism are perturbed in the failing heart.

Figure 1.4 Energy metabolism in the failing heart. In the context of my thesis, I focus on heart failure with reduced ejection fraction. This figure is published in the textbook chapter 'Cardiac energy Metabolism' by Lopaschuk and Ho (in press).



1.4.1 Fatty acid metabolism in heart failure

Myocardial fatty acid oxidation is generally thought to decrease in the setting of heart failure but this is rather controversial. Decreases in absolute cardiac fatty acid oxidation rates may indeed occur due to the overall impaired oxidative capacity of the heart alongside decreases in the transcription of fatty acid oxidative enzymes[48]. However, direct and indirect assessments of fatty acid oxidation in both human and animal models have been inconsistent. Early work from Dr. Richard Bing in heart failure patients with depressed cardiac function found that myocardial fatty acid uptake and oxygen consumption were both increased[49-51]. Furthermore, arterio-venus measurement studies have found that in heart failure, fatty acids remain the primary source of energy for the heart[52-54]. Work from our lab has also found that myocardial fatty acid oxidative rates are not decreased in animal models of heart failure[8,55]. This is not without confusion as other studies have presented decreases in myocardial fatty acid oxidative rates while some observe no differences[56,57].

Therefore, the decisive changes to fatty acid metabolism in the failing heart are still unclear. Differences in findings though may be due to the severity of heart failure in the different study models as well as the presence (or absence) of comorbidities.

1.4.2 Glucose metabolism in heart failure

As previously mentioned, glucose metabolism is broken into two parts: glycolysis and glucose oxidation.

In the setting of heart failure, glycolytic rates increase in the heart in an attempt to compensate for the impairment in the heart's overall oxidative capacity. As such, increases in glucose uptake, GLUT1 expression and phosphofructokinase-1 activity accompany the

increases in glycolytic rates[50,58-61]. Increases in glycolysis though are not enough to compensate for the overall drop in the heart's mitochondrial oxidative capacity due to the fact that glycolysis only produces a net of two ATP molecules (recall that the oxidation of glucose produces 31 ATP molecules while glycolysis alone only produces 2 ATP).

Glucose oxidation, the latter part of glucose metabolism, is significantly impaired in heart failure. Numerous animal studies assessing glucose oxidation rates in various models of heart failure have found decreased glucose oxidation: 3 weeks post abdominal aortic constriction in mice[62], 4-6 weeks post transverse aortic constriction in mice[55,63,64], volume overload heart failure in rats[60,61] and pacing-induced heart failure in pigs[65]. It is interesting to note that 10 weeks post-transverse aortic constriction, mice have been shown to have increases in both glucose oxidation and glycolytic rates[66]. Additionally, dogs with pacing-induced heart failure also presented increases in glucose oxidation[67]. As of yet, no human studies have been conducted to assess cardiac glucose oxidation rates in heart failure. However, cardiac tissue from heart failure patients have decreased expression of the rate limiting enzyme of glucose oxidation PDH, implying decreased myocardial glucose oxidation[68].

1.4.3 Ketone metabolism in heart failure

At the start of my thesis, initial studies that assessed ketone body utilization in both heart failure patients and mice were published[7,69]. Specifically, heart failure patients (congestive heart failure with 2 patients that had ischemic cardiomyopathy) presented increased cardiac ketone body oxidative rates[69]. Similarly, in a pressure overload hypertrophy model of heart failure, mice also had increased expression of ketone body oxidative metabolites and proteins[7]. These observations were again recapitulated in both humans with

hypertrophic heart failure[70]. We were also the first to directly measure ketone body oxidative rates in the heart and this will be presented in Chapter 3 of this thesis.

With others and our own lab finding increased reliance on ketone bodies for energy in the failing heart, questions surfaced about the consequences of increased ketone body usage in heart failure. Was this an adaptive or maladaptive process that the failing heart was using? To answer this question, we set out to investigate the effect of ketone bodies on overall cardiac ATP production in a healthy setting first. This study comprises Chapter 4 of my thesis. During the period of which Chapter 4's study was being conducted, other groups also set out to investigate the role of ketone bodies in heart failure.

In a study with mice that had a cardiomyocyte-specific knockout of SCOT (the rate limiting enzyme of ketone body oxidation), mice had accelerated pathological remodeling in the setting of pressure overload hypertrophy[71]. On the other hand, in mice with an overexpression of BDH1 (the first enzyme in ketone body oxidation), mice had decreased oxidative stress and pathological remodeling in a model of pressure overload hypertrophy[16]. Furthermore, infusion of ketones into dogs that had pacing-induced heart failure as well as heart failure patients resulted in slight improvements in cardiac function[72,73]. As such, these initial studies have given credence to ketone's beneficial role in the setting of heart failure (Table 1.2).The way in which ketones confer these benefits is unclear though ketones have previously been proposed to be a more efficient fuel for the heart but this hypothesis was quickly disproven by work from our lab[8,13,74], much of which will be discussed in Chapter 4 of this thesis. Instead, we have now shown that instead of increasing cardiac efficiency, ketones are simply an extra source of fuel that can supply the heart with additional ATP[74].

With knowledge that ketones can be an extra source of fuel and increase ATP production in a healthy heart (See Chapter 4), the obvious question then became, well can we use ketones to increase cardiac ATP production in an energy-starved failing heart and would this translate to cardiovascular functional benefits? This leads to Chapter 5 of my thesis where we investigate whether increasing ketone delivery to the heart, via a ketogenic diet, can improve the outcomes of heart failure.

Table 1.2 Types of Heart Failure and Myocardial Ketone Body Oxidation

Type of Heart Failure	Cardiac Ketone Body Oxidation Rates	Reference
Dilated cardiomyopathy (Congestive heart failure in humans; only 2 patients had ischemic cardiomyopathy)	Increase	Bedi et al. 2016[69]
Pressure overload hypertrophy (TAC in mice) *though keep in mind that this group uses a CH model in which they combine TAC + apical MI	Increase	Aubert et al. 2016[7]
Pressure overload hypertrophy (Ascending aortic band in mice, primary studies are in rat nenonatal ventricular cardiomyocytes)	Decrease (Based on protein expression, no rates measured)	Nagao et al. 2016[75]
Pressure overload hypertrophy (TAC in mice)	Increase	Uchihashi et al. 2017[16]
HFrEF (Humans) *the type of cardiomyopathy was not specified except that there was hypertrophy	Increase	Voros et al. 2018[70]
Pressure overload hypertrophy (TAC in mice)	Increased when normalized to CW Unchanged absolute rates	Ho et al. 2019[8]
Hypertrophy (Cardiac pacing in dogs)	Increase	Horton et al. 2019[72]

Type of Heart Failure	Cardiac Ketone Body Oxidation Rates	Reference
Ischemic cardiomyopathy (Balloon occlusion of LAD in pigs)	Increase in total ketone body myocardial uptake (this doesn't necessarily mean there is an increase in ketone oxidation rates since a decrease in ketone oxidation rates could result in increased accumulation of ketones in the myocardium)	Santos-Gallego et al. 2019[76]
Ischemic cardiomyopathy (LAD ligation in mice)	Decrease	Karwi et al. 2019[77]
Ischemic cardiomyopathy (LAD ligation in mice)	Decrease	Ho et al. (Unpublished)

1.5 Ketone supplementation in the setting of heart failure

As mentioned in the previous section (Section 1.4.3), part of my thesis helps improve our understanding of the effects of an acute increase in ketones in the healthy heart. While this is discussed in detail in Chapter 4; briefly, ketones can be an extra source of fuel and increase overall cardiac ATP production in a healthy heart. For an energy-starved failing heart with depleted ATP levels[47,78], the possibility of increasing total ATP production by delivering ketones to the heart became an enticing therapeutic venue to investigate.

There are several methods that can be utilized to increase ketone delivery to the heart but can generally be thought of as endogenous and exogenous approaches. Endogenously, ketones are produced in the liver due to a low insulin or fasted state. Through consumption of a ketogenic diet, intermittent fasting, or sodium glucose co-transporter 2 (SGLT2) inhibitors, one can manipulate the body to promote ketogenesis secondary to the decrease in circulating insulin levels. Exogenously, ketone concentration can also be increased in our blood through the consumption of 'lab-made' ketones, namely, ketone salts or ketone esters. In the following subsections, each strategy to increase blood ketone levels (ketosis) is discussed.

1.5.1 Endogenous ketones

1.5.1.1 Ketogenic diet

A portion of this section has been published online in an article I wrote for 'Medscape.com' in collaboration with the 'American College of Cardiology & Medscape' which can be accessed via https://www.medscape.com/viewarticle/943443.

The first method that will be discussed is the most pertinent to my thesis, the ketogenic diet. Heralded as a weight loss strategy, the ketogenic diet has become a 'fad diet' in our society despite the uncertainty surrounding its cardiovascular risk factors. The "keto" diet, short for the ketogenic diet is an eating style in which carbohydrate intake is less than 30-50 grams (5-10% total daily calories) per day while 70-80% of total caloric intake comes from fats and 10-20% from proteins. Reducing carbohydrate intake while increasing fat intake results in a metabolic shift in the body termed 'ketosis'. In this state, blood ketone levels are increased due to the restriction of carbohydrates and a subsequent increase in adipose tissue mobilization to the liver for hepatic ketogenesis. Having been used for almost a century for a range of medical conditions, the ketogenic diet started as an alternative treatment for pediatric epilepsy in the 1920's[79]. While the implementation of a ketogenic diet for epileptic seizures has waned due to the development of anticonvulsant medications, the diet is still pervasive in our society as a therapeutic strategy for: neurodegenerative diseases, diabetes, non-alcoholic fatty liver disease, cancer and most prominently, weight loss[80-89]. Consumption of this high-fat diet has been shown to increase satiety leading to subsequent decreased appetite and calorie consumption[90,91]. However, whether the ketogenic diet causes isocaloric weight-loss is controversial[92]. Furthermore, whether the health benefits extend to individuals with cardiovascular disease remains ambiguous due to the high-fat content in the ketogenic diet and potential increase in cardiovascular risk factors.

In the setting of heart failure, the ketogenic diet has only been recently assessed by a few groups, including our lab. First, in mice with pressure-overload-induced heart failure treated with a high-fat, low-carbohydrate diet for 4-weeks, myocardial ketone levels were increased while the protein expression of key ketone metabolic enzymes were

downregulated[93]. Functionally speaking, the development of cardiac hypertrophy was suppressed in the mice treated with the ketogenic diet due to activation of 'glycogen synthase kinase 3 beta'(GSK-3β)[93]. In another study, genetically modified mice lacking the mitochondrial pyruvate carrier (MPC) developed cardiac hypertrophy and dysfunction. But when treated with a ketogenic diet, either before or after the development of compensated left ventricular hypertrophy, the ketogenic diet prevented or regressed the cardiac dysfunction[94]. Similarly, in cardiac-specific MPC knock-out mice, cardiac dilatation and contractile dysfunction was reversed with the ketogenic diet [95]. In this study, the authors indirectly assessed myocardial ketone metabolic rates and found that the ketogenic diet downregulated cardiac ketone body oxidation[95].

From these studies, the ketogenic diet appears to confer cardioprotective effects in the setting of pressure-overload hypertrophic heart failure. However, none of their studies directly assessed myocardial ketone oxidation rates or investigated ischemic heart failure. In my thesis, I will determine whether the ketogenic diet is beneficial in the setting of ischemic heart failure (Chapter 5).

1.5.1.2 Intermittent fasting

Intermittent fasting is a pattern of eating whereby you eat within a set time period each day, regardless of the amount of calories or source of calories that are being consumed. Variations of intermittent fasting include alternate-day fasting (ex. eat one day, fast next day, repeat) and time-restricted eating (ex. eat only within the time window of 12:00pm and 6:00pm).

Intermittent fasting's effects on the overall metabolic health of an individual leans towards improvements in insulin sensitivity. In adults aged 21-70 not taking any statins or anti-diabetic medications but had modestly elevated low-density lipoprotein (LDL), implementation of a low-frequency intermittent fasting regimen (water-only for 24hrs twice weekly for 4 weeks) resulted in no changes to LDL levels[96]. However, homeostatic model assessment of insulin resistance (HOMA-IR), or a value that approximates insulin resistance (increase in HOMA-IR indicates increase in insulin resistance), was significantly decreased in individuals who performed intermittent fasting versus control individuals that ate *ad libitum*[96]. Improvements in insulin sensitivity are also observed in prediabetic men that practiced early time-restricted feeding in a 5-week randomized, crossover, isocaloric and eucaloric controlled feeding trial[97]. By restricting all meals between 8:00am and 2:00pm, individuals would experience an extended fast of at least 14 hours. In addition to reducing the desire to eat in the evening, this form of intermittent fasting led to reductions in postprandial insulin, blood pressure and oxidative stress, all contributing to improvements in beta-cell function as well as insulin sensitivity[97].

The effects of intermittent fasting on the heart and more specifically, heart failure, remains controversial and unclear[98]. In Wistar rats subjected to an experimental myocardial infarction (MI), rats were intermittently fasted (alternate-day feeding pattern) either before or after induction of MI. Rats that were intermittently fasted presented decreased cardiomyocyte hypertrophy and left ventricular dilatation, with potentiated benefits when the intermittent fasting was initiated pre-MI[99]. The cardiovascular benefits of intermittent fasting can be attributed to improvements in insulin signaling, insulin resistance, low-density lipoprotein cholesterol levels, blood pressure, resting heart rate and triacylglycerol levels[100]. Importantly,

cardioprotection mediated by intermittent fasting may also be mediated by the increased delivery of ketone bodies to the heart although the metabolic switch that occurs in the setting of failing hearts from animals subjected to intermittent fasting remain unknown[101]. A recent study has found that intermittent fasting can activate AMPK in pulmonary hypertensive rats, resulting in restructuring of the mitochondria and improvements in right ventricular function[102]. Altogether, there is growing evidence to suggest that intermittent fasting may be beneficial in the setting of heart failure.

1.5.1.3 SGLT2 inhibitors

Sodium glucose co-transporter 2 (SGLT2) inhibitors garnered attention following the ground-breaking EMPA-REG-OUTCOME trial published in the New England Journal of Medicine in 2015[3]. The discovery that SGLT2 inhibitors significantly reduced deaths from cardiovascular causes, non-fatal myocardial infarctions or non-fatal strokes in type 2 diabetic patients with high cardiovascular risk was extremely exciting. Since then, numerous of trials have been conducted to suggest that other drugs in the SGLT2 inhibitor family also improve cardiovascular outcomes in type 2 diabetic patients[103].

The way in which SGLT2 inhibitors may confer their cardiovascular benefits is complex and multifaceted[104]. Relevant to my thesis, SGLT2 inhibitors' cardioprotection may be due to the secondary ketogenic effect of inhibiting blood glucose uptake in the proximal tubule. In this pseudo-fasted state, blood ketone levels rise and the amount of ketones being delivered to the heart increases. In fact, work from our lab has shown that diabetic mice treated with empagliflozin have improved cardiac ATP production due to an increased reliance on glucose oxidation for energy as well as restoration of ketone body oxidation[105].

1.5.2 Exogenous ketones

1.5.2.1 Ketone salts

Ketone salts are ketones (i.e. beta-hydroxybutyrate) conjugated to a mineral (ex. sodium, potassium or magnesium). They usually come in a powder form that can be mixed with a flavour so that they may be dissolved into solution for drinking. Alternatively, they can be injected or infused.

In the setting of heart failure, ketone salts were infused into heart failure patients (HFrEF) via a pulmonary artery catheter. The authors found that there was a dose-response relationship with cardiac output and the concentration of sodium-beta-hydroxybutyrate being infused. This increase in cardiac output was due to an increase in stroke volume. Thus, infusion of this ketone salt resulted in improvements in cardiac systolic function in HFrEF patients.[73]

1.5.2.2 Ketone esters

Ketone esters were developed by Dr. Richard Veech and his colleague, Dr. Kieran Clarke, at the University of Oxford. Ketone esters are conjugated to an alcohol and are available in the form of a liquid to ingest.

In the setting of heart failure, ketone esters have been shown to prevent and treat heart failure in both mice and rats with TAC/MI-induced heart failure[106]. A custom diet with ketone esters was initiated 1-week pre-surgery and continued for 4-weeks post-surgery. The authors reported significant increases in plasma beta-hydroxybutyrate levels which were accompanied by increases in the protein expression of the rate-limiting-enzyme of ketone body oxidation, SCOT, in left ventricular tissue. Together, the increase in the presumed ketone body oxidation rates in the heart resulted in decreased left ventricular dysfunction and remodeling[106].

1.6 Hypothesis and specific aims

1.6.1 Hypothesis

Increasing myocardial ketone body oxidation (acutely and chronically) will provide an extra source of energy for the starving failing heart, which will be protective and mitigate further pathophysiological remodeling of the failing heart. This is illustrated in Figure 1.5.

1.6.2 Specific Aims

Aim 1: Assess the metabolic profile of the failing heart in response to an acute increase in ketones

My first objective will be to assess ex vivo isolated working heart function from heart failure mice, and to assess the energy metabolic profile of the failing heart in response to an acute increase in ketone body concentrations. To do this, C57BL/6J male mice (12-wk-old) will undergo either sham surgery, or transverse aortic constriction surgery to induce pressure-overload hypertrophy over 4-wks. Isolated working hearts from these mice will then be perfused with radiolabeled glucose (5 mM), palmitate (0.8 mM), and ß-hydroxybutyrate (0 mM or 0.6 mM) to assess carbohydrate metabolism, fatty acid oxidation and ketone body oxidation rates, respectively. The pulmonary artery will also be cannulated during these perfusions, allowing for quantification of myocardial oxygen consumption rates and cardiac efficiency. Hearts will be collected for protein profiling via immunoblotting to assess for changes in protein expression of metabolic-related enzymes.

Aim 1 is addressed in Chapter 3 of my thesis.

Aim 2: Assess the metabolic profile of the healthy heart in response to acute increases in ketone concentrations

My second objective will be to assess ex vivo isolated working heart function from healthy mice, and to assess the energy metabolic profile in response to an acute increase in ketone body concentrations. Isolated working hearts from C57BL/6J male mice (12-wk-old) will be perfused with radiolabeled glucose (5 mM), palmitate (0.8 or 1.2 mM), and ß-hydroxybutyrate (0 mM, 0.6 mM or 2.0 mM) to assess carbohydrate metabolism, fatty acid oxidation and ketone body oxidation rates, respectively. The pulmonary artery will also be cannulated during these perfusions, allowing for quantification of myocardial oxygen consumption rates and cardiac efficiency.

Aim 2 is addressed in Chapter 4 of my thesis.

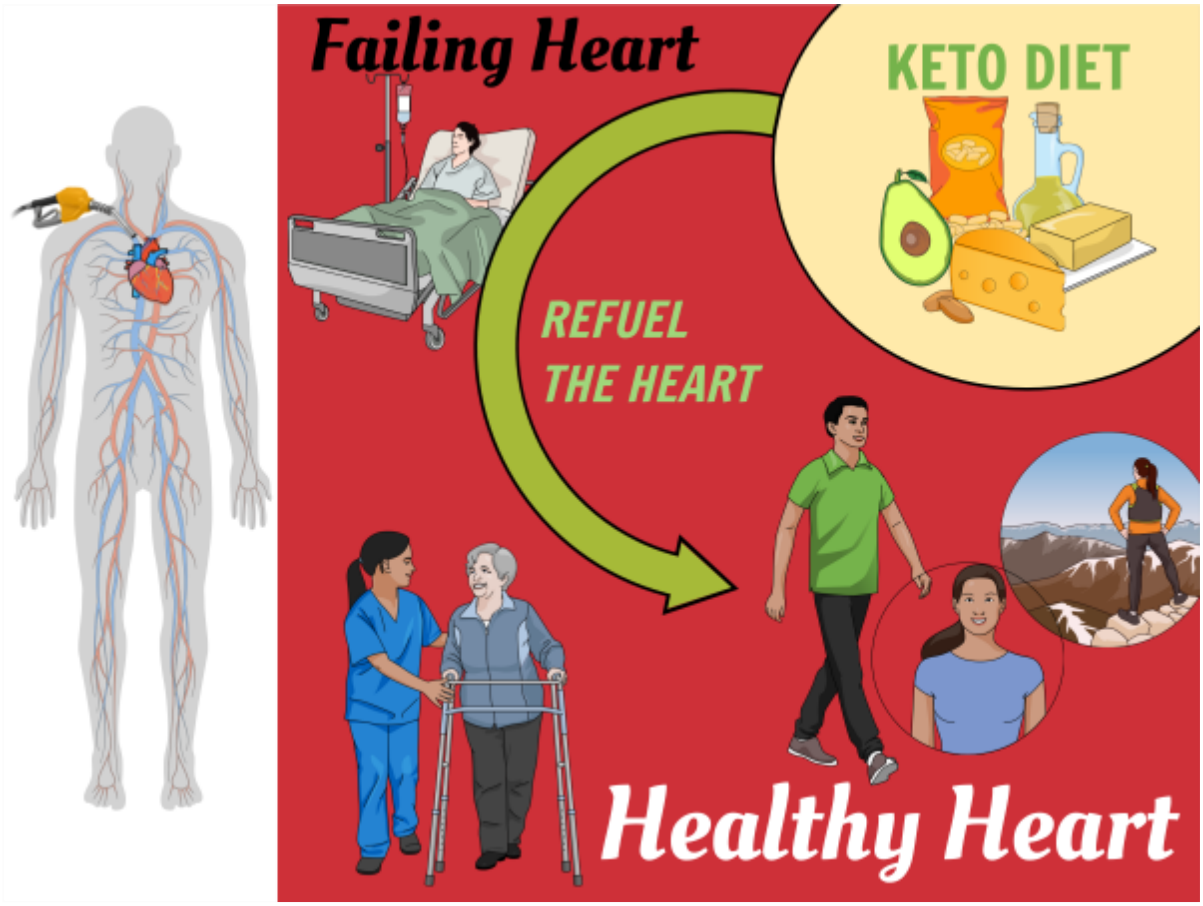
Aim 3: Assess the cardiac function and metabolic profile of the failing heart in response to a chronic increase in ketones

My third objective will be to assess in vivo cardiac function and the energy metabolic profile of the failing heart in response to a chronic increase in ketones. Male mice will be subjected to experimental heart failure (via left anterior descending coronary artery ligation) over a 4-wk period after which mice will be randomized to receive either a control diet (80% carbohydrate, 10% fat, 10% protein) or a ketogenic diet (5% carbohydrate, 85% fat, 10% protein) for 3-wks. In vivo bi-weekly assessment of cardiac function will be carried out via serial ultrasound echocardiography to determine whether chronically increasing in vivo circulating ketone levels, via the ketogenic diet, can attenuate the progression of experimental heart failure. After 3-wks of the ketogenic diet, mice will be euthanized and their hearts

subjected to an isolated working heart perfusion for cardiac function and energy metabolic measurements. Hearts will be collected for protein profiling via immunoblotting to assess for changes in protein expression of metabolic-related enzymes.

Aim 3 is addressed in Chapter 5 of my thesis.

Figure 1.5 An illustration of my hypothesis.



1.6.3 Key Goals of my Thesis

(1) characterize the energy metabolic profile of the failing heart and determine whether ketones acutely improve cardiac function and efficiency in the failing heart

(2) determine whether ketones can acutely improve cardiac function, ATP production and efficiency in the healthy heart

(3) determine whether ketones chronically, via a ketogenic diet, improve cardiac function and efficiency in the failing heart

1.6.4 Significance of my Thesis

Findings from my thesis will help direct novel therapeutic approaches to treat heart failure including whether the ketogenic diet should be used in heart failure patients.

Chapter II
Materials and Methods

Part of this section is published in the online supplement of a paper in Cardiovascular Research (Ho, K. L., et al. (2019). "Increased ketone body oxidation provides additional energy for the failing heart without improving cardiac efficiency." 115(11): 1606-1616.)

Chapter 2 Materials and Methods

2.1 Introduction

My thesis focuses largely on ketone body metabolism in healthy and failing murine hearts. As such, experiments with whole animal studies were carried out involving experiments that assessed blood glucose levels, blood ketone levels, *in vivo* cardiac function via transthoracic echocardiography, and cardiac energy metabolism via *ex vivo* isolated working heart perfusions. Furthermore, *in vitro* biochemical techniques were carried out to assess cardiac protein expression of various signaling molecules in the metabolic pathways are affected and how they correspond with the metabolic data. Immunoprecipitation was also employed to assess protein-specific lysine acetylation levels. Altogether, my thesis employs numerous state-of-the-art experimental techniques at the whole animal and tissue levels to understand how cardiac ketone body oxidation is affected in heart failure and whether this can be therapeutically manipulated to improve the outcomes of heart failure.

2.2 Materials

Any chemicals used for buffers preparations not listed below were obtained from Sigma-Aldrich (St. Louis, Missouri).

2.2.1 Animal Experiment Related Materials

- Both the control diet and the ketogenic diet were custom-made and purchased from Research Diets Inc. (New Brunswick, New Jersey). See Table 2.1 for diet composition.
- 12-week old C57Blk/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine).

- Sodium pentobarbital (euthanyl) was purchased from Bimeda-MTC Animal Health Inc. (Cambridge, Ontario, CA).

Table 2.1. Diet Composition of the Control Diet and Ketogenic Diet formulated by Research Diets, Inc.

Macronutrient	Control Diet (D18072403)		Ketogenic Diet (D18072401)	
	gm%	kcal%	gm%	kcal%
Protein	10	10	16	10
Carbohydrate	77	80	8	5
Fat	4	10	61	85
Total		100		100
kcal/gm	3.8		6.4	
Ingredient	**gm**	**kcal**	**gm**	**kcal**
Casein	100	400	100	400
L-Cystine	1.5	6	1.5	6
Corn Starch	500	2000	0	0
Maltodextrin 10	150	600	0	0
Sucrose	49.9	200	49.9	200
Dextrose	111.95	448	0	0
Cellulose, BW200	50	0	50	0
Soybean oil	25	225	25	225
Cocoa butter	20.2	182	358.8	3229
Mineral mix, S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H_2O	16.5	0	16.5	0
Vitamin Mix, V1000C	1	4	1	4
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0	0	0.05	0
FD&C Red Dye #40	0.025	0	0	0
FD&C Blue Dye #1	0.025	0	0	0
Total	1056.6	4064	633.25	4064

2.2.2 Echocardiograph Related Materials

- Vevo 3100 with an MX550S was obtained from VisualSonics (Toronto, Ontario).
- Oxygen tank (University of Alberta)
- Q-tips (Drugstore)
- Gauze (Amazon)
- Isoflurane (University of Alberta)
- Indus electrode crème (#600-001-01S)
- Sigma gel high conductive multipurpose
- Surgical tape
- Nair with soothing Aloe and Lanolin (Drugstore)
- Systane Night-time relief eye lubricant (Drugstore)
- Aquasonic ultrasound gel (Amazon)

2.2.3 Perfusion Related Materials

- Radiolabeled glucose [U-^{14}C] and [5-^{3}H] were both purchased from Perkin Elmer (Waltham, Massachusetts)
- Radiolabeled fatty acid [9,10-^{3}H] palmitate was purchased from Perkin Elmer (Waltham, Massachusetts)
- Radiolabeled ketone body [3-^{14}C] hydroxybutyrate was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, Missouri)
- Bovine serum albumin (BSA Fraction V, fatty acid free) was purchased from Equitech-Bio Inc. (Kerrville, Texas)

- Human insulin (Novolin™ ge Toronto) was obtained from Novo Nordsik (Mississauga, Ontario)
- Scintillation counting fluid was obtained from MP Biomedicals (Solon, Ohio)
- Hyamine hydroxide was obtained from Curtis Laboratories (Bensalem, Pennsylvania)
- Ecolite™ and Cytoscint™ Aqueous Counting Scintillation fluids were purchased from MP Biomedicals (Solon, Ohio, USA).

2.2.4 Western Blot Related Materials

- Western Lightning® Chemiluminescence Reagents Plus were obtained from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario)
- Nitrocellulose Trans-Blot® Transfer Medium, protein assay dye and Precision Plus Protein™ Dual Color MW marker was purchased from Bio-Rad Laboratories (Hercules, California)
- Medical X-ray FUJI films were obtained from Mandel Scientific (Guelph, Ontario)
- Monoclonal and polyclonal primary antibodies were obtained from:
    - Cell Signaling Technology (Danvers, Massachusetts).
        - Akt (9271)
        - P-Akt (9272)
        - T-PDH (3205S)
        - NLRP3 (15101S)
        - VDAC (4661S)
    - Abcam (Cambridge, Massachusetts)
        - LCAD (ab129711)
        - β-HAD (ab93172)

- Proteintech (Rosemont, Illinois)
  - OXCT1 (12175-1-AP)
- Novus-Biologicals (Centennial, Colorado)
  - BDH1 (NBP1-88673)
- Millipore (Burlington, Massachusetts)
  - P-PDH (ABS204)
- ThermoFisher Scientific (Waltham, Massachusetts)
  - IL-6 (M620)

- Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California)
- Protease inhibitor cocktail was obtained from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA).
- Phosphatase inhibitor cocktails 2 and 3 were obtained from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)

2.2.5 Immunoprecipitation Related Materials

- Protein A/G-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, California)
- Acetyl-lysine antibody was purchased from Millipore (Burlington, Massachusetts)

Plasma Experiment Related Materials

2.2.6 Other Materials

- Free fatty acid (FFA) assay kit was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan)

- Total ketone bodies assay kit was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan)
- HPLC analysis of:
    - Adenine nucleotides utilized a Supelcosil™ LC-18-T Super Guard and Supelcosil™ LC-18-T columns obtained from Sigma-Aldrich (St. Louis, Missouri)
    - Short chain CoA esters utilized a Nova-Pak Supelcosil™ obtained from Waters Company (Milford, Massachusetts) and a Adsorbosphere® C18 obtained from Altech Associates/Mandel Scientific
    - Long chain CoA esters utilized a C18 guard cartridge, C18(2) 100 A column obtained from Phenomenex (Torrance, California)

2.3 Methods

2.3.1 Ethics Approval

All protocols involving our mice were approved by the University of Alberta Institutional Animal Care and Use Committee. All mice received treatment and care abiding by the guidelines set out by the Canadian Council on Animal Care and all animal experiments conformed to the guidelines from the Directive 2010/63/EU of the European Parliament.

2.3.2 Murine model of transverse aortic constriction

At 12 weeks of age, mice were subjected to a transverse aortic constriction (TAC) surgery to induce pressure overload hypertrophy. To carry out this surgery, mice were first anesthetized with an intraperitoneal injection of ketamine/xylozine (100mg/kg / 10mg/kg) to allow for intubation with a 14 gauge polyethylene catheter ventilated with air. Once stage III

(surgical anesthesia) was achieved, the hair was shaved off of the mouse's left parasternal area and a midline sternotomy is performed to expose the aortic arch. A 27-gauge cannula needle is then placed alongside the aorta and using a 6-0 silk suture, the suture is tied to encircle the needle and the aorta between the innominate and left common carotid arteries. The needle is then removed and the surgical site is closed using 6-0 Prolene sutures. Following surgery, mice received doses of analgesia (Metacam, 1 mg/kg subcutaneous injection) and antibiotics (penicillin, 10 mg/kg intramuscular injection). Mice are kept on a warm heating pad until consciousness is regained and then transferred to a clean cage where they are closely monitored for the next 72 hours. Metacam is administered once every 12 hours for the first 72 hours post surgery. Mice are then allocated 1 week to fully recover before being subjected to further experiments.

2.3.3 Murine model of left anterior descending coronary artery ligation

At 12 weeks of age, mice were subjected to a left anterior descending coronary artery (LAD) surgery to permanently ligate the LAD and induce a myocardial infarction (MI). To carry out this surgery, mice were first anesthetized with an intraperitoneal injection of ketamine/xylozine (100mg/kg / 10mg/kg) to allow for intubation with a 14 gauge polyethylene catheter ventilated with air. Once stage III (surgical anesthesia) was achieved, the hair was shaved off of the mouse's chest and a left thoracotomy is performed in the fifth intercostal space to expose the left ventricle. Next, the LAD was encircled with a 7-0 silk suture after which the muscle and skin layers were closed up using a 6-0 suture. Sham mice underwent a similar procedure but the LAD was not ligated following the thoracotomy. Following surgery, mice received doses of analgesia (Metacam, 1 mg/kg subcutaneous injection) and antibiotics (penicillin, 10 mg/kg intramuscular injection). Mice are kept on a warm heating pad until

consciousness is regained and then transferred to a clean cage where they are closely monitored for the next 72 hours. Metacam is administered once every 12 hours for the first 72 hours post surgery. Mice are then allocated 1 week to fully recover before being subjected to further experiments.

2.3.4 Ketogenic diet feeding protocol

Mice were randomized to receive either a control diet (80% carbohydrates, 10% fat, 10% protein) or a ketogenic diet (5% carbohydrates, 85% fat, 10% protein) for 3 weeks. The details of the control and ketogenic diets can be found in Table 2.1.

2.3.5 Blood tests

Blood glucose and ketone measurements were performed via a tail bleed using either an Accu Check system (Roche®) to assess blood glucose levels or a FreeStyle Precision Neo system (Abbott®) to assess blood ketone levels.

2.3.6 Transthoracic echocardiography

Transthoracic echocardiography was carried out to assess *in vivo* heart function pre-surgery (0 weeks), pre-diet (1 weeks) as well as post-diet (5 weeks).

2.3.6.1 Imaging System

In vivo cardiac function was assessed using a Vevo 3100 high-resolution echocardiography. The Vevo imaging system was equipped with a 30-MHz transducer (MX550S, VisualSonics, Toronto, Ontario, Canada). The device settings are as follows: Device: Vevo 3100 MX550S, Application: Mouse (Small) Cardiology, Preset: PSLAX, Transmit: Frequency – 40MHz, Power – 100%, Acquisition: Frame Rate – 171fps, Gain –

31dB, Depth – 15.00mm, Width – 14.08mm, Display: Dynamic Range – 60dB, Display Map – C5, Vevo HD – On.

2.3.6.2 Anesthesia

Prior to anesthetizing mouse, their weight was taken and a new study file was set up on the imaging system with the above settings. The oxygen tank is then turned on and the mouse is added to the chamber. The flow of oxygen is 1L/minute and all valves are adjusted to direct the oxygen to the induction chamber for 1 minute before isoflurane is added to the system. Isoflurane is turned on using the knob at the top of the tank and slowly increased at 0.5% increments (every 30 seconds) until the animal falls unconscious around 2% isoflurane. The isoflurane is then turned off and the chamber is flushed with oxygen for 2-3 seconds before the lid is opened and unconscious mouse is removed.

2.3.6.3 Set-Up of Mouse on Examination Platform

The mouse is then quickly transferred to the pre-warmed examination table. The mouse is positioned properly so that it's nose is carefully placed in the tube delivering oxygen. Isoflurane is then turned back on to 1% to maintain anesthesia and adjusted appropriately if the mouse is visibly waking up. Electrode cream is then placed on the conductive points of the examination table and the limbs are gently taped down to the platform. A rectal probe is then inserted to monitor temperature and eye lubricant is carefully applied to the mouse's eyes to prevent them from drying out during the procedure. Next, the mouse's chest is shaved using Nair and a warm gauze cloth. A warm pre-cut blanket is placed on the lower half of the mouse to keep them warm during the procedure.

2.3.6.4 Transthoracic Echocardiography

Electrode gel is then applied to the shaved chest and any bubbles in the gel are removed to ensure there are no white streaks in the ultrasound images. The transducer is then positioned on the chest and the heart is imaged. Heart rate and respiratory rate are monitored throughout the entire procedure and heart rate is ideally maintained above 400 beats per minute while respiratory rate is maintained around 100 beats per minute. Extremities (paws) are constantly checked to make sure they do not pale or turn blue. Each mouse takes about 15-20 minutes maximum to complete from start of anesthesia to waking up.

2.3.6.5 Ultrasound Imaging

The exact details on how to perform the ultrasound echocardiography are as follows:

2.3.6.5.1 Aortic Arch View

The probe is first positioned in the aortic arch view (high right parasternal view - point towards chin of animal and adjust clockwise if necessary; take the highest frequency) and a B-mode, EKV, colour, PW doppler (ascending and descending aorta) are measured.

2.3.6.5.2 Pulmonary Artery Measurement

The probe is positioned slightly up from the parasternal long-axis (PSLAX) view to visualize the pulmonary artery (the aorta should be tucked in from behind). A B-mode, EKV, colour, PW doppler (at the intersection with the aorta tucked in beneath the pulmonary artery; a blue streak should be observed indicating that blood is moving away from the transducer) are measured.

2.3.6.5.3 Pulmonary Vein Measurement

The probe is next slightly angled towards the atria to visualize the pulmonary vein (this is usually seen beside the pulmonary artery) and a red streak (indicating that blood is moving towards the transducer) should be observed. A B-mode, EKV, colour, PW doppler (at the highest velocity of 300-360mm/s) are measured.

2.3.6.5.4 Parasternal Long Axis (PSLAX) View

Next, the probe is positioned in the parasternal long-axis position such that the apex of the heart, the aorta, and its ventricular walls are clearly defined. A B-mode, EKV, M-mode of the atria, M-mode of the aortic valve and M-mode of the left ventricle (septum at the top and posterior wall at the bottom) are taken. The probe is also slightly adjusted to get a clear outline of the aorta such that the left ventricular outflow tract can be measured.

2.3.6.5.5 Short Axis (SAX) View

The Simpson method was utilized in my study due to the fact that the ejection fraction of a heart that has undergone LAD ligation will be different depending on where you take the measurement (all the cardiac muscle lower than the position at which the coronary artery was ligated will have very poor contractile function while the cardiac muscle above the point of ligation could present normal contractile function). As such, the Simpson method, which accounts for the entire volume of the left ventricle during systole and diastole, is a much better method to determine ejection fraction.

To take images of the ‘elliptical disks’, we start by finding the aortic root with the triangular valves and take a B-mode and colour image. After, the knob controlling the y-axis

of the transducer is the only thing adjusted while the x-axis is locked in position. The papillary muscles should be visible at the animal-relative cardinal positions of east and south. This is known as level 1 and clean EKV must be taken for the measurement to be made. Subsequent measurements are carried out at level 2 after adjusting the y-axis knob such that the papillary muscles are visible at the animal-relative cardinal positions of north-east and south-east. At level 2, another EKV is taken and an M-mode is measured at the level of the papillary muscles. The papillary muscles should be visible as faint 'mushroom caps' in the M-mode images. Lastly, level 3 is located when the y-axis knob is adjusted to the point where the papillary muscles are no longer visible. At level 3, another clean EKV is taken.

2.3.6.5.6 Apical Four Chamber (A4C) View

The position of the platform and mouse are then adjusted such that the head is tilted by about 20 degrees towards the ground and the transducer is placed to 'look up' towards the 4 chambers of the heart. All four chambers of the heart should be visible and if colour is turned on, blood flow from the atria to the ventricles should be observed. A B-mode, EKV, colour, PW doppler (to assess mitral valve flow and tricuspid valve flow), and tissue doppler (to assess mitral valve tissue movement). Since tissue doppler here can be tricky to measure due to the exact position required to get a good measurement, a good tip used is to ensure the e' and a' wave straddle the p-wave (atrial depolarization) in the electrocardiogram.

2.3.7 Isolated working heart perfusions

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (60mg/kg) and once mice are unresponsive during a response test (via pinching their toes and

paws), hearts were excised and subjected to an isolated working heart perfusion. The details of the isolated working heart perfusion are described in the following subsections.

2.3.7.1 Hanging the Heart

As soon as the chest cavity is cut open and the heart is 'popped' out of the chest cavity, it is gently removed and immediately placed in ice-cold Krebs-Henseleit bicarbonate solution (118mM NaCl, 25mM $NaHCO_3$, 5.9mM KCl, 5mM EDTA pH=7.4, 1.2mM $MgSO_4 \cdot 7H_2O$, 2.5mM $CaCl_2 \cdot 2H_2O$, 5mM glucose; gassed with 95% oxygen and 5% carbon dioxide). The heart is then quickly transported to the perfusion rig and the aorta is cannulated using an 18G plastic cannula and perfused in a Langendorff retrograde fashion with Krebs-Henseleit solution that has been warmed to 37ºC. At this point, the hydrostatic pressure is set to 60mmHg and during this period, extraneous tissue is trimmed off of the heart (ex. fat, lung, trachea).

2.3.7.2 Switching from Langendorff to Working Mode

Once the heart has equilibrated and coronary perfusion has been established, the left atrium is cannulated via the pulmonary vein with a 16G steel cannula connected to a preload reservoir (left atrium preload reservoir). The preload reservoir, connected to the cannula via a flexible water-jacketed tube, is heated as close as possible to 37ºC.

Right before switching to the working mode, the aortic inflow line from the Langendorff reservoir was clamped off and the left atrial inflow line from the left atrium preload reservoir is opened.

Contraction of the left ventricle naturally pumps the preload reservoir buffer through the aorta where it can then travel to the afterload line. A compliance chamber is also utilized to simulate arterial compliance and this is located upstream of the aortic cannula. The height

of the preload and afterload columns are set so that the preload is equivalent to 10mmHg and afterload is 50mmHg. The perfusate is constantly being recirculated and its pH is maintained at 7.4 by gassing the perfusate with a glass oxygenator containing 95% oxygen and 5% carbon dioxide.

2.3.7.3 Mechanical Function Measurements

A Gould P21 pressure transducer from Harvard Apparatus is connected to the aortic outflow line to measure heart rate and aortic pressure. A signal is then transmitted from the pressure transducer to a computer that has specialized software to record the data. Left ventricular developed pressure can then be calculated by taking the aortic pressure (systolic pressure) and subtracting the preload pressure (set at 10mmHg).

In order to measure flow through the aorta and determine cardiac output (mL/min), ultrasonic flow probes (Transonic T206) are positioned in the afterload (aorta flow) and preload (cardiac output) lines. By taking the difference between cardiac output and aortic flow, coronary flow can be calculated in mL/min. Cardiac work is then calculated by multiplying cardiac output and the left ventricular developed pressure, and finally normalizing this for the heart's dry weight.

2.3.7.4 Measurement of Oxygen Consumption

Myocardial O_2 consumption was measured using in-line O_2 probes in the left atrial inflow line and cannulated pulmonary artery.

2.3.7.5 Isolated Working Heart Perfusion

Now in the working mode, the isolated working heart is anterogradely perfused, via the left atrial inflow line, with a Krebs-Henseleit solution consisting of 2.5 mM Ca^{2+}, 5 mM glucose, 0.8 mM palmitate (pre-bound to 3% albumin) or 1.2mM palmitate, and 0, 200 or 600 μM ß-hydroxybuturate (β-OHB). The specific concentration of substrates are detailed for each project in their corresponding chapter. If insulin was added into the perfusate, insulin (100 μU/ml) was added to the perfusate via the left atrial inflow line at 30 minutes.

2.3.7.6 Measurement of Metabolic Oxidative Rates

Palmitate and β-hydroxybutyrate oxidation rates were measured simultaneously by collecting $^{3}H_2O$ and $^{14}CO_2$ produced from the oxidation of [9,10-^{3}H] palmitate, and [3-^{14}C] hydroxybutyrate, respectively.

Glycolysis and glucose and oxidation rates were measured by simultaneously measured in a parallel series of hearts by collecting $^{3}H_2O$ and $^{14}CO_2$ produced by [5-^{3}H] glucose, and [U-^{14}C]glucose, respectively.

In order to directly assess the palmitate oxidation and glycolytic rates, $^{3}H_2O$ needs to be separated from the [9,10-^{3}H] palmitate and [5-^{3}H] glucose. To separate them, a vapor transfer method is employed using a lidless 1.5mL microcentrifuge tube placed inside of a 5mL scintillation vial filled with 500μL of water. During the perfusion, 200μL of perfusate is collected from the system at 10 minute intervals throughout the entirety of the 60 minute perfusion. The collected perfusate is then transferred to the lidless 1.5mL microcentrifuge tube and the scintillation vial is capped. Next, the scintillation vials containing the sampled perfusates are incubated at 50ºC for 24 hours and subsequently at 4ºC for 24 hours. After 48

hours of incubation at the specified temperature has elapsed, the microcentrifuge tubes are removed and scintillation fluid (Ecolite) is added to all of the vials. The amount of ^{3}H can then be assessed with a liquid scintillation counter.

In order to directly assess the β-hydroxybutyrate and glucose oxidation rates, $^{14}CO_2$ production needs to be quantified. $^{14}CO_2$ is released as a gas in the oxygenation chamber as well as found as $H^{14}CO_3^-$ in the perfusate itself. To quantify the $^{14}CO_2$ in the oxygenation chamber, gaseous $^{14}CO_2$ is allowed to leave the perfusion system via an exhaust line where it can be subsequently trapped in a hyamine hydroxide solution. Timed samples from the hyamine hydroxide solution can then be taken and counted in the liquid scintillation counter to measure ^{14}C. $H^{14}CO_3^-$ in the perfusate itself is quantified by taking perfusate samples at timed intervals and injecting them into vials under mineral oil. The $^{14}CO_2$ can then be released from the perfusate by acidification using 1mL of H_2SO_4. The acidification step is carried out in a sealed metabolic flask with a hyamine hydroxide saturated filter paper present to capture all of the $^{14}CO_2$.

2.3.7.7 Calculation of ATP Production and Tricarboxylic Acid Cycle Activity

ATP production rates were calculated based on 31 ATP produced from each glucose oxidized, 2 ATP from each glucose passing through glycolysis, 104 moles of ATP produced from each molecule of palmitate oxidized, and 21.25 ATP from each molecule of β-OHB oxidized. This assumes that there is 100% coupling of mitochondrial oxidative phosphorylation. Tricarboxylic acid (TCA) rates were calculated based on 2 acetyl CoA produced from each glucose oxidized, 8 acetyl CoA produced from each molecule of palmitate oxidized, and 2 acetyl CoA from each molecule of β-OHB oxidized.

2.3.7.8 End of Perfusion Procedure

At the end of the 60-minute perfusion period, hearts were snap-frozen in liquid nitrogen and subsequently stored at -80 degrees Celsius.

2.3.8 Western blotting

Approximately 25mg of frozen myocardial tissue was homogenized in a buffer containing 50mM Tris-Hcl (pH 7.5), 0.15M NaCl, 5mM EDTA, 0.5% NP40 (IGEPAL), 1% Tx-100, 10mM sodium butyrate, 5mM nicotinamide,1µM trichostatin A (Sigma), protease and phosphatase inhibitors (Sigma). Following homogenization and extraction of the cellular contents in the lysate via centrifugation at 10,000g for 15 minutes, a Bradford protein assay was conducted (Biorad). For western blot, 30µg of protein was loaded per gel lane and samples were resolved with a 10% gel and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, an overnight wet transfer to a 0.45µM nitrocellulose membrane was carried out after which, membranes were blocked in 5% non-fat milk for 1 hour and probed overnight at 4° with: anti-acetyl-lysine (Millipore, 1/1000 dilution, abbreviated in paper as 'Ac-K'), anti-ACADL (Abcam 129711, 1/2000 dilution, abbreviated in paper as 'LCAD'), anti-OXCT1 (Proteintech 12175-1-AP, 1/1000, abbreviated in paper as 'SCOT'), anti-BDH1 (Novus Biologicals NBP1-88673, 1/1000). All proteins were normalized to anti-VDAC (Cell Signaling 4661S, 1/1000). Membranes were then washed with 1x tris-buffered saline with Tween-20, incubated in the appropriate secondary antibody and finally visualized with Western Lightning Plus-Enhanced Chemiluminescence Substrate (Perkin Elmer). Densitometric analysis was carried out with the software ImageJ.

The specific protocol followed for western blots is as follows (written by myself):

After completing a protein assay, decide how much protein you'd like to load into each well in the gel (Recommended: 30μg; the lower the volume and higher the concentration, the better the resolution of each band).

2.3.8.1 SDS-PAGE

First, prepare your 10-well (recommended) or 15-well gel. If you are not running your gel today, you can store this gel in running buffer (wrapped with presoaked paper towel and in a ziplock bag) at 4°C for until needed. Next, prepare your samples with the appropriate amount of sample buffer, sample and ddH_2O. If you are not running your gel the same day, you can store these samples at RT overnight or store them at -20°C for the future. Next, load your samples onto your gel using the special pipette loading tips. Then run your gel at 75V until you see your samples reach the separating gel and the ladder begin to resolve (~25 minutes). Turn the voltage up to 120V and run the dye off the gel or until need be. While waiting for the gel to finish running, prepare your fresh transfer buffer with methanol and chill this.

2.3.8.2 Transfer of gel to nitrocellulose membrane

Assemble the sandwich in the following order. Take care to make sure that the filter paper on either side of the gel and membrane don't touch each other to make a 'continuous circuit'. The order with which the transfer sandwich should be assembled is: black side down of the gel holder cassette, 1 black sponge, 2 filter papers, gel (cut stacking off), nitrocellulose membrane, 2 filter papers, 1 black sponge, clear side of gel holder cassette should close over the sandwich.

After the transfer sandwich is assembled, place the sandwich into the tank with the black side towards the black cathode (-) and the clear side (of the cassette) towards the red

anode (+). Next, put an ice pack into the tank. I highly recommended that you place the tank into a Styrofoam box filled with ice to keep the tank extra cold during the prolonged transfer. Furthermore, adding a stir bar into the tank also doesn't hurt and can potentially help transfer efficiency (although I've never verified this myself!). Finally, top up the tank with fresh transfer buffer and put the lid onto the tank and move this to the cold room. Transfer for your desired time. (If you are transferring for any time other than overnight, be sure to turn off the power pack and change the ice pack half way into the transfer time). Generally, I follow the following rules when transferring: 2 hours at 90V, 2.5 hours at 85V, 3 hours at 80V, overnight at 25V (recommended!), or 5 hours at 60V. Before leaving the cold room, make sure you see bubbles moving up along the side of the cathode (black) side of the sandwich frame (indicates that the actual power pack is working and isn't dead).

2.3.8.3 Post-Transfer

After the allotted transfer time has elapsed, remove the tank from the cold room. Then carefully remove the cassette(s) and peel the membrane away from the gel. To assess transfer efficiency, stain the gel with some 'Ponceau red stain and submerse your membrane for a few seconds. Do ensure that the membrane is constantly hydrated and doesn't dry out.After immersing your membrane in ponceau, pour the ponceau back into your stock, rinse the membrane with some water to get rid of background, take a picture of the membrane if you want (some people normalize to ponceau stain but I don't recommend to this; I just use ponceau to make sure my transfer worked if I have new parameters that I am implementing to my western blot protocol). Finally, rinse the membrane for ~1 minute with TBST on the shaker to remove the ponceau stain.

Next, block the membrane in 10% milk (or 5% milk/3% BSA, 3.5% BSA, 5% BSA; depending on what future proteins you plan to detect) for a minimum of 30 minutes (and maximum 1 hour) at room temperature. If blocking your membrane with milk, rinse the membrane with TBST for ~1-5 minutes to just remove residual milk and prevent contamination of your primary antibody dilution tube.

2.3.8.4 Primary and Secondary Antibody

Incubate your membrane in primary antibody overnight in the cold room with rotation. Alternatively, incubate your membrane in primary antibody for 2 hours at room temperature. Next, wash your membrane 3 x 10 minutes with TBST at room temperature. Then, incubate your membrane in secondary antibody (HRP – linked Goat anti-rabbit/mouse/chicken in 1% milk or 5% milk) for 1 hour at room temperature or overnight in the cold room with rotation. After secondary antibody incubation, wash your membrane 3 x 10 minutes with TBST at room temperature. To visualize the membrane's secondary antibodies, 'water' the membrane with ECL and put the membranes in a radiography cassette. Finally, develop in the dark room (2 minutes, 1 minutes, 30s, 10s) using X-ray film. And importantly, do not forget to label your molecular weight ladder on your films.

