KETONE METABOLISM IN THE HEART

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I dedicate this Thesis to myself, my late father (Abah), Ma, Dr Rhys and Prof Kieran.

Thank you for always being by my side!

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Declaration

I declare that the work presented in this Thesis is my own and includes study design, experimental procedures, data analysis and data presentation. I acknowledge that initial assistance with experimental methods was given by Dr Brianna Stubbs (Chapter 3), Henry Lee and Dr Cher-Rin Chong (Chapter 4) and Jessica Beeson (Chapter 6). Dr David Hauton and Professor James McCullagh ran the mass spectrometer (MS) and helped with MS data analysis (Chapters 3 and 5).

Abstract

The aphorism "fat burns in the flame of carbohydrate" leads to the question of whether the oxidation of the ketone body, D-β-hydroxybutyrate (βHB), is facilitated by anaplerosis via glycogen and/or glucogenic amino acids. The work in this thesis aimed to investigate the importance of anaplerosis in ketone body metabolism in the isolated heart. The first study (Chapter 3) determined whether glycolysis from glycogen affected the heart's ability to oxidise βHB. The glycogen content of isolated rat hearts was altered by perfusion with or without glucose, before perfusing with Krebs-Henseleit (KH) buffer containing [^{14}C]-βHB or [5-^{3}H]-glucose to measure βHB oxidation and glycolytic rates, respectively. βHB oxidation in hearts with low glycogen (LG) was 2-fold lower than those with high glycogen (HG), and βHB oxidation rates directly correlated with glycogen content. βHB oxidation decreased glycolytic rates to ~0.2 μmol. gww^{-1}.min^{-1} in all hearts, redirecting glucose into glycogen in LG heart, but not in HG hearts. βHB alone or with glucose increased the Krebs cycle intermediates, citrate, 2-oxoglutarate and succinate, and the total nicotinamide adenine dinucleotide phosphate (NADP/H) pool measured using metabolomics. Thus, glycogen is required for βHB oxidation. The second study (Chapter 4) determined the effect of βHB oxidation on cardiac energetics using ^{31}P-NMR spectroscopy. In HG hearts, βHB oxidation increased the free energy of ATP hydrolysis (ΔG_{ATP}) by increasing the concentration of phosphocreatine ([PCr]) and the phosphorylation potential, calculated from the [ATP]/[ADP][Pi] ratio. βHB oxidation was unable to increase the low ΔG_{ATP} in LG hearts due to low glycolytic flux through pyruvate. The third study (Chapter 5) defined the effect of the glucogenic amino acids, asparagine, valine and glutamine, on βHB oxidation in hearts with decreased flux through pyruvate to oxaloacetate (glycogen-depleted and perfused with low glucose buffer). Asparagine increased βHB oxidation 2-fold by increasing the metabolites of the malate-aspartate shuttle and purine nucleotide cycle, aspartate, fumarate and guanosine triphosphate. Neither valine nor glutamine increased βHB oxidation rates. The final study (Chapter 6) determined the effect of substrates and loading conditions on cardiac efficiency (the ratio between hydraulic work and oxygen consumption) in working rat hearts. Fatty acids were found to decrease cardiac efficiency, via increased oxygen consumption and decreased hydraulic work, at both low and high pre- and afterloads. At high, but not at low, pre- and after-loads, βHB plus acetoacetate (AcAc) increased efficiency by increasing hydraulic work. Thus, βHB plus AcAc increased cardiac efficiency more than glucose alone or with fatty acids at high pre- and after-loads. In conclusion, βHB oxidation and glycolysis from glycogen have a synergistic relationship in heart, which serves to increase the ΔG_{ATP} and thereby cardiac efficiency.

Abbreviations

AcAc	Acetoacetate	HDAC	Histone deacetylase
AcAc-CoA	Acetoacetyl-CoA	HG	High glycogen
ADP	Adenosine diphosphate	HMG-CoA	3-hydroxymethylglutaryl-CoA
ANOVA	Analysis of variance	IC-MS	Ion exchange chromatography-mass spectrometry
ATP	Adenosine triphosphate	KB	Ketone body
AU	Arbitary units	KH	Krebs-Henseleit
BDH	D-β-hydroxybutyrate dehydrogenase	LC-MS	Liquid chromatography-mass spectrometry
βHB	D-β-hydroxybutyrate	LCHF	Low-carbohydrate high-fat
CoA	Coenzyme A	LG	Low glycogen
CPT	Carnitine palmitoyltransferase	MS	Mass spectrometry
ΔG_{ATP}	Free energy of ATP hydrolysis	$NAD^+(H)$	Nicotinamide adenine dinucleotide oxidized (reduced)
DNA	Deoxyribonucleic acid	$NADP^+(H)$	Nicotinamide adenine dinucleotide phosphate (reduced)
dCTP	Deoxycytidine triphosphate	PCr	Phosphocreatine
EDTA	Ethylenediaminetetracetic acid	PDH	Pyruvate dehydrogenase
FAD	Flavin adenine dinucleotide	PFK	Phosphofructokinase
FA	Fatty acids	Pi	Inorganic phosphate
FOXO3a	Forkhead box other-3a	Q	Ubiquinone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	QH_2	Ubiquinol
GDP	Guanosine diphosphate	SEM	Standard error of the mean
GTP	Guanosine-5'-triphosphate	SCOT	Succinyl-CoA:3-oxoacid-CoA transferase
GLUT	Glucose transporter	SCS	Succinyl-CoA synthase
GLUT4	Glucose transporter 4	SIRT	Silent information regulator

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Chapter 1: General introduction

1.1 General introduction

The heart continuously produces energy for work by metabolising a variety of energy substrates. Fatty acids (both in non-esterified and esterified (TAG) form) are the predominant cardiac fuel source. Fatty acids and carbohydrates (glucose and lactate) oxidation accounts for approximately 90% of cardiac ATP production, with metabolism of amino acids and ketone bodies (KB: D-β-hydroxybutyrate, acetoacetate, and acetone) supplying the balance (Fig. 1.1) (1-9).