2.3.9 Immunoprecipitation

After homogenizing 20-25mg of frozen ventricular tissue with the previously described buffer and extracting the supernatant from cell lysate, 250µg of protein was pre-cleared with 50µL of protein A/G-agarose beads (Santacruz 2003) and subsequently incubated overnight at 4°C with 2µL of acetyl-lysine antibodies (Millipore 05-515). The next day, 50µL of A/G-agarose beads were added to pull down protein-antibody complexes with a 6-hour incubation

at 4°C. Following pull-down, the bead—antibody-protein complexes were washed 3 times with a buffer containing 150mM NaCl, 50mM Tris-Hcl, 5mM EDTA, 0.5% NP-40 (IGEPAL), 1μM trichostatin-A, 5mM nicotinamide, 5mM sodium butyrate and protease inhibitors. The bead-antibody-protein complexes were then heated at 95°C for 5 minutes in sample buffer to release the acetylated proteins. Western blot was then carried out to detect for long-chain acyl CoA dehydrogenase that was acetylated. Normalization was carried out to the antibody IgG heavy chain of the anti-acetyl-lysine antibody (Millipore 05-515) used to pull down the proteins.

The specific protocol for immunoprecipitation is as follows (written by myself):

2.3.9.1 Homogenization and Extraction of Proteins

Prepare homogenization IP buffer (Pre-chill the correct volume of IP buffer; make sure to prepare EXTRA for sample preparation). Add the following right before use:sodium butyrate, nicotinamide, trichostatin A (TSA), protease inhibitor, and phosphatase inhibitor cocktails I and II. Next, label flat-bottom (for homogenization) tubes and et liquid nitrogen and warm gloves with lab gloves over top. Retrieve samples from -80°C freezer and place (along with homogenization tubes) in liquid nitrogen with a cardboard tube holder. Weigh out between 15 and 50mg of tissue by addition (Taring scale with a cold tube). Then, homogenize tissue with proportional amount of cold complete IP buffer (<50mg = 150μL, 50mg = 200μL, >50mg = 250μL). Next, let samples sit on ice for 10 minutes then centrifuge at 10 000g for 15 minutes at 4°C (centrifuge in cold room). During this time, label coloured 1.5mL Eppendorf tubes and pre-chill on ice. Then, extract supernatant into newly labelled 1.5mL Eppendorf tubes and discard the pellet.

2.3.9.2 Protein Assay and Sample Preparation

Label 1.5mL Safelock Eppendorf tubes and pre-chill on ice. Next, conduct a Bradford protein assay using the 'Biorad' reagent. Once the concentration of your samples has been attained, calculate the volume of cell lysate + IP buffer required so that each sample has between 100-500µg of protein in 100µL. For the **positive control**, prepare a regular western blot sample and use less than 10µg (~3µg is good). You can store the positive control at -80 degrees until you are ready to heat the samples. Next, prepare samples in pre-chilled Safelock Eppendorf tubes. The **negative control** is prepared by pipetting 100µL of IP buffer to a single tube. If pre-clearing is not carried out on the same day, store diluted samples and negative control at -80°C overnight. Optionally, if there is time, cut P-100 pipette tips at first line from the bottom and at a 45° angle.

2.3.9.3 Pre-Clearing and 1°Ab Incubation

If samples were previously stored at -80°C, thaw samples on ice (This will take anywhere between 45 minutes to 2 hours). Re-suspend A/G agarose beads (Use Santa Cruz) by gently flipping the tube up-and-down. Add 20µL (or 40µL if your total volume is 130µL) of A/G agarose beads using the cut P-100 tips (This will prevent bead damage) to each of the samples (including negative control). Next, parafilm the top of these tubes. Then, incubate these tubes using a rotator in the cold room for 4 hours [Lower limit = 2 hours, Upper limit = 4 hours]. During this time, pre-label and chill new safe-lock 1.5-mL Eppendorf tubes. Then, centrifuge samples at 16,000g for 5 minutes. Next, extract supernatant into the prechilled new Safelock tubes. Next, add the IP-compatible antibody that will be used to pull down the

proteins (<300µg = use 2µL of antibody, ≥300µg = use 2.5µL of antibody) and finally, parafilm the tubes and rotate overnight in the cold room (4°C).

2.3.9.4 Pull-Down, Washing and Elution

Retrieve the samples from the cold room on ice. Next, add A/G agarose beads to each sample using the cut P-100 pipette tips. Use 10µL/100µg of protein. Parafilm the tubes. Incubate using the rotator in the cold room (4°C) for 6 hours (or overnight). Meanwhile, prepare the following: regular 1.5-mL Eppendorf tubes for final sample extraction, IP wash buffer (w/ all extra components), IP wash vacuum system w/ tube rack, needle and 30G syringe, 5X sample buffer w/ DTT, turn on the block heater (This will take at least 20 minutes to stably maintain 95°C) and finally, prepare gels for running the gel later or tomorrow.

After incubation with the beads is complete, centrifuge samples at 5,000g for 5 minutes and discard the supernatant (unless the supernatant will be re-used to pull down more proteins). Next, wash the pellet 3 times by adding the complete wash buffer and gently running the tube over a tube holder 5-10 times. Confirm by observation that the pellet has been resuspended in wash buffer (There should be quite a bit of bubbles). Following each wash, centrifuge samples at 5,000g for 5 minutes in the cold room (4°C). The amount of wash buffer to use is dependent on the amount of protein you initially started with (<300µg = use 300µL of wash buffer, ≥300µg = use 500µL of wash buffer). Next, add 5X sample buffer (with DTT) and vortex (on high) for about 3-5 seconds per tube. Heat the samples at 95°C for 5 minutes and immediately put on ice. Centrifuge the samples at 15,000g for 15 minutes (ideally at room temperature but for the time being, use the cold room centrifuge). Extract supernatant into new regular 1.5-mL Eppendorf tubes. (See additional notes for step 33). Make note of the approximate volume you

are able to extract for each tube. Try to take all of the supernatant but take care to not take any beads in the pellet since they will show their own protein-specific bands. Store samples at -20°C if the gel is not being run on the same day.

2.3.9.5 SDS-PAGE Gel and Transfer

Thaw all samples out on ice (or not on ice, it doesn't matter). With your eyes, briefly compare the tubes to determine which one has the least amount of volume available. Measure this volume out and set this as your loading volume for your gel. Continue the steps on the 'Western Blot' protocol.

2.3.9.6 Additional Notes

Ensure that your samples are always on ice following homogenization and right until sample buffer + heat have been applied to denature the proteins.

The less antibody you add the less signal interference from the heavy chain ~50kDa (Especially if you are detecting proteins around 50kDa) so from optimizing, using less than 3μL of antibody may be the best choice unless the protein you are pulling down is not abundant (Highly dependent on the brand of antibody you are using to pull down and the protein you select to pull down).

If pulling down acetylated proteins, it is recommended that you use the 'Millipore anti-acetyl lysine antibody, mouse monoclonal, catalogue # '05-515' since this displayed better results than Cell Signaling's anti-acetyl lysine (Proven May 8, 2017). However, CST's anti-acetyl lysine for western blot detection works well. If pulling down LCAD, it doesn't work as well as the above method because the abcam anti-ACADL ab128566, which is IP-compatible, appears to still require quite a bit of protein (>150μg) in order to display the LCAD band.

If you choose to reuse the supernatant to pull-down additional proteins, it is highly recommended that you start with plenty of tissue (300-500µg) because from my experience (May 8, 2017), only starting with 150µg and reusing the supernatant to pull down acetylated proteins AFTER having pulled down LCAD does not produce ANY signal. Future experiments are required to confirm the lower limit.

If necessary, use the micro-centrifuge in the lab (Room temp.) to pull down the beads more and get more supernatant. *It is important that when you 're-centrifuge' samples, that the tube always stays in the same orientation. My recommendation is to always keep the neck of the tube lid on the outer circumference of the centrifuge (Away from the origin such that the radius b/w the neck of the tube lid and the origin is maximized).

I find it very difficult to be able to extract 100% of the volume of sample buffer you add and typically, the amount of supernatant that I can extract following elution (heating + centrifugation) is anywhere between 50% - 90% of the volume of sample buffer added.

2.3.10 CoA/Nucleotide Extraction

The following protocol (typed up by myself) was utilized to extract CoA esters and nucleotides for UPLC.

2.3.10.1 Preparation

The following need to be completed 1-2 days prior to starting the experiment. First, cut pH strips (for nucleotide extraction) into thin 1-2mm strips and cut the yellow portion off since this isn't a critical portion of the strip and this will decrease the amount of volume taken up by the strip from the extraction. Next, prepare stock solutions: 0.4M (6%) PCA/1mM DTT, 5M K_2CO_3 (aliquot 1mL into 1.5mL V-shaped Eppendorf tubes to prevent inter-experiment

contamination), 50mM EDTA (this needs to be quite concentrated -100x- so that it doesn't affect the final volume). Then label and pre-weigh glass vials with their caps. Then label and pre-weigh Eppendorf tubes. And lastly, pre-chill glass vials and tubes in a tray in the cold room or 4 degree fridge

2.3.10.2 Procedure

Throughout this procedure, it is important to ensure that everything is kept cold because malonyl-CoA is very susceptible to temperature-induced degradation.

2.3.10.2.1 CoA extraction

To begin, get liquid nitrogen and weigh out 25-30mg (or 10mg – 5mg for CoA + 5mg for nucleotide) of tissue and take samples for dry:wet ratios. Be precise and consistent when recording the weight as well as being very careful to not let any tissue thaw. Keep samples in liquid nitrogen until ready. Next, add 1mL 6% PCA/1mM DTT/and EDTA (0.5mM) if these samples also require nucleotides to be quantified (This is stored at 4°C) per 100mg of tissue.

Next, homogenize samples for 20-25 seconds (30 seconds may be too harsh and degrade CoAs) using hand-held homogenizer set at the highest speed. Then leave samples on ice for 10 minutes. Next, centrifuge at 12,000 g for 5 minutes. Then, using the preweighed and labelled glass vials (**or the preweighed and labelled Eppendorf tubes if nucleotide extraction will be carried out**), take out all of the supernatant by pipetting it slowly with gel-loading tips. Record the new weight of the glass vial/Eppendorf tube. This is your total extracted_volume for CoAs. If no nucleotide extraction is required, the CoAs are now ready for HPLC analysis and the following steps are not required. If nucleotide extraction is to be carried out, please refer to the following subsection.

2.3.10.2.2 Nucleotide extraction

First, split the extracted supernatant by pipetting 100uL of the supernatant into labelled glass vials. These glass vials holding 100uL of supernatant are now ready for CoA analysis. The remaining supernatant in the Eppendorf tubes are to be processed as detailed in the following steps. The first step is to calculate the remaining volume for the nucleotide and then top it up to 100uL using the PCA extraction solution. pH to 5-7 (7 is good) with 5M K_2CO_3.

The general rule of thumb is that you can add $1/10^{th}$ of the volume that you are pHing (take the total extracted volume minus 100uL = remaining volume that you are pHing). So if the remaining volume is 130uL, add 13uL of 5M K_2CO_3. However to prevent overshooting, I recommend adding a bit less than the 'rule of thumb' volume such as 10uL. For 100uL of sample (from 10mg of tissue), 10.3uL was established to be a good volume to add 5M K_2CO_3.

After adding your volume of 5M K_2CO_3, write down how much you added, then vortex and using the microcentrifuge, bring all the solution down. Then, using the pre-cut pH strips, gently angle the Eppendorf tube and dip the pH strip into the supernatant quickly to avoid drawing up excessive supernatant (which would ultimately negatively affect the final extraction volume). Compare the pH strip with its box to approximate the pH and write this down. Repeat the above steps until the pH is between 5-7. If minor pH adjustments are required, use the 0.5M K_2CO_3 to pH the supernatant. Next, let the samples sit on ice for 10 minutes then centrifuge at 10,000g for 2 minutes. Finally, take out all of the supernatant by pipetting it slowly with gel-loading tips into the pre-weighed glass vials and record the new weight. The weight difference is the volume extracted for the nucleotides. This supernatant is now ready for HPLC analysis for nucleotides. If the HPLC analysis is not performed the same day, these extracted and neutralized samples can be stored at -80°C until analysis.

2.3.11 Statistical analysis

All values are presented as mean ± SEM. Significant differences were calculated via a student's t-test when comparing two means whereas two-way ANOVAs were used when comparing more than two means followed by a Tukey post hoc test. All statistical analyses were carried out using GraphPad Prism V9. Differences were deemed statistically significant when the p-value < 0.05.

Chapter III
Increased Ketone Oxidation In The Failing Heart

A version of this chapter has been published in Cardiovascular Research (Ho, K. L., et al. (2019). "Increased ketone body oxidation provides additional energy for the failing heart without improving cardiac efficiency." 115(11): 1606-1616.). All figures for this published study can be found at the end of the chapter.

Chapter 3 Increased Ketone Oxidation in the Failing Heart

In this chapter, **Aim 1** is addressed and *ex vivo* cardiac function and metabolic profile of the failing heart in response to an acute increase in ketones are assessed.

We find evidence for increased ketone body oxidation providing additional energy for the failing heart without an improvement in cardiac efficiency.

3.1 Contribution Statement

My role in this project included performing all experiments (except those noted below) alongside the writing of the manuscript. *Ex vivo* isolated working heart perfusions were performed by Mr. Cory Wagg (Lab technician of Dr. Gary Lopaschuk's laboratory) and echocardiography was performed by Ms. Donna Becker (former lab technician from the Cardiovascular Research Centre).

3.2 Abstract

3.2.1 Aims

The failing heart is energy-starved and inefficient due to perturbations in energy metabolism. Although ketone oxidation has been shown recently to increase in the failing heart, it remains unknown whether this improves cardiac ATP production or efficiency. We therefore assessed cardiac metabolism in failing hearts and determined whether increasing ketone oxidation improves cardiac ATP production and efficiency.

3.2.2 Methods and Results

C57BL/6J mice underwent sham or transverse aortic constriction (TAC) surgery to induce pressure overload hypertrophy over 4-weeks. Isolated working hearts from these mice were perfused with radiolabelled ß-hydroxybutyrate, glucose, or palmitate to assess cardiac metabolism. Ejection fraction decreased by 45% in TAC mice. Failing hearts had decreased glucose oxidation while palmitate oxidation remained unchanged, resulting in a 35% decrease in ATP production. Increasing ßOHB levels from 0.2 to 0.6 mM increased ketone oxidation rates from 251±24 to 834±116 nmol·g dry wt^{-1}·min^{-1} in TAC hearts, rates which were significantly increased compared to sham hearts and occurred without decreasing glycolysis, glucose or palmitate oxidation rates. Therefore, the contribution of ketones to ATP production in TAC hearts increased to 18% and total ATP production increased by 23%. However, while overall ATP production increased, cardiac efficiency was not improved.

3.2.3 Conclusions

Increasing ketone oxidation rates in failing hearts increases overall ATP production without compromising glucose or fatty acid metabolism, albeit without increasing cardiac efficiency.

3.3 Introduction

The ultimate catabolic organ, the heart has a very high energy demand albeit having minimal energy reserves. Therefore, the heart continuously produces energy by metabolizing a variety of energy substrates such as fatty acids, carbohydrates (glucose, lactate), ketones and amino acids. This metabolic "flexibility" allows the healthy heart to dynamically alter its metabolic preference to match changes in circulating substrates and hormones, relying primarily on fatty acids in the fasted state and carbohydrates in the fed state. However, this metabolic flexibility is perturbed as the heart undergoes significant metabolic remodelling during the progression of heart failure[11,12,107,108], and the myriad of metabolic perturbations that manifest during heart failure contribute to a decline in cardiac efficiency[109].

Although there is consensus that cardiac ATP production is impaired in the failing heart, there is no consensus as to the actual energy metabolic profile in the failing heart, which may be due in part to the discrepancies in the severity, duration, etiology and comorbidities associated with heart failure[11]. Studies have demonstrated both fatty acid oxidation decreasing[110-112] or not changing[113,114] in the failing heart, whereas fatty acid uptake has been observed to be increased in congestive heart failure patients[115]. In addition, glucose oxidation rates are impaired in heart failure[113,114], which is distinct from the characteristic increase in glycolysis rates observed in the failing heart[20].

More recently, there has been increased interest in the importance of ketone oxidation as a source of energy in heart failure. This is especially in light of the recent findings that myocardial ketone oxidation has been shown to be increased in both experimental[7] and clinical[6] heart failure. However, it has also been suggested that ketone oxidation rates decrease during heart failure, which may contribute to an elevation of circulating ketone levels, such

that a compensatory response can protect the heart against oxidative stress[75]. Furthermore, the implications of changes in myocardial ketone oxidation on cardiac function remain elusive. It is also possible that an increased reliance on ketones during heart failure may increase the mitochondrial acetyl CoA pool and subsequently increase mitochondrial protein acetylation, which could compromise mitochondrial bioenergetics[14]. However, an increase in myocardial ketone oxidation rates has also been postulated to be an adaptive response, as mice with a cardiac-specific deletion for succinyl-CoA:3-ketoacid CoA transferase (SCOT, the rate-limiting enzyme of ketone oxidation) exhibit a marked reduction in ketone oxidation, which leads to accelerated pathological remodelling during experimental heart failure[15]. A recent study has also found that cardiac-specific overexpression of β-hydroxybutyrate dehydrogenase 1 (BDH1, the enzyme that interconverts the ketone bodies acetoacetate and β-hydroxybutyrate) protects mice against TAC-induced heart failure[116]. In the context of diabetic cardiomyopathy, ketones have also been hypothesized to be a "thrifty" fuel, and that increasing ketone oxidation in the non-diabetic failing heart has the potential to increase cardiac efficiency[4,5]. It has also been observed that ketones can inhibit myocardial fatty acid oxidation in healthy swine hearts which may or may not have consequences on the hypertrophic heart's metabolic profile[117]. In light of all of these previous studies' findings and the glaring absence of direct measurements of myocardial ketone oxidation rates throughout the literature, we wanted to directly quantify myocardial ketone oxidation rates in this study. In addition, we wanted to comprehensively elucidate the metabolic profile of the failing heart and in light of our previous study in swine, determine whether increased myocardial ketone oxidation inhibits fatty acid oxidation rates in the failing heart and whether this would affect cardiac ATP production or cardiac efficiency. This involved directly measuring the metabolic profile of the

failing heart at two concentrations of ß-hydroxybutyrate (ßOHB), the primary ketone body in the circulation.

3.4 Methods

Male C57BL/6J mice were randomly assigned, at 15-16 weeks of age, to a sham surgery or transverse aortic constriction (TAC) surgery to induce pressure overload hypertrophy over a 4-week period. After 4 weeks of TAC, transthoracic echocardiography was performed to assess *in vivo* cardiac function. Mice were then anesthetized and hearts were excised and perfused as isolated working preparations to assess *ex vivo* cardiac function as well as the cardiac metabolic profile. Finally, hearts were snap-frozen and used for biochemical analysis. Figure 3.1 outlines the experimental protocol followed for this study.

Unless detailed below, the experimental protocols utilized for this study can be found in Chapter 2. Specific isolated working heart perfusion conditions for this project are as follows.

3.4.1 Perfusion Conditions

Hearts were perfused as isolated working preparations with Krebs-Henseleit solution containing 2.5 mM Ca^{2+}, 100 μU/ml insulin, 5 mM [5-^{3}H/U-^{14}C]glucose, 0.8 mM palmitate (pre-bound to 3% albumin), and either 200 μM or 600 μM ß-hydroxybuturate (β-OHB). A second series of hearts were perfused under identical conditions, except with 100 μU/ml insulin, 5 mM glucose, 0.8 mM [9,10-^{3}H]palmitate (pre-bound to 3% albumin), and either 200 μM or 600 μM [3-^{14}C]ß-OHB. All sham and TAC hearts were perfused for an initial 30 min period with 200 μM β-OHB and an additional 30 minutes at a concentration of 600 μM β-OHB (Supplementary Figure 1B). Glycolysis and palmitate oxidation were determined by the

quantitative collection of $^{3}H_2O$ produced from [5-^{3}H]glucose and [9,10-^{3}H]palmitate, respectively, while glucose oxidation and ß-OHB oxidation was measured by the quantitative collection of $^{14}CO_2$, from [U-^{14}C]glucose and [3-^{14}C]ß-OHB, respectively. At the end of the perfusion, hearts were snap-frozen with liquid nitrogen and stored at -80 degrees Celsius until processed for biochemical analysis.

3.4.2 Statistical Analysis

Data is presented throughout as the mean ± SEM. Significant differences were determined by using an unpaired, two-tailed Student's t-test or a two-way ANOVA when comparing more than two means followed by a Bonferroni post hoc test. Statistical analysis was carried out using commercially available software (GraphPad Prism V7). Differences were deemed statistically significant when $P < 0.05$.

3.5 Results

3.5.1 Experimental Heart Failure Induces Robust Changes in Myocardial Energy Metabolism

Male C57BL/6 mice (12-wk of age) underwent a sham or TAC surgery. As expected, at 4-wk post-surgery, the TAC mice showed an increase in cardiac hypertrophy, as assessed by transthoracic ultrasound echocardiographyFigure 3.8. TAC surgery also resulted in marked reductions in ejection fraction and fractional shortening (Figure 3.2 A,B), as well as cardiac output and stroke volume (Figure 3.8). Furthermore, adverse LV remodelling was evident as seen by an increase in the LV internal diameter and LV posterior wall thickness during diastole (Table 3.1). TAC-induced heart failure also produced diastolic dysfunction, evidenced by a decrease in the mitral deceleration time and an increase in the E/E' ratio (Figure 3.2 C). In parallel with this *in vivo* assessment of cardiac function, assessment of cardiac function in *ex vivo* isolated working hearts obtained from mice 4-wk post-TAC surgery showed a marked decrease in cardiac work (Figure 3.2 D) and heart rate times peak systolic pressure (or rate pressure product). Together, this data demonstrates that the TAC surgery resulted in the development of a marked heart failure in the mice.

The contribution of ketone oxidation, fatty acid oxidation, glucose oxidation, and glycolysis to overall energy metabolism was determined in isolated working hearts from TAC mice, in the presence of 200 μM or 600 μM ß-OHB (Figure 3.9). Increasing ketone levels to which the heart was exposed had no effect on cardiac function in either Sham or TAC mice (Figure 3.2 D). At 200 μM βOHB, we observed no change in absolute rates of βOHB oxidation in TAC versus Sham hearts (Figure 3.3). Increasing ketone levels from 200 μM to 600 μM ß-

OHB resulted in a significant rise in ketone oxidation, but with no difference observed between Sham and TAC hearts. However, because cardiac work is an important determinant of the heart's oxidative rates[118,119], we also normalized these metabolic rates to cardiac work, which showed a significant increase in ketone oxidation rates in the TAC hearts perfused with 600 μM ß-OHB compared to the Sham hearts (Figure 3.4 A). Absolute fatty acid oxidation rates were similar between TAC and sham hearts and were not affected by the concentration of ketones to which the heart was exposed (Figure 3.3 B). Curiously, when normalized for cardiac work, fatty acid oxidation rates increased in the TAC hearts, which was most notable in the presence of 200 μM ß-OHB (Figure 3.4 B). Unlike ketone and fatty acid oxidation rates, absolute glucose oxidation rates were significantly decreased in TAC hearts compared to sham hearts (Figure 3.3 C). When normalized for cardiac work, this decrease in glucose oxidation in TAC hearts was no longer observed but increasing the concentration of ketones did increase the normalized glucose oxidation rates in TAC hearts (Figure 3.4 C). Absolute glycolysis rates were slightly lower in TAC versus sham hearts (Figure 3.3 D) but were not different when normalized for cardiac function (Figure 3.4 D).

3.5.2 The contribution of ketone oxidation to ATP production increases in the failing heart and provides an additional source of energy

As the failing heart is often referred to as "an engine out of fuel"[78], we assessed the actual tricarboxylic acid (TCA) and ATP production rates from exogenous substrates in hearts from sham and TAC mice. Consistent with this notion, TAC mice hearts exhibited a decreased TCA cycle activity that was primarily due to a significant reduction in glucose oxidation-derived acetyl-CoA production (Figure 3.5 A). However, increasing the concentration of ketones to 600 μM resulted in an increase in TCA cycle activity in the hearts from TAC mice,

which was primarily due to an increase in the contribution of both ketone oxidation and glucose oxidation to TCA cycle activity. Specifically, increasing the concentration of ketones from 200 μM to 600 μM ß-OHB resulted in an increase in the contribution of ketone oxidation to TCA acetyl CoA production from 12% to 27% in the hearts from TAC mice (Figure 3.9). Of importance, increasing ketone concentration did not decrease the contribution of either glucose or fatty acid to TCA cycle acetyl CoA production, and actually showed a trend to an increased contribution of glucose oxidation to TCA cycle acetyl CoA production in the hearts from TAC mice (Figure 3.5 A).

ATP production rates from glucose oxidation, glycolysis, fatty acid oxidation, and ketone oxidation were also depressed in failing hearts from TAC mice, regardless of whether hearts were exposed to 200 μM or 600 μM ß-OHB (Figure 3.5 B). However, increasing the concentration of ketones significantly increased total ATP production in TAC hearts and, while not significant, showed a trend to increased ATP production in sham hearts as well. Specifically, increasing the ketone concentration from 200 μM to 600 μM ß-OHB resulted in an increase in the contribution of ketone oxidation to ATP production from 8% to 18% (Figure Figure 3.5 C). Interestingly, at 600 μM ß-OHB, there was a trend towards increased contribution of glucose oxidation to ATP production rates in TAC hearts (Figure 3.5 C).

We have previously demonstrated that cardiac efficiency (cardiac work/acetyl CoA produced) is decreased in the failing heart[114]. Confirming these previous observations, we observed a decrease in cardiac efficiency, at 600 μM ß-OHB, in hearts from TAC mice (Figure Figure 3.5 D). Of interest, increasing ketone concentration from 200 μM to 600 μM ß-OHB, which increased the contribution of ketone oxidation to acetyl CoA production (Figure 3.5 A),

did not improve cardiac work (Figure 3.2 D) and thus, cardiac efficiency remained unchanged (Figure 3.5 D).

3.5.3 Acetylation of long-chain acyl-CoA dehydrogenase is increased in the failing heart

In light of the ability of ketones to increase TCA acetyl CoA production (Figure 3.5 A) and the failing heart's increased ketone oxidation rates (Figure 3.4 A), it has been previously suggested that the failing heart's increased reliance on ketones may be contributing to the mitochondrion acetyl CoA pool and potentiating mitochondrial protein hyperacetylation in the failing heart[14]. In support of this concept, we observed that failing hearts exposed to 600 μM ßOHB, having significantly increased ketone oxidation rates (Figure 3.4 A), also exhibited increased protein lysine acetylation levels compared to sham hearts (Figure 3.6 B).

It has been well established that the mRNA for fatty acid oxidation enzymes is decreased in heart failure[110]. However, as shown here (Figure 3.3B and Figure 3.4B), this is not necessarily accompanied by a decrease in actual fatty acid oxidation rates. Since we observed an increase in mitochondrial acetylation in TAC hearts (Figure 3.6 B) and acetylation of fatty acid oxidative enzymes has previously been associated with both decreased and increased activity[120], we examined the acetylation status of long chain acyl CoA dehydrogenase (LCAD) in hearts from TAC mice (Figure 3.6 C). A significant increase in acetylation of LCAD was observed, which argues against the notion that this protein modification is inhibitory and may partly explain the higher fatty acid oxidation rates we observed in hearts from TAC mice. Interestingly, the protein expression levels of the β-oxidation enzymes, LCAD and β-hydroxyacyl-CoA-dehydrogenase (βHAD), were unchanged (Figure 3.6 A). The protein expression of the ketone oxidation enzyme SCOT was also not

changed. However, the expression of the ketone oxidation enzyme BDH1 was ~3-fold greater in hearts from TAC mice (Figure 3.6 A).

3.6 Discussion

In this study, we are the first to directly measure and quantify absolute myocardial ketone oxidation rates in the failing heart. We provide the novel finding that ketones are not an insignificant source of fuel for the heart and that increased ketone oxidation may be an attempt to reverse the energy deficit seen in the failing heart. To our surprise, increasing ketone oxidation to 600μM also did not compromise other sources of ATP production in the failing heart, namely, glucose and fatty acid oxidation. Furthermore, we demonstrate that cardiac efficiency is decreased in the failing heart and that, although ketones have been proposed to be a "thrifty fuel, increasing ketone body oxidation in the failing heart is not associated with an increase in cardiac efficiency. Lastly and unexpectedly, we also observed that fatty acid oxidation rates were not impaired in failing hearts. Therefore, while the increase in normalized myocardial βOHB oxidation rates is consistent with recent studies in animal models of and humans with heart failure[6,7], increases in myocardial fatty acid oxidation and no change in glycolysis rates contrasts current dogma in the literature[11,121].

At both physiologically and pathophysiologically relevant circulating ketone concentrations [6,122,123], we show that ketone oxidation provides less than 10% of the heart's energy requirements in sham hearts (Figure 3.7 A) and slightly more in the failing heart (Figure 3.7 B). However, if ketone concentrations are increased from 200 μM to 600 μM, the contribution of ketone oxidation to ATP production significantly increases and can provide over 20% of the failing heart's ATP production (Figure 3.7 B). Our observations of increased cardiac ketone oxidation and increased BDH1 protein expression in TAC hearts is consistent with previous studies by Aubert *et al*.[7] and Bedi *et al*.[6], both of which have provided strong evidence to suggest that ketone oxidation is increased in the failing heart. However, the direct

quantitative measurements of ketone oxidation made in our studies confirm that not only is there an increase in ketone oxidation in the pressure overload hypertrophic heart, but that this increase also occurs without inhibiting glucose or fatty acid oxidation, ultimately contributing to increased ATP production in the failing heart. This is particularly topical since the adaptive or maladaptive nature of heart failure-induced increases in ketone oxidation has been debated[124,125]. Logically, a potential negative consequence of increased myocardial ketone oxidation is increased substrate competition and subsequent decreases in the contribution of other energy substrates, such as glucose and fatty acids, to ATP production. Previous studies in swine intravenously infused with βOHB revealed that increasing circulating ketone body concentration can inhibit myocardial fatty acid oxidation rates by up to 54%[117]. Additionally, a more recent positron emission tomography study in humans found that infusion with βOHB decreased myocardial glucose uptake[126]. However, in the present study, exogenous addition of ketones (from 200 to 600μM βOHB) or TAC-induced increases in ketone oxidation rates were not accompanied by significant decreases in either fatty acid or glucose oxidation rates. The discrepancy in results could be due to the range of βOHB concentrations we used in this study, since previous studies administered a ketone concentration such that arterial concentration reached ~870μM in swine[117], almost 50% higher than the concentration we chose to perfuse the hearts with in the current study. Furthermore, the Gormsen et al. study in healthy humans reported circulating βOHB to be between 3000 - 4000μM[126], ten-fold higher than the range of βOHB we chose to use to investigate the failing heart. Therefore, ketone's inhibitory effect on fatty acid or glucose oxidation may occur at a supra-physiological level in the context of heart failure, such as that seen during prolonged fasting or chronic consumption of a ketogenic diet[127]. As such, while the range of 200-600μM βOHB was intentionally chosen because of

pathophysiological established circulating ketone concentrations in humans with and without heart failure[6,122,123], it would be interesting for future studies to investigate a wider range of βOHB doses to study the extent to which ketones add a new layer of complexity to the Randle cycle[128].

Due to the absence of any observed substrate competition in our study, the acute addition of ketones increased overall ATP production and our findings support the concept that increased myocardial ketone oxidation in the failing heart is adaptive by increasing energy supply. In support of the implication that increased myocardial ketone oxidation may be adaptive in the setting of heart failure, Schugar et al. (2014) also similarly found evidence in mice with a cardiac-specific knockout of *Oxct1*[15]. They found that these mice, having blunted myocardial ketone oxidation, exhibited increased pathological ventricular remodeling when subjected to TAC. Therefore, this would suggest that increased myocardial ketone oxidation is adaptive and protective, *in vivo*, against TAC-induced heart failure. However, it is important to note that the authors also found that hearts that were unable to oxidize ketones also had increased fatty acid oxidation. As such, it is difficult to conclusively say whether the accelerated pathological remodeling was due to an increase in fatty acid oxidation or the absence of ketone oxidation. Regardless, a more recent study gives similar convincing evidence to suggest that increased myocardial ketone oxidation is adaptive for the failing heart. Uchihashi et al. (2017) found that mice with cardiac-specific Bdh1 overexpression, possessing accelerated myocardial ketone oxidation, were protected against oxidative stress and had decreased pathological remodeling following TAC-induced heart failure[116]. Together, our findings give more insight and support to these studies' findings[15,116] and suggests that the failing heart's increased reliance on ketones is adaptive.

Recent attention has focused on the possibility that ketones are a "superfuel" or "thrifty' fuel for the heart, based on the concept that the oxidation of ketones is a more efficient source of energy than fatty acids[4,5]. Moreover, it has been suggested that empagliflozin, an SGLT2 inhibitor that improves cardiovascular outcomes in diabetics with cardiovascular disease[3], confers this cardioprotection via increasing circulating ketone levels and subsequent myocardial ketone oxidation[4,5]. However, it is an important point to note that while ketones may be a more efficient substrate than fatty acids, in terms of the P/O ratio, they are not more efficient than glucose as an energy substrate[13]. Nevertheless, our data provide the first direct demonstration as to how ketones impact cardiac efficiency, and particularly in the setting of heart failure. Increasing the concentration of ketones from 200 μM to 600 μM did not augment cardiac efficiency in either the non-failing or failing heart. Likewise, we also demonstrate that cardiac efficiency is decreased in the failing heart compared to the non-failing heart and is not improved by increasing ketone oxidation. It is worth noting that while the ATP produced per oxygen consumed (P/O ratio) is slightly better for ketone oxidation than fatty acid oxidation, the P/O ratio is actually lower for ketone oxidation compared to glucose oxidation. This does not support the concept of ketones being a more "thrifty" fuel. Confirming this, we demonstrate that increasing ketone oxidation does not increase cardiac efficiency in either the failing or non-failing heart, and that the increase in ketone oxidation observed in heart failure is not associated with an improved cardiac efficiency. However, we cannot rule out that applying a higher concentration of ketones could affect cardiac efficiency and therefore, future studies are required to establish a realistic and effective dose of ketones that may affect cardiac efficiency. As a result, the potential benefit of increased ketone oxidation in heart failure would not be to increase cardiac efficiency, but rather to at least partially restore the acetyl-

CoA pool and thereby increase overall ATP production (which may be beneficial in light of the fact that the failing heart is energy starved[78]). Importantly, increasing ketone oxidation in the failing heart did not compete with the oxidation of fatty acids and glucose. Rather, the additional availability of ketones simply increased TCA cycle activity and ATP production rates, thus providing the heart with an additional source of energy. In summary, our findings would suggest that ketones are not a more "thrifty" fuel, but rather are an extra source of fuel for the failing heart.

The contribution of fatty acid oxidation to ATP production in the failing heart has been a topic of considerable debate[11]. It is generally assumed that fatty acid oxidation rates are decreased in the failing heart, due in part to a decreased expression of mitochondrial enzymes involved in fatty acid oxidation[110,121]. However, while some human and experimental studies have shown a decrease in fatty acid oxidation in the failing heart[111,112,129-131], other studies have shown that fatty acid oxidation rates are either unchanged or increased[59,114,115,132-134]. In this study we show that despite the development of severe heart failure in mice subjected to TAC surgery, cardiac fatty acid oxidation rates were not depressed. In fact, when normalized for cardiac work, fatty acid oxidation rates were actually increased in the failing heart. The reason for the maintained fatty acid oxidation rates is not clear but there is a possibility that heart failure-induced increases in mitochondrial protein acetylation[14] could affect the acetylation status of fatty acid oxidation enzymes and activity[120]. In this study, we confirm that total protein lysine acetylation is increased in failing hearts and that concurrently, acetylation of the fatty acid oxidation enzyme, LCAD, is increased. As a result, we cannot rule out the possibility that increased post-translational acetylation may be a mechanism by which fatty acid oxidation rates are maintained in heart failure, despite decreased transcription of mitochondrial fatty acid

oxidation enzymes. Of interest, it has been proposed that an increase in ketone body oxidation in the failing heart could contribute to the overall acetyl CoA pools, increase mitochondrial protein hyperacetylation and consequently, exaggerate mitochondrial respiratory dysfunction[14]. However, we show that increasing the concentration of exogenously supplied ketone bodies to the heart did not compromise mitochondrial respiration, but rather increased overall ATP production.

In terms of absolute rates, the major metabolic change observed in the failing heart was a significant decrease in glucose oxidation rates. This decrease in glucose oxidation parallels what we have seen in previous studies in the murine failing heart[113,114], as well as what others have seen in the failing swine heart[65], and is consistent with recent studies demonstrating that a cardiac-specific impairment in glucose oxidation produces cardiac hypertrophy and diastolic heart failure[135]. One explanation for this reduction in glucose oxidation is due to the insulin resistant state of the failing heart[11,113,136]. Our study suggests that decreased myocardial glucose oxidation is primarily responsible for the decrease in ATP production in the failing heart.

3.6.1 Limitations

A limitation of our study is that metabolic rates measurements do not account for endogenous substrate contribution to ATP production. As such, our calculated ATP and acetyl CoA production as well as cardiac efficiency is not entirely comprehensive, and future studies that simultaneously account for both exogenous and endogenous substrate metabolism in the failing heart are necessary. Moreover, it has been well documented that glycolytic rates significantly increase in the failing heart[137] but in our study, we did not observe any increase in glycolysis. This is likely due to the specific animal model used in our studies. Previous

studies have demonstrated that the mouse heart has accelerated glycolytic rates compared to other animal models such as rats[138]. As such, our glycolytic rates, while important for the overall assessment of cardiac metabolism in the murine heart, cannot be translated to humans with heart failure as the mouse glycolytic rates are already saturating its ~20% contribution to overall ATP production.

3.6.2 Conclusions

Myocardial ketone oxidation rates are increased in the failing heart and when exposed to 600μM βOHB, can contribute over 20% of the hearts' ATP production. Therefore, increasing ketones from 200 to 600μM increases ketone oxidation in the failing heart, contributing to an increased overall ATP production but does so without improving cardiac efficiency. Furthermore, the additional ATP production obtained by increasing ketone oxidation does not occur at the expense of either glucose or fatty acid oxidation, suggesting that the level to which heart failure induces an increase in ketone oxidation may be an adaptive response to increase ATP production in the failing heart.

3.7 Tables

Table 3.1 Working heart measurements 4 weeks post-TAC

	Sham (n=19)	TAC (n=22)
Heart Rate (beats • min^{-1})	294 ± 6	272 ± 8
Peak systolic pressure mmHg)	64 ± 1	59 ± 2
Developed Pressure (mmHg)	24 ± 1	20 ± 1
HR x PSP (bpm • mmHg • 10^{-3})	19 ± 0.4	16 ± 1
HR x DP (bpm • mmHg • 10^{-3})	7 ± 0.2	5 ± 0.3
Cardiac Output (ml/min)	9.8 ± 0.5	6.5 ± 0.7
Aortic Output (ml/min)	6.8 ± 0.5	4.3 ± 0.5
Coronary Flow (ml/min)	3.1 ± 0.2	2.2 ± 0.3
Cardiac Work (ml • mmHg • min^{-1})	6.3 ± 0.2	4.0 ± 0.4
Cardiac Work (joules • g dry wt^{-1}• min^{-1})	2.22 ± 0.15	1.02 ± .12

3.8 Figures

Figure 3.1 Schematic diagram of the methodology employed in this study. **A**, study diagram of the C57Blk/6 mice aged to 16 weeks after which a transverse aortic constriction (TAC) or sham surgery was carried out. Four-weeks post-TAC, in vivo cardiac function was assessed with transthoracic echocardiography after which the mice were sacrificed, and hearts were immediately extracted and subjected to an isolated working heart perfusion to measure metabolic rates. **B**, the outline of the perfusion timeline in which hearts were perfused with 200µM ßOHB for the initial 30 minutes after which 400µM ßOHB was added at time 30 minutes to increase the concentration of ßOHB to 600µM for the remainder of the perfusion.

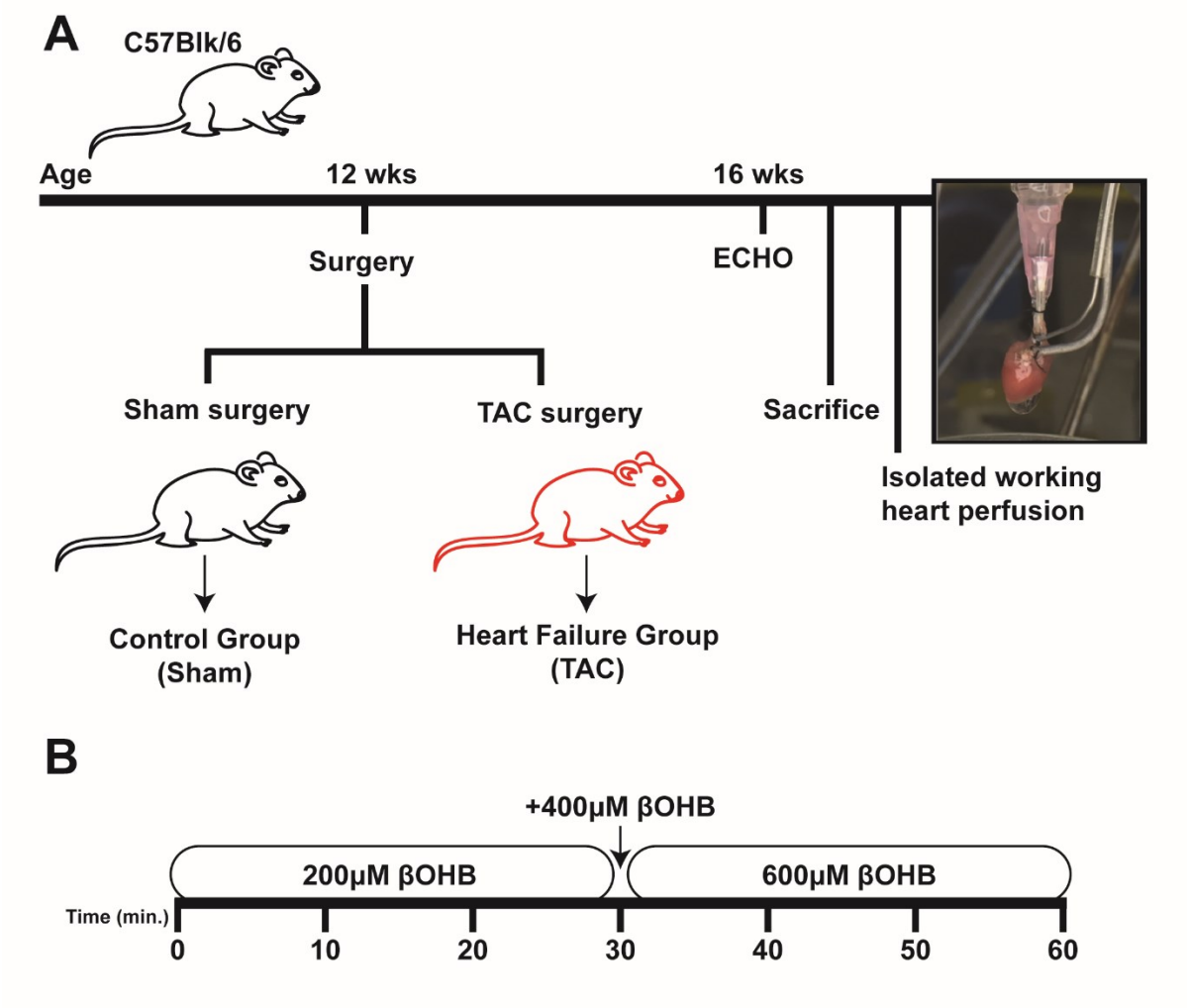
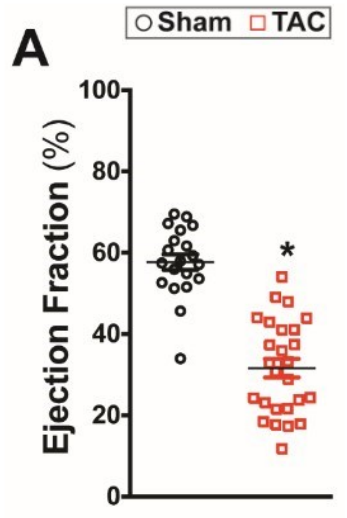
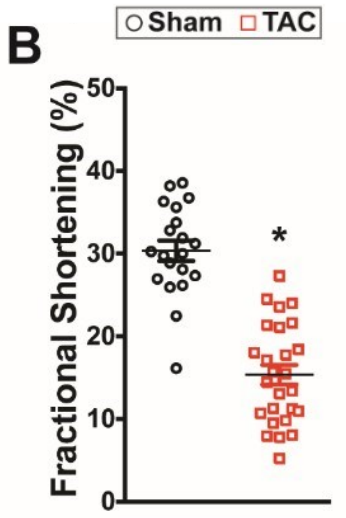

A
C57Blk/6
Age
12 wks
16 wks
Surgery
ECHO
Sham surgery
TAC surgery
Sacrifice
Isolated working heart perfusion
Control Group (Sham)
Heart Failure Group (TAC)
B
+400μM βOHB
200μM βOHB
600μM βOHB
Time (min.)
0
10
20
30
40
50
60

FIGURE 3.1

Figure 3.2 Assessment of in vivo and ex vivo cardiac function and morphology in TAC and Sham C57Blk/6J mice. **A,** Echocardiographic assessment of ejection fraction (Unpaired two-tailed t-test, n = 20 for sham, 26 for TAC). **B,** Echocardiographic assessment of diastolic dysfunction as measured by the 'e' to 'e prime' ratio (Unpaired two-tailed t-test, n = 18 for sham, 23 for TAC). **C,** Echocardiographic assessment of systolic function by a measure of fractional shortening (Unpaired two-tailed t-test, n = 20 for sham, 26 for TAC). **D,** *Ex vivo* assessment of cardiac function in the isolated perfused heart by a measure of cardiac work in which the concentration of ßOHB was increased from 200µM to 600µM at 30 minutes (Multiple t tests with correction for multiple comparisons using the Bonferroni-Dunn method, n = 19 for sham, 22 for TAC). A-C, Sham groups are represented by white circles while TAC groups are represented by red squares. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group.


A
Sham
TAC
Ejection Fraction (%)
100
80
60
40
20
0
*


B
Sham
TAC
Fractional Shortening (%)
50
40
30
20
10
0
*

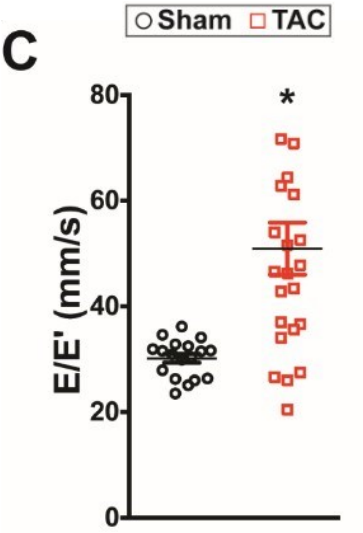

C
Sham
TAC
E/E' (mm/s)
80
60
40
20
0
*

FIGURE 3.2

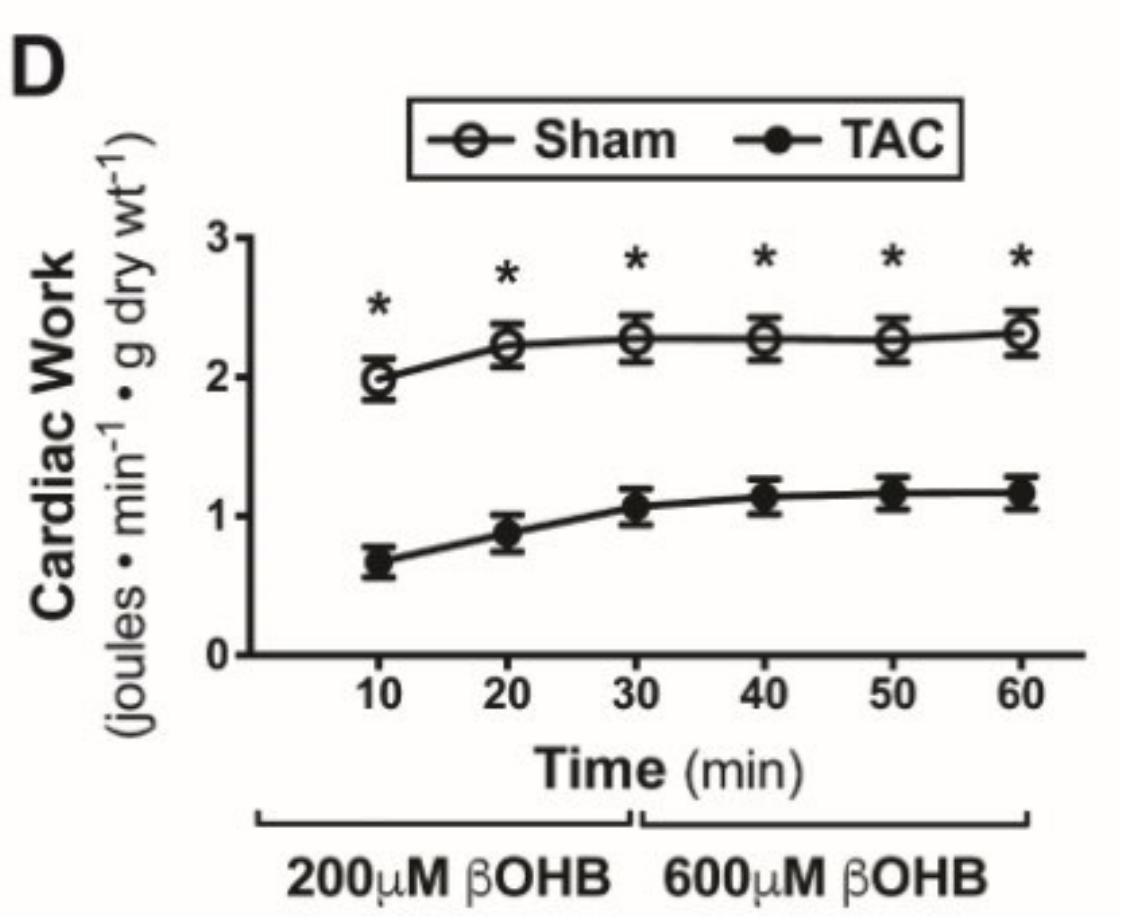

D
Sham
TAC
Cardiac Work
(joules • min-1 • g dry wt-1)
3
2
1
0
*
*
*
*
*
*
10
20
30
40
50
60
Time (min)
200μM βOHB
600μM βOHB

FIGURE 3.2

Figure 3.3 Absolute metabolic rates of the failing heart in the presence of either 200µM ßOHB or 600µM ßOHB. **A,** ßOHB (ketone body) oxidation (n = 6 for sham, n = 6 for TAC). **B,** Palmitate (fatty acid) oxidation (n = 8 for sham, n = 9 for TAC). **C,** Glucose oxidation (n = 13 for sham, n = 16 for TAC). **D,** Glycolysis (n = 10 for sham, n = 13 for TAC). Sham groups are represented by white circles while TAC groups are represented by red squares. A Two-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group. †$p<0.05$ compared to the 200µM ßOHB group.

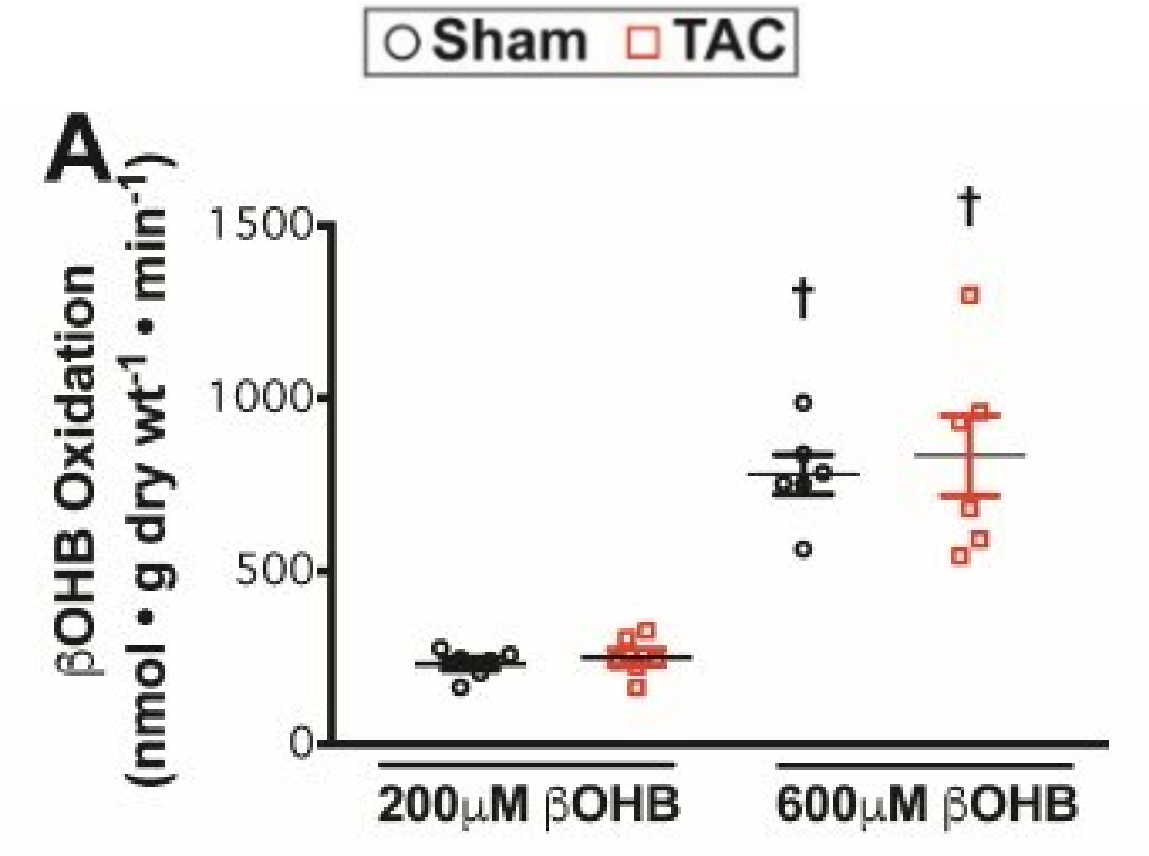

Sham
TAC
A
βOHB Oxidation
(nmol • g dry wt-1 • min-1)
1500
1000
500
0
†
†
200μM βOHB
600μM βOHB

FIGURE 3.3

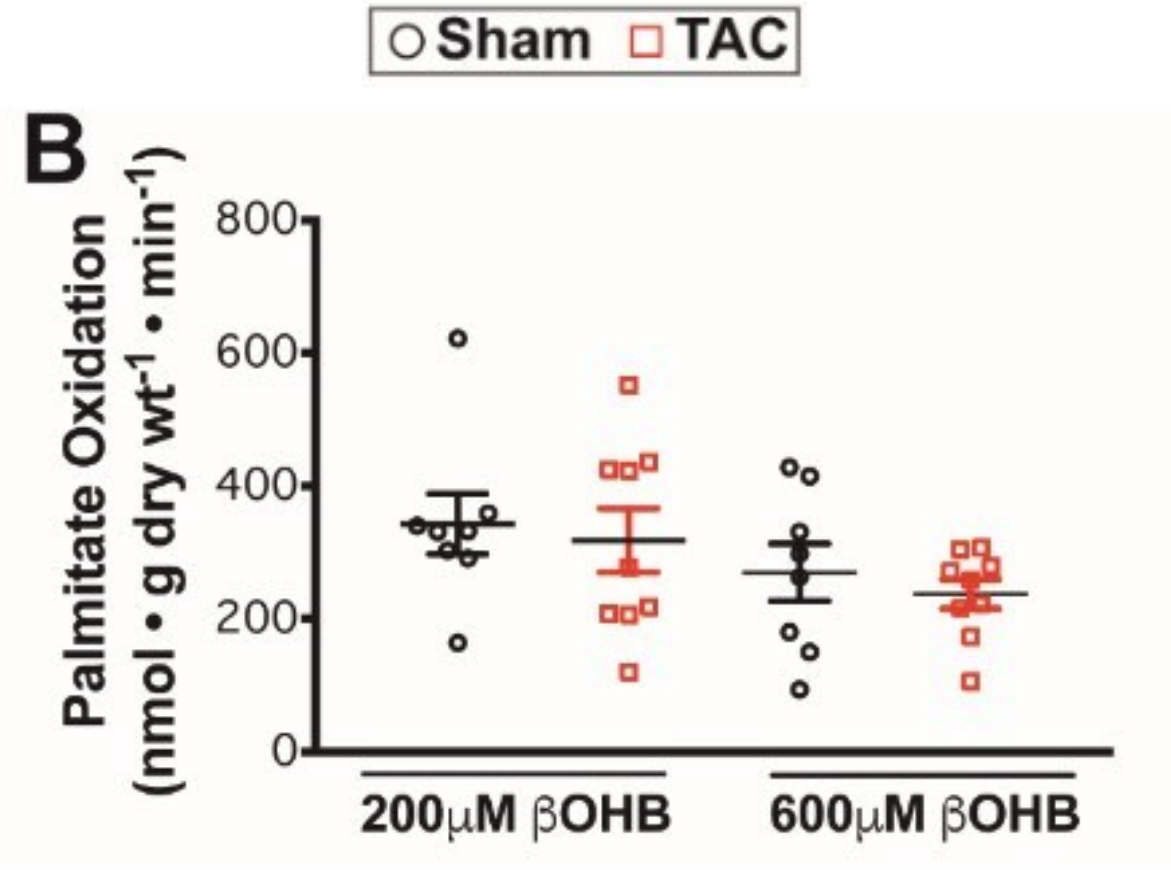

Sham
TAC
B
Palmitate Oxidation
(nmol • g dry wt-1 • min-1)
800
600
400
200
0
200μM βOHB
600μM βOHB

FIGURE 3.3

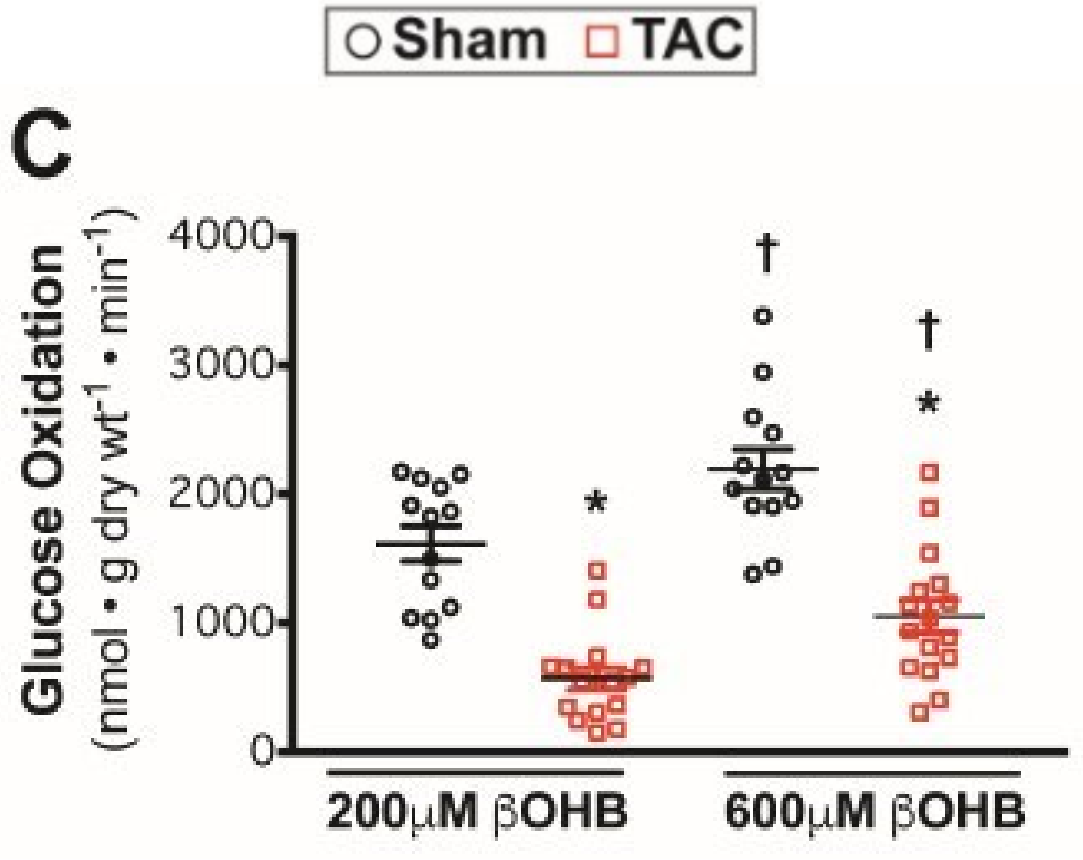

Sham
TAC
C
Glucose Oxidation
(nmol • g dry wt-1 • min-1)
4000
3000
2000
1000
0
†
†
*
*
200μM βOHB
600μM βOHB

FIGURE 3.3

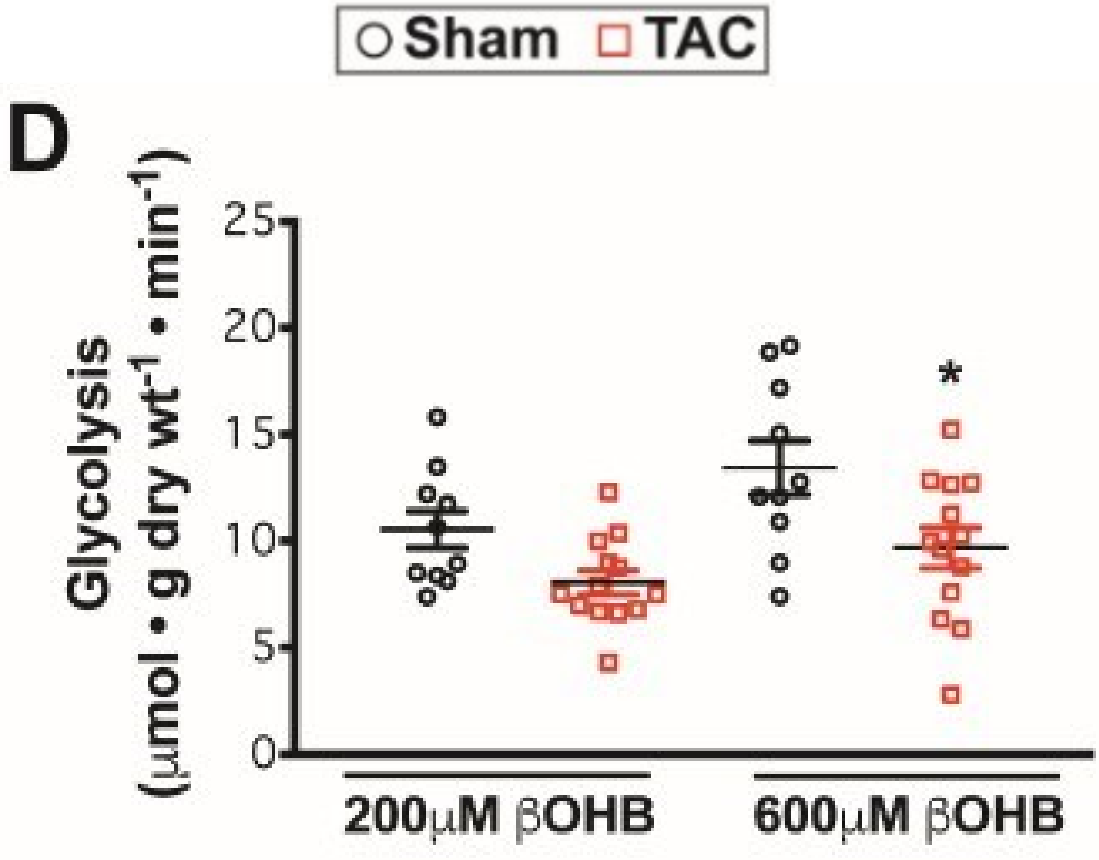

Sham
TAC
D
Glycolysis
(μmol • g dry wt-1 • min-1)
25
20
15
10
5
0
*
200μM βOHB
600μM βOHB

FIGURE 3.3

Figure 3.4 Normalized metabolic rates of the failing heart in the presence of either 200µM ßOHB or 600µM ßOHB. **A,** ßOHB (ketone body) oxidation normalized to cardiac work (n = 6 for sham, n = 6 for TAC). **B,** Palmitate (fatty acid) oxidation normalized to cardiac work (n = 8 for sham, n = 8 for TAC). **C,** Glucose oxidation normalized to cardiac work (n = 13 for sham, n = 13 for TAC). **D,** Glycolysis normalized to cardiac work (n = 10 for sham, n = 11 for TAC). Sham groups are represented by white circles while TAC groups are represented by red squares. A Two-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group. †$p<0.05$ compared to the 200µM ßOHB group.

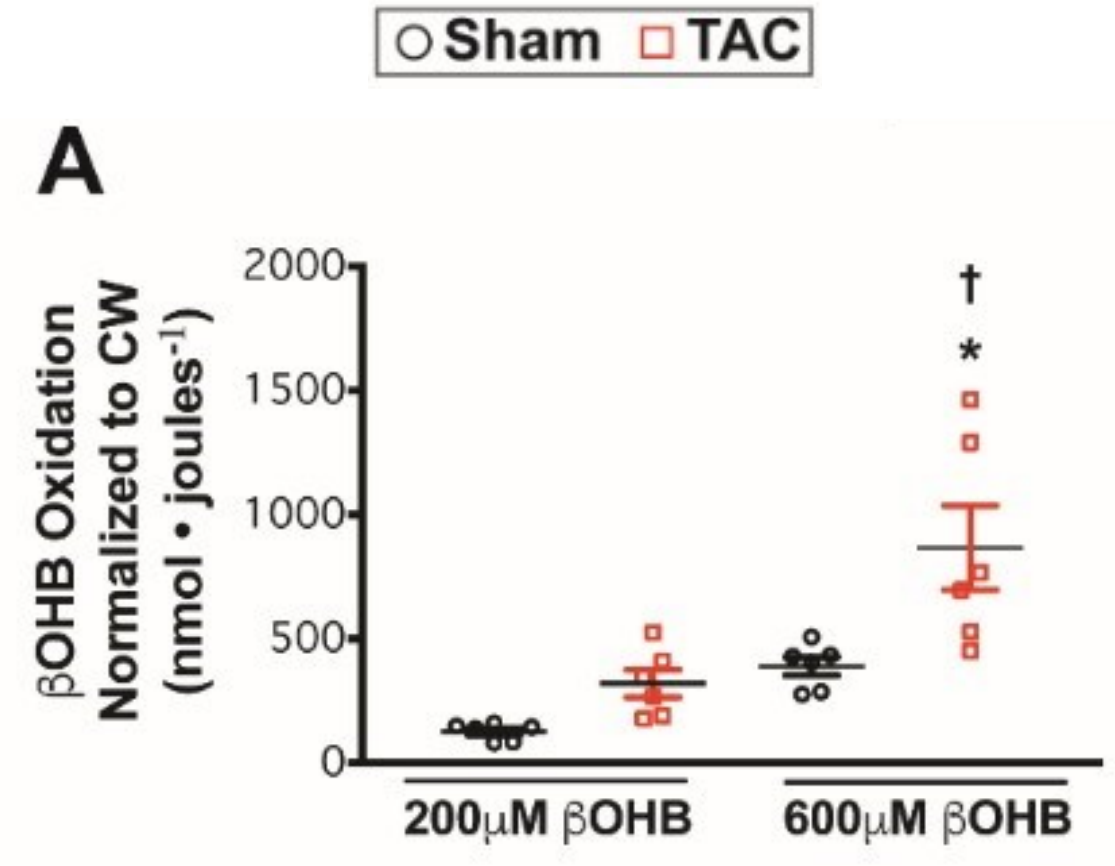

Sham
TAC
A
βOHB Oxidation
Normalized to CW
(nmol • joules-1)
2000
1500
1000
500
0
†
*
200μM βOHB
600μM βOHB

FIGURE 3.4

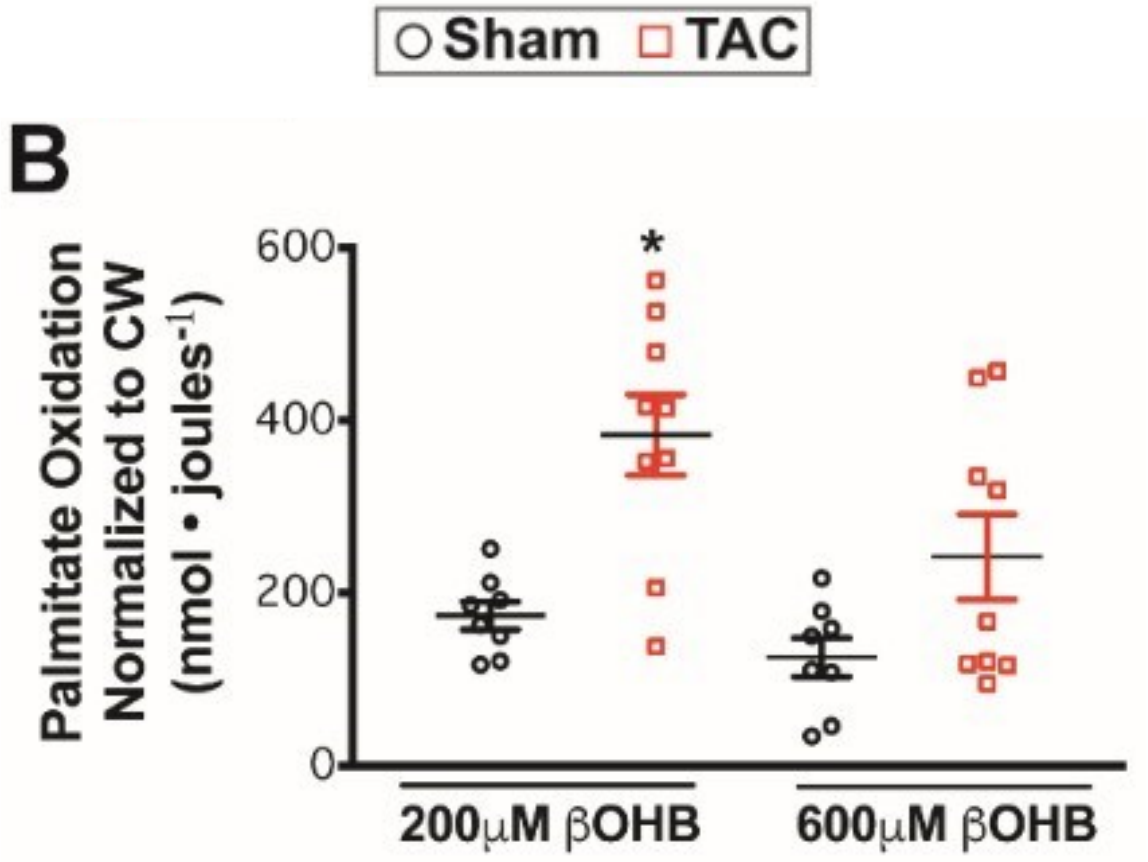

Sham
TAC
B
Palmitate Oxidation Normalized to CW (nmol • joules-1)
600
400
200
0
*
200μM βOHB
600μM βOHB

FIGURE 3.4

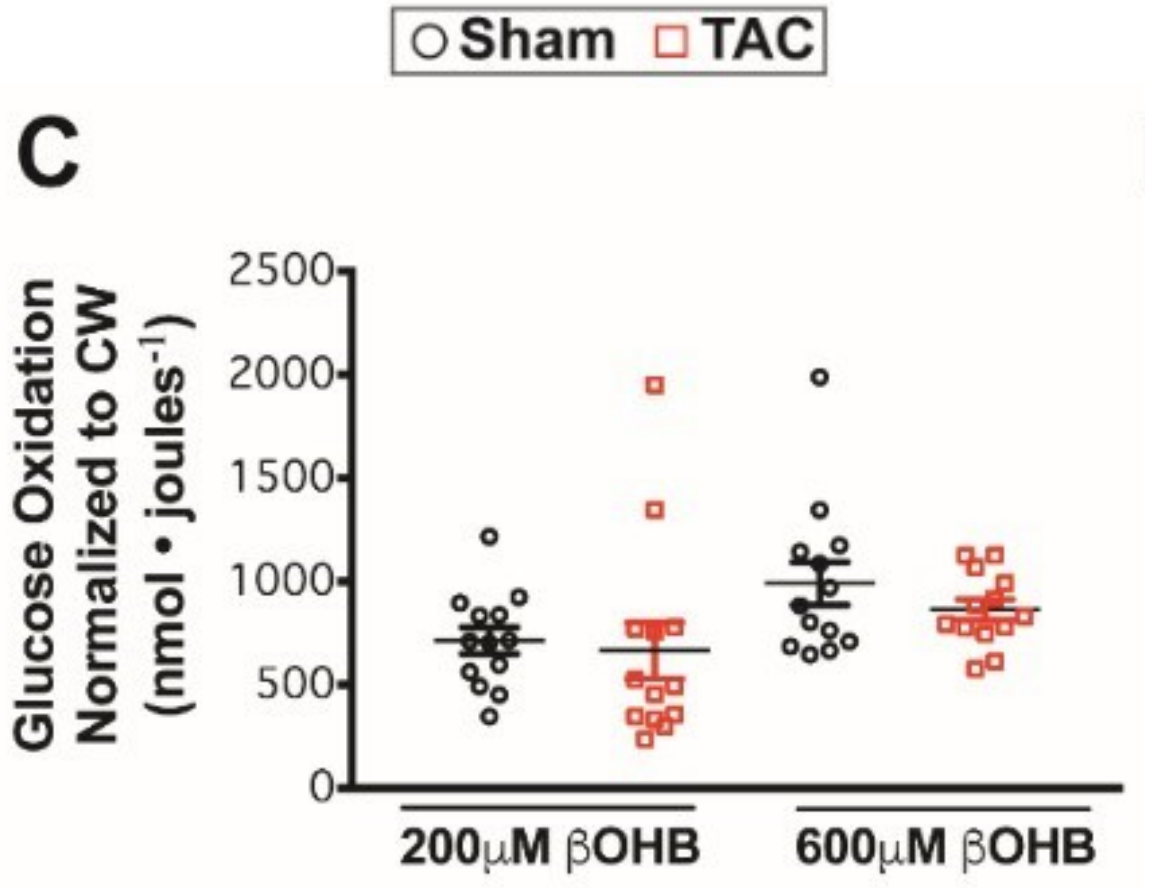

Sham
TAC
C
Glucose Oxidation
Normalized to CW
(nmol • joules-1)
2500
2000
1500
1000
500
0
200μM βOHB
600μM βOHB

FIGURE 3.4

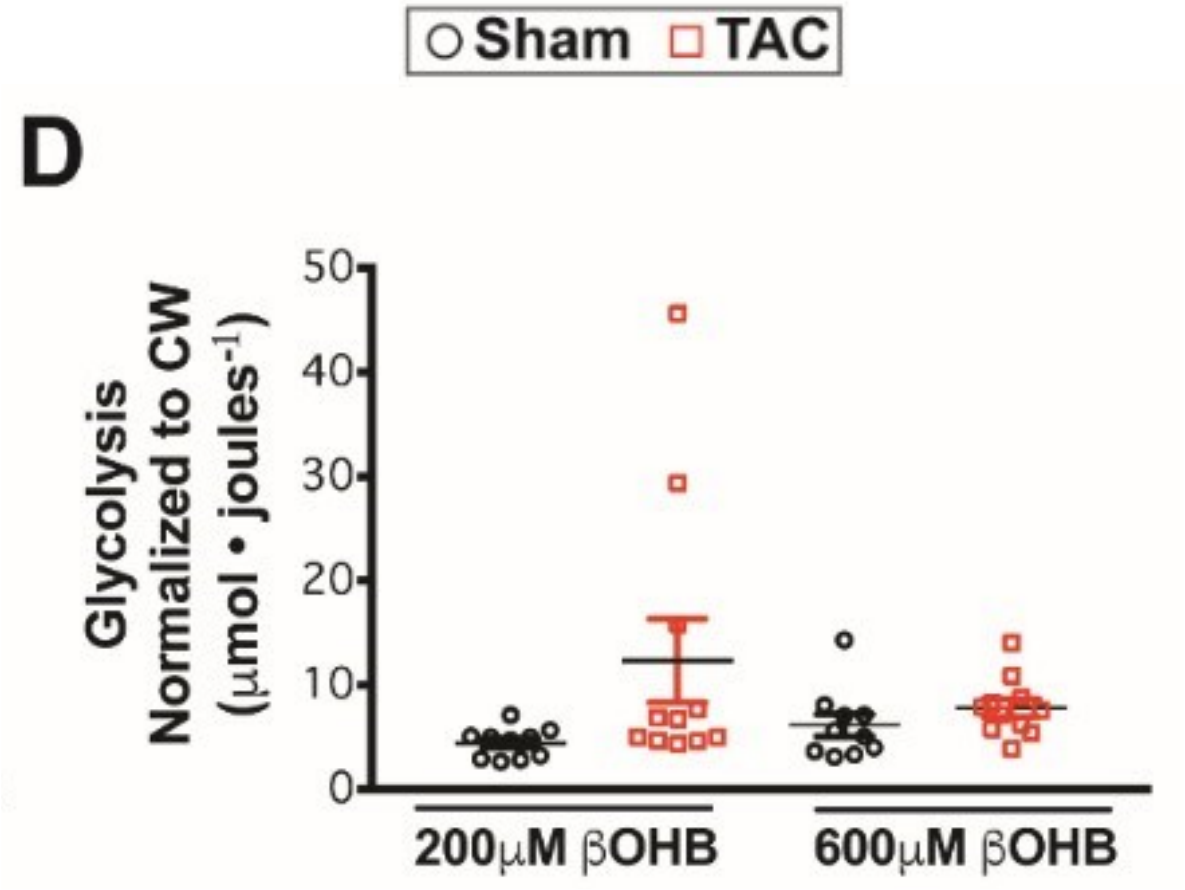

Sham
TAC
D
Glycolysis
Normalized to CW
(μmol • joules-1)
50
40
30
20
10
0
200μM βOHB
600μM βOHB

FIGURE 3.4

Figure 3.5 TCA acetyl CoA production, ATP production and cardiac efficiency in the failing heart in the presence of either 200µM ßOHB or 600µM ßOHB. **A,** TCA acetyl CoA production from glucose oxidation, palmitate oxidation or ßOHB oxidation. **B**, ATP production from glucose oxidation, glycolysis, palmitate oxidation or ßOHB oxidation. **C,** The percent contribution of glucose oxidation, glycolysis, palmitate oxidation or ßOHB oxidation to the total ATP production. **D,** Cardiac efficiency as determined by normalizing cardiac work for total acetyl CoA production (n = 6 for all groups). In panel D, sham groups are represented by white circles while TAC groups are represented by red squares. A Two-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group. †$p<0.05$ compared to the 200µM ßOHB group.

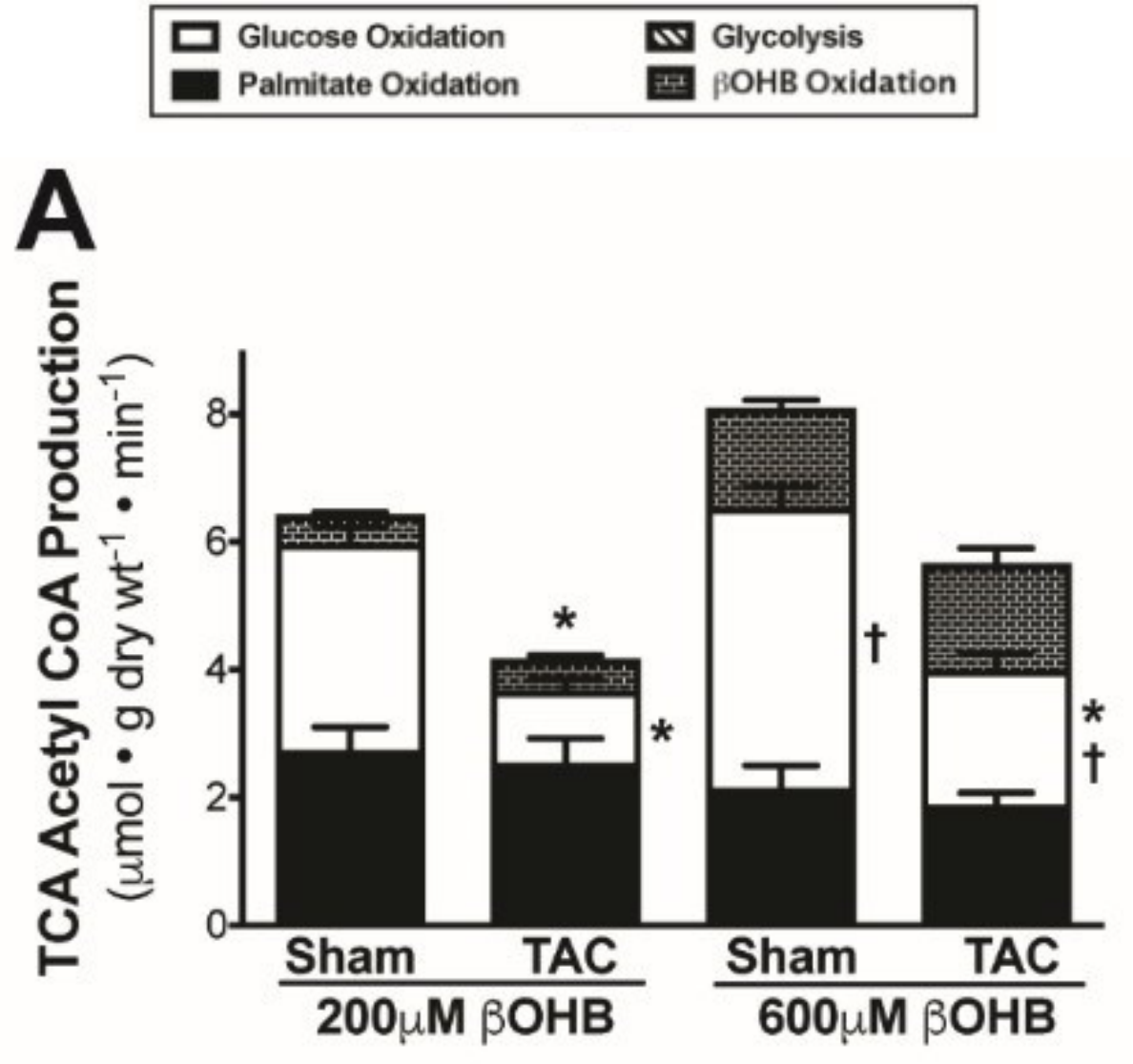

Glucose Oxidation
Palmitate Oxidation
Glycolysis
βOHB Oxidation
A
TCA Acetyl CoA Production
(μmol • g dry wt-1 • min-1)
0
2
4
6
8
*
*
†
*
†
Sham
TAC
Sham
TAC
200μM βOHB
600μM βOHB

FIGURE 3.5

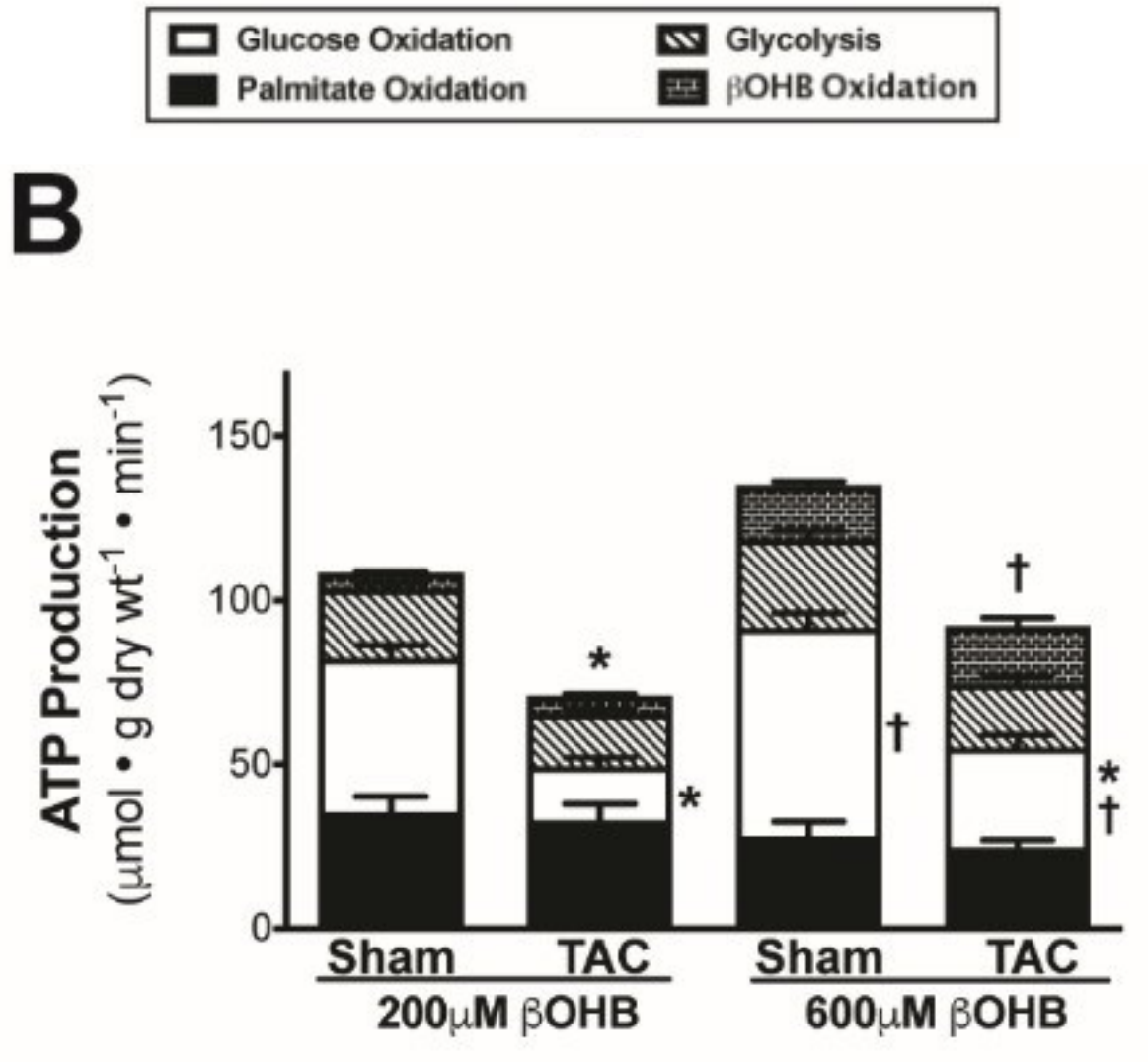

Glucose Oxidation
Palmitate Oxidation
Glycolysis
βOHB Oxidation
B
ATP Production
(μmol • g dry wt-1 • min-1)
150
100
50
0
*
*
†
†
*
†
Sham
TAC
Sham
TAC
200μM βOHB
600μM βOHB

FIGURE 3.5

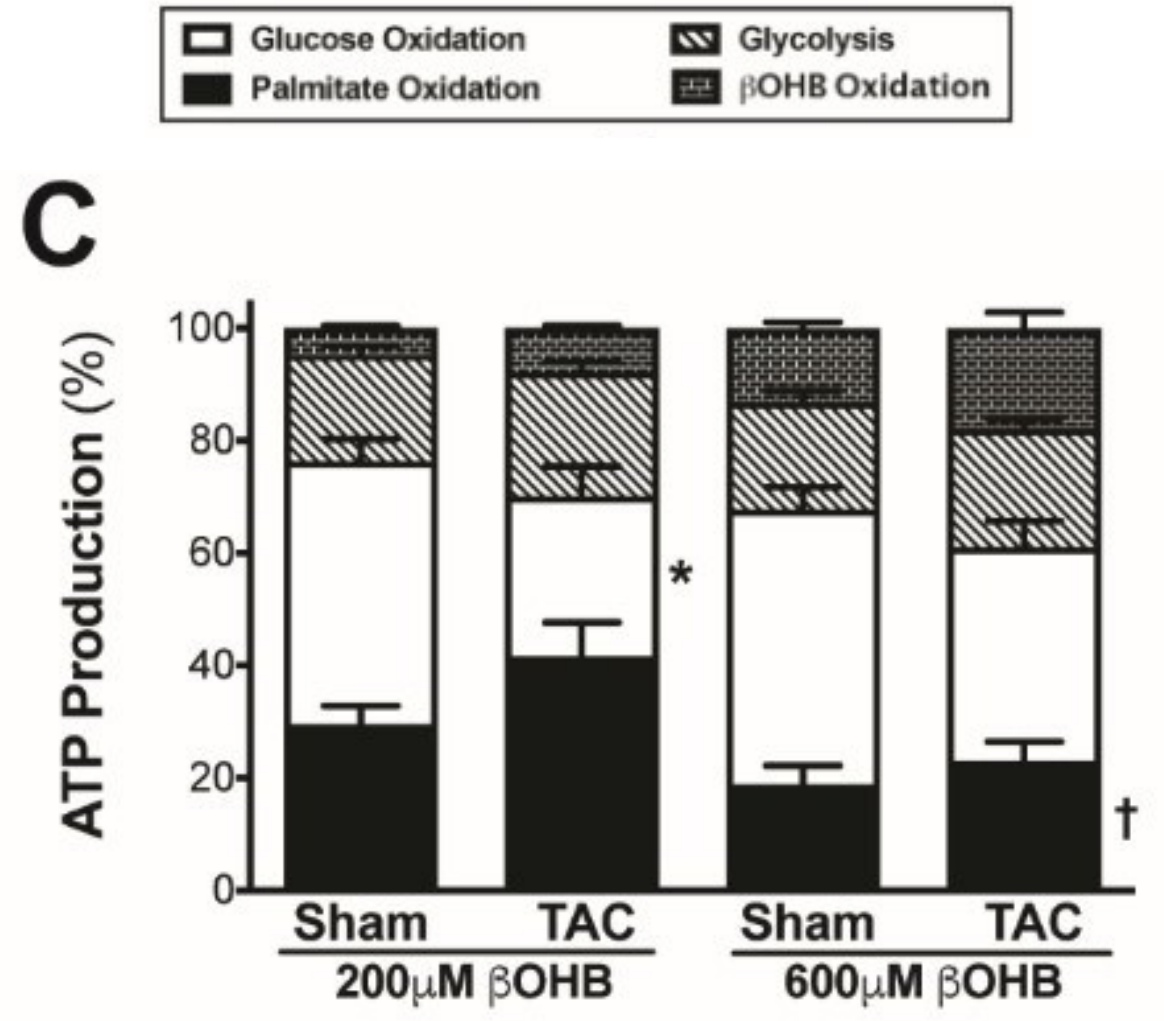

Glucose Oxidation
Palmitate Oxidation
Glycolysis
βOHB Oxidation
C
ATP Production (%)
100
80
60
40
20
0
*
†
Sham
TAC
Sham
TAC
200μM βOHB
600μM βOHB

FIGURE 3.5

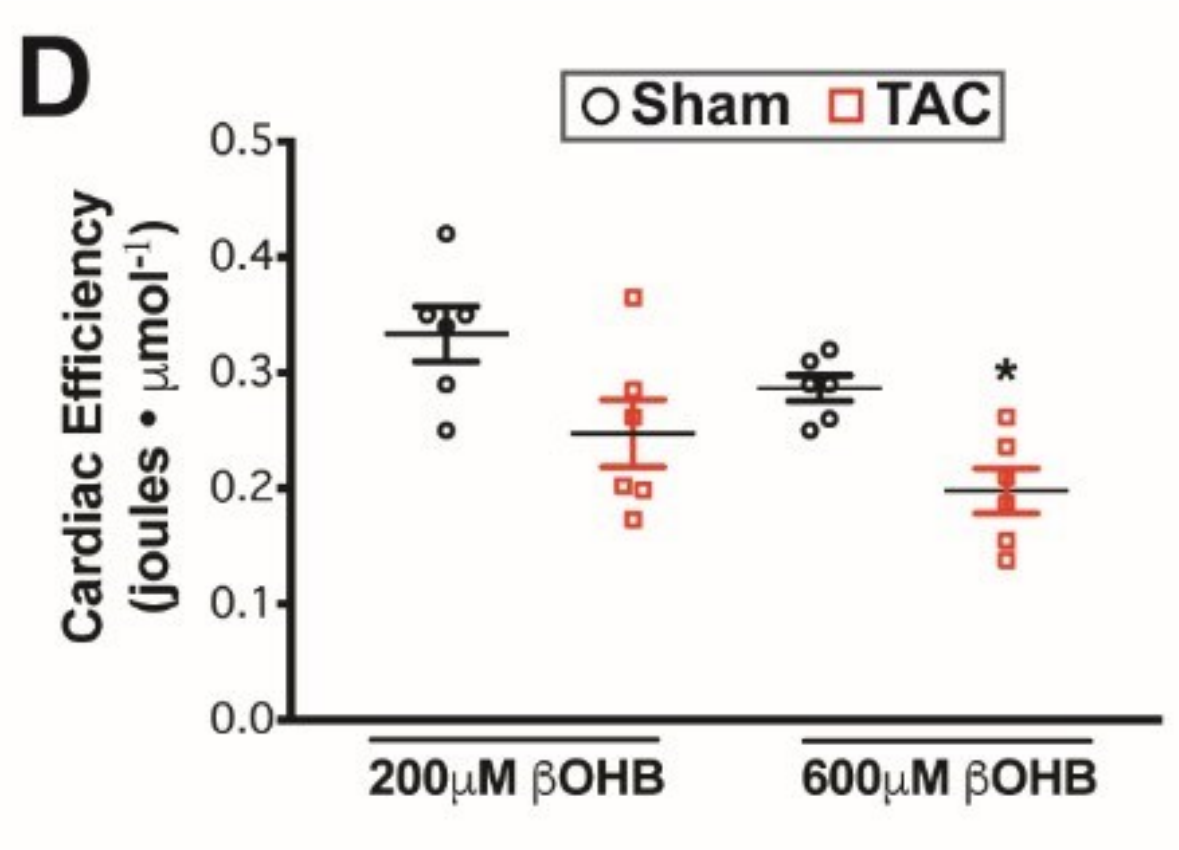

D
Sham
TAC
Cardiac Efficiency
(joules • μmol-1)
0.5
0.4
0.3
0.2
0.1
0.0
*
200μM βOHB
600μM βOHB

FIGURE 3.5

Figure 3.6 Protein expression levels of fatty acid and ketone body oxidative enzymes in the murine pressure overload failing heart. A, Protein expression of all metabolic enzymes assessed at 600μM ßOHB (Top to bottom): long–chain acyl CoA dehydrogenase (LCAD), ß-hydroxy-acyl-CoA-dehydrogenase (ßHAD), succinyl-CoA-3-oxaloacid CoA transferase (SCOT), ß-hydroxybutyrate dehydrogenase 1 (BDH1) and voltage-dependent anion channel (VDAC) (Multiple t tests with correction for multiple comparisons using the Bonferroni-Dunn method, n = 7 for all groups). All proteins were normalized to the loading control, VDAC. **B,** Overall protein lysine acetylation in sham versus TAC hearts at 600μM ßOHB (Unpaired two-tailed t-test, n = 7 for both groups) whereby the loading control used was VDAC. **C,** Assessment of long-chain acyl CoA dehydrogenase (LCAD) acetylation status in sham versus TAC hearts at 600μM ßOHB in which normalization was to the heavy chain of the antibody used to pull down all of the lysine-acetylated proteins (Unpaired two-tailed t-test, n = 4 for sham and 7 for TAC). Densitometric analysis is shown on the right for panels A-C. Sham groups are represented by white circles while TAC groups are represented by red squares. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group.

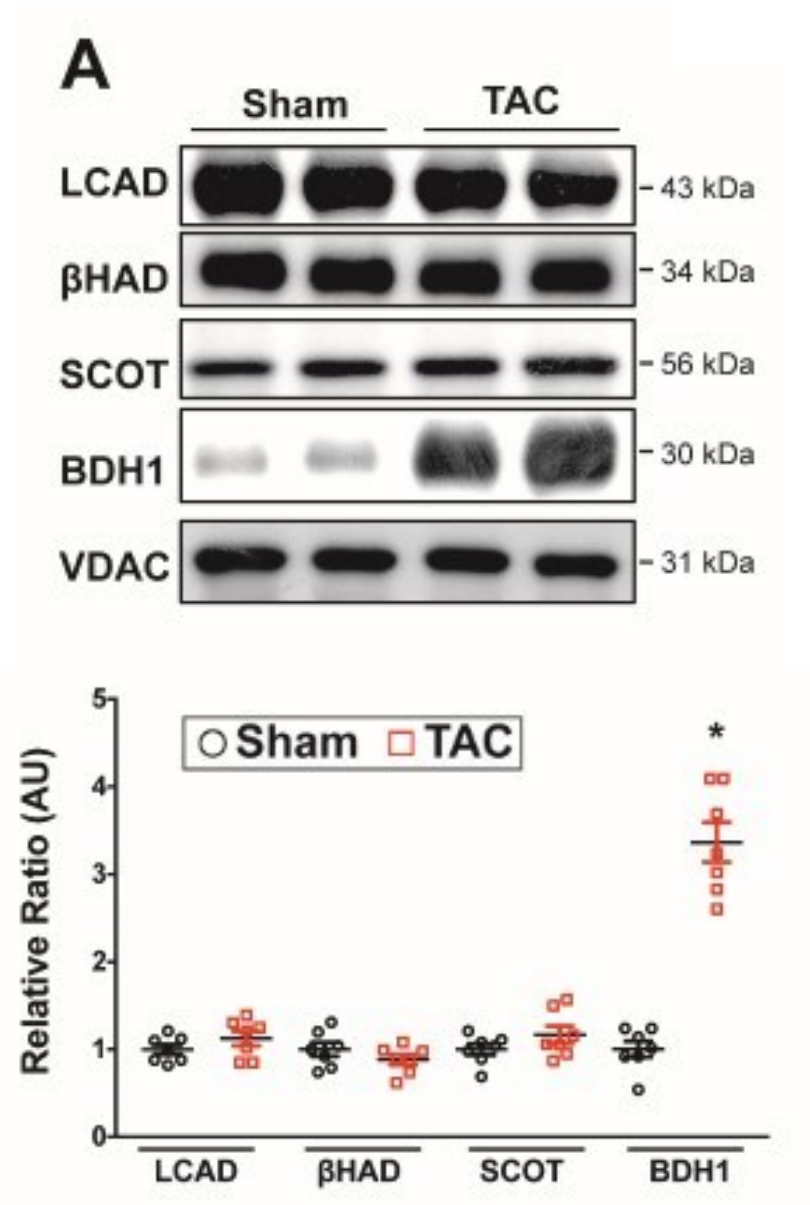

A
Sham
TAC
LCAD
βHAD
SCOT
BDH1
VDAC
43 kDa
34 kDa
56 kDa
30 kDa
31 kDa
Relative Ratio (AU)
Sham
TAC
*
LCAD
βHAD
SCOT
BDH1

FIGURE 3.6

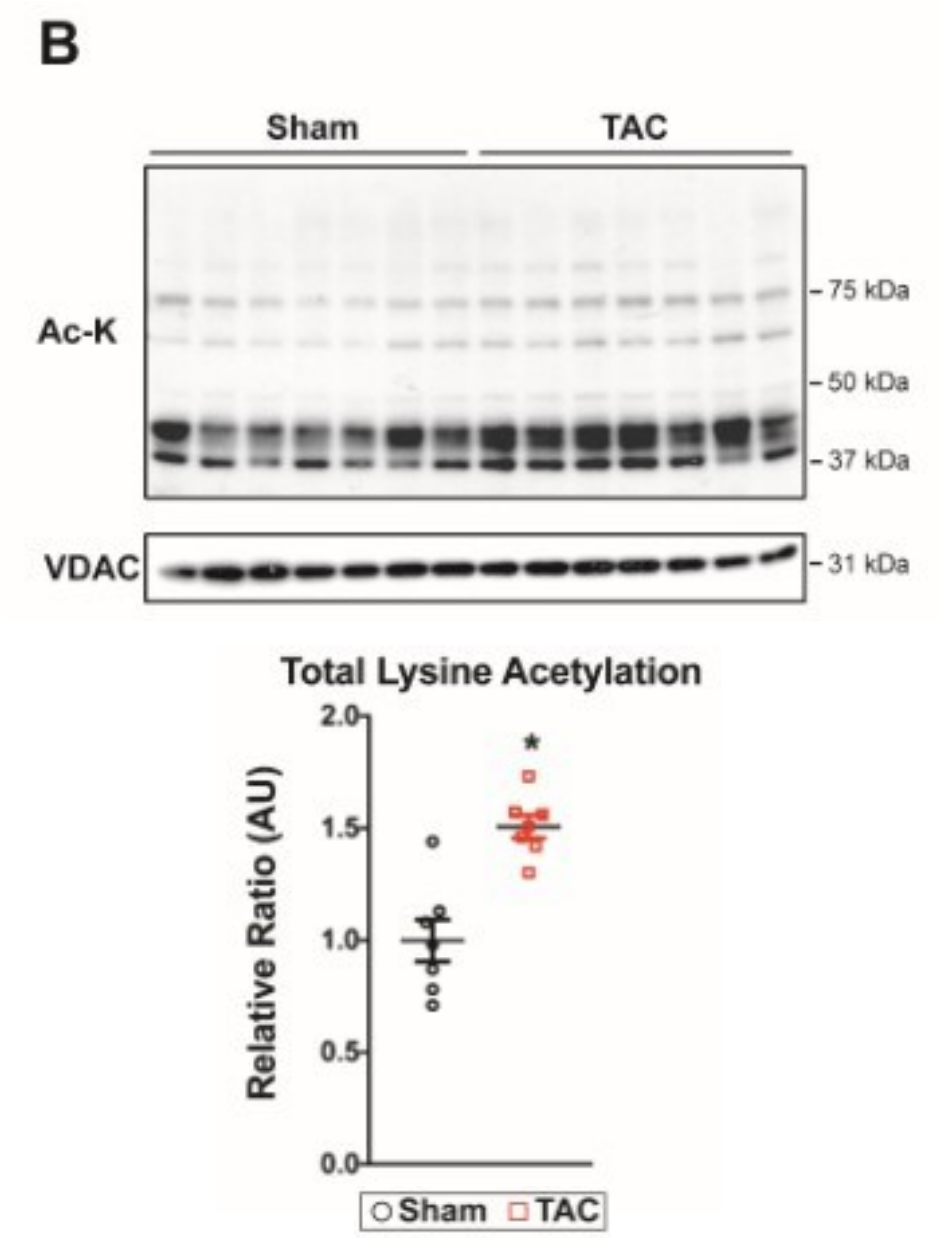

B
Sham
TAC
Ac-K
75 kDa
50 kDa
37 kDa
VDAC
31 kDa
Total Lysine Acetylation
Relative Ratio (AU)
2.0
1.5
1.0
0.5
0.0
Sham
TAC

FIGURE 3.6

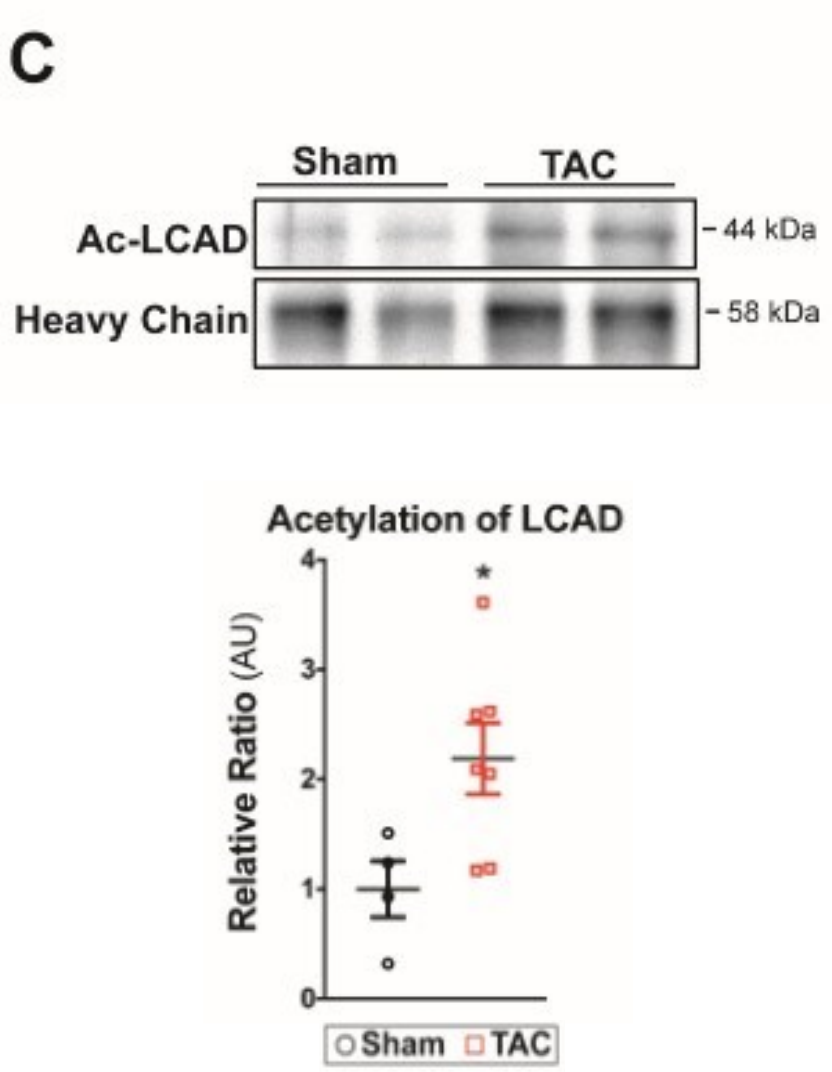

C
Sham
TAC
Ac-LCAD
Heavy Chain
44 kDa
58 kDa
Acetylation of LCAD
Relative Ratio (AU)
0
1
2
3
4
Sham
TAC

FIGURE 3.6

Figure 3.7 Summary diagram of the failing heart's increased reliance on ketone oxidation for ATP production. The percent contribution of ßOHB to total TCA acetyl CoA production in the sham heart **(A)** and TAC heart **(B)** at 200µM ßOHB, and the TAC heart at 600µM ßOHB **(C)**. The total ATP production decreases in TAC hearts (B) compared to sham hearts (A) but increasing ßOHB availability to the failing heart (C) increases its contribution to ATP production by more than 2-fold making it a significant substrate contributing to increased overall ATP production for the energy starved heart.