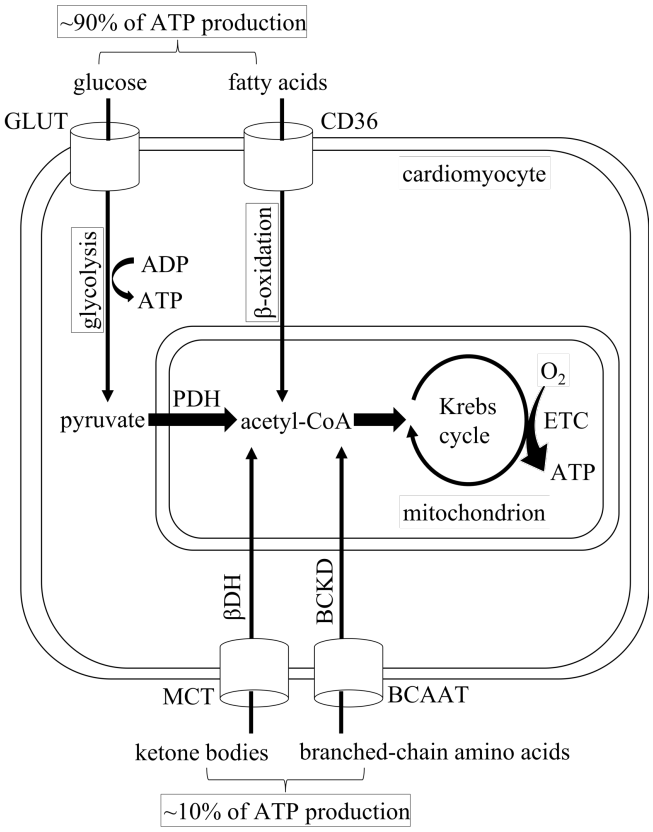


Figure 1.1 *Overview of main metabolic pathways in the heart. The heart (cardiomyocyte) is capable of oxidizing a wide variety of substrates for ATP production. Some of ATP is derived from glycolysis in the cytosol, but most of ATP is produced by oxidative phosphorylation in the mitochondrion. Energy substrates compete for mitochondrial oxidative energy metabolism via generation of acetyl-CoA for the Krebs cycle. Pyruvate from glucose can generate acetyl-CoA via pyruvate dehydrogenase (PDH). Fatty acids can generate acetyl-CoA via β-oxidation. Fatty acids and carbohydrate contribute to ~90% of total ATP production. Branched-chain amino acids (BCAAs) and ketone bodies can generate acetyl-CoA via branched-chain α-ketoacid dehydrogenase (BCKD) and D-β-hydroxybutyrate dehydrogenase (βDH), respectively. Both BCAA and ketone bodies contribute to ~10% of total ATP production. GLUT, glucose transporter; CD36, cluster of differentiation 36; MCT, monocarboxylate transporter; BCAAT, branched-chain amino acid transporter; ETC, electron transport chain.*

The heart is capable of utilising all energy substrates. During increased energy demand or hormonal stimulation, left ventricle contractile power and myocardial oxygen consumption can increase up to 6-fold of the resting values, which requires a proportional increase in the generation of NADH and $FADH_2$ from energy fuel substrates (10). This metabolic "flexibility" allows the healthy heart to alter its metabolic preference to match changes in the availability of circulating substrates. However, the metabolic flexibility is perturbated in heart failure. The aetiology of heart failure is complex (7, 11-15). Although studies in humans provide conflicting results, potentially due to differences in clinical settings of control groups (16-18), most studies have suggested that in the end stages of heart failure, hearts have decreased fatty acid uptake and a switch towards glucose utilisation (12, 19-21), perhaps explained by the greater oxygen efficiency of glucose oxidation compared to fatty acid oxidation (22). By contrast, increased FA oxidation is found in diabetes (23), which has been blamed for the aetiology of diabetic cardiomyopathy as well as the worsened ischaemia-reperfusion characteristics of the diabetic myocardium (24-26). Furthermore, glucose intolerance is a common feature of heart failure, and the non-diabetic failing heart may itself be insulin resistant (27). The conundrum of which comes first, cardiac disease or metabolic derangement (28), has proved difficult to resolve. These efforts have focussed on FA and glucose metabolism, but a role for ketone metabolism in heart disease has recently become apparent (8, 29, 30). A very recent study showed that the failing heart consumed more ketone bodies and lactate and had higher rates of proteolysis (9). Ketone metabolism is clearly present in the failing heart and ketogenesis is primarily occur in the liver, but it is unclear whether enhanced cardiac ketone metabolism causes, is caused by or is unrelated to cardiac dysfunction. However, interest has increased in the importance of ketone bodies as a source of energy in both experimental and clinical heart failure (31-39).

Ketone bodies are soluble 4-carbon compounds derived principally from fatty acids during carbohydrate-depleted conditions, that can be metabolised by many oxidative tissues, including heart, as glucose-sparing energy substrates (40-42). Although ketones can provide a significant energy to the heart (43, 44), they are cataplerotic, causing depletion of Krebs cycle intermediates (45-47). In the Krebs cycle, the 2-carbon acetyl group of acetyl-CoA derived from βHB (by ketolysis) or fatty acid (by β-oxidation) is completely oxidised to two CO_2, with no net change in any of the intermediates. However, Krebs cycle intermediates are removed from the cycle by other pathways a process called cataplerosis. Thus, entry of ≥ 3-carbon substrates (anaplerosis) is important to replenish Krebs cycle intermediates and maintain cycling efficiency, leading to the important question of how replenishing metabolic intermediates via anaplerosis impacts the ability and capacity of hearts to oxidise βHB (48, 49). Anaplerosis is a term that was first used by Kornberg to describe a series of enzymatic reactions that replenish the pools of metabolic intermediates in the Krebs cycle (50). The ultimate success of metabolic-based interventions requires a better understanding of how energy fuel substrates, namely fatty acids, carbohydrates, amino acids, and ketone bodies, are inter-related, via anaplerosis and cataplerosis.

This Chapter (Introduction) aims to describe the background and rationale for the investigation of ketone body metabolism in the heart. Firstly, it will provide concepts of utilisation and efficiency of ketone body metabolism in the heart. Secondly, it will describe the metabolism of fatty acid as a predominant fuel in the normal heart. Thirdly, this Chapter will review the concept of efficient energy transfer through repletive and depletive reactions (anaplerosis and cataplerosis) of the Krebs cycle intermediates, in which availability of anaplerotic substrates, including glycogen, glucose, and glucogenic amino acids may facilitate the heart's ability to oxidise cataplerotic substrate, such as ketone body. Finally, this Chapter will detail a potential role of ketone body as a fuel for the failing heart.

1.2 Ketone body metabolism

Ketone bodies (KB: D-β-hydroxybutyrate, acetoacetate, and acetone) are produced in the liver mitochondria, essentially as a transport form of acetyl-CoA derived from adipose triacylglycerols, and are used by other oxidative organs, such as brain, heart, kidney cortex, and skeletal muscle, for terminal oxidation as a metabolic response to physiological states characterized by limited carbohydrate availability (neonatal period, starvation, and post-exercise), as well as consumption of low-carbohydrate high-fat (ketogenic) diet or ketone supplements. The level of acetoacetate and D-β-hydroxybutyrate in the blood ranges from 0.2 to 0.5 mM, although levels can increase up to 10-20 mM during diabetic ketoacidosis, which are considered pathological (Table 1.1) (51).

Table 1.1 *Plasma ketone levels attained during ketosis. LCHF = low-carbohydrate high-fat, MCT = medium chain triglyceride. Adapted from (31).*

	Condition	Plasma ketone level in human (mM) (Reference)	Plasma ketone level in Wistar rats (mM) (Reference)
Endogenous	Fed	0.05 – 0.1 (40)	0.1 (52)
	Starvation (24 hours)	0.3 – 0.5 (53)	0.9 (52)
	Starvation (10 days)	3 – 6 (40)	
	Starvation (4 weeks)	5 – 8 (53)	
	LCHF diet	4 – 11 (54)	1.5 (55)
Exogenous ketosis	*(R,S)*-1,3-butanediol	0.2 (56)	
	MCT	0.5 (57)	
	Ketone salt	1 – 3 (58)	1.4 (59)
	Ketone ester	1 – 6 (60)	1.5 (59)