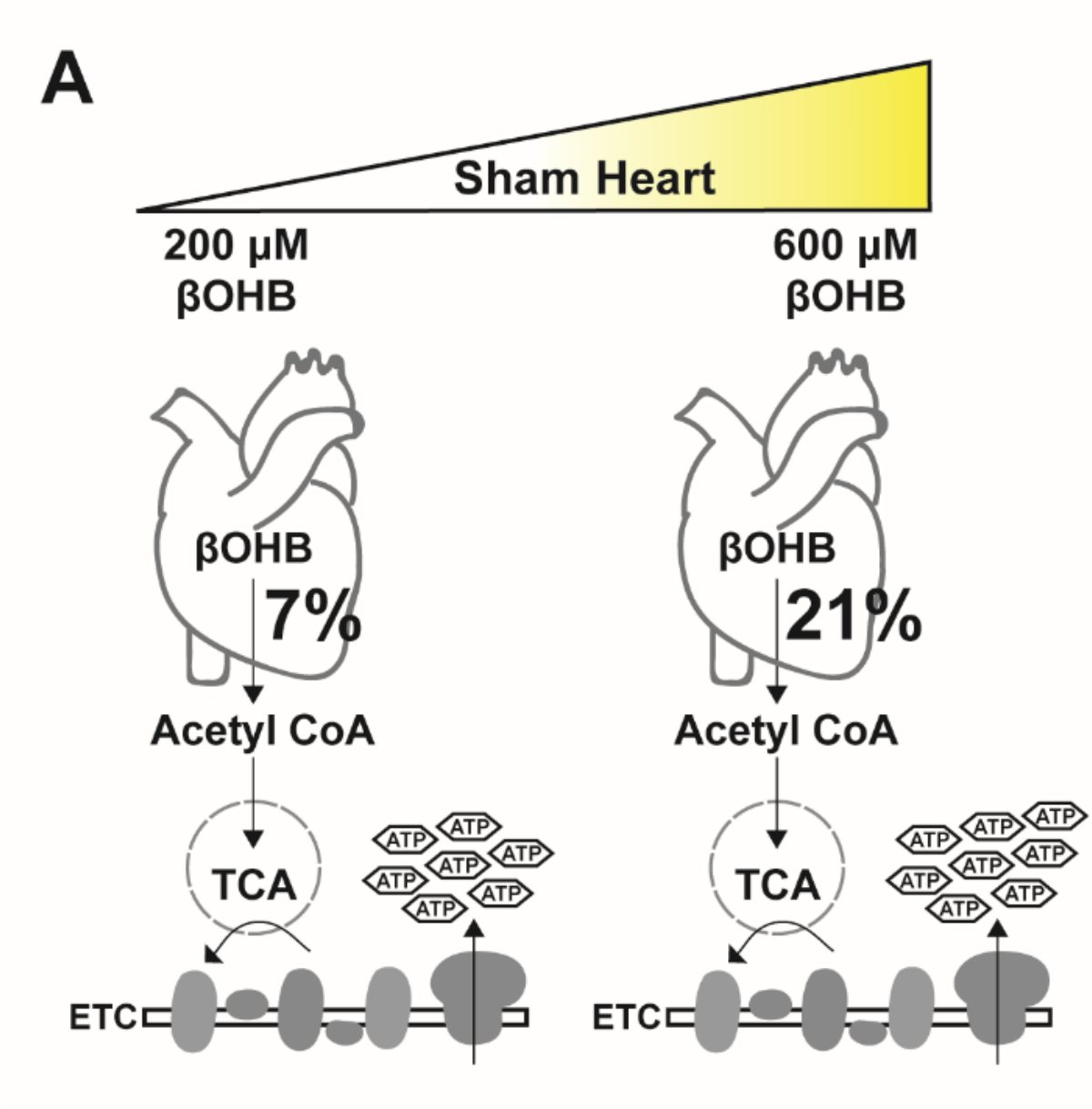

A
Sham Heart
200 μM
βOHB
600 μM
βOHB
βOHB
7%
βOHB
21%
Acetyl CoA
Acetyl CoA
TCA
TCA
ATP
ETC
ETC

FIGURE 3.7

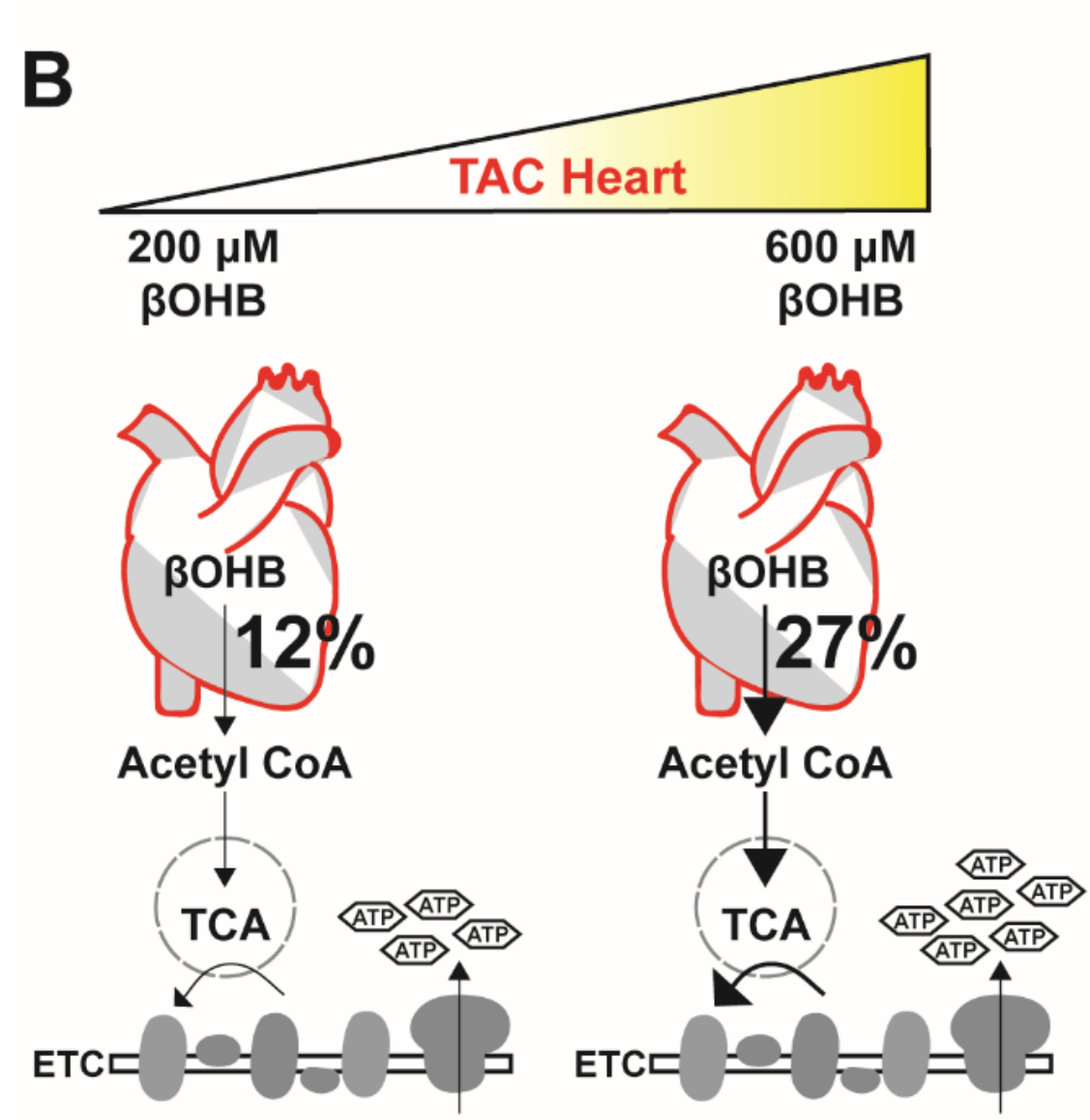

B
TAC Heart
200 µM
βOHB
600 µM
βOHB
βOHB
12%
Acetyl CoA
TCA
ATP
ATP
ATP
ATP
ETC
βOHB
27%
Acetyl CoA
TCA
ATP
ATP
ATP
ATP
ATP
ETC

FIGURE 3.7

Figure 3.8 Assessment of in vivo and ex vivo cardiac function and morphology in TAC and Sham C57Blk/6J mice. Echocardiographic assessment of corrected left ventricular mass (**A**), cardiac output (**B**), stroke volume (**C**), left ventricular internal diameter during diastole (**D**), left ventricular posterior wall during diastole (**E**) and mitral deceleration time (**F**). **G**, Ex vivo assessment of cardiac function in the isolated perfused heart by a measure of heart rate (HR) times peak systolic pressure (PSP) in which the concentration of ßOHB was increased from 200µM to 600µM avt 30 minutes (Multiple t tests with correction for multiple comparisons using the Bonferroni-Dunn method, n = 19 for sham, 22 for TAC). **A-F**, an unpaired two-tailed t test was performed and sham groups are represented by white circles while TAC groups are represented by red squares. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group.

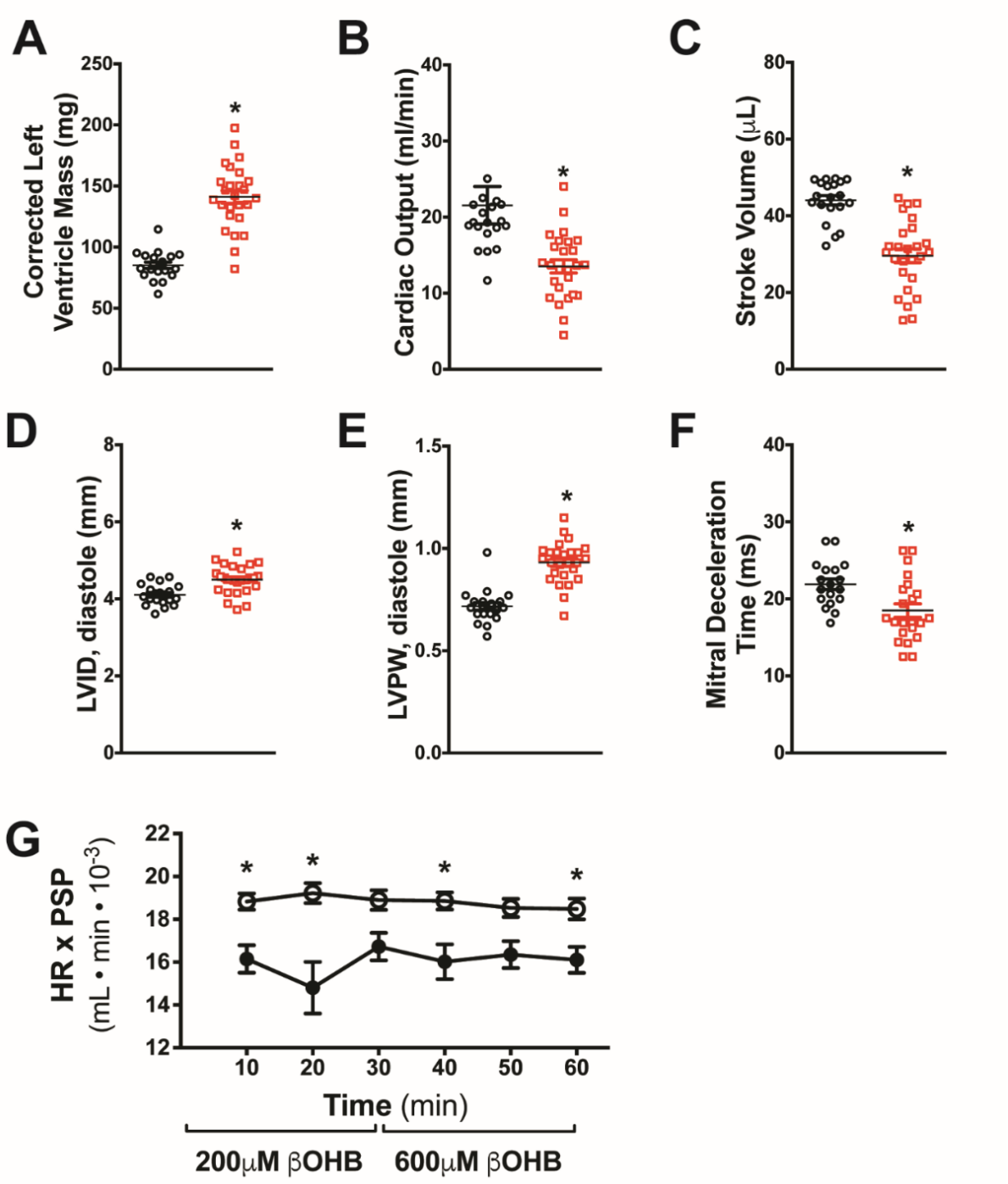

A
Corrected Left Ventricle Mass (mg)
250
200
150
100
50
0
*
B
Cardiac Output (ml/min)
40
30
20
10
0
*
C
Stroke Volume (µL)
80
60
40
20
0
*
D
LVID, diastole (mm)
8
6
4
2
0
*
E
LVPW, diastole (mm)
1.5
1.0
0.5
0.0
*
F
Mitral Deceleration Time (ms)
40
30
20
10
0
*
G
HR x PSP
(mL • min • 10-3)
22
20
18
16
14
12
*
*
*
*
10
20
30
40
50
60
Time (min)
200µM βOHB
600µM βOHB

FIGURE 3.8

Figure 3.9 Percent contribution of glucose oxidation, fatty acid oxidation and ketone oxidation to total TCA acetyl CoA production. A Two-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the sham group. †$p<0.05$ compared to the 200μM ßOHB group.

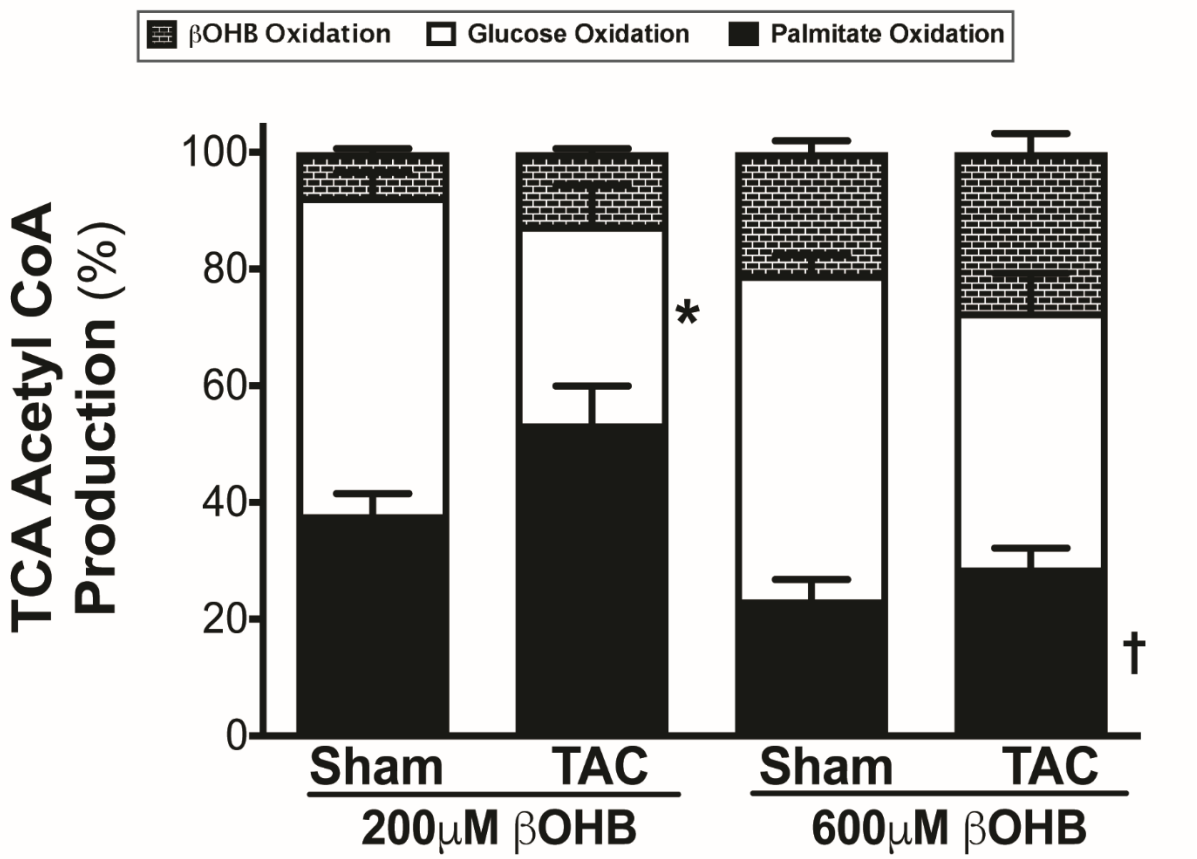

βOHB Oxidation
Glucose Oxidation
Palmitate Oxidation
TCA Acetyl CoA Production (%)
100
80
60
40
20
0
*
†
Sham
TAC
Sham
TAC
200μM βOHB
600μM βOHB

FIGURE 3.9

Figure 3.10 Assessment of the protein expression of proteins related to glucose oxidation and glucose uptake in sham and TAC hearts. Protein expression of (**A**) phosphorylated pyruvate dehydrogenase, the rate limiting enzyme of glucose oxidation, normalized to total pyruvate dehydrogenase protein expression, (**B**) GLUT1 and GLUT4 protein expression normalized to VDAC. Data are all expressed as relative ratio to the sham group and are expressed as mean ± SEM. *$p<0.05$ compared to the sham group.

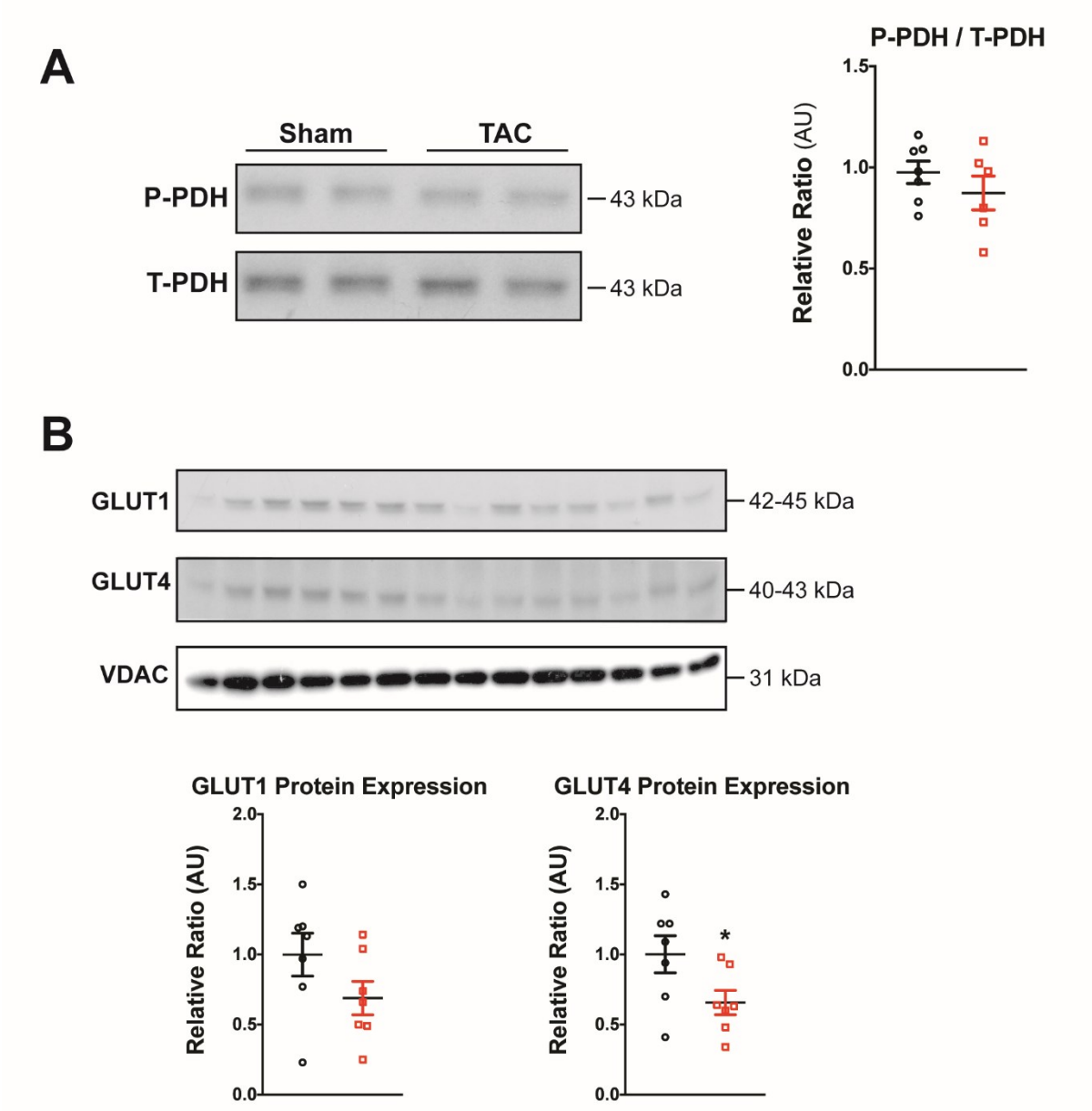

A
Sham
TAC
P-PDH
43 kDa
T-PDH
43 kDa
P-PDH / T-PDH
Relative Ratio (AU)
1.5
1.0
0.5
0.0
B
GLUT1
42-45 kDa
GLUT4
40-43 kDa
VDAC
31 kDa
GLUT1 Protein Expression
Relative Ratio (AU)
2.0
1.5
1.0
0.5
0.0
GLUT4 Protein Expression
Relative Ratio (AU)
2.0
1.5
1.0
0.5
0.0
*

FIGURE 3.10

Chapter IV
Ketones Are An Extra Source of Fuel For The Heart But Do Not Increase Cardiac Efficiency

A version of this chapter has been published in Cardiovascular Research (*Ho, K. L., *Karwi, Q.G., et al. (2021). " Ketones can become the major fuel source for the heart but do not increase cardiac efficiency." 117(4): 1178-1187.). All figures for this published study can be found at the end of the chapter. **Authors contributed equally to this work.*

Chapter 4 Ketones are an Extra Source of Fuel for the Heart but do not Increase Cardiac Efficiency

In this chapter, **Aim 2** is addressed and *ex vivo* cardiac function alongside the metabolic profile of a healthy heart are assessed in response to acute increases in ketone concentration.

We find evidence that ketones do not improve cardiac efficiency and are an extra source of fuel for the heart that do not compete with glucose or fatty acids.

4.1 Contribution Statement

My role in this project included performing all the experiments (except those noted below) alongside the writing of the manuscript. Dr. Qutuba Karwi, a co-first author on this manuscript, assisted in writing a portion of the 'Discussion' section of the manuscript as well as the production of Figure 4.4 in the published manuscript. *Ex vivo* isolated working heart perfusions were performed by Mr. Cory Wagg (Lab technician of Dr. Gary Lopaschuk's laboratory). HPLC analysis of CoA and nucleotides was performed by Ken Strynadka.

4.2 Abstract

4.2.1 Aims

Ketones have been proposed to be a "thrifty" fuel for the heart and increasing cardiac ketone oxidation can be cardioprotective. However, it is unclear how much ketone oxidation can contribute to ATP production in the heart, nor whether increasing ketone oxidation increases cardiac efficiency. Therefore, our goal was to determine to what extent high levels of the ketone body, β-hydroxybutyrate (βOHB), contributes to cardiac ATP production, and whether this influences cardiac efficiency.

4.2.2 Methods and Results

Isolated working mice hearts were aerobically perfused with palmitate (0.8mM or 1.2mM), glucose (5mM) and increasing concentrations of βOHB (0, 0.6, 2.0mM). Subsequently, oxidation of these substrates, cardiac function and cardiac efficiency were assessed. Increasing βOHB concentrations increased myocardial ketone oxidation rates without affecting glucose or fatty acid oxidation rates. Increasing ketone concentration resulted in increased: ketone oxidation rates, TCA cycle activity, reduced equivalents and myocardial oxygen consumption rates. However, the marked increase in ketone oxidation at high concentrations of βOHB was not accompanied by an increase in cardiac work, suggesting that mitochondrial uncoupling was increased in the presence of excess reduced equivalent production from ketone oxidation. Consequently, cardiac efficiency decreased when the heart was exposed to higher ketone levels.

4.2.3 Conclusions

We demonstrate that while ketones can become an extra source of fuel for the heart that do not compete with glucose or fatty acids, they do not increase cardiac efficiency, which underscores the concept that ketones are not a ‘superfuel’.

4.3 Introduction

The heart is the most metabolically demanding organ in the body[139]. To sustain contractile function, the heart meets its high demand for energy (adenosine triphosphate (ATP)) by metabolizing an array of fuels – fatty acids, glucose, lactate, ketones, and amino acids[12,107]. A healthy, insulin-sensitive heart is metabolically versatile and can dynamically adapt to various physiological states (fed vs. fasted) by shifting its reliance primarily between fatty acids and glucose[2]. For example, during a post-prandial state, increases in circulating insulin levels prime the heart to use glucose for energy[2]. Conversely, in a fasted state where circulating levels of blood glucose are low, the heart increases its reliance on fatty acids for energy[2]. Maintenance of this cardiac 'metabolic flexibility' (shifting fuel use from fatty acids to glucose and *vice versa*) is also attributed to the Randle cycle phenomenon, whereby fatty acid and glucose metabolism are reciprocally regulated[128]. Loss of this metabolic flexibility, such as that seen in diabetes and heart failure, can lead to perturbed cardiac metabolic profiles and contractile dysfunction[140,141].

Ketones are a source of energy that our bodies primarily use during fasting/starvation[127], though ketones are also a prominent fuel source during consumption of a ketogenic diet[142], prolonged exercise[143], or in poorly controlled diabetics[144]. There are three different types of ketone bodies: β-hydroxybutyrate (βOHB), acetoacetate and acetone[124], with the predominant ketone body found in our circulation being βOHB[145]. While most studies have focused on the involvement of fatty acids and carbohydrates in cardiac intermediary energy metabolism, there has recently been a growing appreciation for ketones as an important cardiac fuel source[3]. This has become increasingly apparent in light of the proposed concept that ketones are a potential 'super-fuel' for the heart in a diabetic setting[4,5]. The notion that ketones

are a ‘super-fuel’ was suggested following the demonstration that empagliflozin, an SGLT2 inhibitor used to treat type 2 diabetes, exhibited profound cardioprotective effects in diabetic patients with high cardiovascular risk[3]. It has been proposed that empagliflozin-mediated increases in ketone levels could explain the cardioprotection seen in these patients by improvements in the cardiac metabolic state[4,5], specifically an increase in cardiac efficiency. However, we have shown that neither ketones nor empagliflozin improve cardiac efficiency in diabetic mice[105]. Ketones have also been brought to the forefront in the setting of heart failure where we, alongside several other labs, have shown that the failing heart has increased reliance on ketones as a fuel source[6,7,16,146,147].

The heart can be exposed to increased ketone concentrations under numerous physiological (fasting, exercise), supra-physiological (administering ketone esters, SGLT2 inhibitors, or a ketogenic diet) and pathological states (heart failure, uncontrolled diabetes). However, it is not clear to what extent ketones can be used as a source of energy by the heart, what effect increased ketone concentrations have on the metabolism of other cardiac energy substrates, or what effect increased ketones directly have on cardiac efficiency. In this study, we investigated what effect increasing ketone concentrations, specifically βOHB, has on cardiac energetics, to definitively underline its effects on cardiac metabolism, function and efficiency in the healthy heart.

4.4 Methods

Male C57BL/6J mice, aged 12 weeks, were anesthetized and hearts were excised for isolated working heart perfusions. Following perfusion, hearts were snap-frozen and used for biochemical analysis.

Unless detailed below, the experimental protocols utilized for this study can be found in Chapter 2. Specific isolated working heart perfusion conditions for this project are as follows.

4.4.1 Perfusion Conditions

Isolated working hearts from C57BL/6J male mice were perfused aerobically for 60-minutes, with either a low or high concentration of radiolabeled palmitate pre-bound to 3% albumin (low, 0.8mM or high, 1.2mM), glucose (5mM) and three increasing concentrations of βOHB (0, 0.6, and 2.0mM). The βOHB levels used are representative of βOHB concentrations that can acutely change under physiological conditions (fasting, intense exercise) and supra-physiological conditions (ex. with a ketone ester[148]) of healthy hearts with an intact insulin axis. Moreover, all hearts were perfused with and without insulin to assess βOHB's effect on cardiac insulin sensitivity. Alongside *ex vivo* cardiac function, oxidation of these substrates was measured as previously described[146]. In light of the pressing question of whether ketones can improve cardiac efficiency, we also measured cardiac efficiency by cannulating the pulmonary artery, and oxygen concentrations difference in the buffer were measured between the left atria and the cannulated pulmonary artery.

4.4.2 Statistical Analysis

Data is presented throughout as the mean ± SEM. Significant differences were determined by using an unpaired, two-tailed Student's t-test or a two-way ANOVA when comparing more than two means followed by a Bonferroni post hoc test. Statistical analysis was carried out using commercially available software (GraphPad Prism V7). Differences were deemed statistically significant when $P < 0.05$.

4.5 Results

4.5.1 Ketones are an unregulated fuel source in the heart and are oxidized in proportion to their availability independent of the Randle Cycle

Circulating ketone levels have the capacity to significantly increase under various physiological and pathophysiological conditions[149]. Using methodology we developed to directly measure ketone oxidation in the heart[146], we subjected isolated working mice hearts to increasing concentrations of βOHB that were physiologically relevant to a mildly ketotic (0.6mM) and a higher ketotic state (2.0mM)[149]. Furthermore, to mimic a ketogenic condition (high fat), we perfused hearts at two concentrations of palmitate, 0.8 and 1.2mM[150]. This was also an important and intentional aspect of the study because physiologically speaking, one would not see high blood ketone levels unless high fat was available[151]. Increasing βOHB concentrations increased myocardial ketone oxidation rates in a first-order kinetics-manner (Figure 4.1 A,E), agreeably with previous findings from Sultan and colleagues[152].

As expected, increasing the fatty acid concentration to 1.2mM palmitate resulted in elevated cardiac palmitate oxidation rates (Figure 4.1 F) compared to 0.8mM palmitate (Figure 4.1 B), although this increase in palmitate concentration did not affect βOHB oxidation rates (Figure 4.1 E vs. A). However, glucose oxidation rates were lower at 1.2mM palmitate (Figure 4.1 G) than at 0.8mM palmitate (Figure 4.1 C), consistent with classical Randle cycle regulation[128]. The addition of insulin in these hearts also confirmed that these hearts were insulin sensitive as seen by significant increases in glucose oxidation rates (Figure 4.1 C, G) and decreases in palmitate oxidation rates (Figure 4.1 B, F). It should also be noted that our cardiac energy metabolism profiles in the presence of low/high βOHB or palmitate

concentrations were independent of changes in the content of short chain CoA esters (e.g. succinyl CoA, malonyl CoA, acetyl CoA, etc.) (Figure 4.5).

Surprisingly, despite an intact Randle cycle and cardiac metabolic flexibility in response to insulin, increasing βOHB to 2.0mM (which markedly increased ketone oxidation rates) had no effect on glucose oxidation rates (Figure 4.1 C, 1G), although there was a trend to a decrease glucose oxidation rates at 2.0mM but did not reach the threshold of statistical significance ($p>0.05$). Glycolysis rates were also unaffected by increasing βOHB concentrations (Figure 4.1 D,H). Palmitate oxidation rates were also unaffected by increasing concentrations of βOHB at 0.8mM palmitate (Figure 4.1 B), but at 1.2mM palmitate, 2.0mM βOHB did have an inhibitory effect on palmitate oxidation rates (Figure 4.1 F). Since endogenous triacylglycerol (TAG) turnover is a potential source of fatty acids for β-oxidation, we also measured the TAG content in the hearts at the end of the perfusion (Figure 4.6 A,D), as well as radiolabelled fatty acid content of palmitate in myocardial TAG (Figure 4.6 B,E). No changes in TAG content or the radiolabelled content in TAG was seen with high ketones, suggesting that high ketone concentrations were not affecting TAG contribution of fatty acids for β-oxidation.

Since cardiac work is an important determinant of the heart's oxidative capacity and oxidative rates[118,119], we normalized all absolute metabolic rates (Figure 4.1) to cardiac work (Figure 4.7) and found no significant differences between absolute and normalized metabolic rates.

While glucose oxidation and fatty acid oxidation are reciprocally regulated via the Randle cycle, myocardial ketone oxidation appears to be unregulated by this cycle, and

consequently the heart has a powerful capacity to increase the oxidation of ketones in proportion to its availability.

4.5.2 Increasing ketone concentrations markedly increases cardiac TCA cycle activity and ketones are an extra source of fuel for the heart

Since our isolated working heart perfusion method of assessing cardiac energy metabolism allows for direct quantification of acetyl CoA entering the TCA cycle, the consequence of increasing βOHB concentration's on TCA cycle activity was directly assessed. Increasing βOHB concentration to 2.0mM resulted in an increase in total TCA cycle activity, with βOHB oxidation having a significant contribution of acetyl CoA for the TCA cycle at both 0.8mM and 1.2mM palmitate (Figure 4.2 A,F). This robust increase in myocardial ketone oxidation rates at higher ketone concentrations (Figure 4.1 A,E) were translated into increases in TCA cycle activity and NADH + $FADH_2$ production (Figure 4.2 B,G).

Myocardial oxygen consumption was also assessed throughout the heart perfusions. Increasing βOHB concentration to 2.0mM resulted in a significant increase in oxygen consumption at 0.8mM palmitate (Figure 4.2 C) and a trend to also increase oxygen consumption at 1.2mM palmitate (Figure 4.2 H). However, while myocardial oxygen consumption proportionally increased alongside the increased TCA cycle activity (Figure 4.2 A,F) and NADH + $FADH_2$ production (Figure 4.2 B,G), this was not translated into changes in cardiac ATP levels (Figure 4.2 D,I) or cardiac work (Figure 4.2 E,J). As a result, βOHB-mediated TCA cycle activity increases in reducing equivalents were accompanied by increases in myocardial oxygen consumption but did not translate into increases in cardiac ATP content or cardiac function.

4.5.3 Increasing ketone concentrations decreases cardiac efficiency

Since significant increases in myocardial ketone oxidation rates, TCA cycle activity and NADH + $FADH_2$ production were uncoupled from changes in cardiac work, we analyzed cardiac efficiency. Cardiac efficiency was determined by two ways: cardiac work normalized to total TCA cycle activity and cardiac work normalized to oxygen consumption. As βOHB concentration increased, cardiac efficiency significantly decreased (Figure 4.3 A,B) at 0.8mM palmitate and trended to decrease at 1.2mM palmitate (Figure 4.3 C,D). This shows that while the heart can readily oxidize ketones, it is not a more efficient source of fuel.

4.6 Discussion

This study provides several important novel observations. **First,** through direct measurement of myocardial ketone oxidation rates and competing energy substrate rates, we found that ketones can be an extra source of fuel for the healthy heart. **Second**: Our experimental model is novel in that it allows for the first time measurement of the coupling of myocardial NADH and $FADH_2$ production and oxygen consumption to contractile function in the heart. **Third,** while our study confirms that glucose and fatty acid metabolism are reciprocally regulated, ketones had no inhibitory effect on glucose oxidation and its oxidation increases in an unregulated manner proportional to its availability. **Fourth,** through direct measurement of reducing equivalent production rates simultaneously with cardiac oxygen consumption and cardiac work, we found that increasing ketone availability to the heart was not accompanied by changes in cardiac function despite the heart's high capacity to oxidize ketones. As a result, we show that increasing ketone availability to the heart decreases cardiac efficiency, challenging the notion that ketones are a "thrifty fuel" for the heart. **Fifth,** we demonstrate that when the heart is provided with an excess of energy substrates, such as high ketones, the enhanced supply of reducing equivalents becomes uncoupled from ATP production rates, leading to a decrease in cardiac efficiency.

Despite a growing and overwhelming interest in the role of ketones in the failing and diabetic heart, no previous study has directly assessed the actual contribution of ketones to cardiac energy metabolism. The importance of this is further implicated when considering that many scenarios can physiologically, supra-physiologically and pathologically increase circulating ketone levels and thus, its availability to the heart. Ketones have also been proposed to be a "thrifty fuel" or "super fuel" for the heart[4,5], although we have previously

challenged this concept[13]. In this study, we demonstrate that the heart can oxidize ketones in proportion to its availability. Of importance, we also show that despite marked increases in the contribution of ketone oxidation to TCA cycle activity at high concentrations of ketones, this was not accompanied by decreases in the contribution of glucose oxidation or fatty acid oxidation to TCA cycle activity. This could have important implications in the setting of heart failure, where providing ketones could be a potential extra source of energy for a starved heart[78], without the concern that other sources of ATP production would be inhibited. A previous study by Stanley et al. [117] investigated the impact of co-infusion of βOHB with fat emulsion on myocardial fatty acid uptake *in vivo* using an anesthetized pig model. An important point of this study is that the measurement of myocardial fatty acid oxidation was based on the assumption that fatty acid uptake equals fatty acid oxidation. However, a growing body of evidence has shown that this is not always the case and that enhanced myocardial uptake of fatty acid does not always mean it is going to be oxidized by the heart since it can be accumulated in the myocardium or stored at triacylglycerol. Considering that neither cardiac ATP nor triacylglycerol were measured in that study, it is not clear how these changes in myocardial uptake fatty acid and βOHB actually influenced cardiac fatty acid and βOHB oxidation, cardiac ATP levels or endogenous stores of fatty acids in the heart. However, the Stanley et al. [117] study does supports the novel findings of our submitted study that ketone's contribution to cardiac ATP production can be increased by enhancing circulating levels of ketone with no inhibitory effect on cardiac glucose oxidation or no changes to cardiac function.

We also demonstrate that ketones are not a more efficient energy substrate compared to other carbon substrates, and that increases in ketone oxidation in the healthy heart are accompanied by decreases in cardiac efficiency. Increasing ketone concentrations in the

healthy heart had no impact on cardiac function despite the fact that both TCA cycle activity and oxygen consumption increased, the result of which was an actual decrease in cardiac efficiency. In the failing heart, we showed that the increase in TCA cycle and oxygen consumption seen with increasing ketone concentrations is matched by an increase in cardiac work, resulting in no change in cardiac efficiency[146]. Combined, these results suggest that ketones are an extra source of fuel for the heart that do not increase cardiac efficiency.

Through reciprocal regulation of glucose and palmitate oxidation, one would expect that an increase in ketone oxidation, as with increased fatty acid oxidation, would result in competition for acetyl CoA supply for the TCA cycle. However, to our surprise, significantly increasing ketone oxidation did not result in any form of glucose oxidation inhibition, and palmitate oxidation was only inhibited at 1.2mM palmitate by 2.0mM βOHB. This suggests that ketones are not subjected to the classic Randle Cycle phenomena that glucose and fatty acids are. Indeed, while raising fatty acid concentrations significantly decreased glucose oxidation as expected (Figure 4.1 C,G), both in the presence or absence of insulin, raising ketone concentrations did not alter glucose oxidation rates. Interestingly, we found that increasing βOHB or palmitate concentration had no effect on the phosphorylation of pyruvate dehydrogenase, a key 'feedback inhibited' enzyme that contributes to the interplay between glucose and palmitate oxidation (Figure 4.9). Regardless, the fact that ketones had no effect on glucose oxidation or the phosphorylation of its rate-limiting enzyme (pyruvate dehydrogenase), has potential significance in the setting of heart failure, where stimulating glucose oxidation can benefit the failing heart[153]. It suggests that increasing ketone oxidation may not compromise glucose oxidation, and therefore heart function, in the failing heart. Our findings, while in a healthy setting, are not consistent with a recent study claiming that the

failing myocardium treated with empagliflozin (increases circulating ketone levels) switches its fuel use away from glucose to ketones. Their findings suggest that ketones decrease glucose use and this results in an increase in cardiac efficiency. However, the authors do not measure glucose oxidation and only measure glucose uptake[76]. Therefore, further studies are still needed to directly assess the interaction between ketone oxidation and glucose oxidation in the failing heart.

We also demonstrate that the main determinant of how much ketones are used as an energy source by the heart is the concentration of ketones to which the heart is exposed. Insulin is an important regulator of energy metabolism in the heart, with increasing insulin concentrations resulting in an increase in glucose oxidation (Figure 4.1 C,G) and a decrease in fatty acid oxidation (Figure 4.1 B,F)[107,154]. In contrast, at lower concentrations of ketones, insulin had no effect on ketone oxidation rates (Figure 4.1 A)[77,152]. Interestingly, insulin did have a mild stimulatory effect on ketone oxidation at high ketone concentrations (Figure 4.1 A,B), although the mechanism by which this occurred is not clear.

The consequence of an unregulated and increased reliance on ketones for energy ultimately resulted in depressed cardiac efficiency (Figure 4.3). Through the metabolism of glucose, palmitate and βOHB, the hydrogen carriers NAD^+ and FAD are reduced to NADH and $FADH_2$, respectively. The reduced equivalents then carry the hydrogens to the electron transport chain where they are used to reduce oxygen to water. Their oxidation also results in proton pumping and the subsequent protonmotive force which is used for ATP synthesis. We found that high βOHB causes an excess supply of NADH and $FADH_2$ that was directly measured for the first time in this study. Interestingly, this excess supply was not accompanied with an increase in ATP levels (Figure 4.2 I) or cardiac function (Figure 4.2 J). We also found

that there was an increase in myocardial oxygen consumption in the high βOHB perfused hearts, which supports an increase in electron transport chain (ETC) activity that accompanied the increase in NADH and $FADH_2$ supply. Interestingly, this increase in oxygen consumption was not accompanied by an increase or a decrease in cardiac work, suggesting there is no change in ATP use by the contractile proteins. Together, there are two possibilities that may explain this disassociation between the excess supply of reducing equivalents and no change in cardiac ATP levels. One possibility is that ATP production increased in the presence of high levels of βOHB, but that high ßOHB's are decreasing the efficiency of the contractile proteins causing an excessive ATP hydrolysis. The second possibility is that the increase in ETC activity, evidenced by an increase supply of NADH and $FADH_2$ and oxygen consumption, becomes uncoupled from ATP production. However, we believe the first possibility is unlikely, and, to the best of our knowledge, there is no evidence in the literature that supports this hypothesis. Cardiac work, which is the major consumer of ATP in the heart, did not change in the presence of excess ßOHB levels, and there no evidence in the literature that ketones decrease the efficiency of the contractile proteins. As a result, this suggests that excess NADH and $FADH_2$ supply from the high ketone oxidation rates became uncoupled from ATP production in the ETC. In line with our findings, perfusing rat hearts with high levels of lactate (8mM) and palmitate (1.2mM) also causes an excessive supply of reducing equivalents, with no effect on the heart's contractile function[155]. Therefore, it seems plausible to suggest that the heart is equipped with a machinery to uncouple reduced equivalent supply from ATP production to handle the excess supply of energy in times of "super plenty" (excessive supply of reducing equivalents).

While mitochondrial uncoupling has been well described [156], it is still unclear exactly how this occurs. A likely scenario is that the protonmotive force that normally drives ATP production is reduced by allowing H^+ to flow to the mitochondrial matrix, instead of building up in the mitochondrial intermembrane space. Uncoupling proteins (UCPs) are mitochondrial inner membrane proteins that are regulated proton channels. UCPs are thus capable of dissipating the proton gradient generated by reducing equivalents-powered pumping of protons between mitochondrial intermembrane space and the mitochondrial matrix. UCPs have also been implicated in regulating the mitochondrial membrane potential by channeling H^+ to the mitochondrial matrix. UCP 2 and 3 are present in the heart mitochondria and are classically known for their roles in thermogenesis and regulation of mitochondrial production of reactive oxygen species, energy expenditure and obesity[157-159]. The role of UCPs in the healthy heart is not likely to be to promote gross thermogenesis, since the heart is not a 'thermogenic organ' like brown adipose tissue. Instead, UCP2 and UCP3's roles in fatty acid export and attenuation of mitochondrial reactive oxygen species are thought to be important in protecting the heart in pathological conditions and can affect cardiac efficiency [160,161]. Taken together, UCPs are potential candidates that can allow H^+ to flow to the mitochondrial matrix to decrease the protonmotive force and consequently ATP production in times of super plenty such as high ketone supply to the heart (Figure 4.4). In a number of conditions expression of UCPs increases under conditions suggestive of mitochondrial uncoupling [160,162,163]. However, in our study, increasing ßOHB supply resulted in an immediate increase in reduced equivalent supply, suggesting that an increased expression of UCPs could not explain the proposed uncoupling that we observed. The possibility exists that excess ketone supply could directly affect the activity of the UCPs, although this has yet to be determined.

Another potential candidate contributing to uncoupling is the mitochondrial permeability transition pore (PTP). PTP is a non-selective channel between the inner membrane and mitochondrial matrix that allows the passage of molecules <1.5 kDa including H^+ [164-166], and it regulates mitochondrial membrane potential [167,168]. Although still controversial, recent studies have suggested that PTP could be an uncoupling channel within the c-subunit ring of the mitochondrial ATP synthase (Complex V) that allows H^+ to flow to the mitochondrial matrix in time of stress [169,170]. While ATP synthase normally functions as a H^+ pore that couples proton motive force to ATP synthesis, this H^+ flux may theoretically become uncoupled from ATP synthesis. It is possible that ATP synthase allows H^+ to flow from the intermembrane space to the mitochondrial matrix uncoupled from ATP synthesis in time of super plenty, such as excess ketone oxidation rates. This is an exciting area to be explored in future investigations.

These proposed mechanisms do not exclude the possibility that there could be another undefined candidate(s) which can be responsible for mitigating the sudden supraphysiological supply of reducing equivalents due to an unregulated fuel source (such as ketones) when there is no increase in cardiac work demand. The fact that cardiac work neither increased nor decreased with the addition of ketones suggests that the increases in the oxidation of this excess supply of reducing equivalents resulted in a subsequent "mild" uncoupling in which there was a lowered protonmotive force, but ATP synthesis was still normal. As the law of thermodynamics states that energy cannot be created or destroyed [171], the "mild" decrease of this protonmotive force is likely dissipated as heat (Figure 4.4).

While our study looked at the acute effects of βOHB on cardiac metabolism, function and efficiency, it is important to note that scenarios that result in chronic elevations in ketone

levels (as with a ketogenic diet or long-term ketone ester consumption) introduce another layer of complexity due to eventual physiological compensatory adaptations in transcriptional machinery as previously reported by Wentz and colleagues[172]. Instead, the acute design of our study allows for us to definitively assess the effect of ketones on cardiac energetics independent of transcriptional compensatory changes. Therefore, regardless of what ketones are doing acutely or chronically, the direct effect of βOHB on cardiac efficiency still stands – it does not improve cardiac efficiency.

To conclude, we present novel data in an animal model showing that βOHB is oxidized in an unregulated manner proportional to its delivery. Importantly, increasing βOHB concentration resulted in marked increases to TCA cycle activity without any significant decrease in glucose oxidation or palmitate oxidation. As a result, increasing βOHB led to an accumulation of reduced equivalents, and increases in myocardial oxygen consumption, all of which were uncoupled from ATP production and cardiac function, ultimately decreasing cardiac efficiency. Our findings not only indicate that acute ketotic milieus can significantly impact cardiac energetics, but also underscore the importance of appreciating ketones as an extra fuel source for the heart that do not exhibit substrate competition or increase cardiac efficiency.

4.7 Figures

Figure 4.1 The metabolic profile of the healthy heart perfused at increasing concentrations of βOHB in the presence of 0.8mM or 1.2mM palmitate. A, E, βOHB (ketone body) oxidation (n=4-7). **B, F,** Palmitate (fatty acid) oxidation (n=3-7). **C, G,** Glucose oxidation (n=6-7). **D, H,** Glycolysis (n=5-9). Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group except for Figure 1A where *$p<0.05$ compared to the 0.6mM βOHB. †$p<0.05$ compared to the without insulin group.

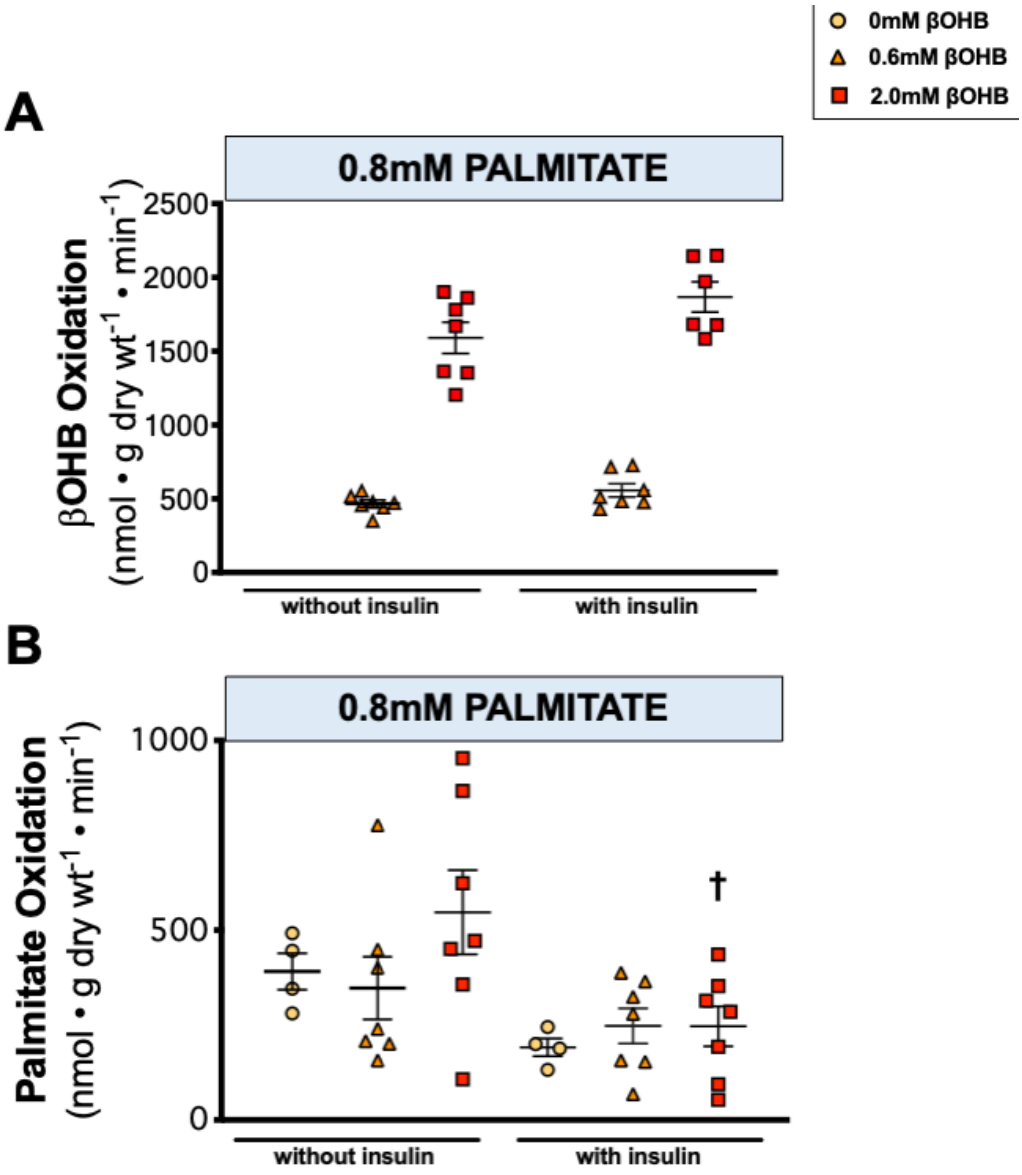

0mM βOHB
0.6mM βOHB
2.0mM βOHB
A
0.8mM PALMITATE
βOHB Oxidation
(nmol • g dry wt-1 • min-1)
2500
2000
1500
1000
500
0
without insulin
with insulin
B
0.8mM PALMITATE
Palmitate Oxidation
(nmol • g dry wt-1 • min-1)
1000
500
0
†
without insulin
with insulin

FIGURE 4.1

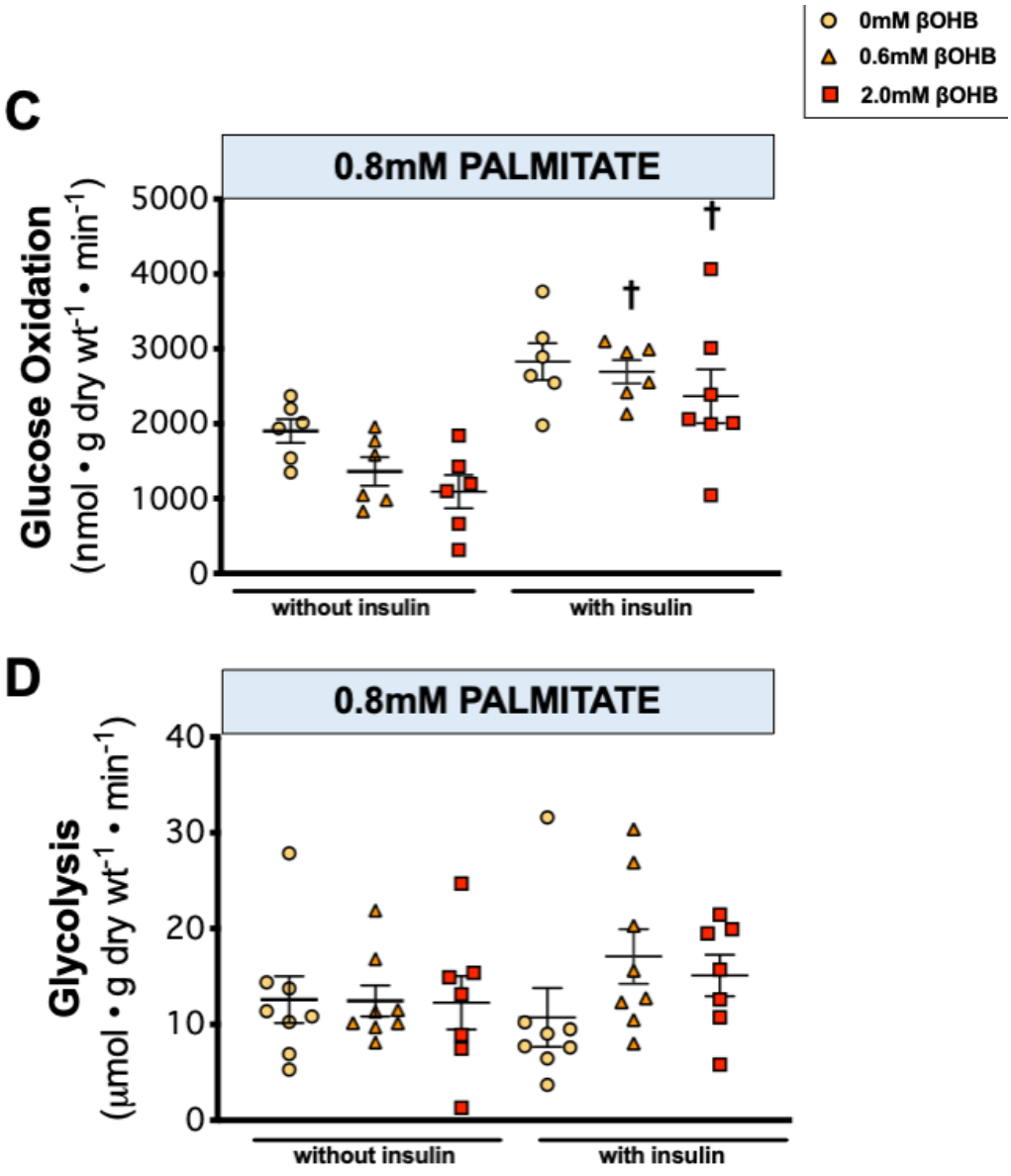

0mM βOHB
0.6mM βOHB
2.0mM βOHB
C
0.8mM PALMITATE
Glucose Oxidation
(nmol • g dry wt-1 • min-1)
5000
4000
3000
2000
1000
0
†
†
without insulin
with insulin
D
0.8mM PALMITATE
Glycolysis
(µmol • g dry wt-1 • min-1)
40
30
20
10
0
without insulin
with insulin

FIGURE 4.1

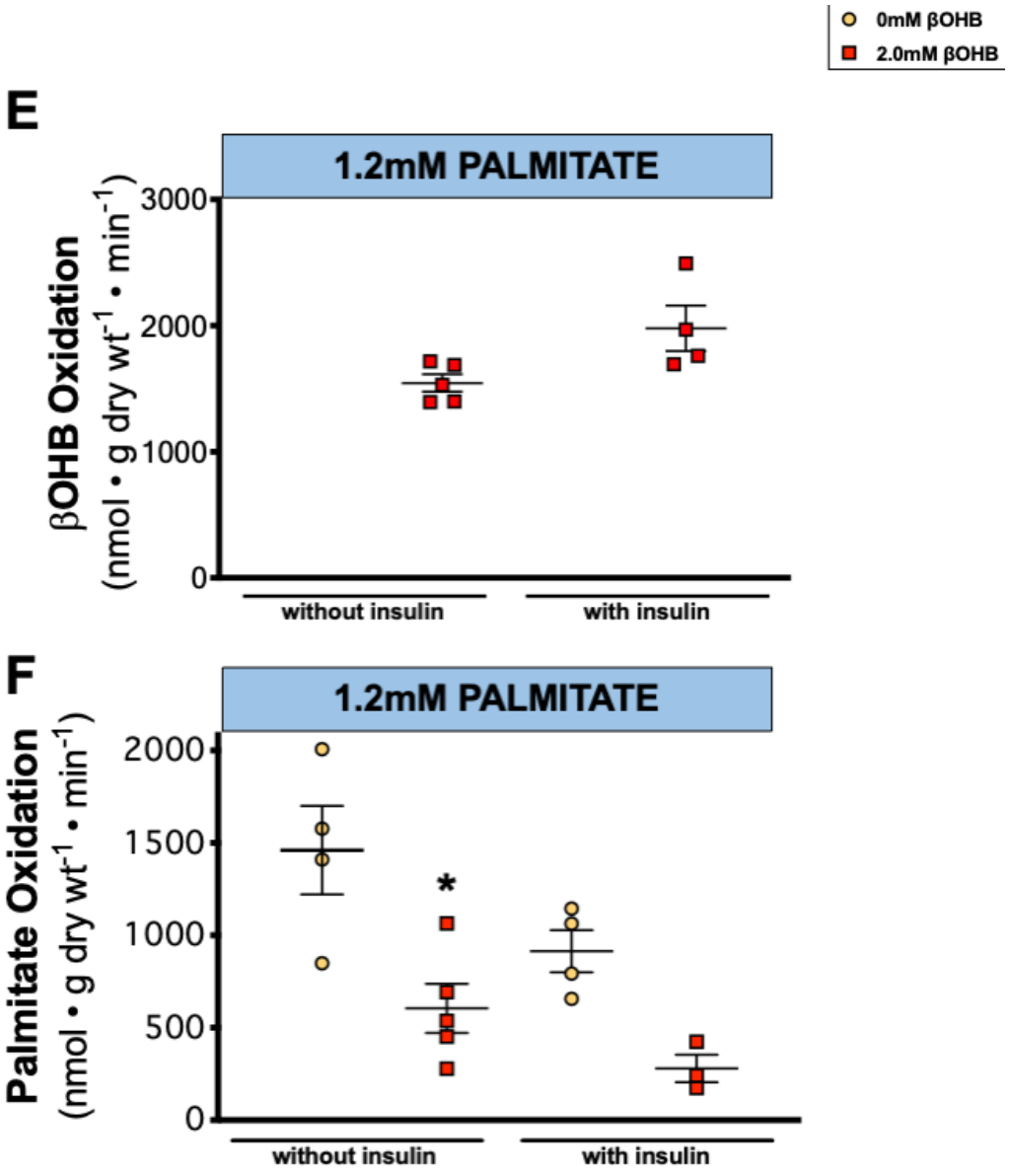

0mM βOHB
2.0mM βOHB
E
1.2mM PALMITATE
βOHB Oxidation
(nmol • g dry wt-1 • min-1)
3000
2000
1000
0
without insulin
with insulin
F
1.2mM PALMITATE
Palmitate Oxidation
(nmol • g dry wt-1 • min-1)
2000
1500
1000
500
0
*
without insulin
with insulin

FIGURE 4.1

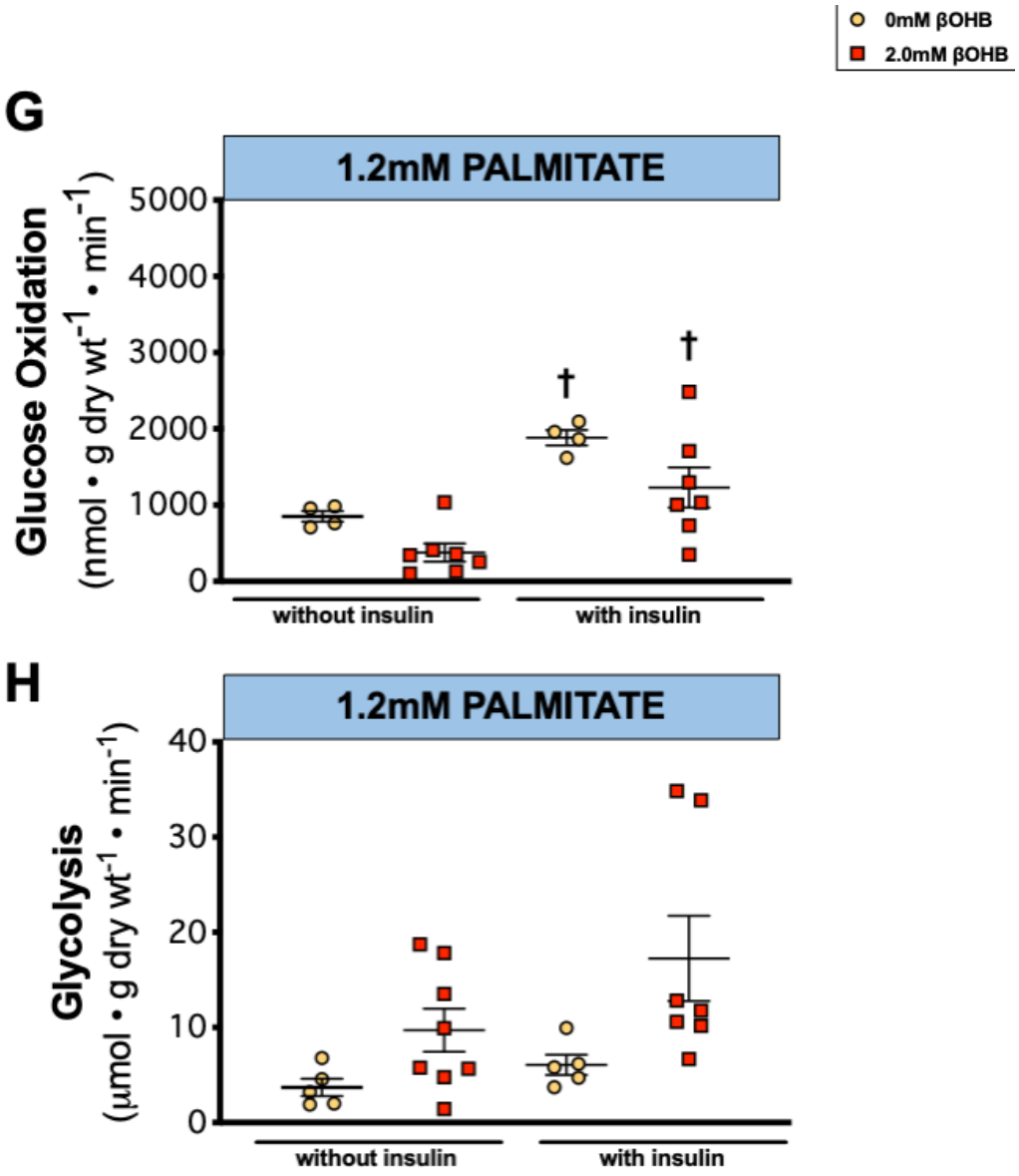

0mM βOHB
2.0mM βOHB
G
1.2mM PALMITATE
Glucose Oxidation
(nmol • g dry wt-1 • min-1)
5000
4000
3000
2000
1000
0
†
†
without insulin
with insulin
H
1.2mM PALMITATE
Glycolysis
(µmol • g dry wt-1 • min-1)
40
30
20
10
0
without insulin
with insulin

FIGURE 4.1

Figure 4.2 The effect of increasing ketone levels on TCA cycle activity, oxygen consumption, cardiac ATP content and cardiac work. **A, F,** TCA cycle activity from glucose oxidation (n=4-7), palmitate oxidation (n=3-7) or βOHB oxidation (n=4-7). **B, G,** Total NADH and $FADH_2$ produced as calculated from glycolysis (n=5-8), glucose oxidation (n=4-7), palmitate oxidation (n=3-7) or βOHB oxidation (n=4-7) rates. **C, H,** Myocardial oxygen consumption rates at 10-minute intervals for the duration of the perfusion (n=9-13). **D, I,** Total cardiac adenosine triphosphate (ATP) content as determined from heart tissue snap-frozen at the end of the perfusion by UPLC (n=10-14). **E, J,** *Ex vivo* cardiac work measured at each time interval during the perfusion (n=10-15). Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A Two-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group (except in panels A, B, F, G where *$p<0.05$ compared to the 0.6mM βOHB group when comparing βOHB oxidation). †$p<0.05$ compared to the without insulin group.

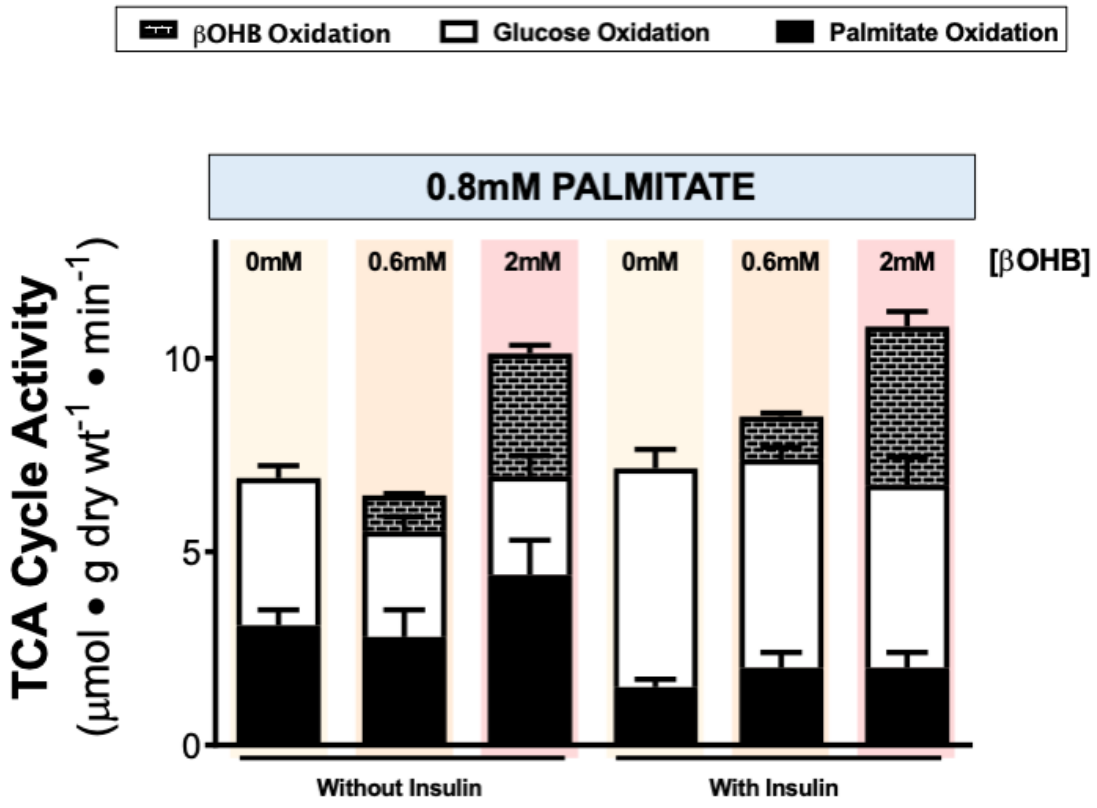

A
βOHB Oxidation
Glucose Oxidation
Palmitate Oxidation
0.8mM PALMITATE
0mM
0.6mM
2mM
0mM
0.6mM
2mM
[βOHB]
TCA Cycle Activity
(μmol • g dry wt-1 • min-1)
10
5
0
Without Insulin
With Insulin

FIGURE 4.2

B

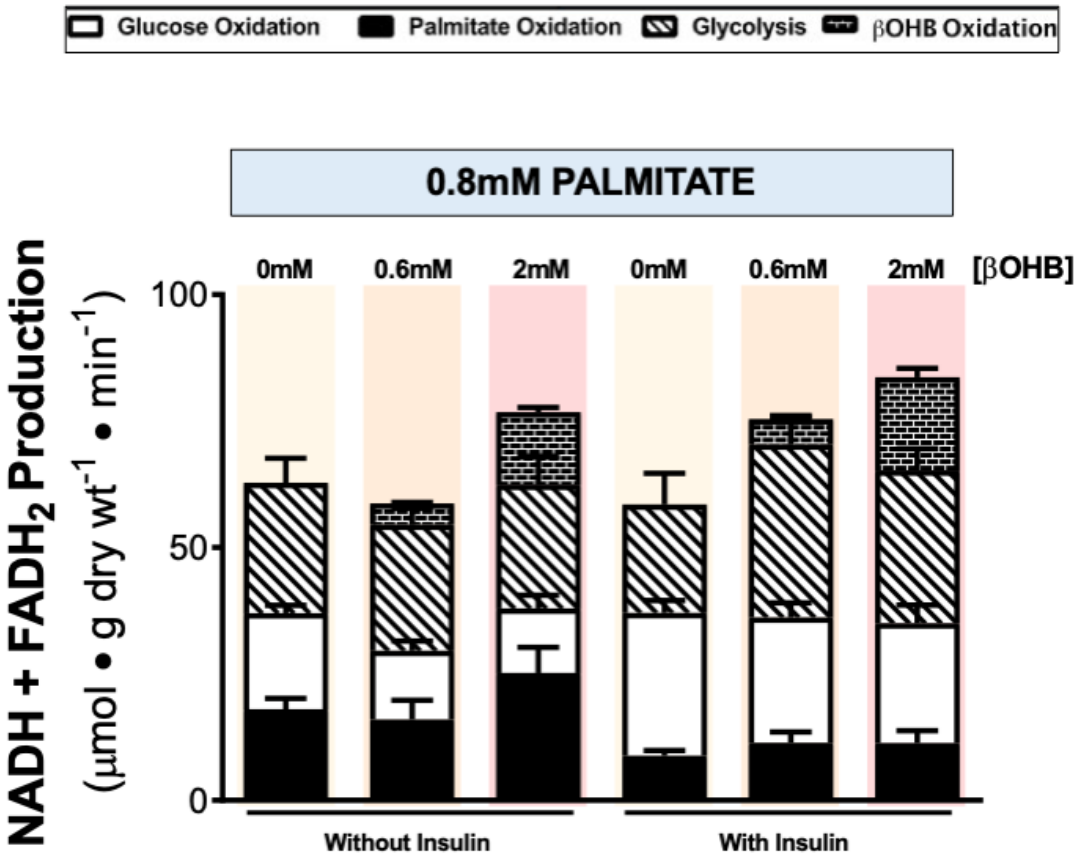

Glucose Oxidation
Palmitate Oxidation
Glycolysis
βOHB Oxidation
0.8mM PALMITATE
0mM
0.6mM
2mM
0mM
0.6mM
2mM
[βOHB]
NADH + FADH2 Production
(μmol • g dry wt-1 • min-1)
100
50
0
Without Insulin
With Insulin

FIGURE 4.2

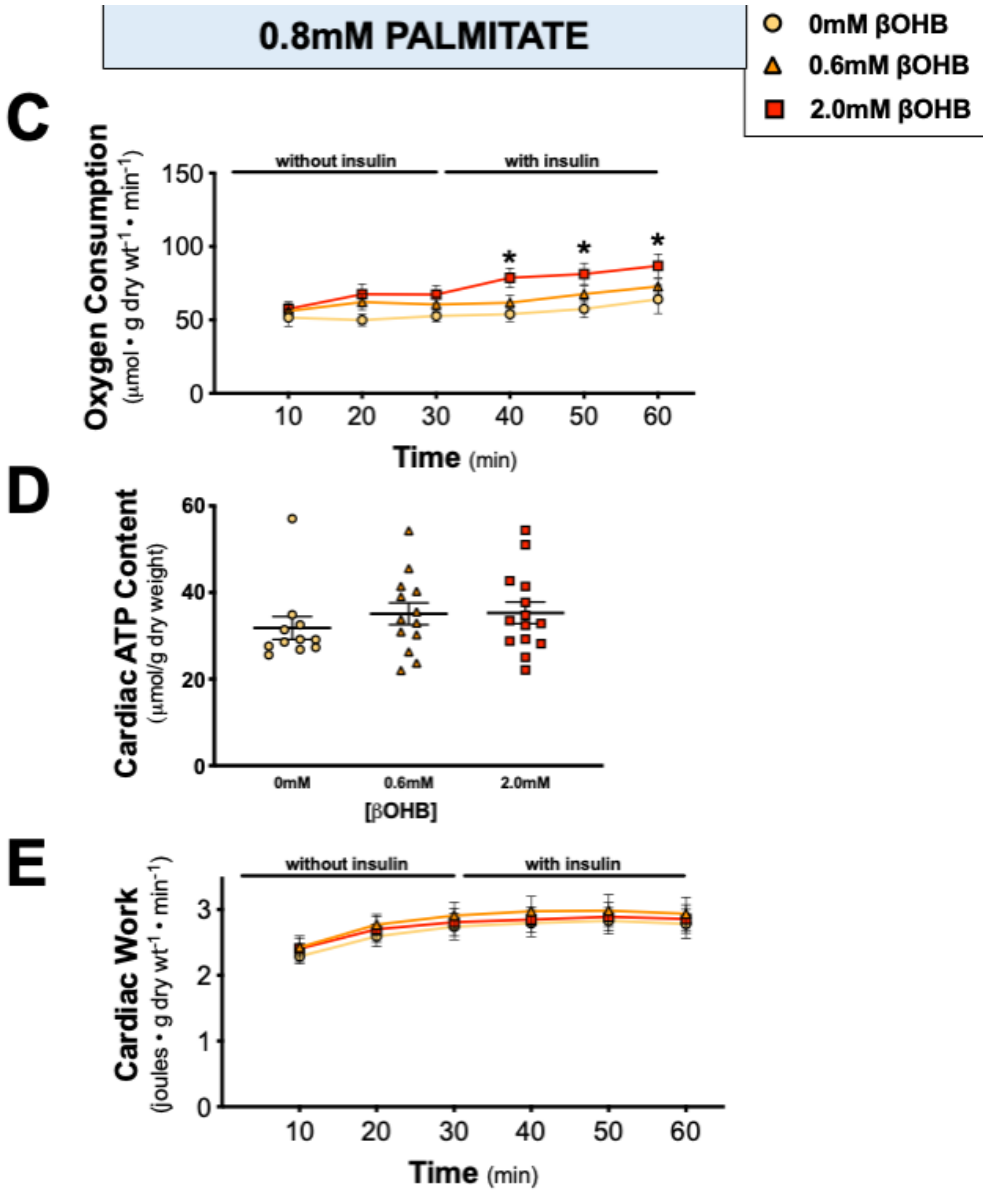

0.8mM PALMITATE
0mM βOHB
0.6mM βOHB
2.0mM βOHB
C
Oxygen Consumption
(µmol • g dry wt-1 • min-1)
without insulin
with insulin
150
100
50
0
10
20
30
40
50
60
Time (min)
D
Cardiac ATP Content
(µmol/g dry weight)
60
40
20
0
0mM
0.6mM
2.0mM
[βOHB]
E
Cardiac Work
(joules • g dry wt-1 • min-1)
without insulin
with insulin
3
2
1
0
10
20
30
40
50
60
Time (min)

FIGURE 4.2

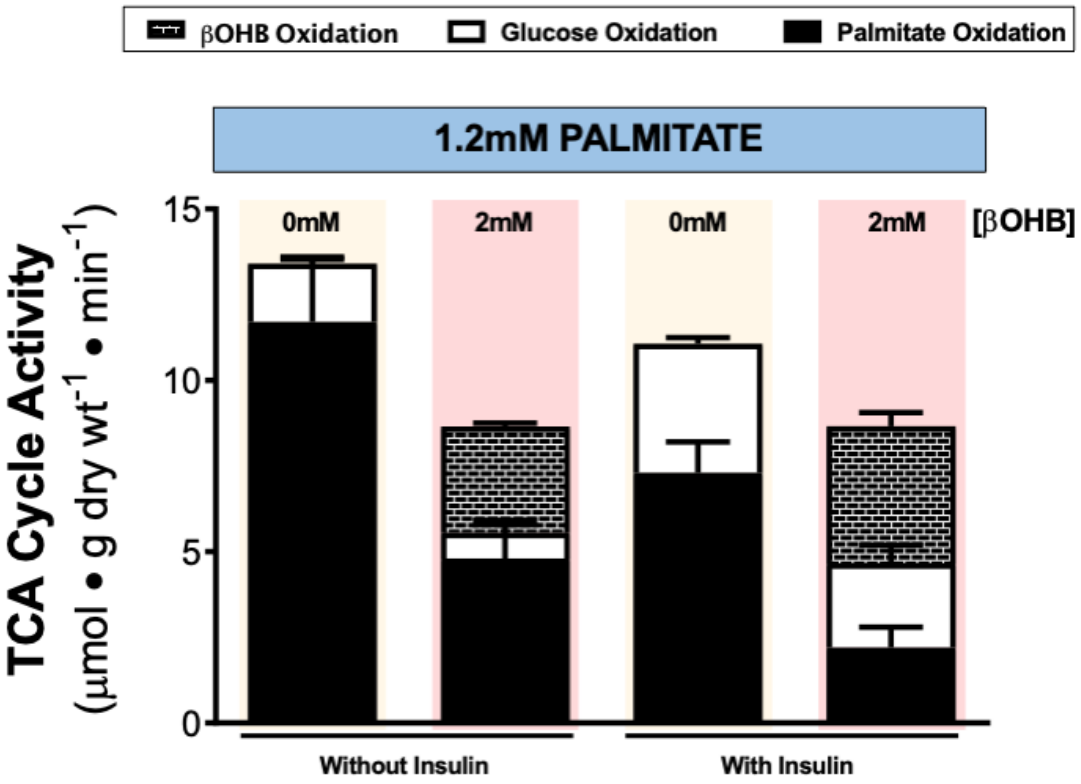

βOHB Oxidation
Glucose Oxidation
Palmitate Oxidation
1.2mM PALMITATE
TCA Cycle Activity
(µmol • g dry wt-1 • min-1)
15
10
5
0
0mM
2mM
0mM
2mM
[βOHB]
Without Insulin
With Insulin

FIGURE 4.2

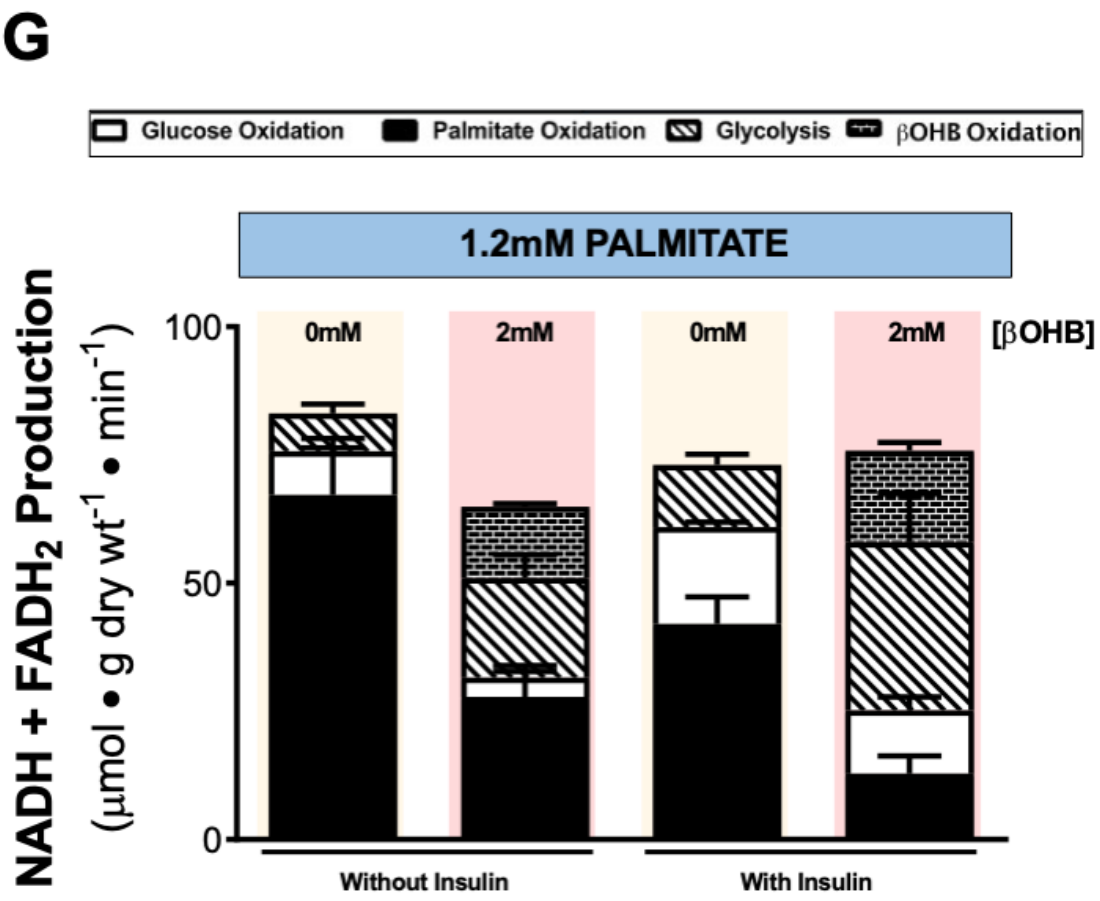

G
Glucose Oxidation
Palmitate Oxidation
Glycolysis
βOHB Oxidation
1.2mM PALMITATE
NADH + FADH2 Production
(μmol • g dry wt-1 • min-1)
100
50
0
0mM
2mM
0mM
2mM
[βOHB]
Without Insulin
With Insulin

FIGURE 4.2

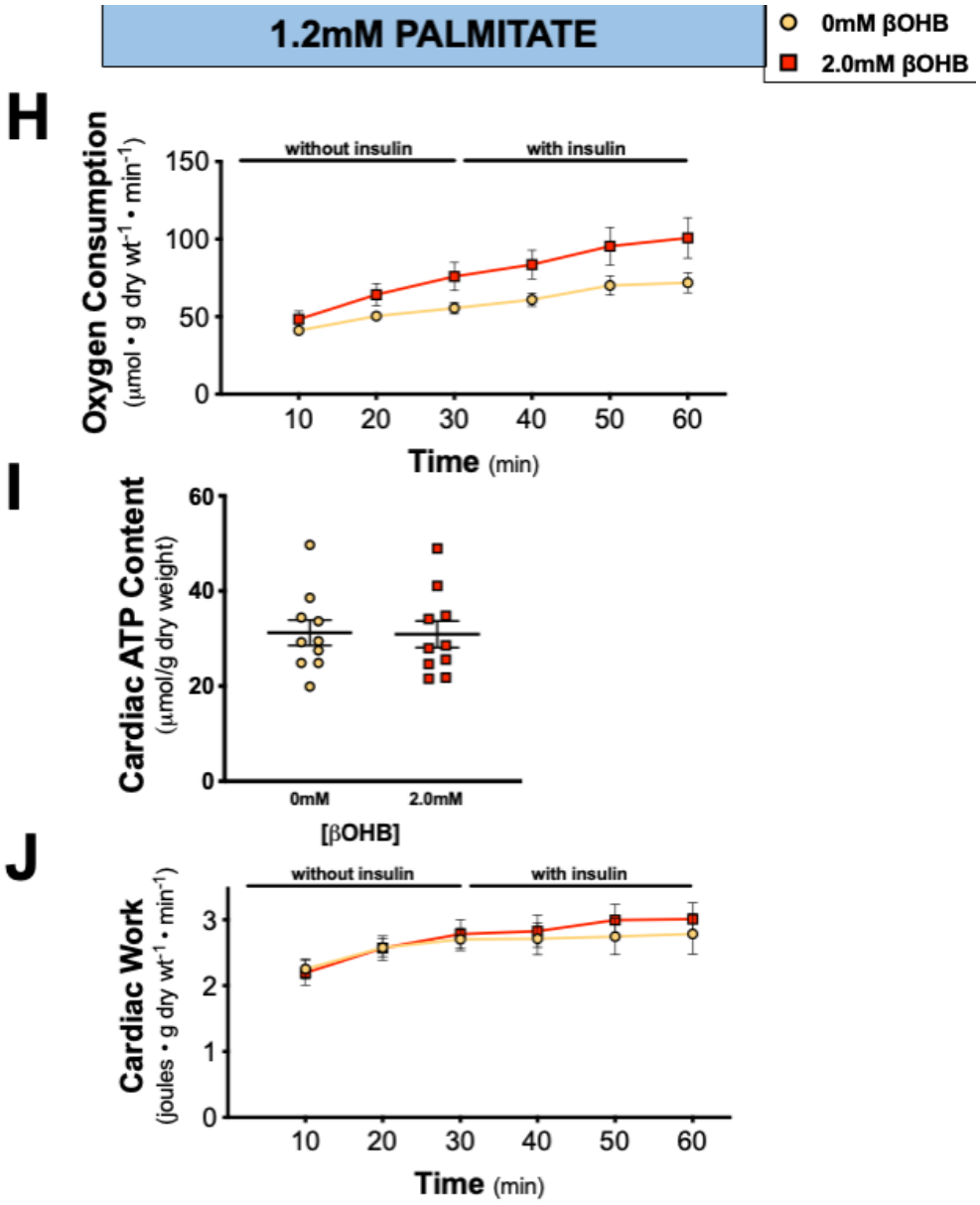

1.2mM PALMITATE
0mM βOHB
2.0mM βOHB
H
Oxygen Consumption
(μmol • g dry wt-1 • min-1)
without insulin
with insulin
150
100
50
0
10
20
30
40
50
60
Time (min)
I
Cardiac ATP Content
(μmol/g dry weight)
60
40
20
0
0mM
2.0mM
[βOHB]
J
Cardiac Work
(joules • g dry wt-1 • min-1)
without insulin
with insulin
3
2
1
0
10
20
30
40
50
60
Time (min)

FIGURE 4.2

Figure 4.3 Ketones decrease cardiac efficiency. **A, C,** Cardiac efficiency as calculated by cardiac work normalized to total TCA cycle activity (n=3-7). **B, D,** Cardiac efficiency as calculated by cardiac work normalized to oxygen consumption (n=8-13). Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for panels A & C while a Two-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for panels B &D. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group. †$p<0.05$ compared to the without insulin group.

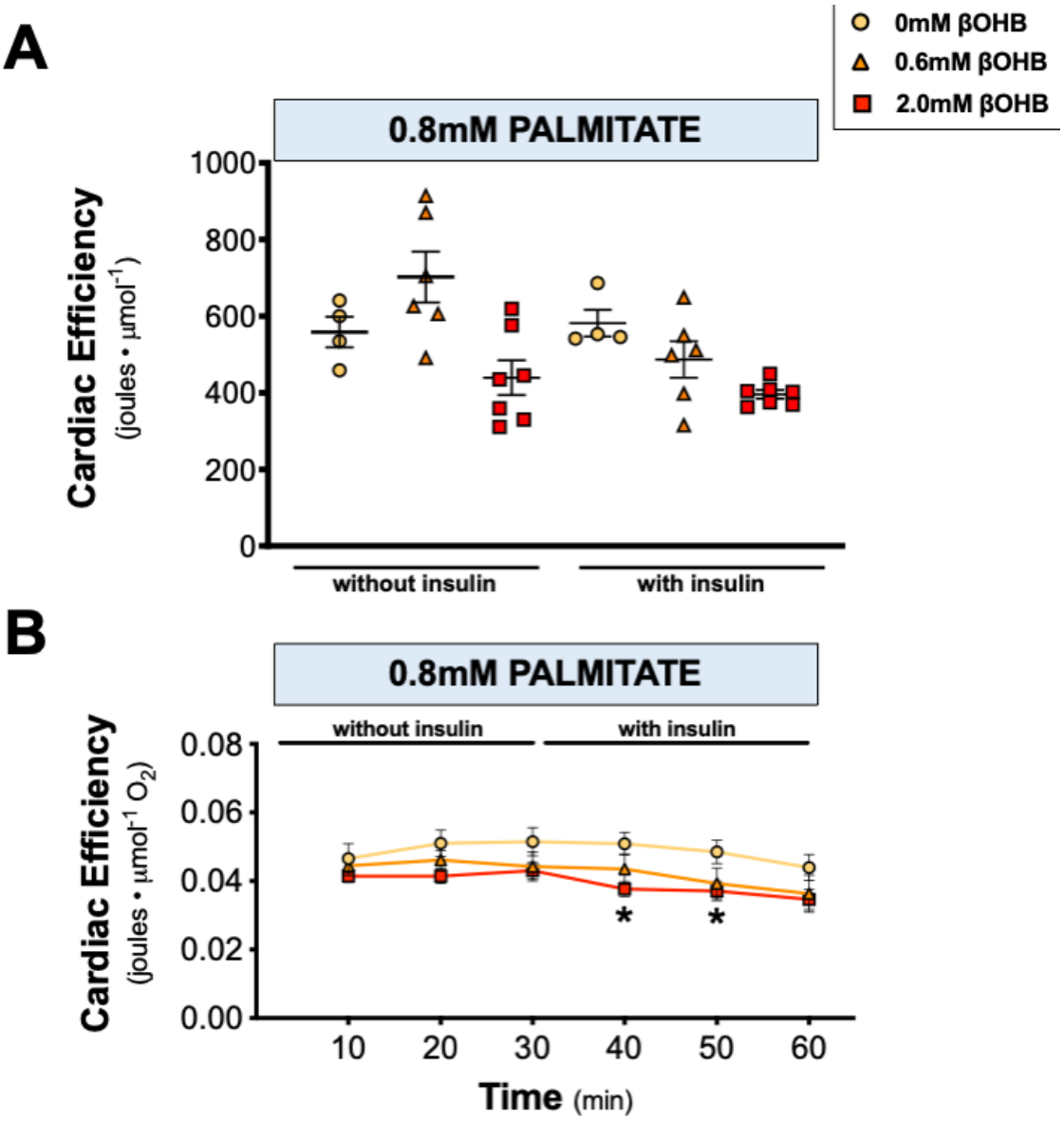

A
0mM βOHB
0.6mM βOHB
2.0mM βOHB
0.8mM PALMITATE
Cardiac Efficiency
(joules • µmol-1)
1000
800
600
400
200
0
without insulin
with insulin
B
0.8mM PALMITATE
without insulin
with insulin
Cardiac Efficiency
(joules • µmol-1 O2)
0.08
0.06
0.04
0.02
0.00
*
*
10
20
30
40
50
60
Time (min)

FIGURE 4.3

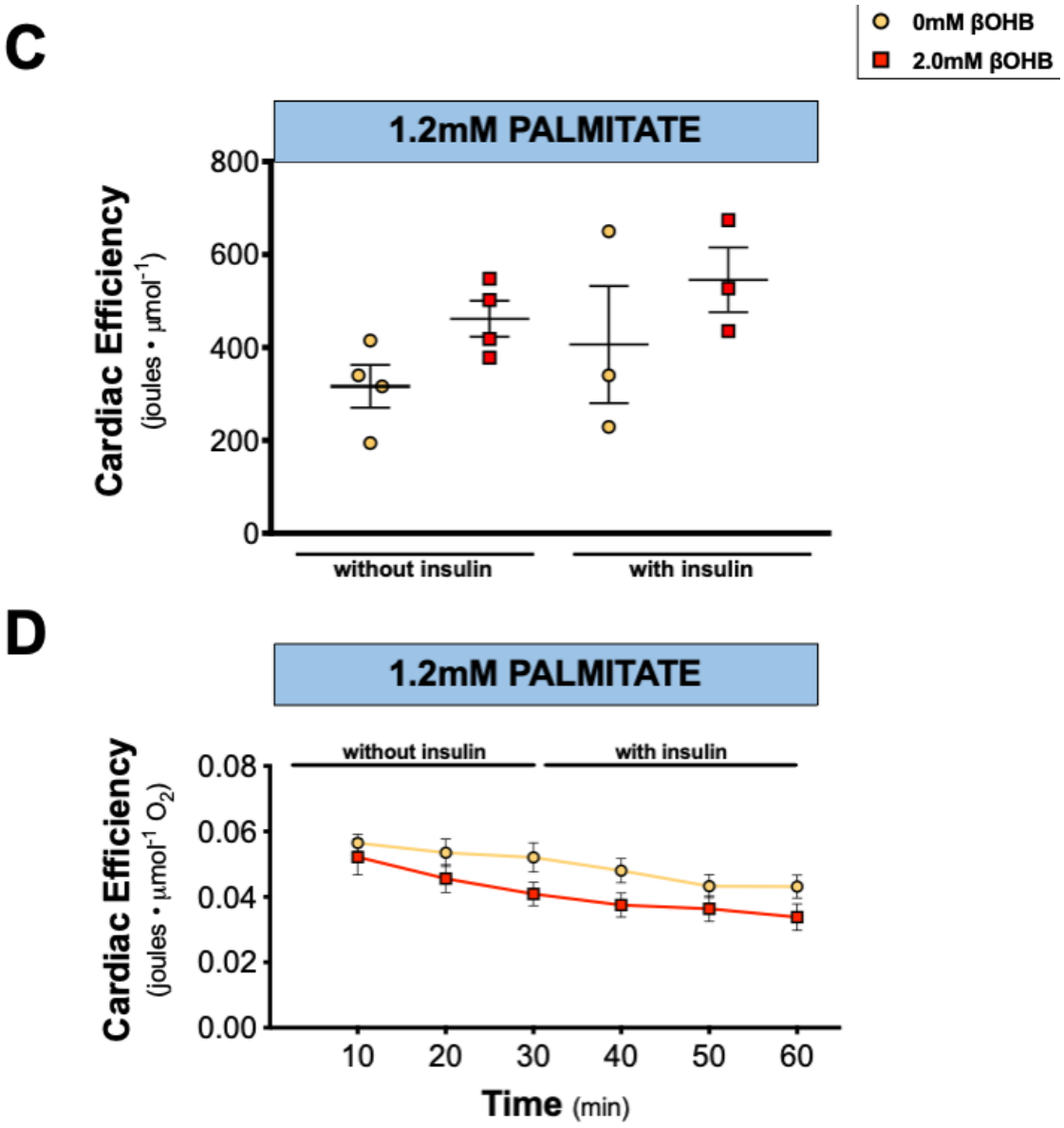

C
0mM βOHB
2.0mM βOHB
1.2mM PALMITATE
Cardiac Efficiency
(joules • µmol-1)
800
600
400
200
0
without insulin
with insulin
D
1.2mM PALMITATE
Cardiac Efficiency
(joules • µmol-1 O2)
without insulin
with insulin
0.08
0.06
0.04
0.02
0.00
10
20
30
40
50
60
Time (min)

FIGURE 4.3

Figure 4.4 Proposed mechanism for preserving the capacity of ATP production in the event of high ketone-derived excess of reducing equivalents and the absence of cardiac work-load. Part **(A)** represents the flow of reducing equivalents to the electron transport chain to be oxidized to provide hydrogen (H^+) and electron to build up the protonmotive force hat drives the production ATP. Part **(B)** represents our proposed role of uncoupling proteins (UCPs) and/or ATP synthase in preserving the mitochondrial capacity of ATP production in the event of a high supply of reducing equivalents in the absence of cardiac work demand for ATP production. We proposed that such excess in reducing equivalents supply, drived from unregulated oxidation of substrates like ketones, triggers the activity of UCPs and/or ATP synthase to reduce protonmotive force and allow H^+ to flow to the mitochondrial matrix. This will prevent excessive production of ATP when it is not needed and preserve the limit, unreplaceable amount of nucleotide (i.e. ADP) that is available for ATP production. This proposed mechanism do not exclude the possibility that there could be another undefined candidate(s) that can be responsible for mitigating the sudden supraphysiological supply of reducing equivalents due to an unregulated fuel source (such as ketones) when there is no cardiac work demand.