1.2.1 Ketone body production in the liver - Ketogenesis

Ketone bodies are naturally occurring four carbon substrates that are synthesized in liver mitochondria (hepatic ketogenesis) from fatty acids, which in turn are produced by the action of lipases (adipose tissue triglyceride lipase; hormone-sensitive lipase) on triacylglycerols stored in adipose tissue. Following transport from adipose tissue to liver in the plasma and uptake into hepatocytes, long-chain acyl-CoA derived from the fatty acids cross the mitochondrial double membrane, mediated by the carnitine shuttle (carnitine

palmitoyltransferase 1 (CPT1), carnitine acylcarnitine translocase (Tr) and CPT2). Malonyl coenzyme A (CoA) inhibits CPT1 for mitochondrial fatty acid entry (61). In liver mitochondria, 3-hydroxymethylglutaryl-CoA synthase 2 (encoded by the nuclear gene *Hmgcs2*) catalyses a condensation of β-oxidation-derived acetoacetyl-CoA and acetyl-CoA to generate 3-hydroxymethylglutaryl-CoA (HMG-CoA), which is cleaved by HMG-CoA lyase (HMGCL) to liberate acetoacetate and acetyl-CoA. Acetoacetate (AcAc) is reduced to D-β-hydroxybutyrate (βHB), in an NAD^+/NADH coupled equilibrium catalysed by the mitochondrial enzyme D-β-hydroxybutyrate dehydrogenase (BDH1). BDH1 modulates the mitochondrial redox potential in liver and heart (62) (Fig. 1.2).

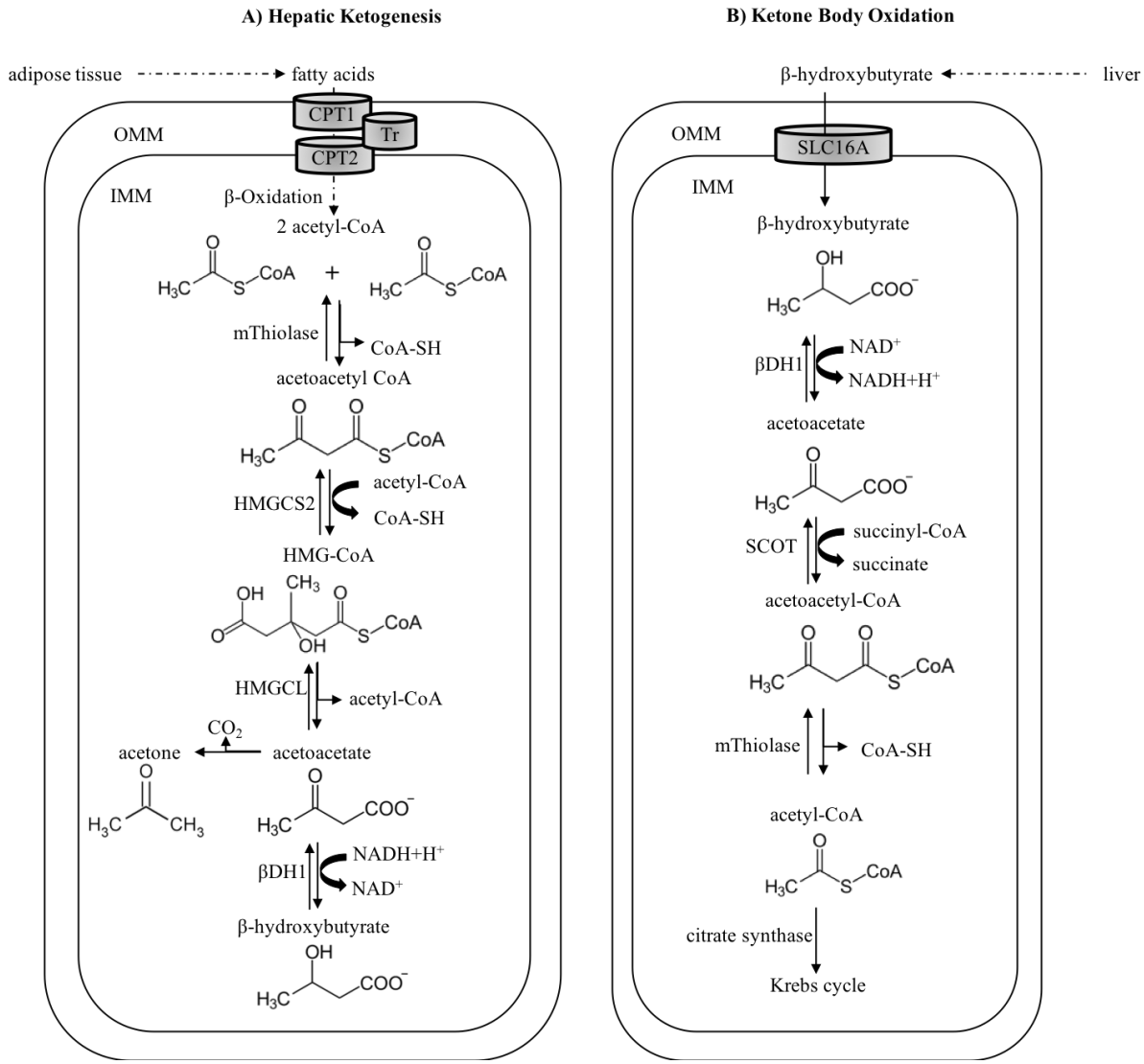


Figure 1.2 *Ketone body metabolism pathway. The production of ketone bodies primarily in liver mitochondria (A). Ketone body oxidation within mitochondria of extrahepatic tissues via D-β-hydroxybutyrate dehydrogenase (BDH1) and succinyl-CoA:3-oxoacid-CoA transferase (SCOT) (B). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; CPT, carnitine palmitoyltransferase; CoA-SH, free coenzyme A; HMGCS2, 3-hydroxymethylglutaryl-CoA synthase 2; HMG-CoA, 3-hydroxymethylglutaryl-CoA; HMGCL, HMG-CoA lyase; NAD$^+$(H), nicotinamide adenine dinucleotide oxidized (reduced); solute ligand carrier protein (SLC) 16A (SLC16A); mThiolase, mitochondrial thiolase. Thiolase activity is encoded by at least 6 genes: ACAA1, ACAA2 (encoding an enzyme known as T1 or CT), ACAT1 (encoding T2), ACAT2, HADHA, and HADHB. CPT1, carnitine palmitoyltransferase-1; CPT2, carnitine palmitoyltransferase-2; Tr, carnitine acylcarnitine translocase.*

1.2.2 Ketone body oxidation in the normal heart

Ketone bodies are released by the liver via solute carrier 16A (SLC 16A) (the monocarboxylate transporter family member 1, 6, and 7) and circulate to extrahepatic tissue for oxidation (63, 64). The oxidation of βHB to AcAc is catalysed by BDH1 in the mitochondria of extrahepatic tissue. AcAc is activated to acetoacetyl-CoA by CoA transferase [succinyl-CoA:3-oxoacid-CoA transferase (SCOT)]. SCOT is expressed in all tissues except liver (65). SCOT catalyses a near-equilibrium reaction, which exchanges coenzyme A between succinate and AcAc (66). Succinyl-CoA and succinate are both Krebs cycle intermediates. A reversible mitochondrial acetoacetyl-CoA thiolase (mThiolase) reaction yields two molecules of acetyl-CoA that enter the Krebs cycle catalysed by citrate synthase (61, 67-69). Ketone bodies can only enter the Krebs cycle as 2-carbon (2C) intermediates, via condensation of acetyl-CoA (2C) with oxaloacetate (4C) to form citrate (6C). These carbons replace two carbons that are lost as CO_2 molecules produced with each full turn of the Krebs cycle. Since ketone bodies are 2-carbon-equivalent molecules, and since they cannot be used to synthesise any (≥ 4 carbon) Krebs cycle intermediate, the oxidation of ketone bodies is considered cataplerotic (70-72) (Fig. 1.2).