Figure 4

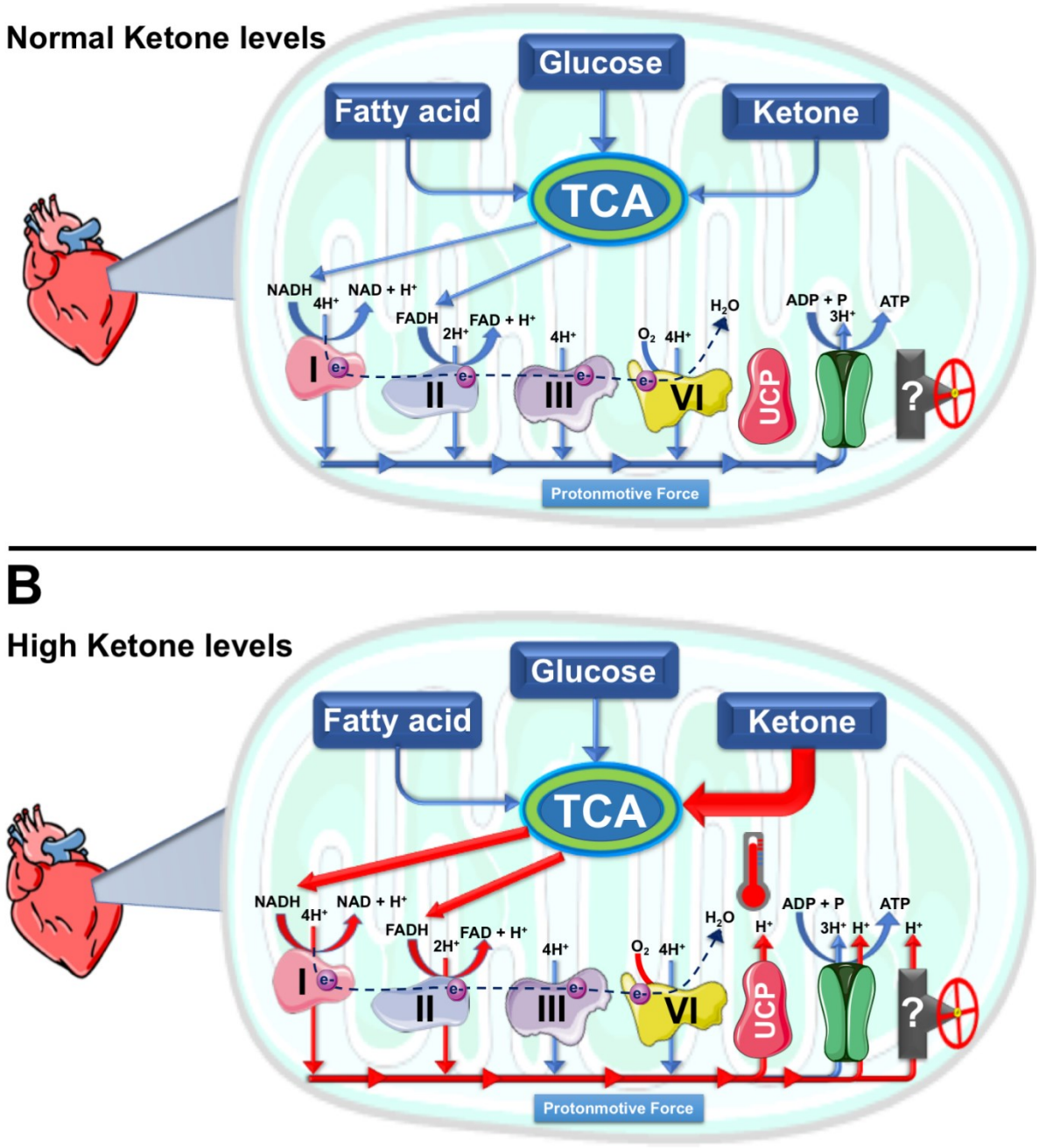


FIGURE 4.4

Figure 4.5 Assessment of the Coenzyme-A (CoA) profile in the hearts at the end of the perfusion. **A, F,** Free CoA content. **B, G,** Succinyl CoA content. **C, H,** Malonyl CoA. **D, I,** Acetyl CoA content. **E, J,** Acetyl CoA to CoA ratio. Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for panels A-E while a student's unpaired t-test was carried out for panels F-J. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group.

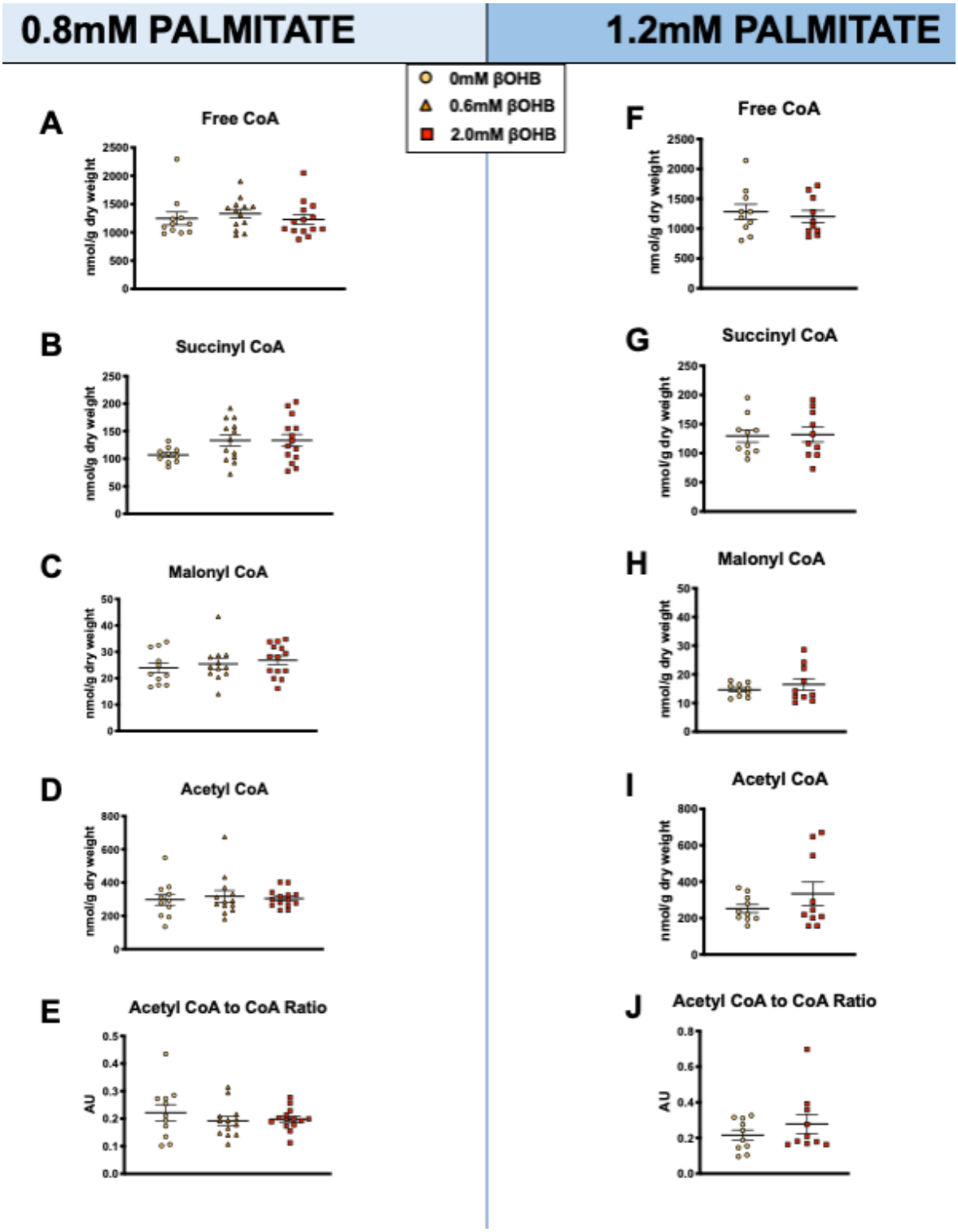

0.8mM PALMITATE
1.2mM PALMITATE
0mM βOHB
0.6mM βOHB
2.0mM βOHB
A
Free CoA
nmol/g dry weight
B
Succinyl CoA
nmol/g dry weight
C
Malonyl CoA
nmol/g dry weight
D
Acetyl CoA
nmol/g dry weight
E
Acetyl CoA to CoA Ratio
AU
F
Free CoA
nmol/g dry weight
G
Succinyl CoA
nmol/g dry weight
H
Malonyl CoA
nmol/g dry weight
I
Acetyl CoA
nmol/g dry weight
J
Acetyl CoA to CoA Ratio
AU

FIGURE 4.5

Figure 4.6 Assessment of triacylglycerol content and radiolabeled content in triacylglycerols. A, D, Triacylglycerol content measured from hearts collected at the end of the perfusion. **B, E,** The radiolabeled palmitate content in the triacylglycerol which is indicative of the incorporation of exogenous palmitate into triacylglycerol. **C, F,** Normalization of radiolabeled palmitate incorporation into triacylglycerol to the heart-specific triacylglycerol content. Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for panels A-C while a student's t-test was carried out for panels D-F. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group.

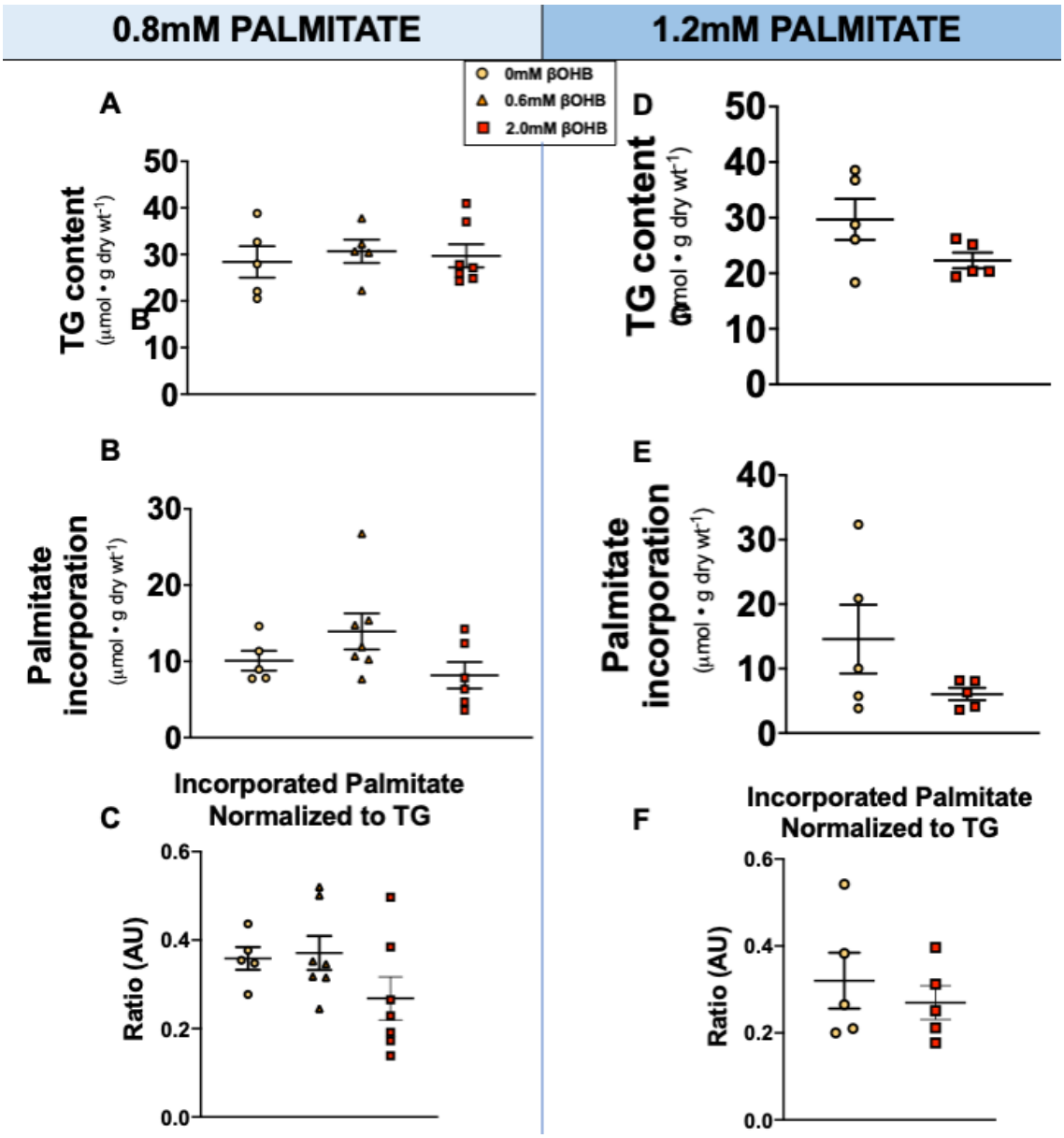

0.8mM PALMITATE
1.2mM PALMITATE
0mM βOHB
0.6mM βOHB
2.0mM βOHB
A
TG content
(µmol • g dry wt-1)
D
B
Palmitate incorporation
(µmol • g dry wt-1)
E
C
Incorporated Palmitate Normalized to TG
Ratio (AU)
F

FIGURE 4.6

Figure 4.7 The metabolic profile of the healthy heart normalized to cardiac work. A, E, βOHB (ketone body) oxidation normalized to cardiac work. **B, F,** Palmitate (fatty acid) oxidation normalized to cardiac work. **C, G,** Glucose oxidation normalized to cardiac work. **D, H,** Glycolysis normalized to cardiac work. Hearts perfused with 0mM ßOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM ßOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group except for Figure 1A where *$p<0.05$ compared to the 0.6mM βOHB. †$p<0.05$ compared to the without insulin group.

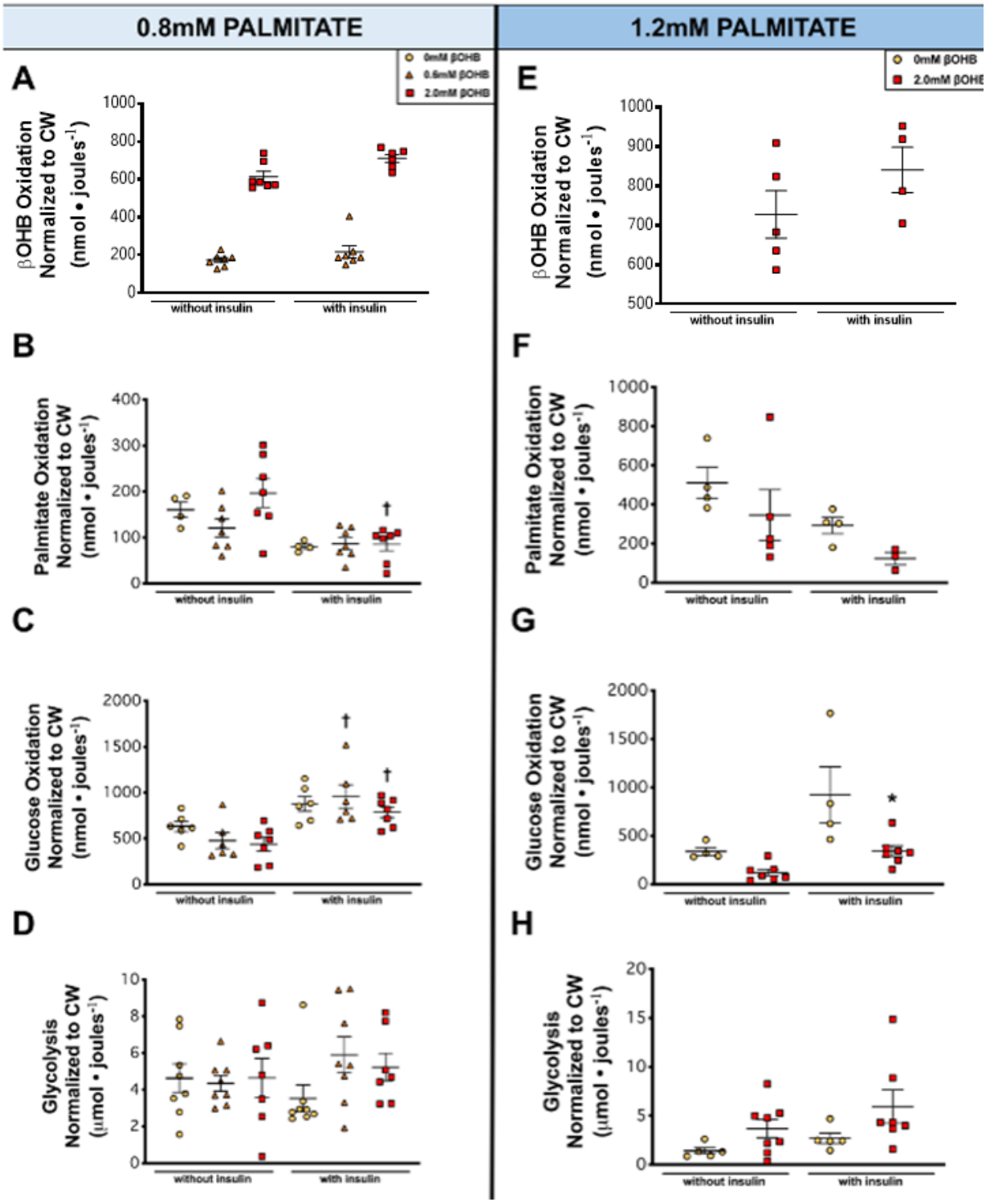

0.8mM PALMITATE
1.2mM PALMITATE
A
βOHB Oxidation Normalized to CW (nmol • joules⁻¹)
0mM βOHB
0.8mM βOHB
2.0mM βOHB
without insulin
with insulin
B
Palmitate Oxidation Normalized to CW (nmol • joules⁻¹)
C
Glucose Oxidation Normalized to CW (nmol • joules⁻¹)
D
Glycolysis Normalized to CW (μmol • joules⁻¹)
E
F
G
H

FIGURE 4.7

Figure 4.8 The effect of increasing ketone levels on the percent contribution of each substrate to total TCA cycle activity.

A, B, The percent contribution of glucose oxidation, palmitate oxidation, βOHB oxidation to total TCA cycle activity at increasing concentrations of βOHB and either at 0.8 or 1.2mM palmitate. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for each panel in this figure. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group (except *$p<0.05$ compared to the 0.6mM βOHB group when comparing βOHB oxidation). †$p<0.05$ compared to the without insulin group.

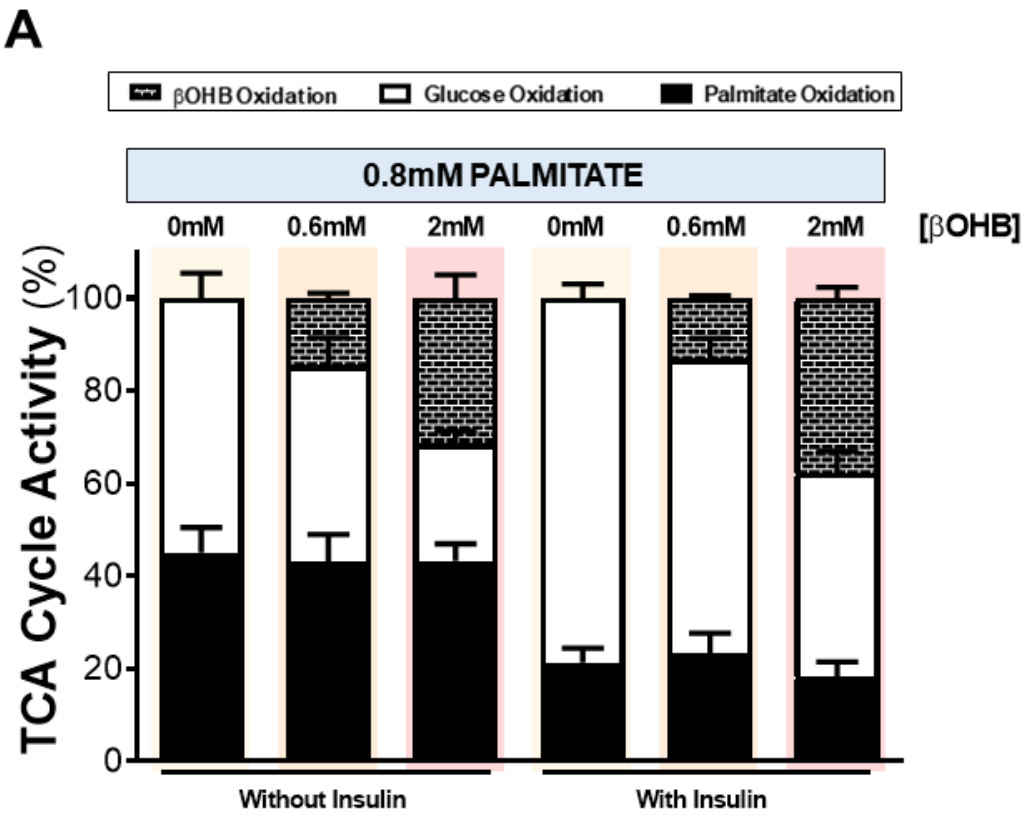

A
βOHB Oxidation
Glucose Oxidation
Palmitate Oxidation
0.8mM PALMITATE
0mM
0.6mM
2mM
0mM
0.6mM
2mM
[βOHB]
TCA Cycle Activity (%)
100
80
60
40
20
0
Without Insulin
With Insulin

FIGURE 4.8

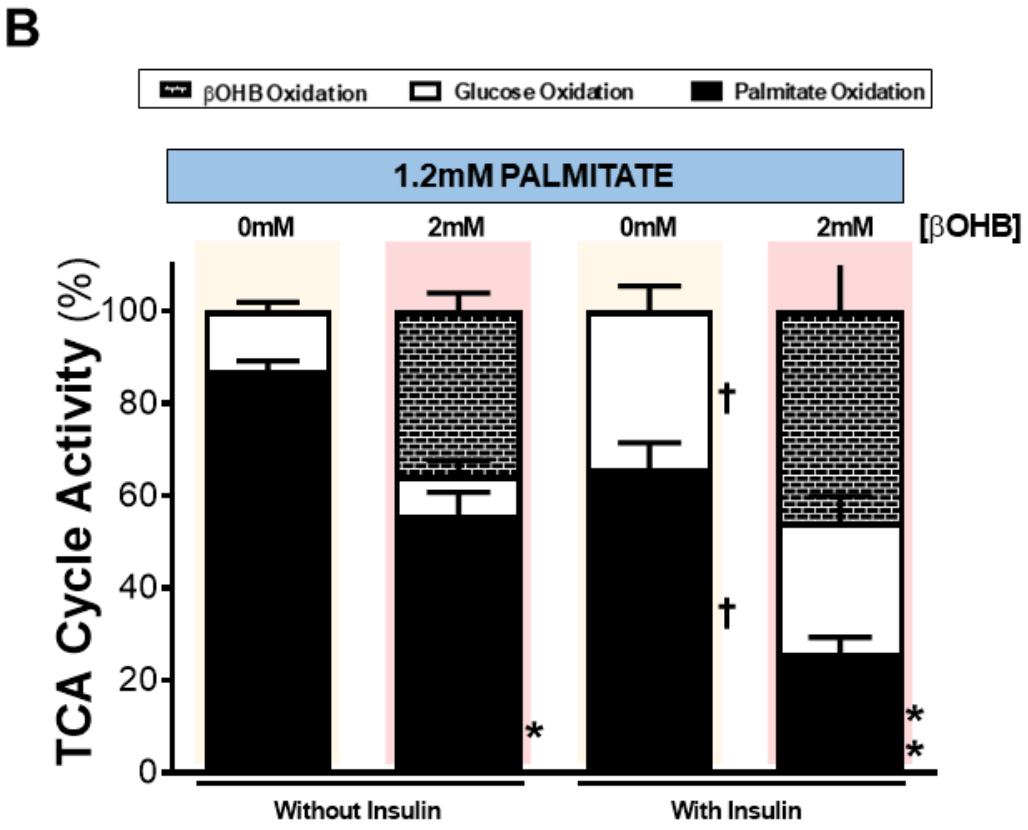

B
βOHB Oxidation
Glucose Oxidation
Palmitate Oxidation
1.2mM PALMITATE
0mM
2mM
0mM
2mM
[βOHB]
TCA Cycle Activity (%)
100
80
60
40
20
0
†
†
*
*
*
Without Insulin
With Insulin

FIGURE 4.8

Figure 4.9 Assessment of phosphorylation of pyruvate dehydrogenase via immunoblotting. **A,** Immunoblot of P-PDH E1-alpha subunit (S293), T-PDH and alpha-tubulin from cardiac tissue (n=6-8). **B,** Densitometric analysis of the immunoblot of P-PDH normalized to T-PDH. Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group.

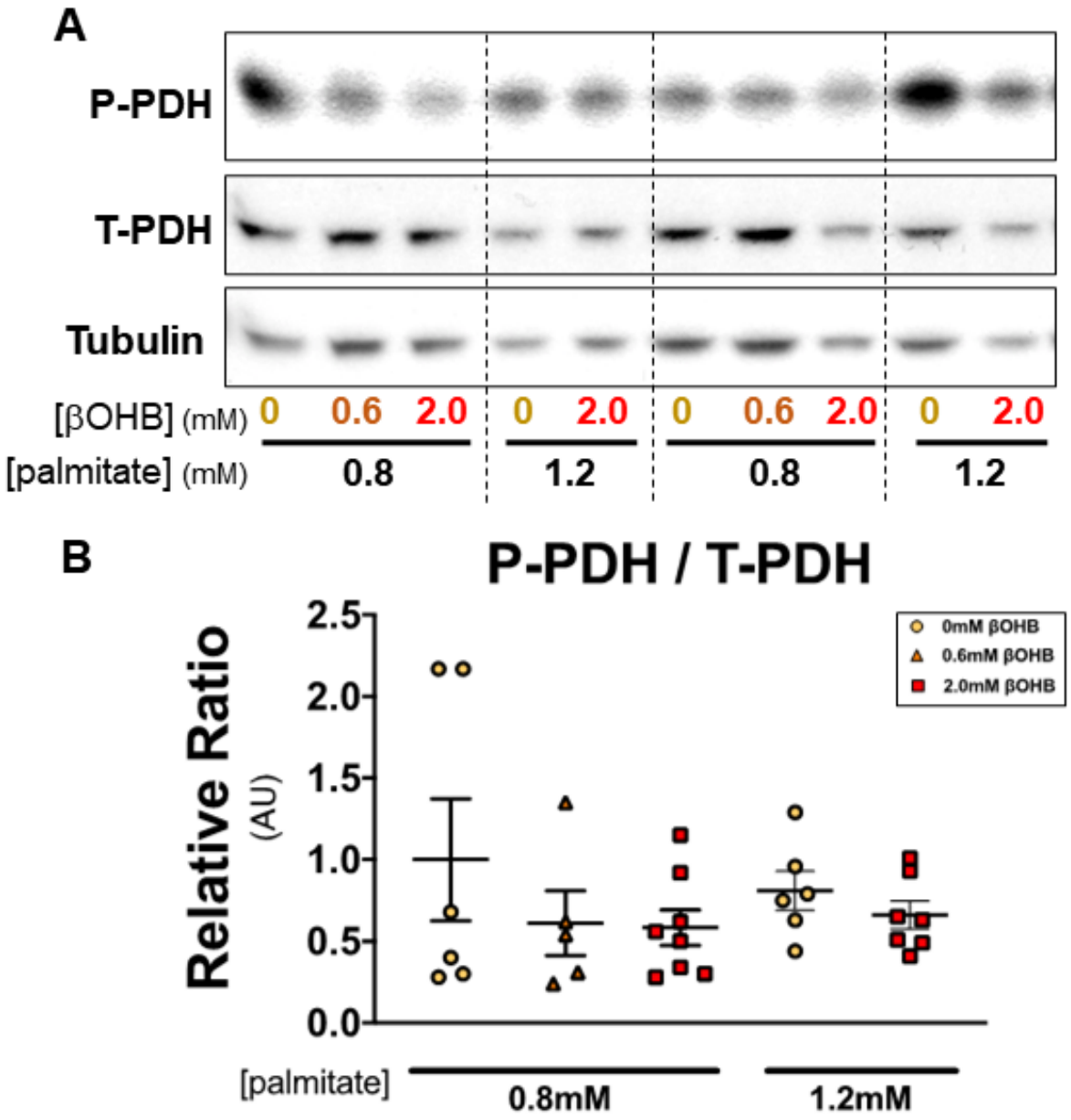

A
P-PDH
T-PDH
Tubulin
[βOHB] (mM) 0 0.6 2.0 0 2.0 0 0.6 2.0 0 2.0
[palmitate] (mM) 0.8 1.2 0.8 1.2
B
P-PDH / T-PDH
Relative Ratio
(AU)
2.5
2.0
1.5
1.0
0.5
0.0
0mM βOHB
0.6mM βOHB
2.0mM βOHB
[palmitate]
0.8mM
1.2mM

FIGURE 4.9

Figure 4.10 Assessment of the nucleotide profile in the hearts at the end of the perfusion. **A, F,** Nicotinamide adenine dinucleotide content. **B, G,** Adenosine monophosphate content. **C, H,** Adenosine diphosphate content. **D, I,** Creatine content. **E, J,** Creatine phosphate content. Hearts perfused with 0mM βOHB are represented by yellow circles, 0.6mM βOHB by orange triangles and 2.0mM βOHB by red squares. A One-Way ANOVA with Bonferroni correction for multiple comparisons was carried out for panels A-E while a student's unpaired t-test was carried out for panels F-J. Data is expressed as mean ± SEM. *$p<0.05$ compared to the 0mM βOHB group.

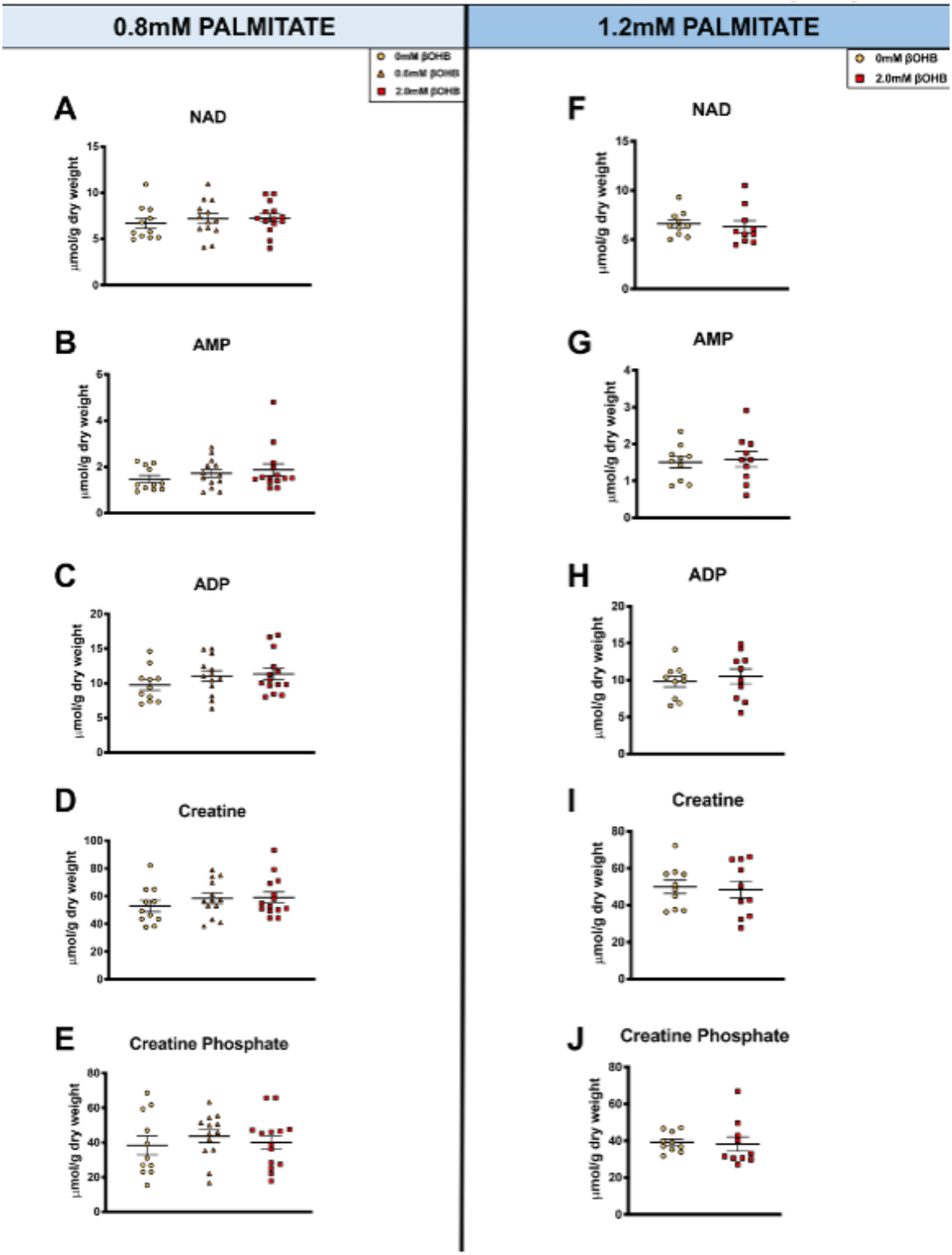

0.8mM PALMITATE
1.2mM PALMITATE
0mM βOHB
0.8mM βOHB
2.0mM βOHB
A
NAD
B
AMP
C
ADP
D
Creatine
E
Creatine Phosphate
F
NAD
G
AMP
H
ADP
I
Creatine
J
Creatine Phosphate
μmol/g dry weight

FIGURE 4.10

Chapter V
The Ketogenic Diet Blunts Glucose Oxidation in Heart Failure

Manuscript Status: This manuscript is in preparation for submission to 'Cardiovascular Research' for publication as an original research article (Ho, K. L., et al.).

Chapter 5 The Ketogenic Diet Blunts Glucose Oxidation in Heart Failure

In this chapter, **Aim 3** is addressed and *in vivo and ex vivo* cardiac function and metabolic profile of the failing heart are assessed in response to a chronic increase in ketones.

We find evidence that the ketogenic diet does not improve heart function in failing hearts likely due to its inability to increase cardiac ketone oxidation rates as well as blunting glucose oxidation following consumption of a ketogenic diet.

5.1 Contribution Statement

My role in this project included performing all the experiments (except those noted below) alongside the writing of the manuscript. *Ex vivo* isolated working heart perfusions were performed by Mr. Cory Wagg (Lab technician of Dr. Gary Lopaschuk's laboratory).

5.2 Abstract

5.2.1 Aims

Cardiac energy metabolism is perturbed in heart failure and is characterized by a shift from mitochondrial oxidative metabolism to glycolysis. Notably, the failing heart relies more on ketones for energy than a healthy heart, an adaptive mechanism that improves the energy-starved status of the failing heart. However, whether this can be implemented therapeutically remains unknown. Therefore, our aim was to determine if increasing ketone delivery to the heart via a ketogenic diet can improve the outcomes of heart failure.

5.2.2 Methods and Results

C57BL/6J male mice underwent either a sham surgery or left anterior descending (LAD) coronary artery ligation surgery to induce heart failure. After 2 weeks, mice were then treated with either a control diet or a ketogenic diet for 3 weeks. Transthoracic echocardiography was then carried out to assess *in vivo* cardiac function. Finally, isolated working hearts from these mice were perfused with appropriately ^{3}H or ^{14}C labelled glucose (5 mM), palmitate (0.8 mM), and ß-hydroxybutyrate (0.6 mM) to assess mitochondrial oxidative metabolism and glycolysis. Mice with heart failure exhibited a 56% drop in ejection fraction which was not improved with a ketogenic diet. Interestingly, mice fed a ketogenic diet had a dramatic decrease in cardiac glucose oxidation. Despite increasing blood ketone levels, cardiac ketone oxidation did not increase, probably due to decreased expression of key ketone oxidation enzymes. Furthermore, in mice on the ketogenic diet no increase in overall cardiac ATP production was observed, and instead there was a shift to an increased reliance

on fatty acids as a source of cardiac ATP production. This resulted in a decrease in cardiac efficiency in heart failure mice fed a ketogenic diet.

5.2.3 Conclusions

We conclude that the ketogenic diet does not improve heart function in failing hearts, possibly due to a lack of increase in cardiac ketone oxidation and a decrease in insulin-stimulated glucose oxidation.

5.3 Introduction

Heart failure is a major burden to society, affecting approximately 8% of all older adults[173]. Alterations in cardiac energy metabolism contribute to the development and progression of heart failure[19,3]. Briefly, while the healthy heart is normally flexible as to which energy substrates it can use for ATP production, the failing heart is metabolically inflexible as well as energy starved[47]. The failing heart is characterized by a shift from mitochondrial oxidative metabolism to glycolysis for its energy[46]. While controversy exists around whether fatty acid oxidation rates are increased, unchanged or decreased in the setting of heart failure, there is more consensus with regards to glucose oxidation being decreased in heart failure[11].

Ketone oxidation in heart failure has generated considerable recent interest in the last few years due to the observation that the failing heart relies more on ketones as a source of energy[7,8,69]. This increase in ketone oxidation is thought to be an adaptive process, that provides the "energy starved" failing heart with an additional source of energy[16,71,72,174]. We showed that increasing ketone oxidation in the failing heart can increase overall cardiac ATP production[74]. Thus, we wanted to investigate whether increasing ATP production in the setting of heart failure via increasing ketone delivery to the heart would be beneficial for cardiac energetics and function.

There are various methods to increase ketone delivery to the heart – ketone salts, ketone esters, medium chain triacylglycerols, sodium glucose co-transporter 2 (SGLT2) inhibitors, intermittent fasting, or the ketogenic diet[175,176]. The ketogenic diet is a popular diet that primarily consists of a high-fat, low-carbohydrate diet that causes blood ketone levels to increase due to the restriction of carbohydrates and decreased insulin signaling[177,178]. The ketogenic diet is practiced mostly for the purposes of weight loss since carbohydrate restriction

promotes mobilization of fat stores. However, the effects of a ketogenic diet on heart function and metabolism remain unclear due to mixed results in various rodent and human models[179-182]. Therefore, whether the ketogenic diet can be implemented in the setting of heart failure to increase ketone delivery to the heart and confer cardiovascular benefits remains unknown. As such, we set out to investigate the functional and metabolic effects of the ketogenic diet in a murine model of ischemic heart failure.

5.4 Methods

Male C57BL/6J mice were randomly assigned, at 15-16 weeks of age, to a sham surgery or a left anterior descending coronary artery surgery to permanently ligate the left anterior descending coronary artery and induce a myocardial infarction (MI), as described previously[183]. Permanent ligation of the left anterior descending (LAD) coronary artery or a sham surgery was carried out in a blinded manner. After anaesthetization, mice undergo a thoracotomy after which the LAD is ligated with a 6-0 silk suture. Sham mice similarly underwent a thoracotomy but the LAD was not ligated. Mice were then carefully inspected over the course of the next 7 days while they recovered. After mice recovered, they were subjected to further experiments.

Mice were randomized 2 week post-surgery to receive either a control diet (80% carbohydrates, 10% fat, 10% protein) or a ketogenic diet (5% carbohydrates, 85% fat, 10% protein) for 3 weeks. During this period, transthoracic echocardiography was carried out to assess *in vivo* heart function pre-surgery (0 weeks), pre-diet (1 weeks) as well as post-diet (5 weeks).

Five-weeks post MI, mice were anesthetized and hearts were excised and perfused as isolated working preparations to assess *ex vivo* cardiac function as well as the cardiac metabolic profile. Finally, hearts were snap-frozen and used for biochemical analysis. Figure 5.1 outlines the experimental protocol followed for this study.

Unless detailed below, the experimental protocols utilized for this study can be found in Chapter 2. Specific isolated working heart perfusion conditions for this project are as follows.

5.4.1 Perfusion Conditions

Isolated working hearts were prepared in a Krebs-Henseleit solution consisting of 2.5 mM Ca^{2+}, 5 mM glucose, 0.8 mM palmitate (pre-bound to 3% albumin), and 600 µM ß-hydroxybuturate (β-OHB). Insulin (100 µU/ml) was added to the perfusate at 30 min

5.4.2 Statistical Analysis

Data is presented throughout the paper as mean ± SEM. Significant differences were calculated via two-way ANOVAs when comparing more than two means followed by a Tukey post hoc test. All statistical analyses were carried out using GraphPad Prism V9. Differences were deemed statistically significant when the p-value < 0.05.

An asterisk indicates that there is statistical significance between the specified group compared to the control group (sham healthy group) while the cross indicates that there is statistical significance between the specified group with insulin versus the specified group without insulin.

5.5 Results

5.5.1 The ketogenic diet does not improve in vivo or ex vivo cardiac function in mice with heart failure

Mice were subjected to a left anterior descending coronary artery surgery in order to develop ischemic heart failure (herein referred to as MI [myocardial infarction] mice). Mice were then randomized to either receive a control diet or a ketogenic diet for 3 weeks after which fasted body weight decreased in mice that consumed a ketogenic diet versus those that consumed a control diet (Figure 5.1A). This drop in body weight was accompanied by a significant decrease in blood glucose levels alongside an increase in βOHB (the primary circulating ketone in the blood) levels (Figure 5.1 C,D). While the ketogenic diet had the predicted effects on body weight and blood ketones, this was not accompanied by an improvement in cardiac function in the MI heart failure mice. MI control mice exhibited a 57% drop in ejection fraction (Figure 5.2 A,B, Figure 5.7A) and a 55% increase in diastolic dysfunction (e/e' ratio) (Figure 5.2 C,D,Figure 5.7 B) compared to sham mice. The decreased %EF was found to be unchanged between MI mice fed a control diet versus a ketogenic diet (Figure 5.2 A,B), signifying that the ketogenic diet does not affect systolic function. Diastolic dysfunction, assessed by using the e-wave to e-prime wave ratio was also not different between MI mice on a control diet and MI mice on a ketogenic diet (Figure 5.2 C,D), signifying that the ketogenic diet also does not affect diastolic function. In addition to the *in vivo* echocardiographic assessment of heart function, *ex vivo* cardiac function of the mice hearts was also assessed in isolated working heart perfusions. Cardiac work dropped by 42% in MI Control mice compared to Sham Control mice (Figure 5.2 E). However, mirroring the in vivo

studies, the ketogenic diet had no effect on cardiac work in either Sham mice, or MI mice (Figure 5.2 E). As such, while the ketogenic diet led to a decrease in body weight, decrease in blood glucose levels and an increase in blood ketone levels, there was no significant effect of the ketogenic diet on *in vivo* or *ex vivo* cardiac function.

5.5.2 The ketogenic diet does not increase cardiac ketone oxidation rates

Because increasing cardiac ketone oxidation can benefit the failing heart, we determined what effect the ketogenic diet had on ketone oxidation rates in the failing heart. Intriguingly, myocardial ketone body oxidative rates were not significantly different between mice fed a control diet versus a ketogenic diet, regardless of whether mice had heart failure (Figure 5.3 A). Recapitulating previous findings in our lab, absolute ketone body oxidation rates were unchanged between sham and heart failure mice, regardless of whether insulin was present or not (Figure 5.3 A). However, normalization of the absolute ketone body oxidation rates to cardiac work (to account for the significant drop in the MI heart's oxidative capacity) suggests that the ischemic failing heart relies more on ketone metabolism for energy (Figure 5.8 A).

5.5.3 The ketogenic diet blunts insulin-stimulated glucose oxidation rates

Of interest, insulin's ability to stimulate cardiac glucose oxidation can be used to assess the heart's insulin sensitive state. Therefore, all hearts were perfused without insulin for 30 minutes after which insulin was added to assess the heart's metabolic response to insulin. As expected, glucose oxidation rates in sham control hearts were significantly increased with the addition of insulin (Figure 5.3 B, Figure 5.8 B). Furthermore, MI control hearts, while trending to have lower glucose oxidation rates than sham control hearts, exhibited a less pronounced,

albeit significant, increase in glucose oxidation upon insulin stimulation (Figure 5.3 B). In contrast, sham and MI hearts from mice that consumed the ketogenic diet presented significantly reduced glucose oxidative rates regardless of whether insulin was added or not, suggesting a lack of insulin sensitivity following consumption of a ketogenic diet (Figure 5.3 B).

Palmitate oxidation rates, while trending to increase with the ketogenic diet in both sham and MI mice, did not significantly change between any groups (Figure 5.3 C). Similarly, glycolytic rates were not significantly different between experimental groups (Figure 5.3 D).

5.5.4 BDH1 protein expression increases in MI hearts but decreases with the ketogenic diet

To further investigate the metabolic changes and their underlying mechanisms, we assessed the protein expression of several metabolic and inflammatory targets via immunoblotting. Consistent with a reduction in myocardial ketone body oxidation rates (Figure 5.3 A), two important enzymes in the ketone oxidative pathway, β-hydroxybutyrate dehydrogenase (BDH1) and succinyl-CoA-oxoacid transferase (SCOT), had decreased protein expression in MI mice on the ketogenic diet versus MI mice on a control diet (Figure 5.4 A,B). Furthermore, BDH1 protein expression was significantly increased in MI control hearts compared to sham healthy hearts, recapitulating our work and others' findings on the failing heart's increased reliance on ketone metabolism for energy.

Long-chain acyl-CoA dehydrogenase (LCAD) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD), important enzymes in the oxidation of fatty acids, both trended to increase in MI hearts compared to sham hearts though the ketogenic diet had no effect on their

expression. Similarly, phosphorylation of the serine/threonine protein kinase Akt or pyruvate-dehydrogenase (PDH), the rate-limiting enzyme of glucose oxidation, was not significantly different between any of the experimental groups.

Lastly, assessment of several markers of inflammation, namely interleukin-1β (IL-1β), NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome, and interleukin 6 (IL-6) were also found to be unchanged with the ketogenic diet.

5.5.5 The ketogenic diet did not increase overall cardiac ATP production and shifts the heart's reliance on energy to fatty acids

Since ketones have been postulated to be another source of energy for the heart, we next wanted to assess whether the ketogenic diet had an effect on the total ATP production in the heart. Therefore, total adenosine-triphosphate (ATP) production was assessed in isolated working hearts. Healthy control hearts relied primarily on palmitate oxidation for its ATP production followed by glucose oxidation, glycolysis and ketone oxidation (Figure 5.5 A,B). In both healthy and MI mice, implementation of the ketogenic diet increased the heart's reliance on fatty acids for energy. This shift in energy reliance to fatty acids did not increase overall cardiac ATP production due to a concurrent decrease in the contribution of glucose oxidation to total ATP production (Figure 5.5 B).

As expected, total cardiac ATP production was substantially lower in MI hearts and while the ketogenic diet increased the failing heart's reliance on fatty acids, there was no parallel increase in overall cardiac ATP production.

5.5.6 The ketogenic diet decreases cardiac efficiency

To ascertain whether the shift in reliance to fatty acids for energy alongside the decreased glucose oxidation affects cardiac efficiency, oxygen consumption was measured in isolated working hearts. While MI hearts had lower oxygen consumption rates than sham hearts (Figure 5.6 A), normalization of cardiac work to oxygen consumption rates (the gold standard to assess cardiac efficiency) revealed that MI hearts had decreased cardiac efficiency which was worsened with the ketogenic diet (Figure 5.6 B).

5.6 Discussion

The ketogenic diet has become concerningly pervasive in our society as a non-pharmacological approach for weight loss despite the lack of clarity surrounding its cardiovascular and metabolic effects. The lack of certainty is compounded by the paucity of direct measurements of myocardial ketone body oxidation in the setting of heart failure. Here we report the first direct measurements of ketone body oxidation in the setting of ischemic heart failure through the application of carbon-14 radiolabelled β-hydroxybutyrate. Using a murine model of ischemic heart failure, we have determined that the ketogenic diet does not improve cardiac function, does not increase cardiac ketone oxidation rates, blunts insulin-stimulated myocardial glucose oxidation rates, shifts the heart's reliance on energy to fatty acids and notably, decreases cardiac efficiency.

The healthy heart is metabolically flexible and able to dynamically draw different proportions of energy from fatty acids, glucose, ketones, lactate and amino acids depending on the metabolic state of the body. This inherent ability to switch between fuel substrates differentiates a healthy heart from a failing heart. In the failing heart, a lack of understanding and confusion persists in the literature regarding the decisive metabolic changes that occur. Generally speaking, the failing heart is metabolically inflexible and switches its reliance on energy from oxidative metabolic processes to glycolysis[11]. As a consequence of mismatched ATP supply and demand, phosphocreatine, creatine and ATP levels drop in the failing myocardium, leading to an 'energy-starved' state[47]. Therefore, therapeutic strategies to restore the energy levels of the failing heart are of interest.

The sodium-glucose-co-transporter-2 inhibitor empagliflozin, and specifically, its ground-breaking clinical trial (EMPA-REG) catalyzed a plethora of interest in ketone bodies

and their potential beneficial role in the setting of cardiovascular disease[3]. Moreover, studies from our lab and others determined that the pressure overload hypertrophic heart relies more on ketones for energy, a process which is likely adaptive[7,8,16,69,72]. We have also previously determined that ketones are readily metabolized by the healthy heart and can be used as extra source of fuel for the heart[74]. Therefore, we hypothesized that increasing ketone delivery, via a ketogenic diet, to the failing heart would improve its energy-starved state and potentially improve cardiac function.

After 3-weeks of the ketogenic diet, our mice exhibited decreased fasting glucose levels and increased fasting ketone levels. These changes in blood parameters were, however, not accompanied by changes *in vivo* or *ex vivo* cardiac function in either sham or MI mice. This contrasts the findings of a study investigating the ketogenic diet in the setting of pressure overload hypertrophy[93]. In this transverse-aortic-constriction model of heart failure, mice were fed a 90%kcal% fat, 3%kcal carbohydrate, 7%kcal protein ketogenic diet for 4-weeks and exhibited decreased cardiac hypertrophy[93]. While the diet the authors used is comparable to ours in terms of %kcal composition, the primary source of fat in their ketogenic diets was lard while ours was composed of cocoa butter. Therefore, the lack of improvement in cardiac function in our study could be attributed to the different model of heart failure (and thus, type of heart failure being studied) as well as the decreased proportion of saturated fat in their ketogenic diet versus ours.

In a model utilizing a cardiac specific knock-out of mitochondrial pyruvate carrier (MPC), mice developed dilated cardiomyopathy that was reversed after 3-weeks of a 93.4%kcal fat, 1.8%kcal carbohydrate and 4.7%kcal protein ketogenic diet[95]. While it is not entirely clear why our findings contradict theirs, it could be due to the different model of heart

failure being studied as well as the primary source of the fat in their ketogenic diet. The authors had a slightly higher %kcal of fat in their ketogenic diet but importantly, sourced their fat from lard which has a higher proportion of unsaturated fatty acids than cocoa butter[95].

In a similar study, another group used cardiac-specific MPC knock-out mice and found that 15-weeks of a ketogenic diet reversed cardiac dysfunction induced by the lack of MPC[94]. Interestingly, Zhang et al. also carried out isolated working heart perfusions to assess glucose oxidation, glycolysis and fatty acid oxidation. While no ketone body oxidative rates were presented, they found that glucose oxidation was significantly decreased while fatty acid oxidation was significantly increased in their $cMPC^{-/-}$ mice (ie. heart failure mice). This gives evidence that the failing heart does switch from oxidative metabolism to glycolysis. As such, it seems counter-intuitive to implement a high fat diet in the setting of heart failure. Nevertheless, the authors found that implementation of a high-fat diet actually rescued cardiac hypertrophy from their $cMPC^{-/-}$ mice[94]. Unfortunately, they did not measure cardiac efficiency in their ketogenic-diet fed $cMPC^{-/-}$ mice but oddly, they found that their $cMPC^{-/-}$ had improved cardiac efficiency compared to their control healthy hearts. Our results contradict theirs as we determined that the ketogenic diet decreases cardiac efficiency in MI hearts. However, Zhang et al. also utilized the exact same ketogenic diet as McCommis et al. and therefore, results could have differed due to the different model of heart failure as well as source of fat in the ketogenic diet[94,95].

Assessment of the metabolic profile in our study revealed that while a ketogenic diet increases blood ketone levels and ketone delivery to the heart, this may not correspond to an increase in ketone body oxidation in the heart. In fact, ketone oxidation rates were unchanged between control-fed and ketogenic-fed mice regardless of whether they had heart failure.

Specifically, in MI mice fed a control diet, BDH1 protein expression was significantly increased compared to healthy sham mice fed a control diet. This supports previous findings published by our lab and others[7,8]. However, implementation of the ketogenic diet decreased BDH1 protein expression in MI hearts. A possible explanation for this could be the compensatory decrease in BDH1 protein expression. Previous work by Wentz et al. have found that cardiac energy metabolism can adapt to a ketogenic nutritional state by promoting transcriptional suppression of ketolytic enzymes (specifically SCOT)[44]. We also found that the ketolytic enzyme SCOT trended to decrease in ketogenic diet-fed MI mice hearts versus control diet-fed MI mice hearts though this did not reach statistical significance. As such, a compensatory transcriptional downregulation of ketone metabolic enzymes may explain the absence of increased ketone body oxidative rates in our study.