In the heart, oxidation of βHB decreased mitochondrial free nicotinamide adenine dinucleotide (NAD^+/NADH) and oxidation of ubiquinone ($CoQ/CoQH_2$) ratios, increasing the redox span between site I and site III, making more energy available for the synthesis of ATP, increasing the free energy of cytosolic ATP hydrolysis (ΔG_{ATP}) (Fig. 1.3) (44). NAD plays an important role as an electron carrier and also as a substrate for signalling enzymes involved in the response to energy and oxidative stress, such as silent information regulator (SIRT)- mitochondrial NAD^+-dependent deacetylation/ADP-ribosylation enzymes. In line with this, sirtuins can increase HMGCS2 activity, which was correlated with elevated serum

βHB levels (73). Ketone body oxidation at cellular level is regulated by the gene encoding the CoA transferase, SCOT (*Oxct1*) (Fig. 1.3).

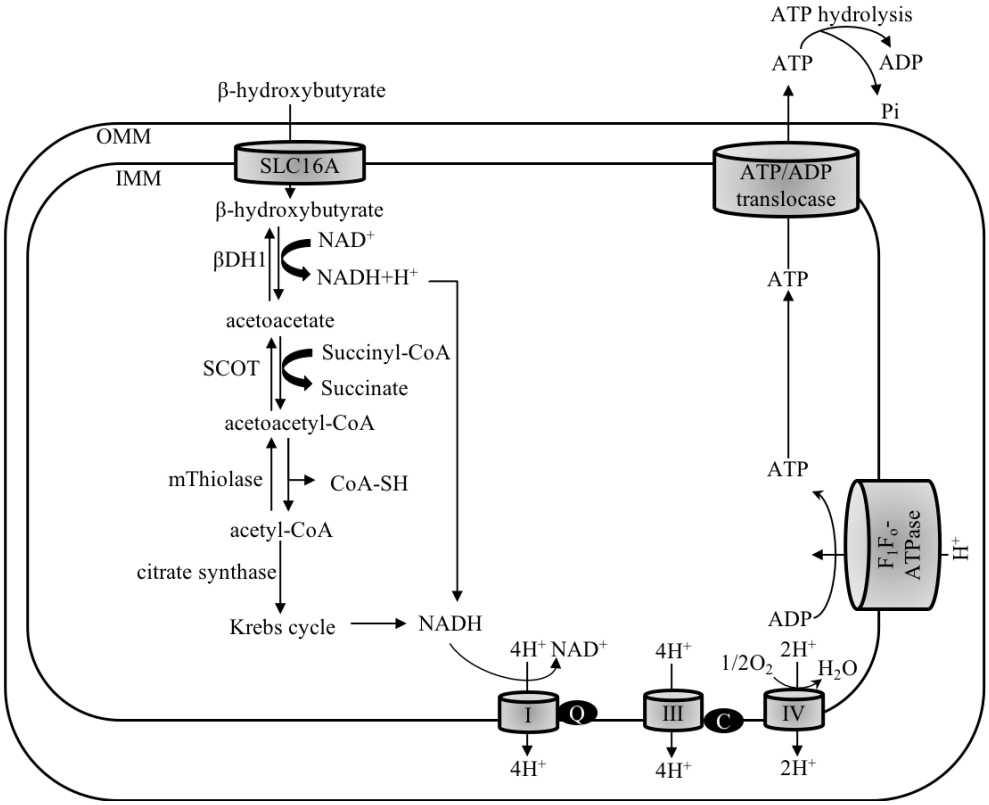


Figure 1.3 *Oxidation of D-β-hydroxybutyrate (βHB) in the extra-hepatic mitochondria. βHB crosses the mitochondrial membrane via solute ligand carrier protein (SLC) 16A (SLC16A) family members. βHB is oxidized to acetoacetate by the inner-membrane bound and phosphatidylcholine-dependent D-β-hydroxybutyrate dehydrogenase I (BDHI). Succinyl-CoA:3-oxoacid-CoA transferase (SCOT) catalyzes a near-equilibrium reaction by which CoA is exchanged between succinate and acetoacetate. The oxidation of ketone bodies increases the free energy of ATP hydrolysis (yielding more ATP per molecule of oxygen) by generating more nicotinamide adenine dinucleotide (NADH) to be delivered to complex I within the electron transport chain. By decreasing NAD^+/NADH and oxidation of ubiquinone ($CoQ/CoQH_2$) ratios, βHB increases the redox span between complexes I and III. Electrons are passed down from NADH to Q resulting in more protons pumped into the intermembrane space to generate more ATP by chemiosmosis. CoA-SH, free coenzyme A; NAD^+(H), nicotinamide adenine dinucleotide oxidized (reduced); Pi, inorganic phosphate; ADP, adenosine diphosphate; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; mThiolase, mitochondrial thiolase; C, cytochrome C; e^-, electron; H^+, hydrogen ion, F_o/F_1 ATPase, ATP synthase; Q, ubiquinone; QH_2, ubiquinol; I, II, III, IV, complexes I-IV of the electron transport chain, respectively.*

1.2.3 Effect of exogenous and endogenous ketones on blood glucose, lipids and insulin

Endogenous ketosis, such as low-carbohydrate high-fat (LCHF) can lower blood glucose and elevate free (non-esterified) fatty acids (FFA) concentrations. Meanwhile, exogenous ketosis, such as ketone salt (sodium hydroxybutyrate or sodium acetoacetate) and ketone ester (R-1,3-butanediol-R-3-hydroxybutyrate) can lower both blood glucose and FFA concentrations (Table 1.1) (31). During endogenous ketosis, low insulin and elevated cortisol stimulate adipose tissue lipolysis; released FFA is the precursor for ketone body production. βHB activates the nicotinic acid receptor, leading to inhibition of adipocyte adenylyl cyclase, results in a suppression of adipocyte lipases, such as hormone-sensitive lipase and adipose triglyceride lipase. The suppression of the lipase decreases the amount of FFA (74). Exogenous ketones from ketone drinks (75, 76) or intravenous infusions (77, 78) can inhibit lipolysis by the same mechanism while elevating blood βHB concentration. Endogenous and exogenous ketosis can decrease blood glucose concentrations by different mechanisms. During endogenous ketosis, low blood glucose results from reduced hepatic gluconeogenesis due to limited carbohydrate availability. This leads to increased ketone body production from lipolysis. During exogenous ketosis, carbohydrate is abundant. Ketone bodies lower the blood glucose by limiting hepatic gluconeogenesis and increasing peripheral glucose uptake (78). Moreover, glycogen exhaustion impairs endurance exercise capacity in man (79-81). An exogenous ketone ester increased postexercise glycogen synthesis in human skeletal muscle (82) and the same may be true for cardiac muscle. In prolonged fasting, ketone bodies replace glucose as the primary fuel consumed by the brain (83).