Due to the nature of the ketogenic diet, circulating insulin levels remain low and it has been speculated that this could lead to improvements in glycemic regulation[184]. However, recent work from our lab has found that a ketogenic diet does not improve glucose homeostasis or promote weight loss in obese mice isocalorically fed a ketogenic diet or control diet[185]. Additionally, other animal models have found that a ketogenic diet can cause dyslipidemia and glucose intolerance[186,187]. Accordingly, at the level of the heart, we also found impaired cardiac insulin responsivity. While insulin can stimulate glucose oxidation in sham and MI hearts, hearts from mice fed a ketogenic diet had blunted glucose oxidation rates that did not respond to the addition of insulin to the heart. This suggests that there may be insulin resistance at the level of the heart which may exacerbate the energy deficit seen in failing heart, ultimately contributing to a decrease in cardiac efficiency.

An increased reliance on fatty acid oxidation can decrease cardiac efficiency due to increased oxygen consumed per ATP molecule produced, reciprocal inhibition of glucose oxidation exacerbating the uncoupling of glycolysis and glucose oxidation, which increases proton levels and intracellular calcium and sodium. As such, ATP must be redirected to maintain ionic homeostasis and thus, cardiac efficiency decreases[188]. This would explain why the ketogenic diet decreased cardiac efficiency in our study. By shifting the energy profile of the heart to rely more on fatty acids, the ketogenic diet decreases cardiac efficiency.

5.6.1 Limitations

Several limitations must be acknowledged in our study which include the use of only male mice. Sex-specific differences unquestionably affect the regulation of cardiac energy metabolism so future studies will need to include female mice. Additionally, glycolytic rates are extremely high in mice so future studies utilizing rats or mammals closer to humans would be ideal. As previously mentioned, there were discrepancies between our ketogenic diet and what others have used in their studies. A drawback to studying the ketogenic diet is the lack of diet standardization. Interestingly, a recent study demonstrated that alternate-day feeding of a ketogenic diet may be beneficial while continuous ketogenic diet feeding aggravates diastolic dysfunction in a model of pressure overload hypertrophy[88]. Therefore, future studies that assess cardiac energy metabolism while also applying an alternate-day feeding model are warranted.

5.6.2 Conclusions

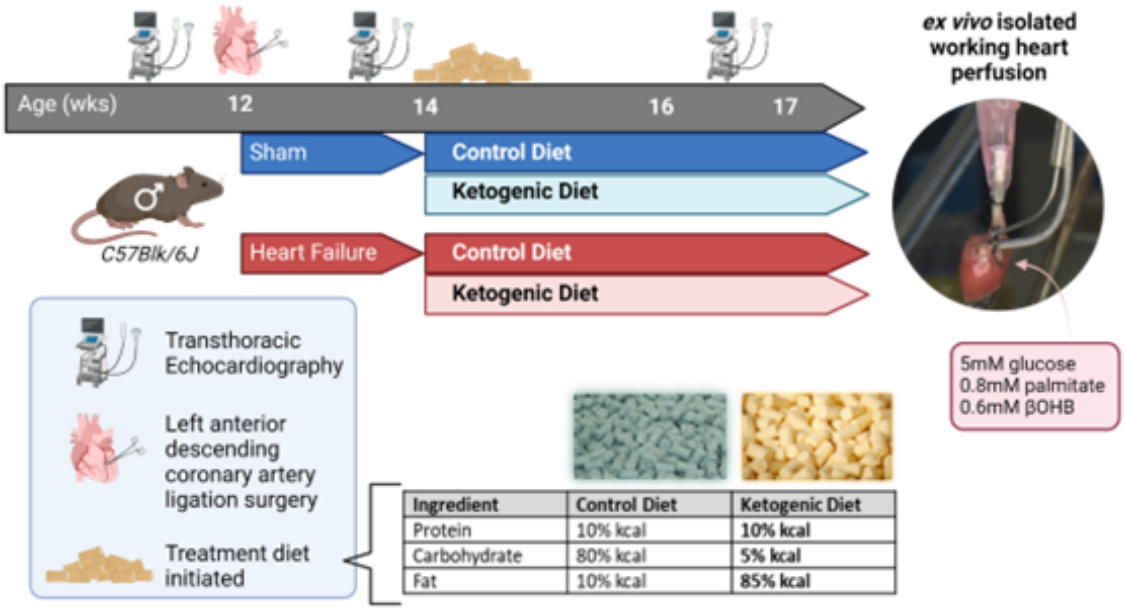
Taken together, we present the first study to directly assess ketone body metabolism in the setting of ischemic heart failure and show that the ketogenic diet does not improve cardiac

function and instead, shifts the heart's reliance to fatty acids for energy, blunts glucose oxidation rates and ultimately, decreases cardiac efficiency.

5.7 Figures

Figure 5.1 Experimental protocol and effect of the ketogenic diet on body weight, blood glucose and blood ketones. **A**, Experimental protocol. Please see methods for details. **B**, Fed and fasted body weight of mice pre-ketogenic diet and post-ketogenic diet. **C**, Fed and fasted blood glucose pre-ketogenic diet and post-ketogenic diet. **D**, Fed and fasted blood beta-hydroxybutyrate levels pre-ketogenic diet and post-ketogenic diet.

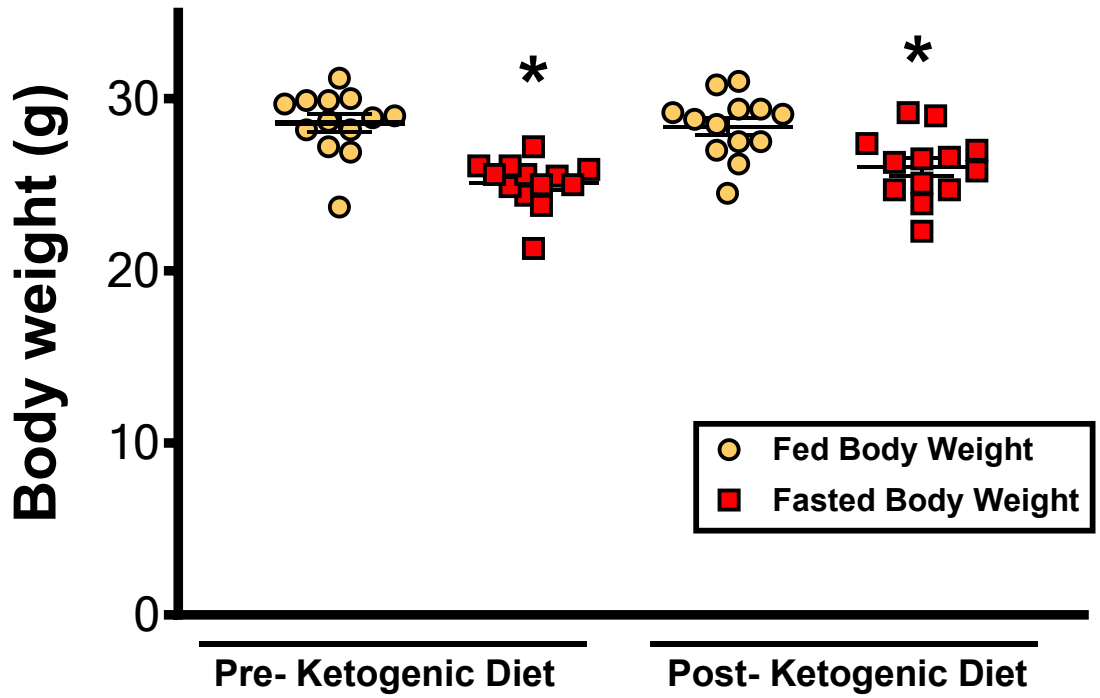
A



Ingredient	Control Diet	Ketogenic Diet
Protein	10% kcal	**10% kcal**
Carbohydrate	80% kcal	**5% kcal**
Fat	10% kcal	**85% kcal**

FIGURE 5.1

B



*: p<0.05 compared to the fed group within the pre/post-diet group.
†: p<0.05 compared to the pre-diet group

FIGURE 5.1

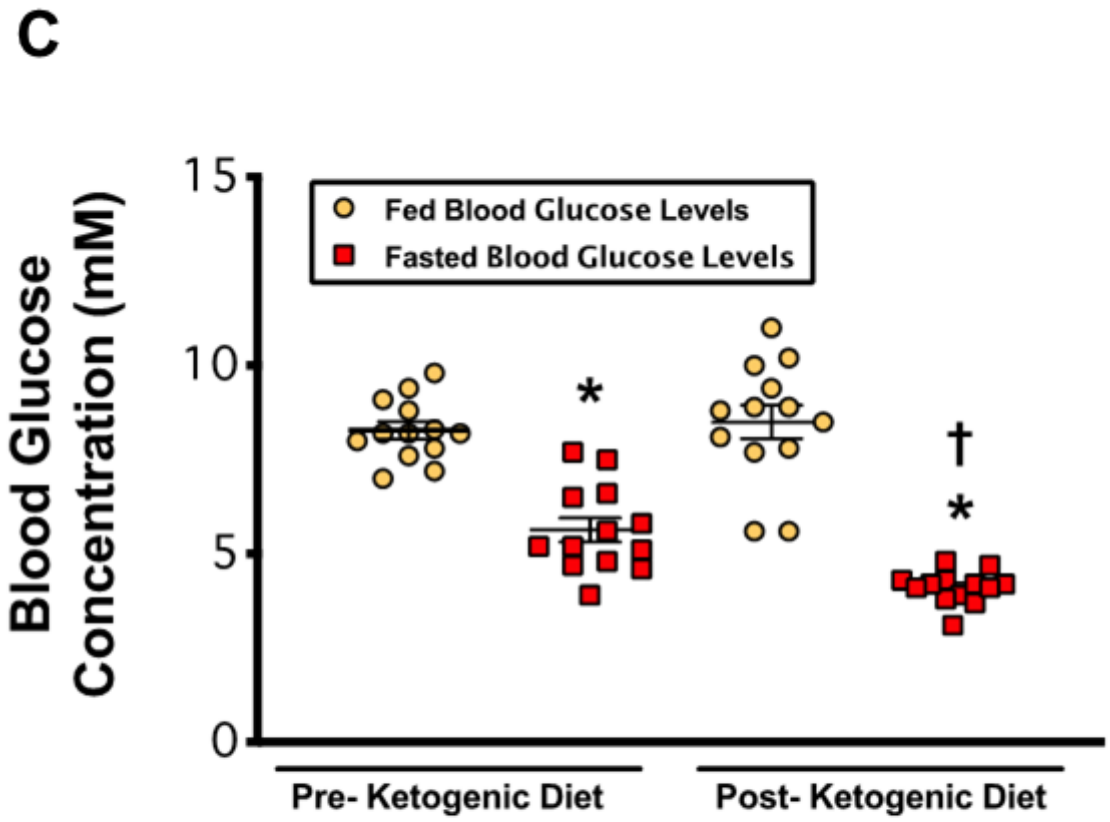

C
Blood Glucose Concentration (mM)
15
10
5
0
Fed Blood Glucose Levels
Fasted Blood Glucose Levels
*
†
*
Pre- Ketogenic Diet
Post- Ketogenic Diet

FIGURE 5.1

D

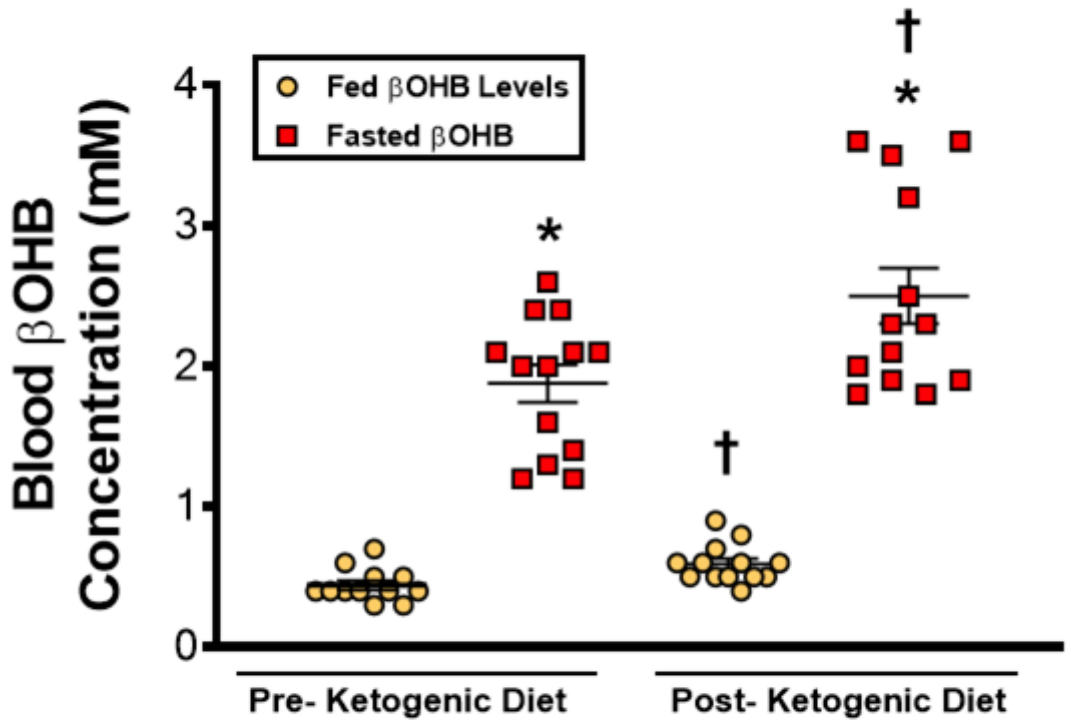

Blood βOHB Concentration (mM)
Fed βOHB Levels
Fasted βOHB
0
1
2
3
4
*
*
†
†
Pre- Ketogenic Diet
Post- Ketogenic Diet

FIGURE 5.1

Figure 5.2 The ketogenic diet does not improve in vivo or ex vivo cardiac function. Transthoracic echocardiographic measurements of in vivo cardiac function: (A,B) Ejection fraction, (C,D) diastolic function. (E) *Ex vivo* measurement of cardiac function via isolated working heart perfusion.

A

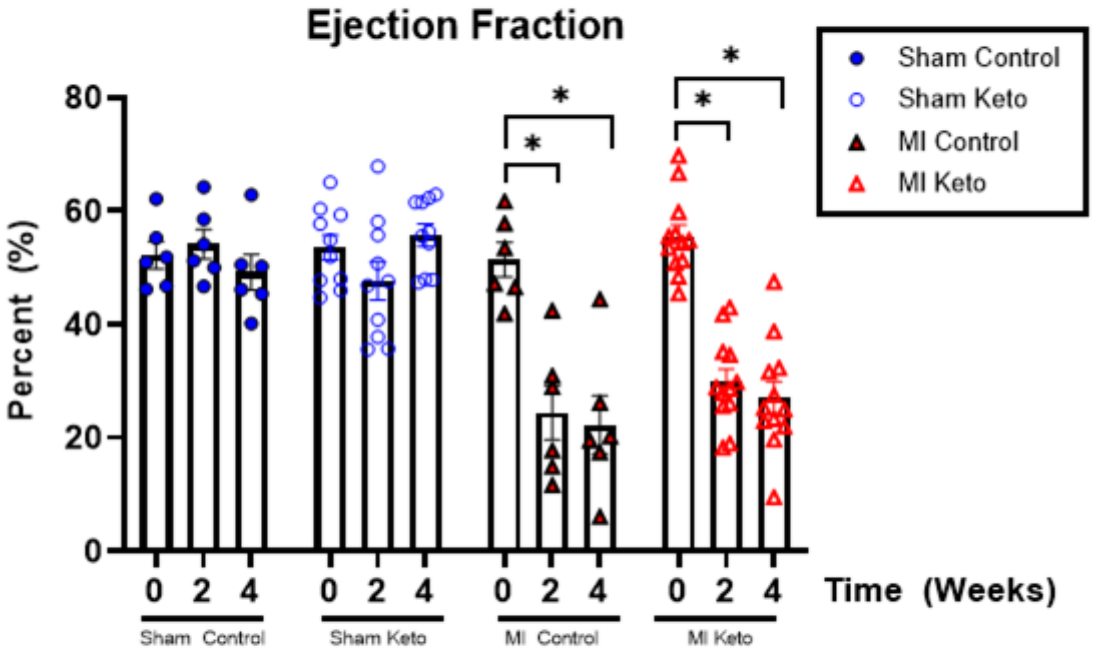

Ejection Fraction
Percent (%)
80
60
40
20
0
*
*
*
0 2 4 0 2 4 0 2 4 0 2 4
Time (Weeks)
Sham Control
Sham Keto
MI Control
MI Keto
Sham Control
Sham Keto
MI Control
MI Keto

FIGURE 5.2

B

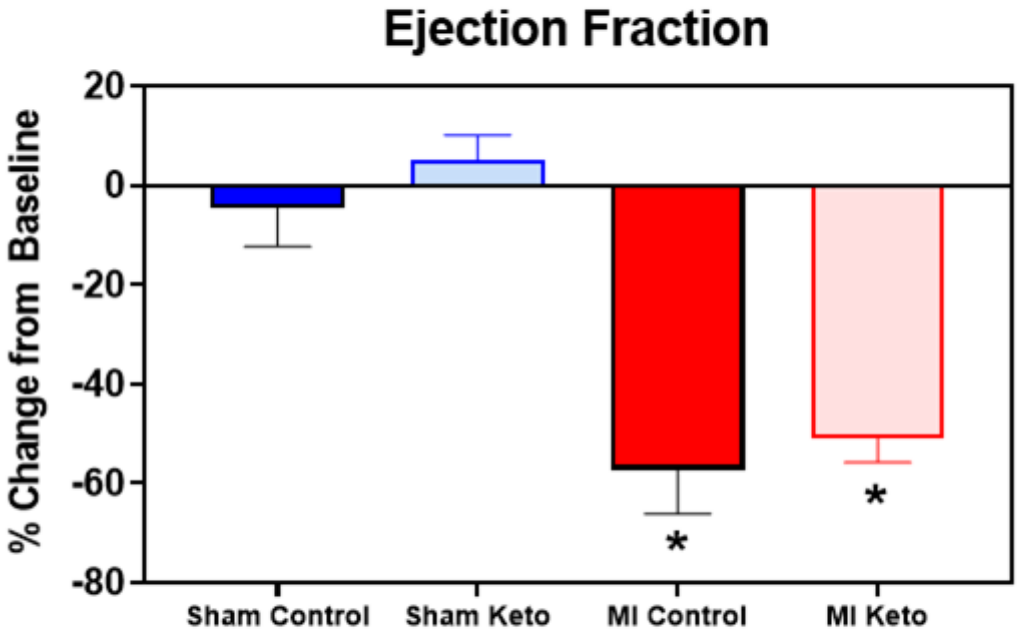

Ejection Fraction
% Change from Baseline
20
0
-20
-40
-60
-80
*
*
Sham Control
Sham Keto
MI Control
MI Keto

FIGURE 5.2

C

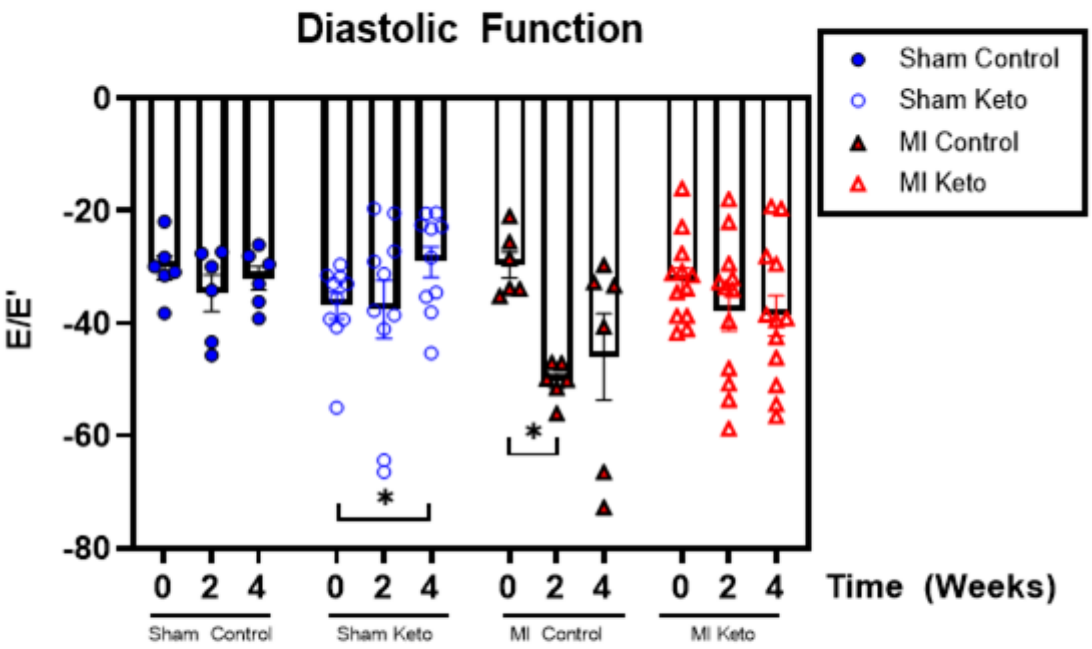

Diastolic Function
Sham Control
Sham Keto
MI Control
MI Keto
E/E'
0
-20
-40
-60
-80
0 2 4 0 2 4 0 2 4 0 2 4
Time (Weeks)
Sham Control
Sham Keto
MI Control
MI Keto
*
*

FIGURE 5.2

D

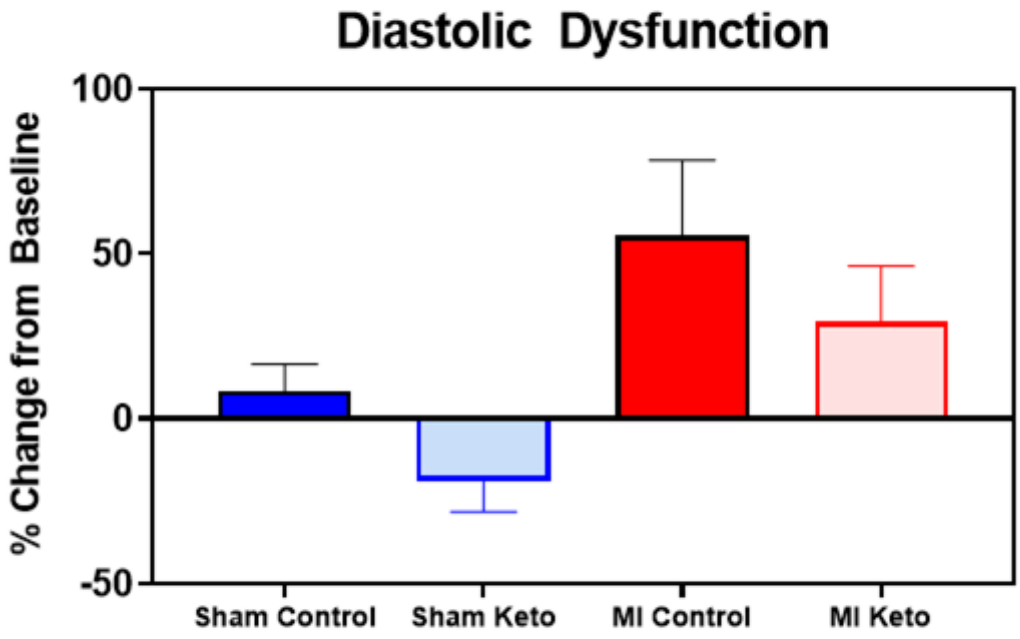

Diastolic Dysfunction
% Change from Baseline
100
50
0
-50
Sham Control
Sham Keto
MI Control
MI Keto

FIGURE 5.2

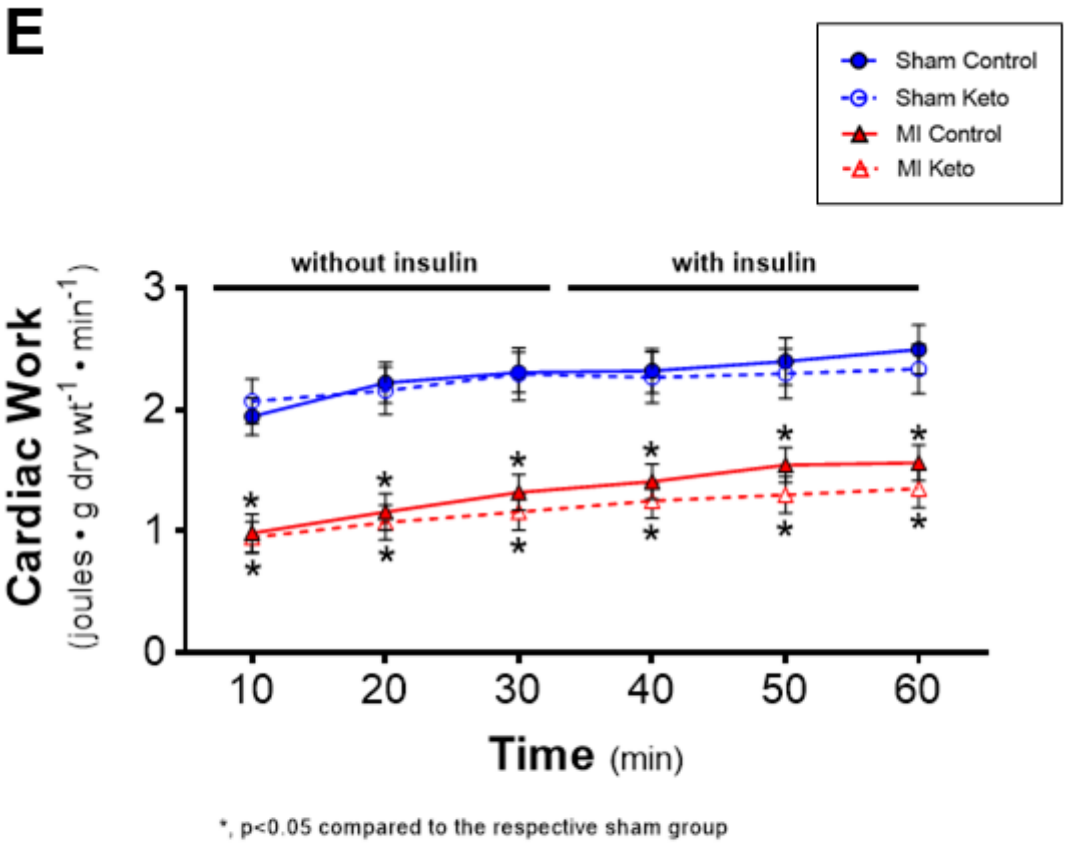

Sham Control
Sham Keto
MI Control
MI Keto
without insulin
with insulin
Cardiac Work
(joules • g dry wt-1 • min-1)
0
1
2
3
10
20
30
40
50
60
Time (min)
*, p<0.05 compared to the respective sham group

FIGURE 5.2

Figure 5.3 The ketogenic diet blunts insulin-stimulated glucose oxidation. Cardiac energy metabolic rates as measured in the isolated working heart. (A) Beta-hydroxybutyrate oxidative rates without and with insulin (ketone body oxidation rates), (B) glucose oxidation rates without and with insulin, (C) palmitate oxidation rates without and with insulin, (D) glycolytic rates without and with insulin.

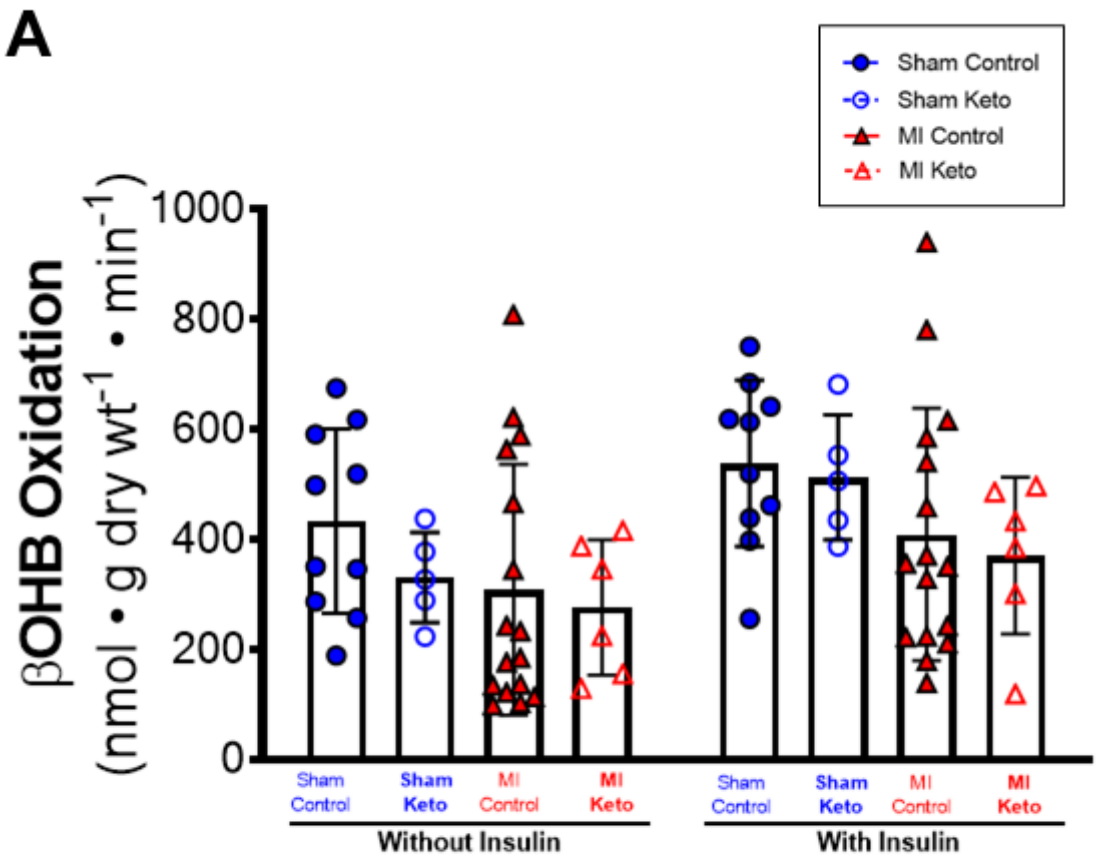
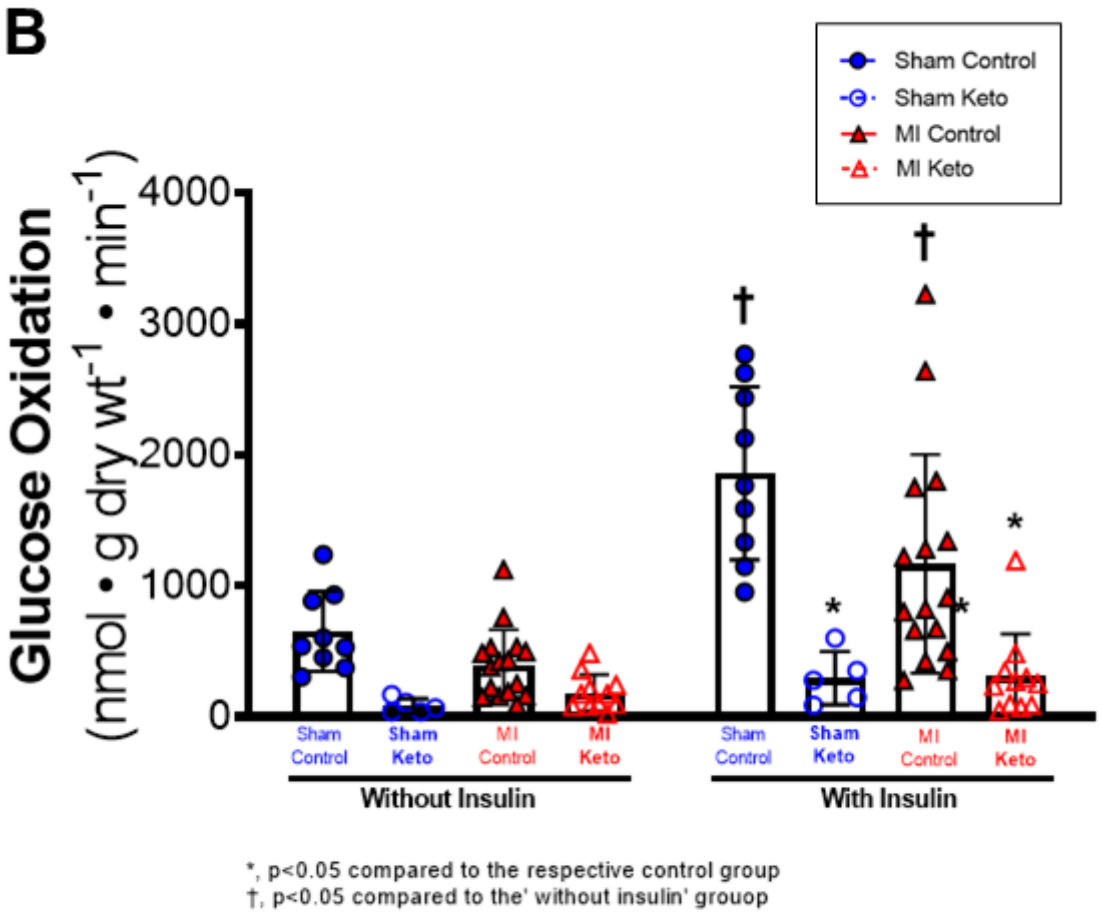

A
Sham Control
Sham Keto
MI Control
MI Keto
βOHB Oxidation
(nmol • g dry wt-1 • min-1)
1000
800
600
400
200
0
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
Sham Control
Sham Keto
MI Control
MI Keto
With Insulin

FIGURE 5.3



*, p<0.05 compared to the respective control group
†, p<0.05 compared to the' without insulin' grouop

FIGURE 5.3

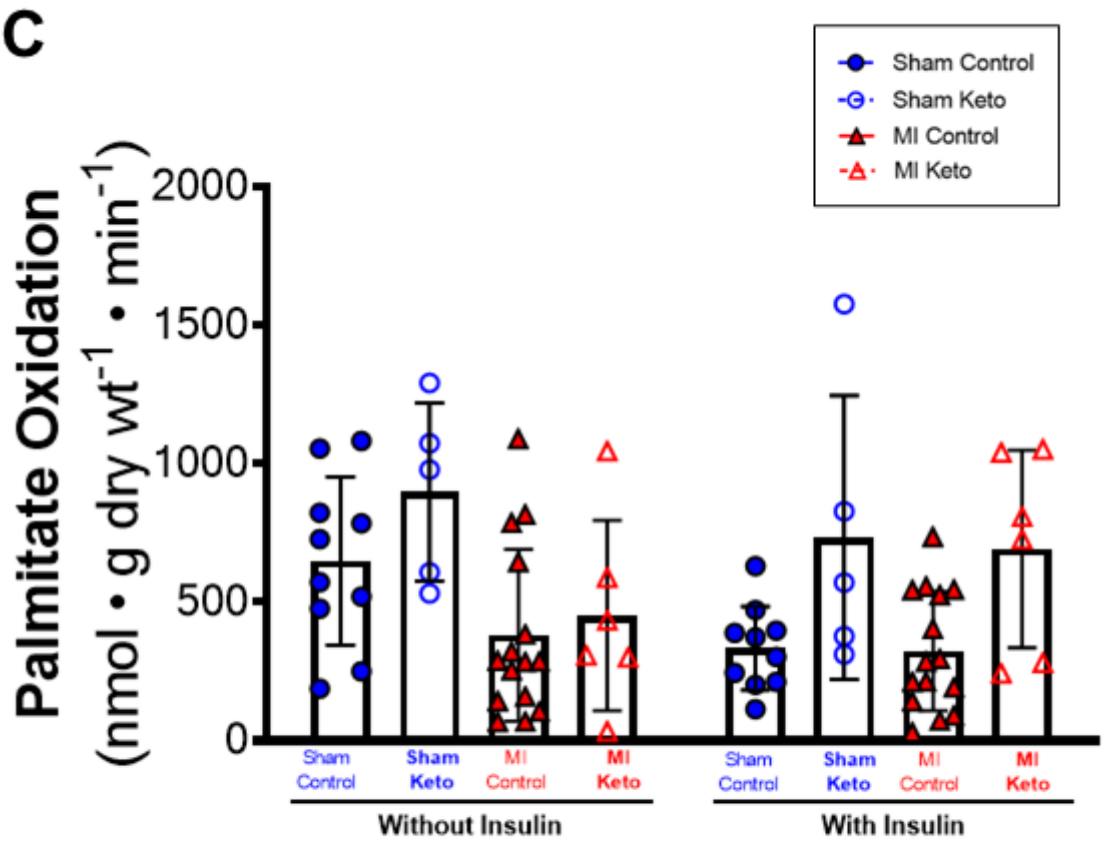

C
Sham Control
Sham Keto
MI Control
MI Keto
Palmitate Oxidation
(nmol • g dry wt-1 • min-1)
2000
1500
1000
500
0
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
With Insulin

FIGURE 5.3

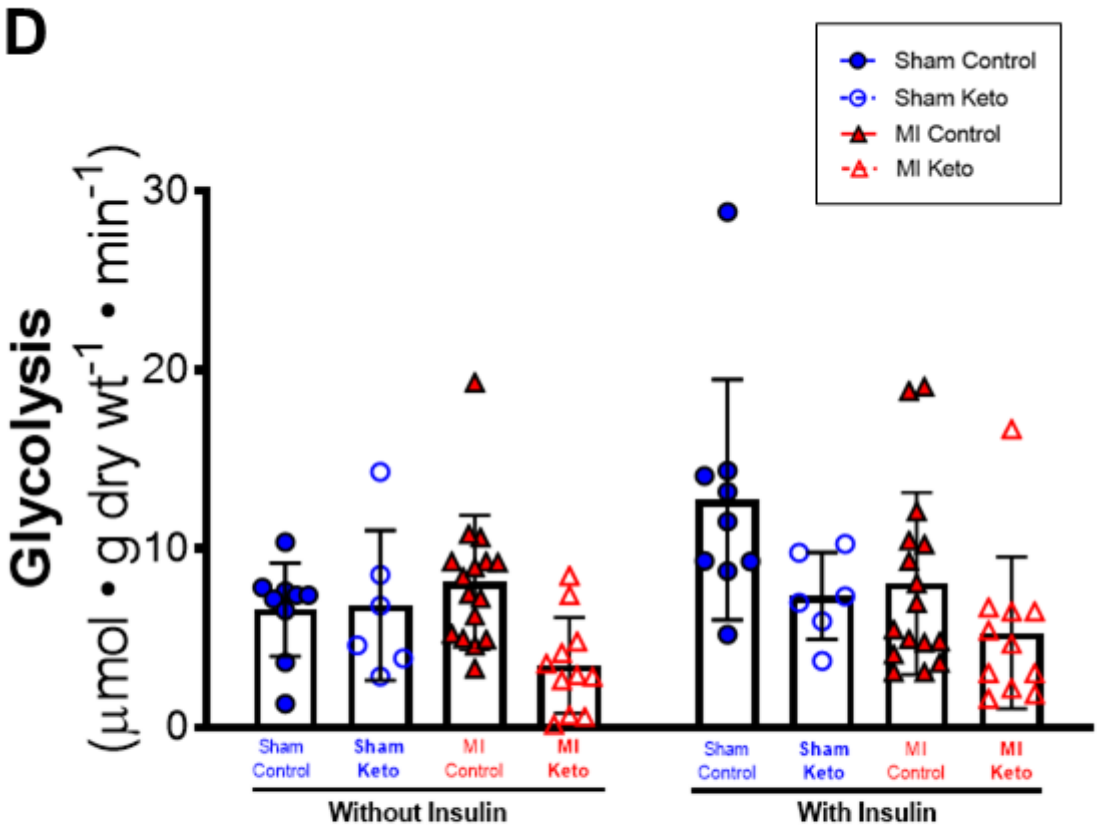

D
Sham Control
Sham Keto
MI Control
MI Keto
Glycolysis
(µmol • g dry wt-1 • min-1)
30
20
10
0
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
With Insulin

FIGURE 5.3

Figure 5.4 Cardiac protein expression of various metabolic and inflammatory targets, namely: long-chain acyl-CoA dehydrogenase (LCAD), β-hydroxyacyl-CoA-dehydrogenase (β-HAD), pyruvate-dehydrogenase (PDH), serine/threonine protein kinase Akt (Akt), succinyl-CoA-oxoacid transferase (SCOT), β-hydroxybutyrate dehydrogenase (BDH1), interleukin-1β (IL-1β) NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome, and interleukin 6 (IL-6).

A

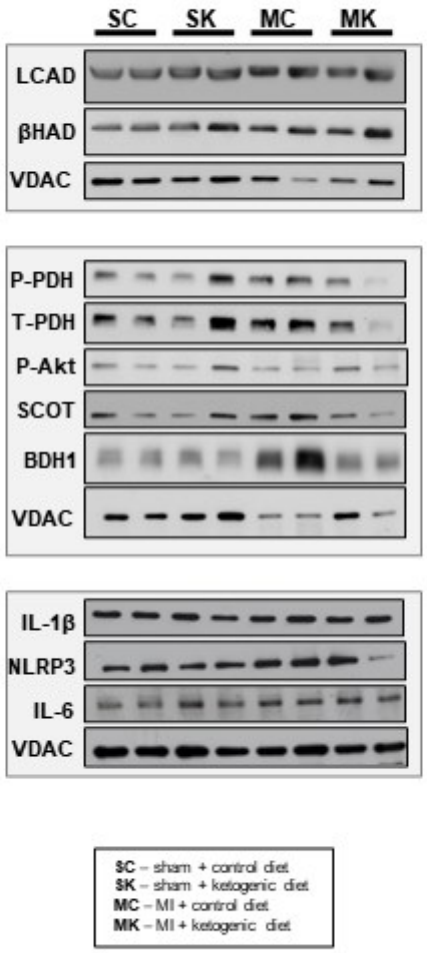

SC
SK
MC
MK
LCAD
βHAD
VDAC
P-PDH
T-PDH
P-Akt
SCOT
BDH1
VDAC
IL-1β
NLRP3
IL-6
VDAC
SC – sham + control diet
SK – sham + ketogenic diet
MC – MI + control diet
MK – MI + ketogenic diet

FIGURE 5.4

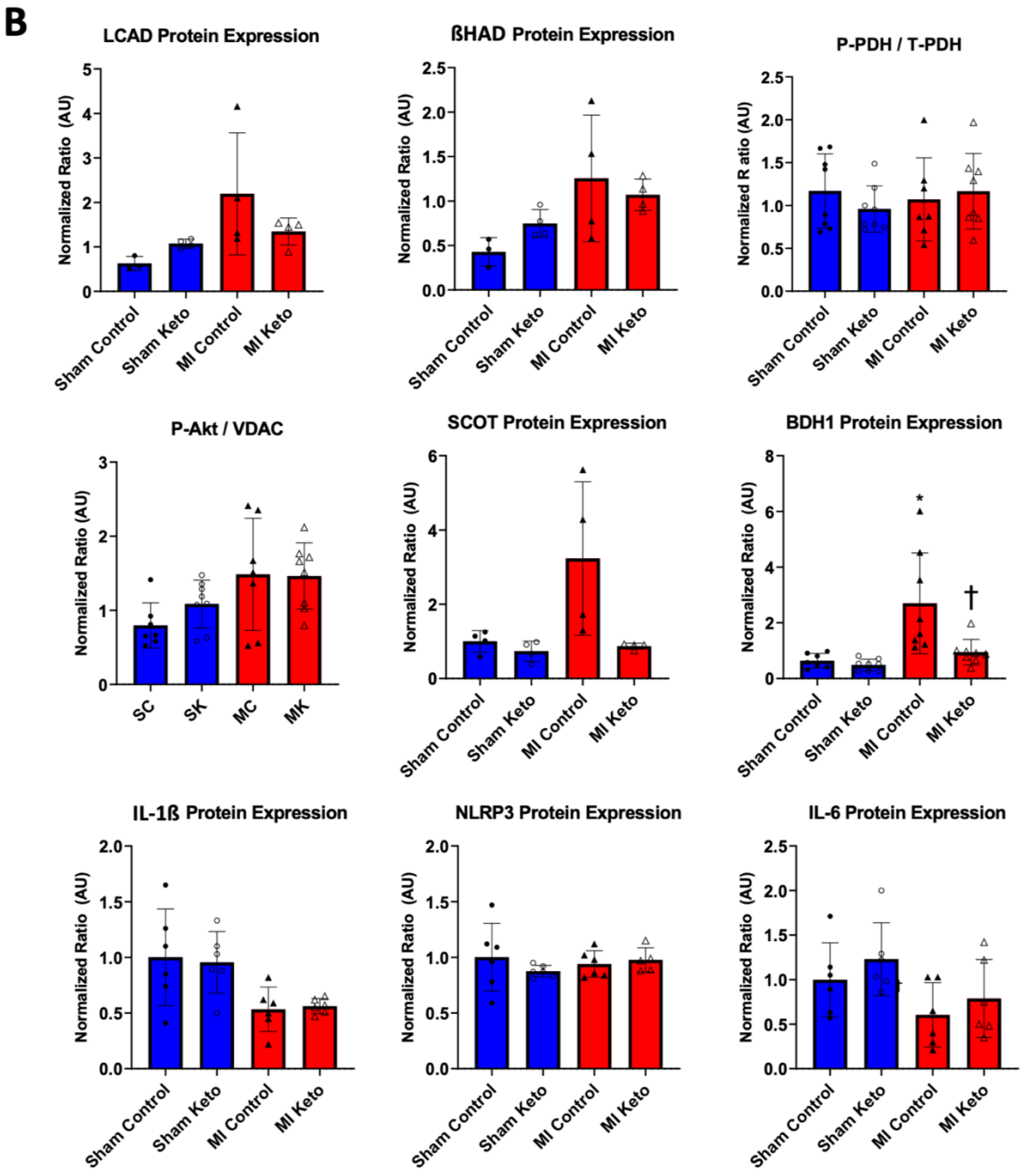

B
LCAD Protein Expression
Normalized Ratio (AU)
Sham Control
Sham Keto
MI Control
MI Keto
ßHAD Protein Expression
Normalized Ratio (AU)
P-PDH / T-PDH
Normalized R atio (AU)
P-Akt / VDAC
Normalized Ratio (AU)
SC
SK
MC
MK
SCOT Protein Expression
Normalized Ratio (AU)
BDH1 Protein Expression
Normalized Ratio (AU)
IL-1ß Protein Expression
Normalized Ratio (AU)
NLRP3 Protein Expression
Normalized Ratio (AU)
IL-6 Protein Expression
Normalized Ratio (AU)

FIGURE 5.4

Figure 5.5 The ketogenic diet causes a shift in reliance to fatty acids for energy. (A) Absolute ATP production rates coming from palmitate oxidation, glucose oxidation, ketone oxidation and glycolysis without and with insulin. (B) Percent contribution of palmitate oxidation, glucose oxidation, ketone oxidation and glycolysis to total (100%) ATP production without and with insulin.

A

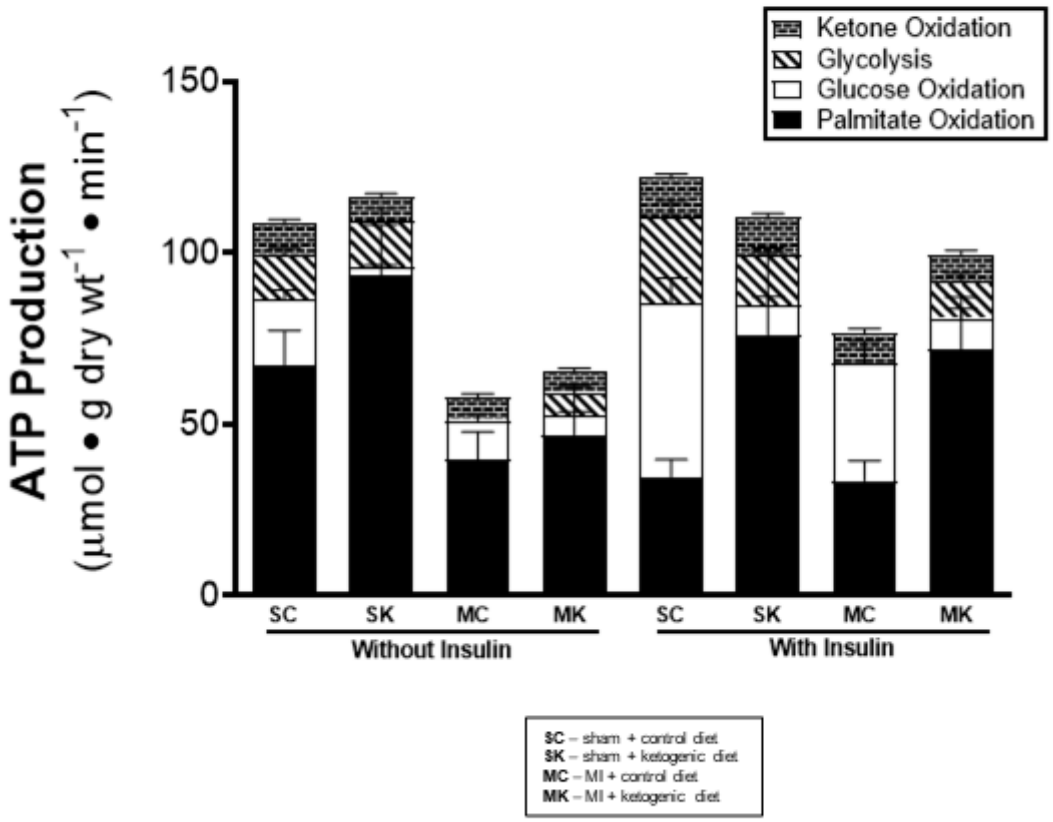

Ketone Oxidation
Glycolysis
Glucose Oxidation
Palmitate Oxidation
ATP Production
(µmol • g dry wt^{-1} • min^{-1})
150
100
50
0
SC
SK
MC
MK
SC
SK
MC
MK
Without Insulin
With Insulin
SC – sham + control diet
SK – sham + ketogenic diet
MC – MI + control diet
MK – MI + ketogenic diet

FIGURE 5.5

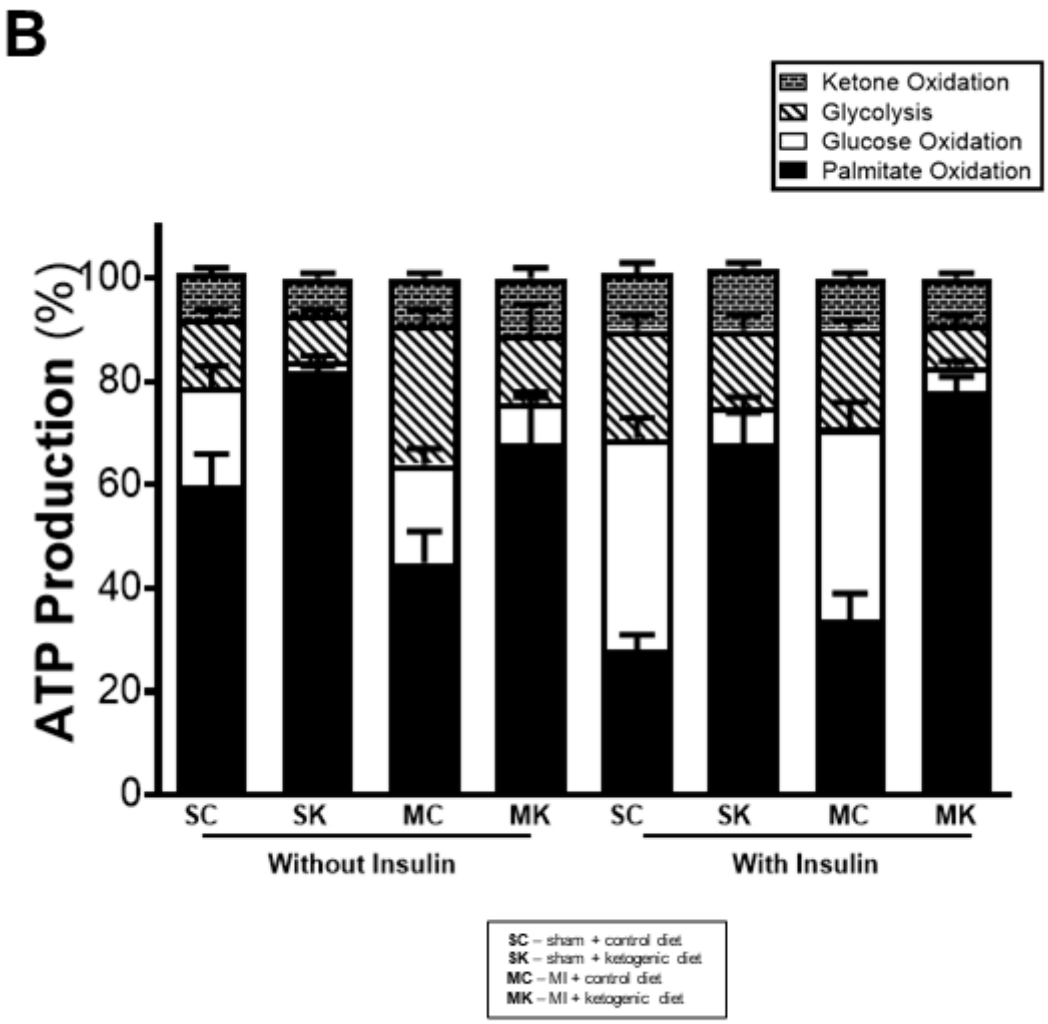
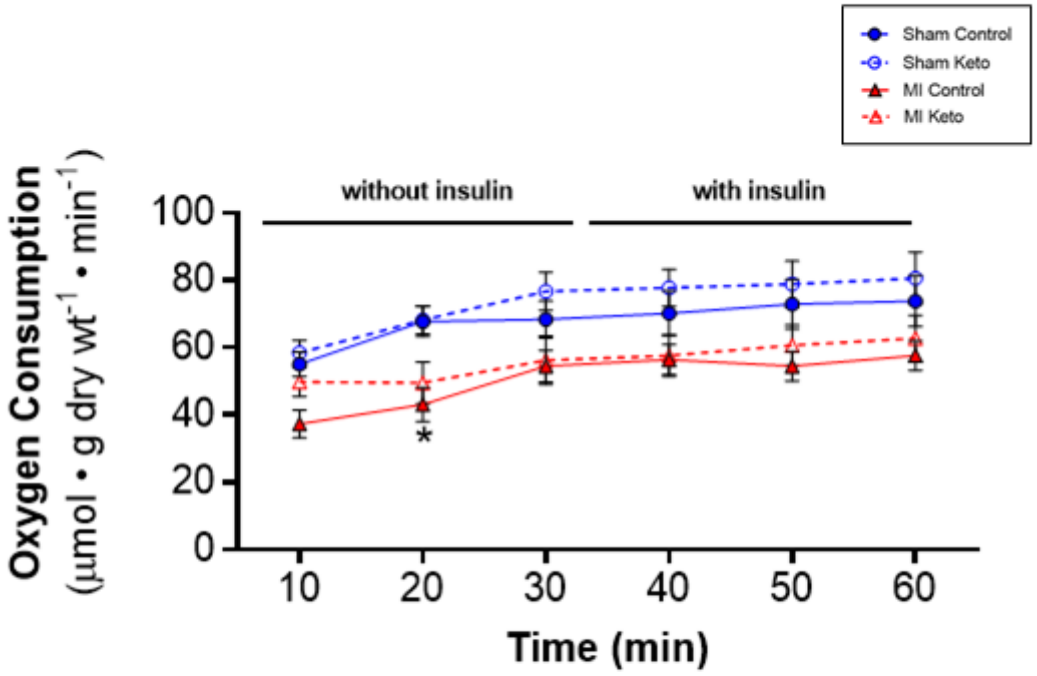

B
Ketone Oxidation
Glycolysis
Glucose Oxidation
Palmitate Oxidation
ATP Production (%)
100
80
60
40
20
0
SC
SK
MC
MK
SC
SK
MC
MK
Without Insulin
With Insulin
SC – sham + control diet
SK – sham + ketogenic diet
MC – MI + control diet
MK – MI + ketogenic diet

FIGURE 5.5

Figure 5.6 The ketogenic diet does not improve cardiac efficiency. (A) Oxygen consumption rates as measured by cannulation of the pulmonary artery in the isolated working heart. (B) Cardiac efficiency as determined by normalizing *ex vivo* cardiac work to oxygen consumption rates.

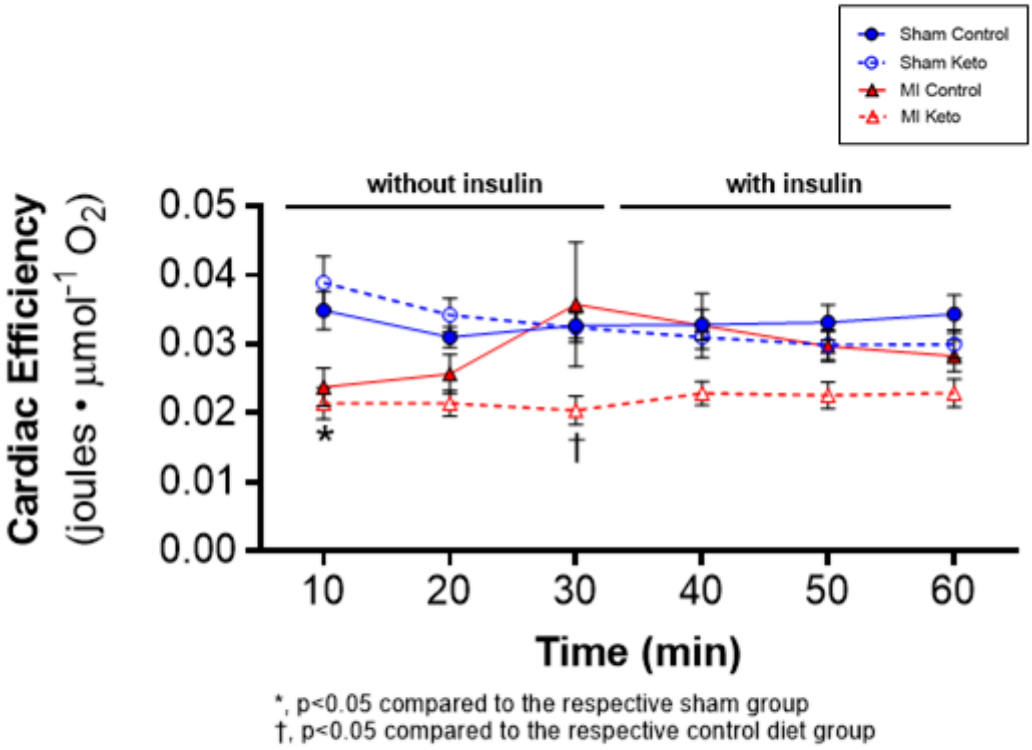
A



*, p<0.05 compared to the respective sham group

FIGURE 5.6

B



*, p<0.05 compared to the respective sham group
†, p<0.05 compared to the respective control diet group

FIGURE 5.6

Figure 5.7 Echocardiographic data plotted as changes over time. Serial transthoracic echocardiographic assessment of cardiac function in sham control, sham keto, MI control and MI keto mice at 0 weeks post MI-surgery, 2 weeks post-MI surgery and 4 weeks post-MI surgery. (A) Ejection fraction, (B) Diastolic dysfunction as assessed by the e/e' ratio.

A

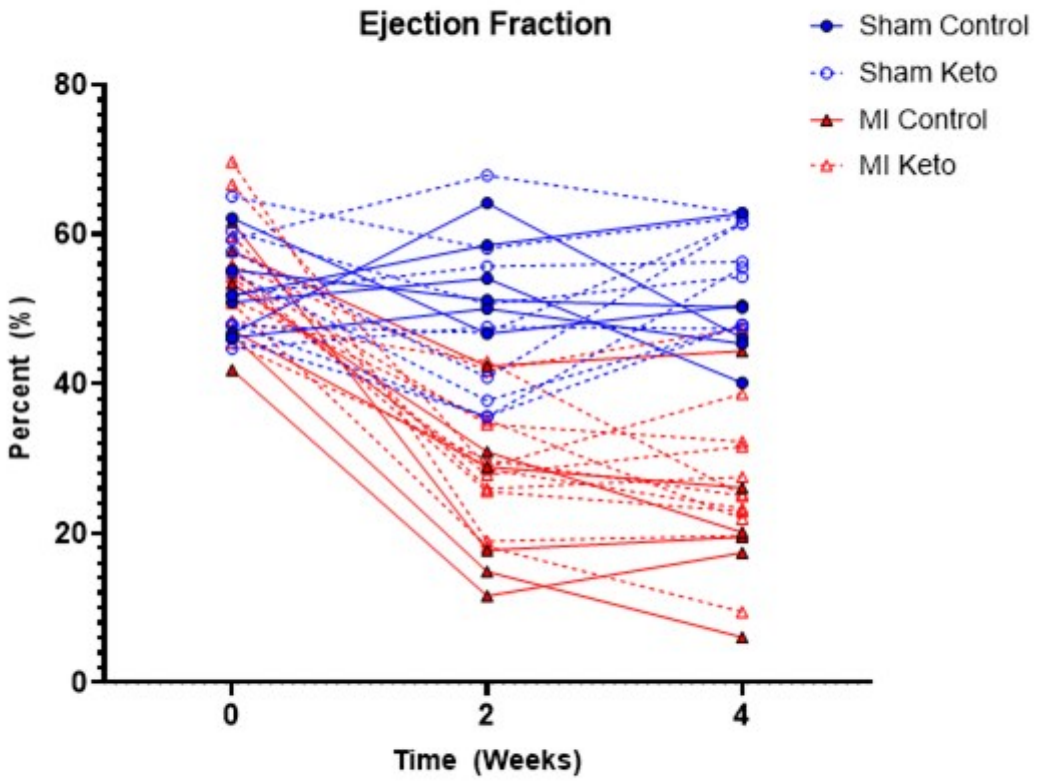

Ejection Fraction
Sham Control
Sham Keto
MI Control
MI Keto
Percent (%)
80
60
40
20
0
0
2
4
Time (Weeks)

FIGURE 5.7

B

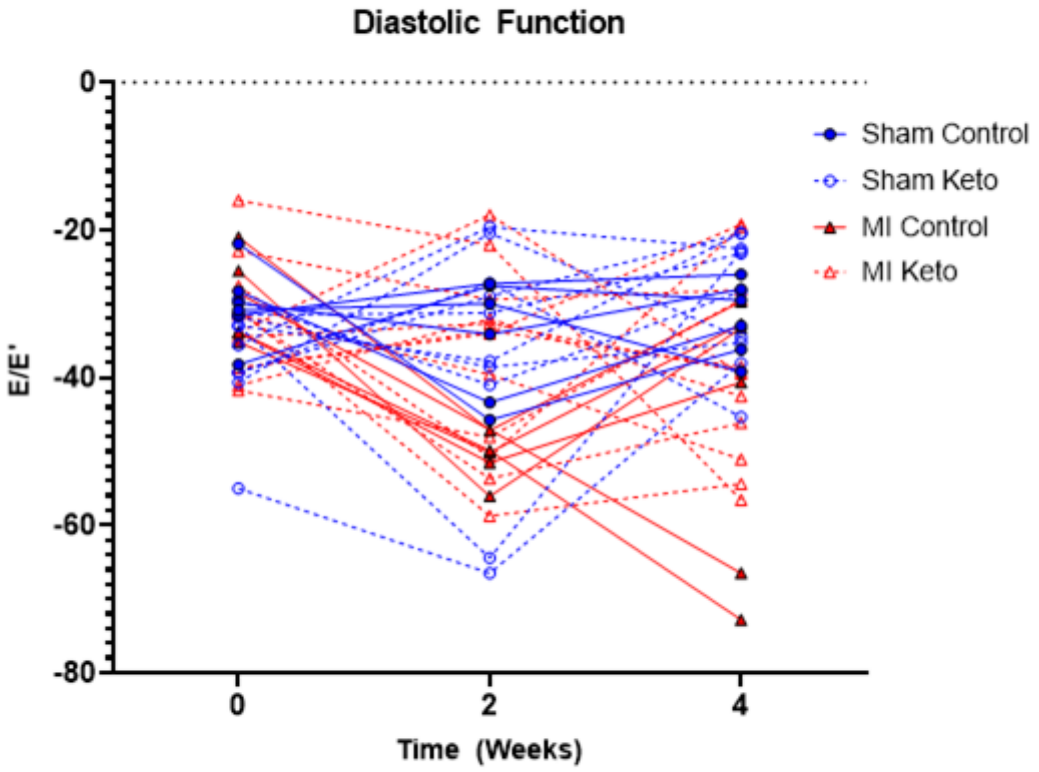

Diastolic Function
0
-20
-40
-60
-80
E/E'
0
2
4
Time (Weeks)
Sham Control
Sham Keto
MI Control
MI Keto

FIGURE 5.7

Figure 5.8 Metabolic rates normalized to cardiac work. Cardiac energy metabolic rates as measured in the isolated working heart are now normalized to their corresponding cardiac work. (A) Beta-hydroxybutyrate oxidative rates normalized to cardiac work without and with insulin (ketone body oxidation rates), (B) glucose oxidation rates normalized to cardiac work without and with insulin, (C) palmitate oxidation rates normalized to cardiac work without and with insulin, (D) glycolytic rates normalized to cardiac work without and with insulin.

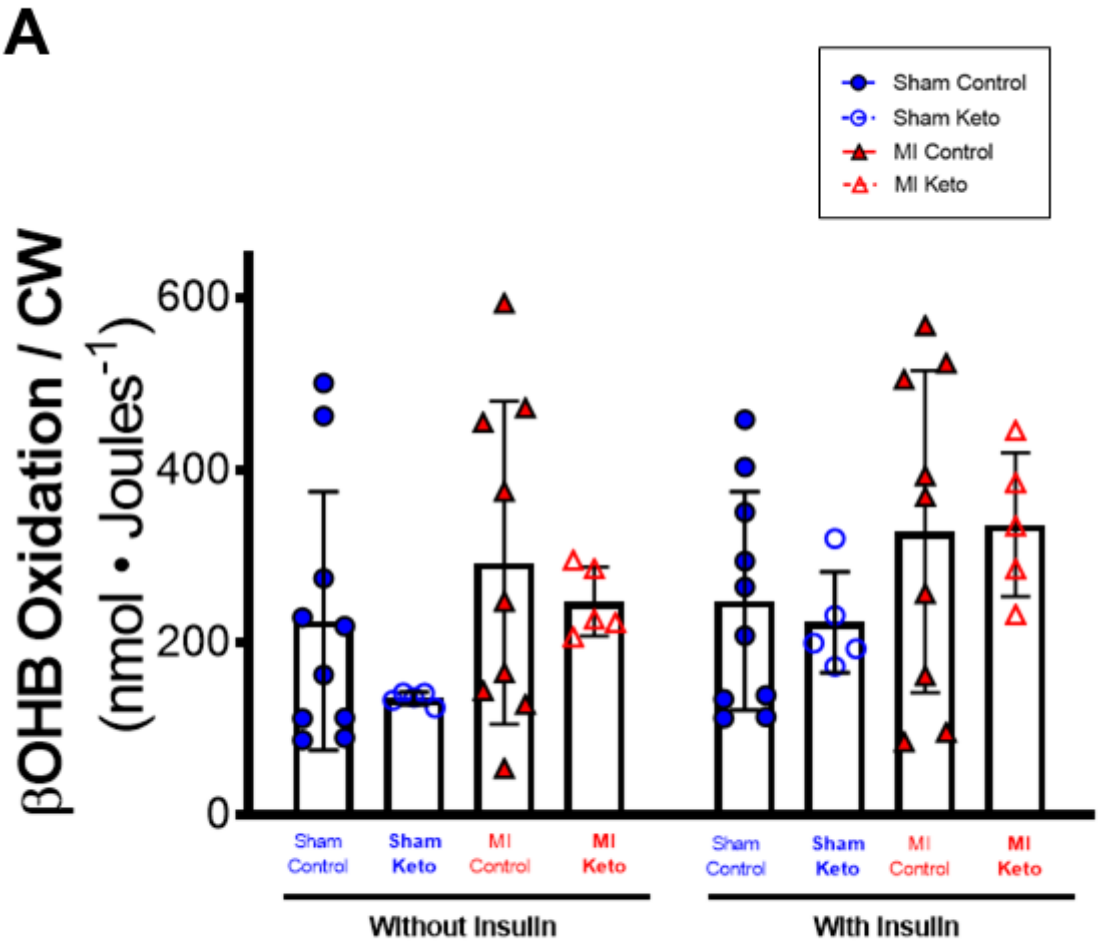

A
Sham Control
Sham Keto
MI Control
MI Keto
βOHB Oxidation / CW
(nmol • Joules-1)
600
400
200
0
Sham Control
Sham Keto
MI Control
MI Keto
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
With Insulin

FIGURE 5.8

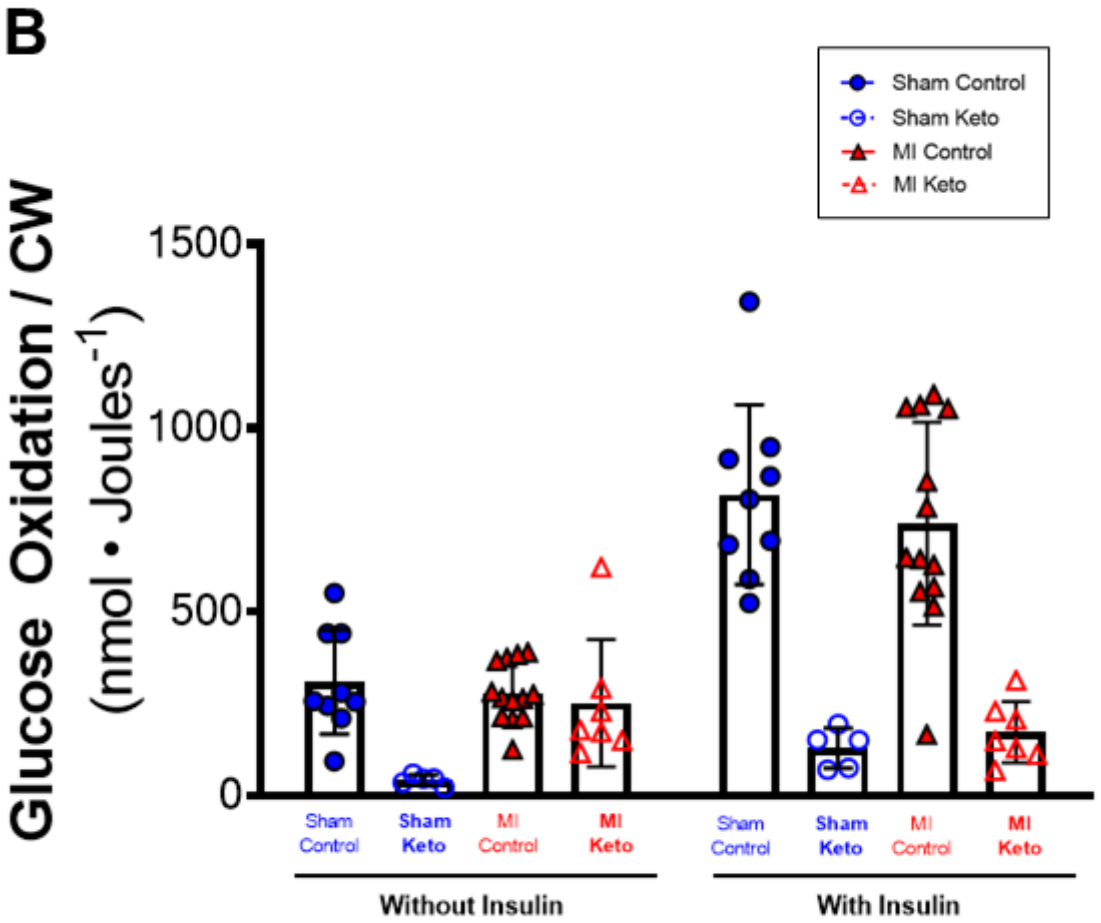

B
Glucose Oxidation / CW
(nmol • Joules-1)
1500
1000
500
0
Sham Control
Sham Keto
MI Control
MI Keto
Sham Control
Sham Keto
MI Control
MI Keto
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
With Insulin

FIGURE 5.8

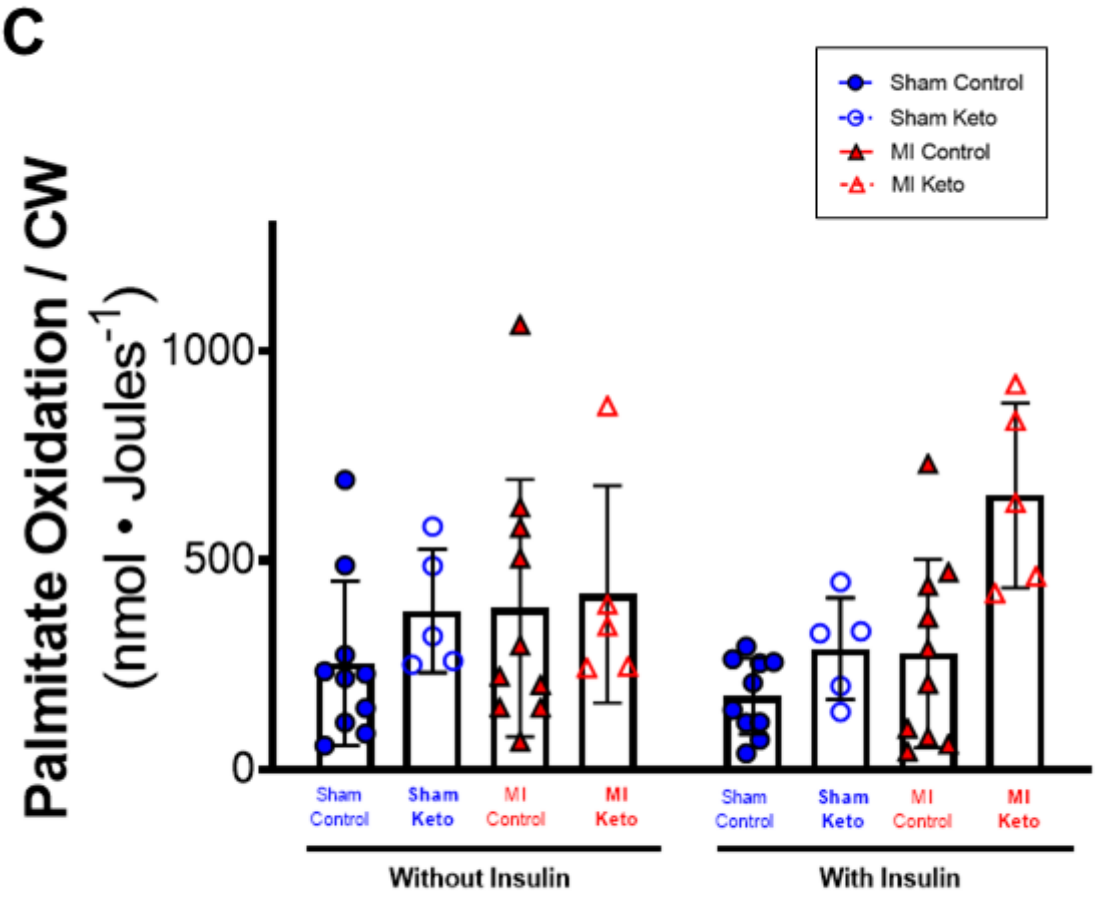

C
Sham Control
Sham Keto
MI Control
MI Keto
Palmitate Oxidation / CW
(nmol • Joules-1)
1000
500
0
Sham Control
Sham Keto
MI Control
MI Keto
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
With Insulin

FIGURE 5.8

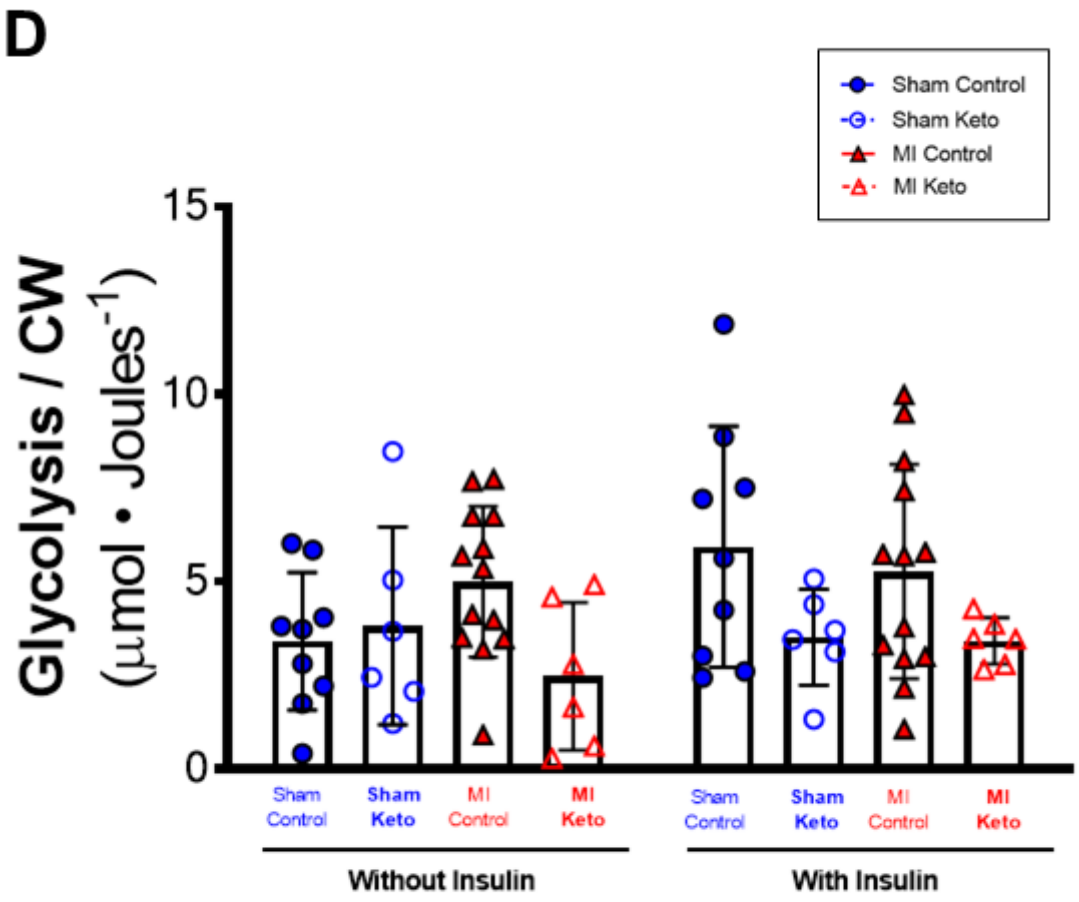

D
Sham Control
Sham Keto
MI Control
MI Keto
Glycolysis / CW
(µmol • Joules-1)
15
10
5
0
Sham Control
Sham Keto
MI Control
MI Keto
Sham Control
Sham Keto
MI Control
MI Keto
Without Insulin
With Insulin

FIGURE 5.8

Chapter VI
Discussion and Conclusions

Chapter 6 Discussion and Conclusions

With growing interest in the importance of ketone body metabolism alongside popularity of the ketogenic diet and evidence that SGLT2 inhibitors increase circulating ketone bodies while conferring cardiovascular benefits, my thesis is highly topical as it investigates whether increasing myocardial ketone body oxidation (acutely and chronically) will provide an extra source of energy for the starving failing heart, which will be protective and mitigate further pathophysiological remodeling of the failing heart (Figure 1.5).

6.1 Aim 1 (Chapter 3)

6.1.1 Novelty

This is the first study to directly measure myocardial ketone body oxidation rates in a failing heart.

6.1.2 Summary

In chapter 3, **Aim 1** was addressed and *ex vivo* cardiac function and metabolic profile of the failing heart in response to an acute increase in ketones were assessed.

In male mice subjected to a TAC surgery to induce pressure overload hypertrophy over 4-weeks, mice hearts perfused with radiolabeled glucose, palmitate and β-hydroxybutyrate revealed robust changes in the overall metabolic profile of the failing heart. The following changes in the metabolic profile were observed (Figure 3.3, Figure 3.4):

I. Absolute rates of glucose oxidation decrease in heart failure
II. Normalized rates of fatty acid oxidation increase in heart failure
III. Normalized rates of ketone oxidation increase in heart failure

By measuring TCA cycle activity and ATP production, we also found that the increased reliance on ketones for energy in the failing heart resulted in an overall increase in ATP production despite the failing hearts having blunted total ATP production (Figure 3.5, Figure 3.9). In this study, we also increased the concentration of ketones delivered to the perfused heart in an acute manner. When the heart was exposed to 600μM βOHB, ketones can contribute to over 20% of the heart's ATP production. This was not accompanied by any changes in cardiac efficiency though.

Lastly, we also found that the failing heart has increased overall lysine acetylation and importantly, hyperacetylation of the fatty acid oxidative enzyme LCAD. Previous work from our lab has shown that hyperacetylation of fatty acid oxidative enzymes increase its activity[33], supporting our observation of increased normalized rates of fatty acid oxidation. We also found a significant increase in the protein expression of the ketone body oxidative enzyme BDH1, supporting our observation of increased normalized rates of ketone oxidation.'

6.1.3 Absolute Versus Normalized Metabolic Rates

In chapter 3, you may recall the discussion of absolute metabolic rates versus normalized metabolic rates. The importance behind normalizing metabolic rates is not acknowledged in the literature enough. The rationale of normalizing metabolic rates is based on the fact that oxidatively derived energy contributes to a large portion of the contractile energy of the heart. As such, normalizing metabolic rates accounts for changes in the oxidative capacity of the heart between different disease states (ie. Healthy versus failing heart).

The lack of normalization of data leads to miscommunications regarding cardiac metabolism between different disease states and models. Since our absolute rates of fatty acid oxidation were unchanged (Figure 3.3 B), one might conclude from seeing this graph that heart failure results in no changes to myocardial fatty acid metabolism. However, this is incorrect. When accounting for the fact that the failing heart has almost half of the cardiac power of a healthy heart, one must consider, then, that the failing heart also has half the oxidative capacity as a healthy heart. Thus, normalization of absolute metabolic rates to cardiac power/work allow us to compare metabolism between a healthy and failing heart when the differences in oxidative capacity are accounted for. Following normalization, one can see that the failing heart has increased fatty acid oxidative rates (Figure 3.4 B). Considering the failing heart has significantly decreased contractile power and is working at half the power of a healthy heart, in relation to its overall energetic state, the failing heart is oxidizing fatty acids more on a per Joule basis.

6.1.4 Controversy Surrounding the Metabolic Profile of the Failing Heart

As previously discussed in the introduction, there is a lot of controversy surrounding the actual changes that occur to cardiac metabolism in the setting of heart failure. This could be due to numerous reasons including the type of heart failure being studied and the model of heart failure being used. And as we have just seen, normalization of metabolic rates is both underrated despite its consequential effect on comparing data between a healthy and diseased heart.

In chapter 3's study, we concluded that the failing heart has an increased reliance on fatty acids for energy due to the increases in normalized rates of fatty acid oxidation alongside the hyperacetylation of the fatty acid oxidative enzyme LCAD. However, a bulk of the literature and a quick search will yield a plethora of studies that have published that "the failing heart switches from fats to glucose". This would imply that fatty acid metabolism decreases while glucose metabolism increases, which is, in fact, not entirely correct. We have written a number of comprehensive reviews to go over all of the studies that have led to the confusion surrounding the failing heart's metabolic profile [11,46,108].

Briefly, the lack of normalization of metabolic rates causes confusion when comparing studies. Furthermore, 'glucose metabolism' is a misnomer in this situation since glycolytic rates do increase in the failing heart but glucose oxidation rates decrease. Since glycolysis and glucose oxidation are both part of glucose metabolism, it is incorrect to generalize that glucose metabolism increases in heart failure as this would imply that glucose oxidation increases. This is further complicated by the fact that in chapter 3's study, we only saw a decrease in absolute glucose oxidation rates but when normalized for cardiac work, normalized rates of glucose oxidation exhibited no changes between a healthy and failing heart. As such, one might see

that data and assume that glucose oxidation is not changed if considering the normalized values. It is therefore important to specify whether one is discussing the absolute rates of metabolism or normalized rates of metabolism when comparing to other studies. Another layer of confusion is introduced when studies indirectly assess metabolism.

Despite the myriad of results for the failing heart's metabolism, the most accurate statement would likely be that the failing heart switches from oxidative metabolism to glycolysis.

6.1.5 Heart Failure and Ketone Metabolism

In this study, we looked at a model of pressure overload hypertrophy. Clinically, heart failure presents in so many different ways that it complicates the matter. Firstly, pressure overload hypertrophy presents in the earlier stages of heart failure while progression through the disease leads from concentric to eccentric remodeling of the heart. The progression from a compensatory state to a decompensatory state of heart failure (cardiac dilatation) is important to consider when comparing both absolute and normalized myocardial metabolic rates.

Note that in our study, we saw no changes in the absolute rates of ketone oxidation but when normalized to cardiac work, myocardial ketone body oxidation increased. The only other lab that has directly assessed myocardial ketone oxidation rates via ^{13}C-labeled βOHB in heart failure did not actually present any data for a heart with depressed function (they only present data for their compensated hypertrophy group which is not their heart failure group)[7]. This is further complicated by the fact that their units of measurement are unclear and since they did not normalize the data, it is hard to compare. Moreover, their study only presents indirect evidence to suggest that ketone metabolism is increasing since ketone-derived intermediates

accumulated. Accumulation of intermediates does not necessarily mean there is an acceleration in ketone body oxidation. The former could also be true were there a decrease in ketone body oxidation. Therefore, our method of directly assessing myocardial ketone body oxidation is superior and our study is the first to clearly demonstrate that the failing heart relies more on ketone oxidation than a healthy heart.

6.1.6 Take Home Message

Take Home Message

Increased ketone body oxidation provides additional energy for the failing heart without an improvement in cardiac efficiency.

6.2 Aim 2 (Chapter 4)

6.2.1 Novelty

This is the first study to assess the heart's metabolic profile (namely, myocardial ketone body oxidation rates) in response to acute increases in the concentration of ketones and fatty acids.

6.2.2 Summary

In chapter 4, **Aim 2** was addressed and *ex vivo* cardiac function alongside the metabolic profile of a healthy heart are assessed in response to acute increases in ketone and fatty acid concentration.

Healthy male mice hearts were subjected to an isolated working heart perfusion with radiolabeled glucose (5 mM), palmitate (0.8 or 1.2 mM), and ß-hydroxybutyrate (0 mM, 0.6 mM or 2.0 mM) to assess carbohydrate metabolism, fatty acid oxidation and ketone body oxidation rates, respectively. The pulmonary artery was also cannulated during these perfusions, allowing for quantification of myocardial oxygen consumption rates and cardiac efficiency.

In light of the errors that were noticed post-publication, I have amended the conclusions from this paper and this can be found in Table 6.1.

Table 6.1 Summary of the Former Versus Amended Conclusions for *Ho et al. 2020* (Chapter 4)

Previous Conclusion	Amended Conclusion
Ketones are an unregulated fuel source in the heart and are oxidized in proportion to their availability independent of the Randle Cycle	Ketones are oxidized in proportion to their availability independent of the Randle Cycle but do not become the major fuel.
Increasing ketone concentrations markedly increases cardiac TCA cycle activity and ketones can become the major fuel of the heart	Increasing ketone concentration increases cardiac TCA cycle activity and the production of reduced equivalents, signifying that if available, ketones can be an extra source of energy for the heart.
Increasing ketone concentrations decreases cardiac efficiency	Despite being a potential extra source of energy for the heart, cardiac efficiency (assessed via O2 consumption) still decreases with increasing concentrations of ketones.

As one can see in Table 6.1, the amended conclusions correct our previous observation that ketones could be the major fuel for the heart. As such, future studies should take care in making any sort of extrapolation from the statement "ketones can become the major fuel". In fact, at a physiological concentration of ketones (~0.6mM), the contribution of ketones to ATP production is not nearly as much as fatty acids (without insulin) or glucose (with insulin).

While the previous conclusion about the contribution of ketones to overall ATP production was incorrect, it still holds true that ketones are metabolized in a dose-dependent manner when administered in this acute setting. With an increase in ketone concentration and increase in ketone oxidation, total TCA cycle activity as well as the production of NADH and $FADH_2$ also increase. However, this does not translate into any improvement in cardiac efficiency. As previously discussed, this lack thereof (cardiac efficiency when assessed as cardiac work normalized to TCA cycle activity) or decrease (cardiac efficiency when assessed as cardiac work normalized to oxygen consumption; considered the gold standard measurement for cardiac efficiency) in cardiac efficiency is likely due to the 'mild uncoupling' characterized by an accumulation of reduced equivalents, no change in cardiac work (energy demand) and thus, decreases in the protonmotive force and subsequent dissipation as heat.

6.2.3 Take Home Message

Take Home Message

We find evidence that ketones do not improve cardiac efficiency and are an extra source of fuel for the heart that do not compete with glucose or fatty acids.

6.3 Aim 3 (Chapter 5)

6.3.1 Novelty

This is the first study to assess the effects of a ketogenic diet on cardiac function and metabolism in a model of ischemic heart failure.

6.3.2 Summary

In this chapter, **Aim 3** was addressed and *in vivo and ex vivo* cardiac function and the metabolic profile of the ischemic failing heart are assessed in response to a chronic increase in ketones via a ketogenic diet.

Male mice were subjected to ischemic heart failure (via left anterior descending coronary artery ligation) after which mice were randomized to receive either a control diet (80% carbohydrate, 10% fat, 10% protein) or a ketogenic diet (5% carbohydrate, 85% fat, 10% protein) for 3 weeks. Cardiac function was assessed *in vivo* via transthoracic echocardiography and after 3-weeks of the diet, hearts were subjected to an isolated working heart perfusion with radiolabeled glucose (5 mM), palmitate (0.8), and ß-hydroxybutyrate (0.6 mM) to assess carbohydrate metabolism, fatty acid oxidation and ketone body oxidation rates, respectively.

In this study, we found that the ketogenic diet does not improve *in vivo* or *ex vivo* cardiac function in mice with ischemic heart failure (Figure 5.2). To our surprise, mice that were consuming the ketogenic diet did not exhibit increases in myocardial ketone oxidation rates and instead, had significantly decreased rates of cardiac glucose oxidation (Figure 5.3). Even when insulin was added to the hearts to stimulate glucose oxidation, hearts from mice that were on the ketogenic diet had no apparent insulin-stimulated increases in glucose

oxidation rates. This could imply that the ketogenic diet could cause insulin resistance at the level of the heart but more work is required to confirm this.

Due to the fact that cardiac ketone oxidation rates were not increased with the ketogenic diet, it makes sense that the ketogenic diet did not increase overall cardiac ATP production either. Decreased BDH1 protein expression also gave evidence to the fact that chronically increasing ketone delivery to the heart may cause a compensatory decrease in the transcriptional machinery associated with ketone metabolism. Lastly, we observed a decrease in cardiac efficiency in failing hearts from mice that were on the ketogenic diet compared to failing hearts from mice on a control diet.

6.3.3 Ketogenic Diets and Cardiovascular Risk Factors

A portion of this section has been published online in an article I wrote for 'Medscape.com' in collaboration with the 'American College of Cardiology & Medscape' which can be accessed via https://www.medscape.com/viewarticle/943443[189].

The effect of the ketogenic diet on cardiovascular risk factors is also important to weigh in when considering this as a therapeutic treatment for heart failure. Studies have demonstrated mixed results regarding the cardiovascular risks of high-fat, low-carbohydrate diets[179,190,191]. In a meta-analysis of eleven randomised controlled trials, Mansoor and colleagues found that ketogenic diets increased low-density-lipoprotein (LDL) cholesterol despite promoting greater weight loss than a low-fat diet[180]. In contrast, multiple studies have reported that the ketogenic diet improves cardiovascular risk factors and no deleterious effects were observed on the lipid profiles of men, women or adolescents[192-195]. However, one study found that the ketogenic diet improved triacylglycerol and high-density lipoprotein cholesterol values but concurrently

worsened LDL cholesterol when compared to a low-fat diet[181]. These findings are further complicated by the fact that the effects of a ketogenic diet are distinct between rodents and humans[182]. Thus, the contradictory results regarding ketogenic diets and their effects on cardiovascular risk factors are likely due to the fact that there are many variations of the ketogenic diet and compliance to a standardized ketogenic diet is low. The source of fatty acids may also play a key role in the cardiovascular risk factors that may surface if an individual consumes a ketogenic diet[196]. Future high-quality clinical trials are warranted to underline the effect of a ketogenic diet on cardiovascular risk factors[197].

6.3.4 Ketogenic diets and heart failure

A portion of this section has been published online in an article I wrote for 'Medscape.com' in collaboration with the 'American College of Cardiology & Medscape' which can be accessed via https://www.medscape.com/viewarticle/943443[189].

Since increased ketone metabolism is adaptive in the failing heart but the ketogenic diet presents controversial cardiovascular risks, whether ketogenic diets can convey these benefits in the failing heart remains enticingly unclear. In a cross-over study with 16 healthy subjects assigned to a ketogenic or control diet, subjects on the ketogenic diet had a decreased cardiac phosphocreatine to ATP (PCr/ATP) ratio, signifying impaired ATP production – similar to what is seen in heart failure patients[47,198].

To date, only a handful of studies have investigated the effect of a ketogenic diet on cardiac function. In mice with pressure overload hypertrophy, consumption of a low-carbohydrate diet for 4 weeks resulted in anti-hypertrophic mechanisms, thus suppressing pathological cardiac hypertrophy[93]. In this study however, it is important to note that the

authors did not directly assess myocardial ketone body oxidation rates and supplemented their 'low-carbohydrate' (they never explicitly call it a ketogenic diet) with protein or fat. It is therefore possible that while the ketogenic diet confers anti-hypertrophic effects, this may not be mediated by ketones at the level of the heart if their oxidation is, in fact, blunted. Interestingly, the authors presented a blot of BDH1 protein expression being blunted with consumption of the low-carbohydrate diet, in line with our results in chapter 5. This would suggest that myocardial ketone body oxidation rates are depressed and glucose oxidation blunted too. However, they did not assess myocardial insulin signaling or glucose metabolism[93].

In two studies published side-by-side, two different groups presented data to suggest that the ketogenic diet, or a high-fat-diet, could reverse experimental heart failure caused by deletion of the mitochondrial pyruvate carrier in the heart[94,95]. While they did assess ketone body metabolism at the level of the heart, they only did so indirectly with the measurement of its metabolites/intermediates. Even though their rates are all indirect measures of metabolism, they find similar results to us in that the ketogenic diet downregulates myocardial ketone body oxidation. They conclude that enhancing fatty acid oxidation at the level of the heart can reverse cardiac dysfunction. However, this contradicts what one would extrapolate from our findings – if glucose oxidation is impaired in heart failure and the ketogenic diet blunts glucose oxidation, it is counter-intuitive that enhancing fatty acid oxidation would improve function. And considering that we found the ketogenic diet blunts insulin-stimulated glucose oxidation, introduction of a high-fat diet would only worsen this and potentially impair insulin signaling. Unfortunately, the authors do not assess insulin sensitivity at the level of the heart in response

to their beneficial high fat diets. Nevertheless, their findings present an interesting concept and bring more attention to the ketogenic diet as we prepare our paper for submission.

6.3.5 Take Home Message

Take Home Message

The ketogenic diet does not improve heart function in failing hearts and blunts myocardial glucose oxidation.

6.4 The Big Picture

Taken together, all three aims that were investigated in this thesis have drawn an important picture for the field concerning the role of ketone metabolism in heart failure.

In arc 1 of the thesis, we found that the failing heart relies more on ketones for energy and glucose oxidation rates are impaired in the failing heart. While there is confusion in the field about the fatty acid oxidative changes that occur in heart failure, it is clear that heart failure results in an impairment in glucose oxidation rates. Therefore, we next wondered whether ketones affect glucose oxidation rates in a healthy setting and what effect ketones had on cardiac efficiency.

In arc 2 of the thesis, we originally hypothesized that if we increase ketone supply to the heart, this would potentially compete for acetyl CoA production and decrease glucose oxidation, consequently decrease cardiac efficiency. That's why we were quite surprised to see that despite delivering a high concentration of beta-hydroxybutyrate to the heart (2.0mM), there was very little effect on glucose oxidation. Instead, we only saw a slight increase in oxygen consumption without any effect on function. Furthermore, we did not see any improvement in cardiac efficiency despite the increase in cardiac ATP production and this goes against the concept that ketones are a 'superfuel'. Knowing that ketones can increase ATP production without inhibiting glucose oxidation led us to question whether we could therapeutically implement ketones in a heart failure setting.

In arc 3 of the thesis, we investigate what the ketogenic diet, a high-fat, low-carbohydrate diet, does to the metabolic milieu of a failing heart and whether it can improve cardiac function. We found that the ketogenic diet did not increase myocardial ketone

oxidation rates and actually blunted glucose oxidation rates regardless if insulin was present. Inhibition of glucose oxidation rates in the presence of higher blood ketone levels (via a ketogenic diet) is not desired in the setting of heart failure. The decrease in glucose oxidation rates is likely a secondary effect of increased reliance on fatty acids for energy. Therefore, we found that despite increasing blood ketone levels, the ketogenic diet is not a valid approach to increase myocardial ketone oxidation since it is offset by increased ketone body oxidative enzyme expression (namely, BDH1). Furthermore, the heart shifts its reliance to fatty acids for energy most likely due to the high fat content in the ketogenic diet. As a result, we suspect that the shift to fatty acids for energy is competing with glucose for ATP production and thus, glucose oxidation rates are significantly decreased in hearts from mice on the ketogenic diet.

Taken together, these arcs come together to tell a story about how ketone metabolism is perturbed in heart failure, and this may be an adaptive process to improve cardiac ATP production as opposed to improving cardiac efficiency. Furthermore, the ability for ketones to improve ATP production without competing with other substrates, namely glucose, allows it to be potential therapeutic strategy to treat heart failure's impaired energetic state. To test whether the ketogenic diet is a valid approach to apply this therapeutic strategy, we treated mice with ischemic heart failure a ketogenic diet but did not find any improvements in cardiac function. Instead, we found that these mice developed symptoms of cardiac insulin resistance due to the high fat nature of the ketogenic diet and cardiac ketone body oxidation was not increased due to compensatory increases in transcriptional regulatory machinery (BDH1 protein expression). As a result, myocardial ketone body oxidation did not increase in hearts from ketogenic diet-fed mice and overall cardiac ATP production was not improved. Thus,

due to the high-fat nature of the ketogenic diet, it is not a valid therapeutic approach to increase cardiac ketone body oxidative rates and may cause cardiac insulin resistance.

6.5 Future Directions

Numerous future experiments are warranted in light of the exciting data presented in my thesis. Firstly, since my thesis focused on heart failure with reduced ejection fraction (HFrEF), it is important to consider that a large portion of heart failure patients present as heart failure with preserved ejection fraction (HFpEF). Thus, all the studies outlined in this thesis should be repeated to assess cardiac energy metabolism in the setting of HFpEF. This is especially topical following the recent development of a representative animal model of HFpEF by Dr. Joseph Hill's laboratory[199]. The administration of N-nitro-L-arginine methyl ester (L-NAME) in conjunction with a high-fat diet results in concomitant hypertensive and metabolic stress similar to what is seen in HFpEF patients. Future application of this model of HFpEF alongside assessment of myocardial ketone body oxidation rates would be of interest.

Important to note is the multi-faceted and complex nature of heart failure. In my thesis, I focused on two models of heart failure: transverse aortic-constriction-induced pressure overload hypertrophy (Chapter 3) and left anterior descending coronary artery ligation-induced ischemic heart failure (Chapter 5). However, there are other types of heart failure that exist – volume overload, tachycardia cardiomyopathy, diabetic cardiomyopathy and genetic-related cardiomyopathies[200]. To note, there is overlap with the above types of heart failure and the classification of heart failure by HFrEF and HFpEF or right-sided heart failure (congestive heart failure) and left-sided heart failure. Due to the numerous types of heart failure that can be studied, there are also a number of animal models of heart failure that need to be considered for future studies[201]. In addition to the possibility of using a transverse aortic constriction model to study pressure overload hypertrophy, studies can also utilize spontaneously hypertensive rats, carry out an abdominal aortic constriction or utilize a mouse with

supravalvular aortic stenosis[202]. Animal models that can recapitulate volume overload include the arteriovenous or aortocaval fistula models[203,204], as well as mitral regurgitation models (via mitral valve chordae tendinae rupture)[205]. Animal models that can recapitulate a myocardial infarction include coronary ligation (ex. left anterior descending coronary artery ligation)[206] or coronary embolization (via injection of polystyrene microspheres into the heart)[207].To study tachycardia cardiomyopathy, one can employ a pacing-induced tachycardic model in animals. To study diabetic cardiomyopathy, one can use a high fat diet with a low dose of streptozotocin among many other diabetic animal models which is outside the scope of this thesis[208]. Lastly, to study genetic-related cardiomyopathies, one can use genetically modified mice alongside the cre-lox system to produce cardiac-specific genetic knockouts. All of the above models of heart failure underline the complexity of heart failure and need for more studies in varying models of heart failure.

Furthermore, in light of the findings of chapter 5 and the potential cardiac insulin resistance that the ketogenic diet may cause, it would be curious to see what would happen to *db/db* mice on a ketogenic diet. Were diabetic mice subjected to a ketogenic diet, would their cardiac insulin resistance be potentiated? Another possible experiment would be utilizing cardiac-specific BDH1 (or SCOT) knock-out mice and determining if they have inherent blunted levels of myocardial glucose oxidation due to their inability to oxidize ketones. To add, treating heart failure mice with cardiac specific overexpression of BDH1 a ketogenic diet would allow us to see whether mice with accelerated myocardial ketone oxidation rates protect against the ketogenic diet-induced decreases in glucose oxidation. This would be interesting in light of the fact that BDH1 overexpression in mice have been shown to be cardioprotective[16].

To add, since the high fat nature of the ketogenic diet likely caused impairments in myocardial glucose oxidation, other forms of ketone delivery need to be studied (ex. ketone esters, ketone salts). It would be interesting to see whether other modes of ketone delivery can increase myocardial ketone oxidation rates and improve cardiac ATP production in the setting of heart failure.

6.6 Key Findings of my Thesis

My thesis has made several key advances for the field:

1. The failing heart relies more on ketones for energy
2. Ketones do not increase cardiac efficiency and can be an extra source of fuel for the heart without competing with glucose or fatty acids
3. The ketogenic diet is not a valid approach to increase cardiac ketone oxidation rates and instead, blunts myocardial glucose oxidation.

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