1.3 Fatty acid β-oxidation in the normal heart

Fatty acid oxidation contributes 70% of ATP production in the normal heart, with the metabolism of glucose, lactate, amino acids, and ketone bodies (acetoacetate, β-hydroxybutyrate) supplying the remainder. The utilisation of fatty acid by the heart is governed by the availability of plasma substrates and circulating hormones, such as insulin and catecholamines (adrenergic signalling) (84), and type of fatty acid delivered to the heart (12). Fatty acids are supplied to the heart as either fatty acids released from triacylglycerol (TAG) contained in the core of chylomicrons (triglycerides, phospholipids, cholesterols, and proteins) or very-low-density lipoprotein (VLDL) or as FFA bound to albumin (4, 85, 86).

The rate of myocardial fatty acid uptake and oxidation is dependent on arterial fatty acid availability, itself a function of plasma fatty acid concentration and fatty acid delivery (coronary blood flow). The majority of fatty acids undergoing β-oxidation are monounsaturated (oleic acid) or polyunsaturated fatty acids (arachidonic acid), saturated fatty acids (palmitatic acid). Oleate is the most abundant fatty acid in the blood (87). However, β-oxidation rates of oleate and palmitate are the same (88, 89). Palmitate and oleate are extremely water insoluble, thereby binding of fatty acids to albumin can increase the solubility of fatty acids in aqueous solutions (90).

The majority of fatty acids utilised by the heart that originate from TAG are predominantly from chylomicron-TAG, with only a small portion derived from VLDL (91, 92). The activity of lipoprotein lipase (LPL) is responsible for the hydrolysis of the chylomicron-TAG to release fatty acid and glycerol. Chylomicron-TAG is an efficient source of fatty acids, which can compete with FFAs bound to albumin in the plasma (91-93). In contrast, VLDL-TAG uptake is at least partly mediated by VLDL/apolipoprotein E (apoE) receptors) (92) (Fig. 1.4).

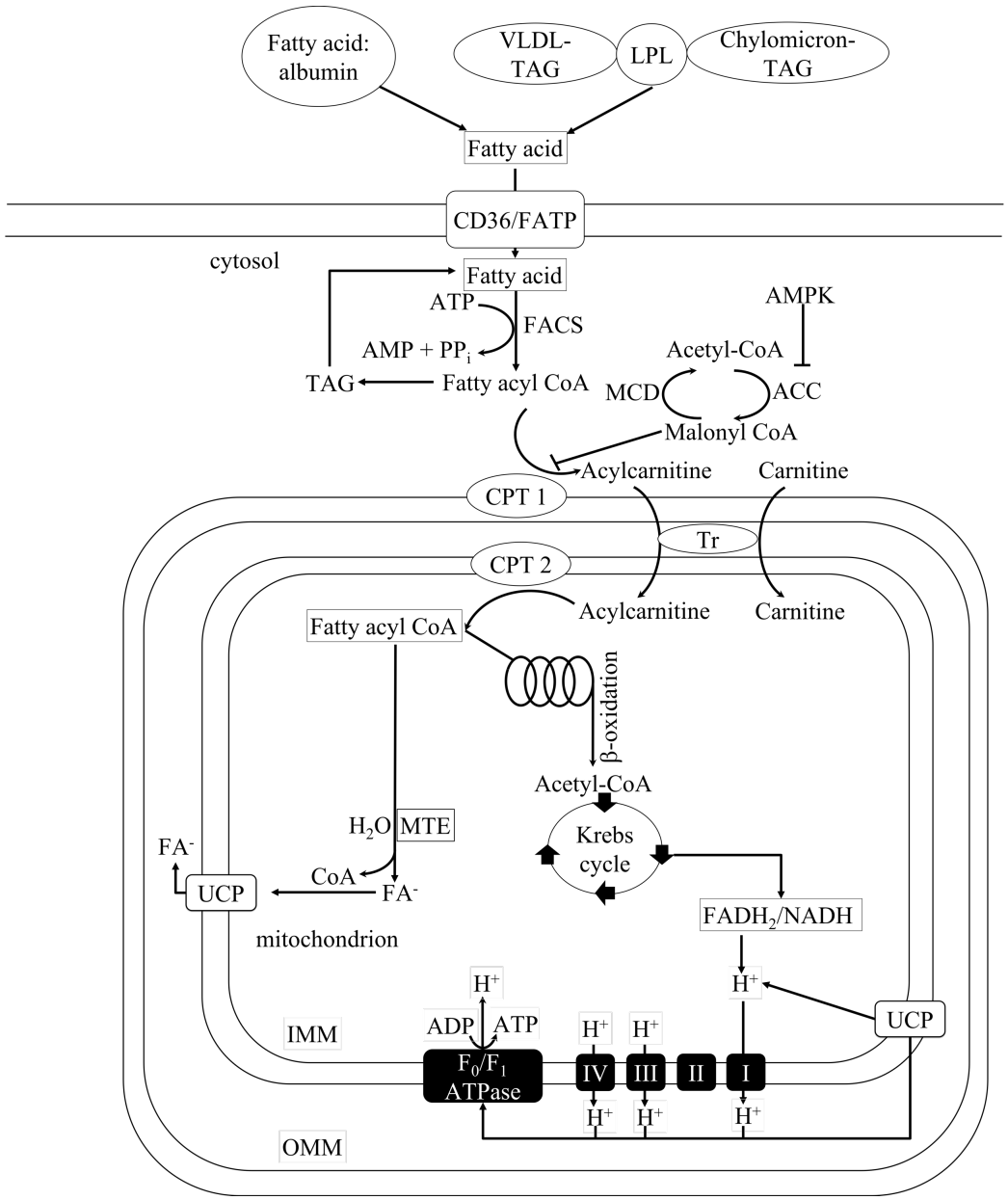


Figure 1.4 *Overview of fatty acid β-oxidation in the heart. TAG-Fatty acid uptake occurs at the level of lipoprotein lipase (LPL), fatty acid uptake into the cardiomyocyte via a protein carrier-mediated pathway (fatty acid translocase protein (FATP) or cluster of differentiation 36 (CD36)), esterification to coenzyme A, mitochondrial uptake via carnitine palmitoyltransferase (CPT) activity, and β-oxidation. Fatty acid β-oxidation rate is also very dependent on the metabolic demand and the activities of the Krebs cycle and electron transport chain (ETC). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; LPL, lipoprotein lipase. VLDL, very-low-density lipoprotein; TAG, triacylglycerol; FACS, fatty acyl-CoA synthase; CPT, carnitine palmitoyltransferase; Tr, carnitine-acylcarnitine translocase; NADH, nicotinamide adenine dinucleotide; $FADH_2$, flavin adenine dinucleotide; UCP, uncoupling protein; ACC, acetyl CoA carboxylase; MCD, malonyl-CoA decarboxylase; AMPK, AMP-activated protein kinase; FA^-, fatty acid anion; MTE, mitochondrial thiesterases; CoA, coenzyme A; ADP, adenosine diphosphate; F_o/F_1 ATPase, ATPases; H^+, hydrogen ion; I, II, III, IV, complexes I-IV of the electron transport chain, respectively.*

Fatty acids enter the cardiac myocyte either via passive diffusion or via a protein carrier-mediated pathway (39, 94-96). Fatty acid translocase (FAT)/ cluster of differentiation 36 (CD36) is a protein carrier, which is responsible for the translocation of fatty acid across the sarcolemmal membrane of cardiomyocytes (97-99) (Fig. 1.4). In the cytosolic compartment of the cardiomyocyte, fatty acyl-CoA synthase (FACS) (100) catalyses the esterification of fatty acids to fatty acyl-CoA. Fatty acyl-CoA can be esterified to TAG by glycerolphosphate acyltransferase (3, 39, 101) (Fig. 1.4). It is postulated that ~20% of fatty acyl-CoA enters the intramyocardial TAG pool (2, 7). Hormone-sensitive lipase activated by cyclic adenosine monophosphate (cAMP) catalyses the breakdown of intracellular TAG (102, 103). It is reported that ~80% of the fatty acids taken up by the normal heart are immediately oxidised (2, 3, 104).

Furthermore, the acyl group from fatty acyl-CoA can be transferred to carnitine via carnitine palmitoyltransferase (CPT)-1 to form acylcarnitine (12). Moreover, malonyl-CoA negatively regulates CPT1 activity (100, 105-112). Acetyl-CoA can synthesise malonyl-CoA, via acetyl CoA carboxylase (ACC) (113-117). In the heart, AMPK (118) regulates ACC activity, via phosphorylation (118-120). There is a close correlation between increased AMPK activity, decreased ACC activity, and increased fatty acid oxidation (121-123). Malonyl-CoA decarboxylase (MCD) can regenerate acetyl-CoA in tissues, such as heart, which lack of a lipogenic pathway to dispose of the malonyl-CoA (39, 124-128) (Fig. 1.4).

Acylcarnitine is shuttled into the mitochondria via carnitine-acylcarnitine translocase (Tr). Acylcarnitine is converted back to fatty acyl-CoA via CPT2. Fatty acyl-CoA produces acetyl-CoA via β-oxidation, involving sequential metabolism of acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-OH acyl dehydrogenase, and 3-ketoacyl-CoA thiolase (129). Each cycle of fatty acid β-oxidation causes a shortening of the fatty acyl moiety by two carbons, as well as the production of acetyl-CoA. Acetyl-CoA enters the

Krebs cycle, producing reduced nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide ($FADH_2$). Fatty acid β-oxidation is regulated by nuclear receptor transcription factors, including peroxisome proliferator-activated receptor (PPAR) α and peroxisomal proliferator-activated receptor γ coactivator-1 (PGC-1) α (130-135) (Fig. 1.4). Although the utilisation of fatty acids can generate a greater amount of ATP, it comes at the expense of a greater oxygen requirement than the use of βHB. This is discussed later (Chapter 6).

Normal circulating FFA concentrations range between 0.2 to 0.6 mM (7). Circulating FFA is very low during the perinatal period (136) and the level rises to about 1-2 mM during starvation, and to over 2 mM during severe stress, such as uncontrolled diabetes and myocardial ischaemia (127, 137). In the physiological and pathophysiological setting of fasting, diabetes, and obesity, the increased circulating FFA and VLDL-derived triacylglycerol increase lipid supply to the cardiomyocyte, which increases the availability of fatty acid ligands for binding to PPAR (α/β/δ/ γ) to form heterodimeric complexes with the retinoid X receptor (RXR) (130-135). The PPAR-RXR heterodimer complex then translocates into the nucleus where it binds to its appropriate response elements. PPAR- α coactivator 1 α (PGC1 α) enhances the ability of the PPARs to increase transcription of their target genes. PPARα is abundantly expressed in heart muscle, and its target genes include those encoding proteins involved in fatty acid uptake (FAT, CD36, FATP1), cytosolic fatty acid binding and esterification (FABP, FACS, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase), malonyl CoA metabolism (MCD), mitochondrial fatty acid uptake (CPT 1), fatty acid β-oxidation [very-long-chain acyl CoA dehydrogenase, long-chain acyl CoA dehydrogenase, medium-chain acyl CoA dehydrogenase (MCAD), 3-KAT], mitochondrial uncoupling [including mitochondrial thies- terases (MTE-1) and uncoupling proteins (UCP3)], and glucose oxidation [PDH kinase (PDK) 4] (12). In patients with heart

disease, the increase in circulating free fatty acids is positively correlated with content of cardiac UCP3, but inversely correlated with myocardial phosphocreatine-to-ATP ratio, an indicator of cardiac energy deficit (138). The consumption of a high fat diet also upregulates UCP3 in cardiac muscle (139). UCP3 transports proton only when activated by fatty acids. UCP dissipate the high proton concentration in the mitochondrial intermembrane space by transferring the proton (H^+) back into the mitochondrial matrix, thereby uncouple it from ATP synthesis (140, 141). When the supply of fatty acyl-CoA exceeds the rate of fatty acid β-oxidation, excess fatty acyl-CoA can be hydrolysed to yield free CoA and a fatty acid anion (FA^-) by mitochondrial thiesterases (MTE). FA anion may leave the mitochondrial matrix via UCP3 (142, 143). The investigation of physiological role of UCP3 in transporting fatty acid anion did not achieve a conclusive result. It was reported that FA anion export occurred independently of UCP3 during fasting (144). In the cytosol, cytosolic thioesterase (CTE) catalyses the reesterification of FA anion to CoA, which consumes ATP for noncontractile purposes (futile cycling) (12) (Fig. 1.4).

1.4 Cardiac anaplerosis and cataplerosis in health and disease

The Krebs cycle is possibly the best example for the concept of efficient energy transfer through metabolic cycles. The mechanism by which oxidation of acetyl-CoA to two CO_2 by the Krebs cycle occurs is the central process in the generation of the reducing equivalents NADH and $FADH_2$ for the electron transport chain. In the Krebs cycle, the 2-carbon acetyl group of acetyl-CoA is completely oxidised to two CO_2, with no net change in any of the intermediates. Hence, the intermediates of the Krebs cycle are essentially 'catalytic' in nature and may be present in very low concentration (48-50, 145, 146). It is not possible for acetyl-CoA itself (nor, hence, for fatty acids or ketone bodies, nor ketogenic amino acids, such as leucine which produce only acetyl-CoA on their degradation) to supplement Krebs cycle intermediates, as their utilisation necessarily involves loss of 2 corresponding (though

not identical) carbon atoms. These substrates are hence cataplerotic in nature, their utilisation leading to a gradual diminution in the Krebs cycle intermediates pool (147-149). Since intermediates of the Krebs cycle are also involved in other many biosynthetic pathways that occur in the mitochondria, and also in the cytosol following their efflux from the mitochondria (48, 49). For instance, citrate can leave the mitochondrion for *de novo* lipogenesis (DNL) (150) although the expression of fatty acid synthase is relatively low in heart (151). The entry of carbons as intermediates, rather than as 2-carbon acetyl-CoA into the cycle, via anaplerosis is very important to balance this efflux (cataplerosis) (48, 49, 152). Pyruvate carboxylase is a biotin-containing enzyme that catalyses the biocarbonate and magnesium ATP-dependent carboxylation of pyruvate to form oxaloacetate (153, 154). It is the major anaplerotic enzyme in the heart (155).

The oxidation rate of acetyl-CoA in the Krebs cycle, which critically determine the formation of NADH and $FADH_2$ for ATP production and turnover time of individual Krebs cycle intermediates, are both variable and very rapid (48). Hence, it has only recently been recognised how critically important maintenance of the Krebs cycle capacity is to oxidative efficiency in oxidative tissues, such as myocardium, and how utilisation of 'cataplerotic' substrates, such as fatty acids and ketone bodies depends on simultaneous provision of anaplerotic substrate ($\geq$ 3-carbon equivalents). Figure 1.5 depicts reactions involved in anaplerosis (blue arrows) and cataplerosis (red arrows).

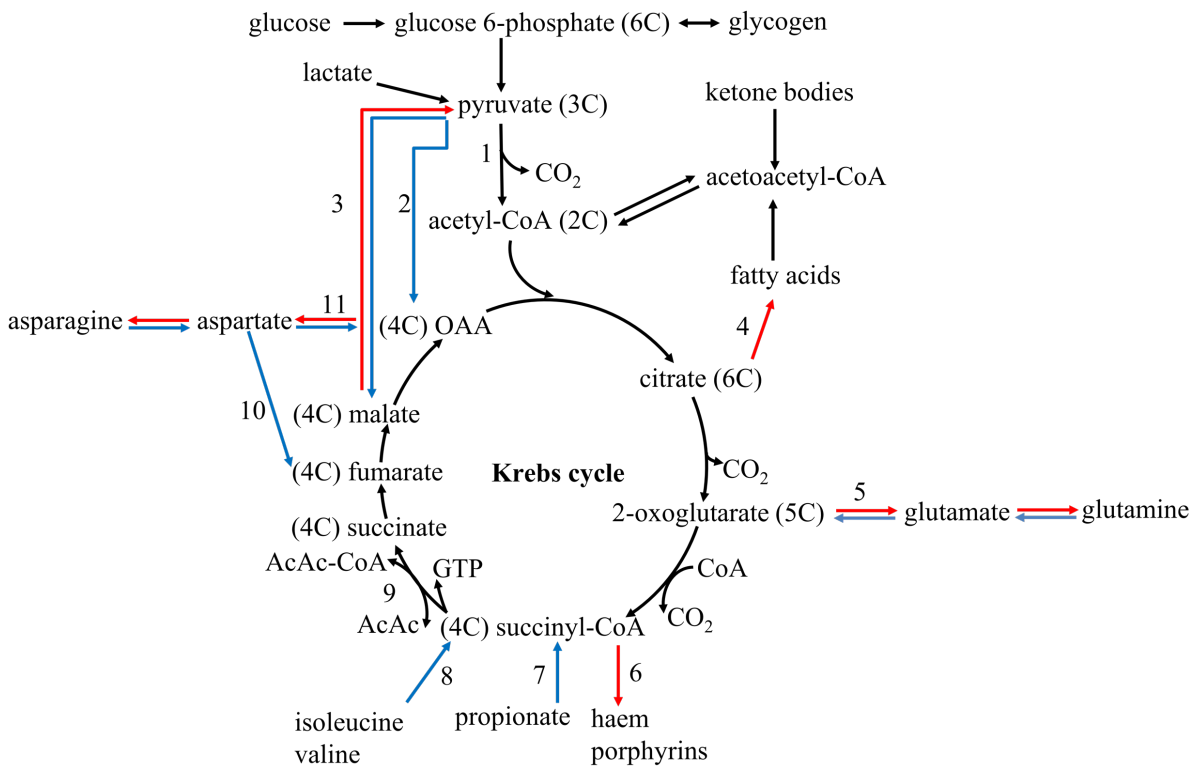


Figure 1.5 *Overview of pathways and reactions that are related to anaplerosis and cataplerosis. Krebs cycle intermediates entry (blue arrows) or removal (red arrows). Number refers to the enzymes catalyzing the following reactions that will be referred to in the text: 1, pyruvate decarboxylation; 2 and 3, pyruvate carboxylation by pyruvate carboxylase and malic enzyme, respectively; 4, ATP-citrate lyase; 5, glutamate dehydrogenase; 6, delta-aminolevulinic acid synthase; 7, propionyl-CoA carboxylase; 8, branched-chain alpha-ketoacid dehydrogenase; 9, succinyl-CoA-3-oxoacid-CoA transferase (SCOT); 10, adenylosuccinate synthetase (purine nucleotide cycle); 11, aspartate aminotransferase;2-oxoglutarate; AcAc, acetoacetate; OAA, oxaloacetate.*

Anaplerotic flux must occur to balance cataplerotic efflux. Rates of citrate efflux and succinate efflux each represented at most 20% of anaplerotic pyruvate carboxylation. The efflux of the Krebs cycle intermediates equivalent to 60% of total pyruvate anaplerosis could be represented by other sites of cataplerotic removal of the Krebs cycle intermediates (48). Anaplerosis from pyruvate via pyruvate carboxylation has been shown to maintain cardiac function and decrease infarct size following experimental myocardial ischaemia in the pig (149, 156).

In mitochondria, citrate can leave mitochondria to generate malonyl-CoA by cytosolic cleavage of citrate catalysed by ATP-citrate lyase (157); this results in inhibition of fatty

acid oxidation via inhibition of carnitine palmitoyl transferase-1 (CPT1). This beneficial effect improved cardiac efficiency in myocardial ischaemia associated with elevated free fatty acids (147, 158-160). Succinate is a key metabolite produced by during ischaemia-reperfusion (IR) injury. During ischaemia, succinate is accumulated from reversal of succinate dehydrogenase (SDH). Upon reperfusion, the accumulated succinate is oxidised by SDH, driving reverse electron transport chain (RET) at mitochondrial complex I, which leads to damaging reactive oxygen species (ROS) generation (161-164). Alternatively, dimethyl malonate, a membrane-permeable precursor of the SDH competitive inhibitor malonate, can decrease ischaemia-generated succinate (162). Moreover, fumarate is cardioprotective via activation of the transcriptional regulator Nrf2 (165, 166)

The contribution of propionate ($CH_3.CH_2.COOH$: an odd number carbon short chain fatty acid) or BCAA to generate succinyl-CoA is low under normal physiological conditions. Myocardium can generate succinyl-CoA from propionate via propionyl-CoA carboxylase, methylmalonyl-CoA racemase and mutase (167-169), but odd number fatty acids are scarce (since most fatty acids are assemblies of acetyl group derived from acetyl-CoA-2 carbon units and hence even numbered). A beneficial effect of an anaplerotic medium-*odd*-chain fatty acids (including precursors of acetyl-CoA and anaplerotic propionyl-CoA, such as triheptanoin and heptanoate) compared to medium-even-chain fatty acids has been seen in patients with the cardiomyopathic form of the very-long-chain acyl-CoA dehydrogenase (VLCAD), fat oxidation disorder. This is because heptanoate can replenish succinyl-CoA via propionyl-CoA, which facilitates acetyl-CoA oxidation, NADH production and hence ATP production to improve muscle and heart contraction (170). In contrast, a study has showed that heptanoate treatment increased the Krebs cycle intermediates without affecting contractile function following ischaemia-reperfusion in pig heart (171). The contractile dysfunction could be due to increased workload rather than reduced anaplerosis.

Moreover, heart failure is associated with deficiencies in cofactors, such as biotin and thiamine that are required for anaplerotic enzymes, such as pyruvate carboxylase and 2-oxoglutarate dehydrogenase, respectively (172, 173). It was reported that administration of propionate alone as an anaplerotic source of succinate induced toxicity in heart failure patients. Alternatively, propionyl-L-carnitine was used to treat heart failure patients by improving exercise capacity and oxygen consumption (174). Moreover, cytosolic malic enzyme is increased in rats with end-stage heart failure (175). Whether the change in malic enzyme is maladaptive or adaptive is unknown.

The relative contributions of anaplerotic pathways feeding oxaloacetate (pyruvate carboxylation) and acetyl-CoA (pyruvate decarboxylation) for citrate synthesis can be determined in hearts perfused with a mix of ^{13}C- substrates, such as [U-$^{13}C_3$] lactate, [U-$^{13}C_3$] pyruvate and 1-^{13}C-octanoate. In combination, gas chromatography-mass spectrometry (GCMS) is used to directly detect ^{13}C-labeling of Krebs cycle intermediates and determine the flux ratios from measured mass isotopomer distributions (48, 148, 176-179). However, quantifying anaplerotic flux remains a major technical challenge. Radioactive and stable isotope (^{13}C isotopomer) labelling techniques can label the Krebs cycle intermediates without net anaplerotic flux due to metabolism via the Krebs cycle, exchange reactions between the Krebs cycle intermediates, and other metabolites, including aspartate and glutamate (48, 177, 180). Oxaloacetate is present in minuscule concentrations (5-10 nmol/g) and its turnover rate is very rapid, up to 800 times per minute (48), making it difficult to quantify (181).

Although recent emergence of the hyperpolarized ^{13}C magnetic resonance (MR) technique, in which ^{13}C-labelled metabolites are followed using MR imaging (MRI) or spectroscopy (MRS) are potentially able to improve sensitivity (>10 000-fold) (182-185) and quantify anaplerotic reactions in the failing heart (using ^{13}C-pyruvate labelling; (186)),

a number of important technical challenges remain. These includes the requirement for a very rapid (within 2 to 3 minutes) hyperpolarized sample transfer and infusion (178, 187). However, hyperpolarization by dynamic nuclear polarization (DNP) can be used as an effective *in vivo* probe of ketone ([1-^{13}C]acetoacetate and [1-^{13}C] β-hydroxybutyrate) metabolism in the heart (188). Perfusion of the heart with the ketone body acetoacetate decreased tissue content of aspartate and glutamate and inhibited 2-oxoglutarate dehydrogenase activity, indicating the importance of anaplerotic substrates, including glucogenic amino acids and odd chain fatty acids, to maintain contractile function (45, 47). Recent findings showed that the increased ketone body oxidation in hypertrophied heart was correlated with an increase in the entry of anaplerotic carbon flux into the Krebs cycle (32).

1.5 Glucose metabolism in the normal heart

In the myocardium, fatty acid β-oxidation contributes ~70% of the acetyl-CoA production, and ~10-40% derives from pyruvate oxidation, which in turn originates from glycolysis and lactate oxidation (189-193). Glucose is an important fuel for all organisms through common metabolic pathways namely, glycolysis (194), the Krebs cycle (146), electron transport chain and oxidative phosphorylation (195). D-glucose ($C_6H_{12}O_6$) is the most abundant organic monomer in nature (196). In the heart, lacking significant gluconeogenic capacity, glucose is derived either from exogenous glucose or from intracellular stores of glycogen.

Insulin stimulates glucose uptake. Insulin binds to its receptor leading to auto-phosphorylation of tyrosine residues in the receptor protein, which interacts with insulin receptor substrates (IRSs). IRSs become phosphorylated and interact with enzyme phosphatidylinositol-3-kinase (PI3K). PI3K generates phosphatidylinositol (3', 4', 5')-triphosphate (PIP_3), which acts through the enzyme 3'-phosphoinositide dependent kinase-1 (PDK1) to phosphorylate and activate protein kinase B (PKB) or Akt. Activated PKB increases glucose transport.

The transport of glucose into cardiomyocytes occurs along a steep concentration gradient and requires specific glucose transporters that belong to the GLUT family (197-199) recruited from intracellular depots to the plasma membrane (200-202). Michaelis-Menten constant, K_m is the concentration of substrate, such as glucose to achieve half of the maximum rate of a reaction. An insulin-sensitive transporter, (K_m = 5 mM) (203, 204) GLUT-4, is predominantly expressed at the surface of adult cardiomyocytes (202).

The insulin-independent transporter, GLUT-1 (K_m = 1-2 mM) is predominantly expressed in foetal myocardium (200), but also occurs in the mature myocardium. Both transporters have a K_m that is in the range of plasma glucose concentration under fasting condition (205). In the overnight-fasted state, plasma concentration of glucose and insulin are 5 mmol l^{-1} and 50-100 pmol l^{-1}, respectively (206, 207).

Insulin and work increases glucose transport into cardiac muscle, via increasing the translocation of GLUT-4 from the endoplasmic reticulum to the plasma membrane (208-213). Glucose is phosphorylated by hexokinase to glucose 6-phosphate, requiring one molecule of ATP. Hexokinase I is predominantly expressed in the foetal and newborn heart. Insulin-regulated hexokinase II is predominantly expressed in the adult heart (214). Phosphoglucose isomerase catalyses the conversion of glucose 6-phosphate to fructose 6-phosphate. The first step committing glucose to the glycolytic pathway is 6-phosphofructo-1-kinase (PFK-1) that catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. This is a rate-limiting step for glycolysis due to complex allosteric regulation of PFK (215). Fructose 6-phosphate is also converted to fructose 2,6-bisphosphate by PFK-2. Fructose 2,6-bisphosphate is a potent activator of PFK-1 for the production of fructose 1,6-bisphosphate and hence the glycolytic pathway (216). AMP is also a positive allosteric effector (215). The reaction consumes one molecule of ATP. ATP, citrate, and protons are negative allosteric effectors (216-218). Fructose 1,6-bisphosphate is then split into

dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (two 3-carbon sugars). These two substances (triose phosphates) are biochemically interconvertible. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) catalyses the oxidation and phosphorylation of glyceraldehyde 3-phosphate to form an energy-rich intermediate, 1,3-bisphosphoglycerate, and the NADH formed is shuttled into the mitochondrial matrix. Furthermore, phosphoglycerate kinase catalyses the reaction of 1,3-bisphosphoglycerate with ADP to form two molecules of ATP and 3-phosphoglycerate.

During increased heart work, glucose transport is regulated by adenosine monophosphate kinase (AMPK) (219). Opie and colleagues reported that tissue content of ATP, phosphocreatine and citrate fell with increased heart work. The increase in adenosine monophosphate (AMP) and inorganic phosphate (Pi) increased glycolysis at the level of phosphofructokinase-1 activity (220, 221). When normal heart is stressed, it oxidises glycogen, glucose and lactate, in order of preference (103). Glycogen has a high surface area, providing a capacity for rapid glycogenolysis during the increase in workload (222-226). During increased heart work, the pyruvate dehydrogenase complex (PDC) is activated by dephosphorylation via pyruvate dehydrogenase phosphatase in the presence of mitochondrial Ca^{2+}, thereby increasing glucose oxidation (10, 227).

Pyruvate kinase catalyses the irreversible (far-from equilibrium) reaction of phosphoenolpyruvate with ADP to form two molecules of ATP and pyruvate. Two molecules of ATP (net) derive from substrate-level phosphorylation in the glycolytic pathway. ATP derived from glycolysis is also used to maintain the sarcolemma sodium-potassium pump for contractile function (228). The control of glycolysis is variably distributed between enzymes depending upon substrate, hormone, and oxygen availability, or other variable conditions (43). Pyruvate and NADH derived from glycolysis are shuttled into the mitochondrial matrix to produce CO_2 and NAD^+, thereby completing the process of

aerobic oxidative glycolysis, or converted to lactate and NAD^+ in the cytosol (anaerobic non-oxidative glycolysis) (Fig. 1.6).

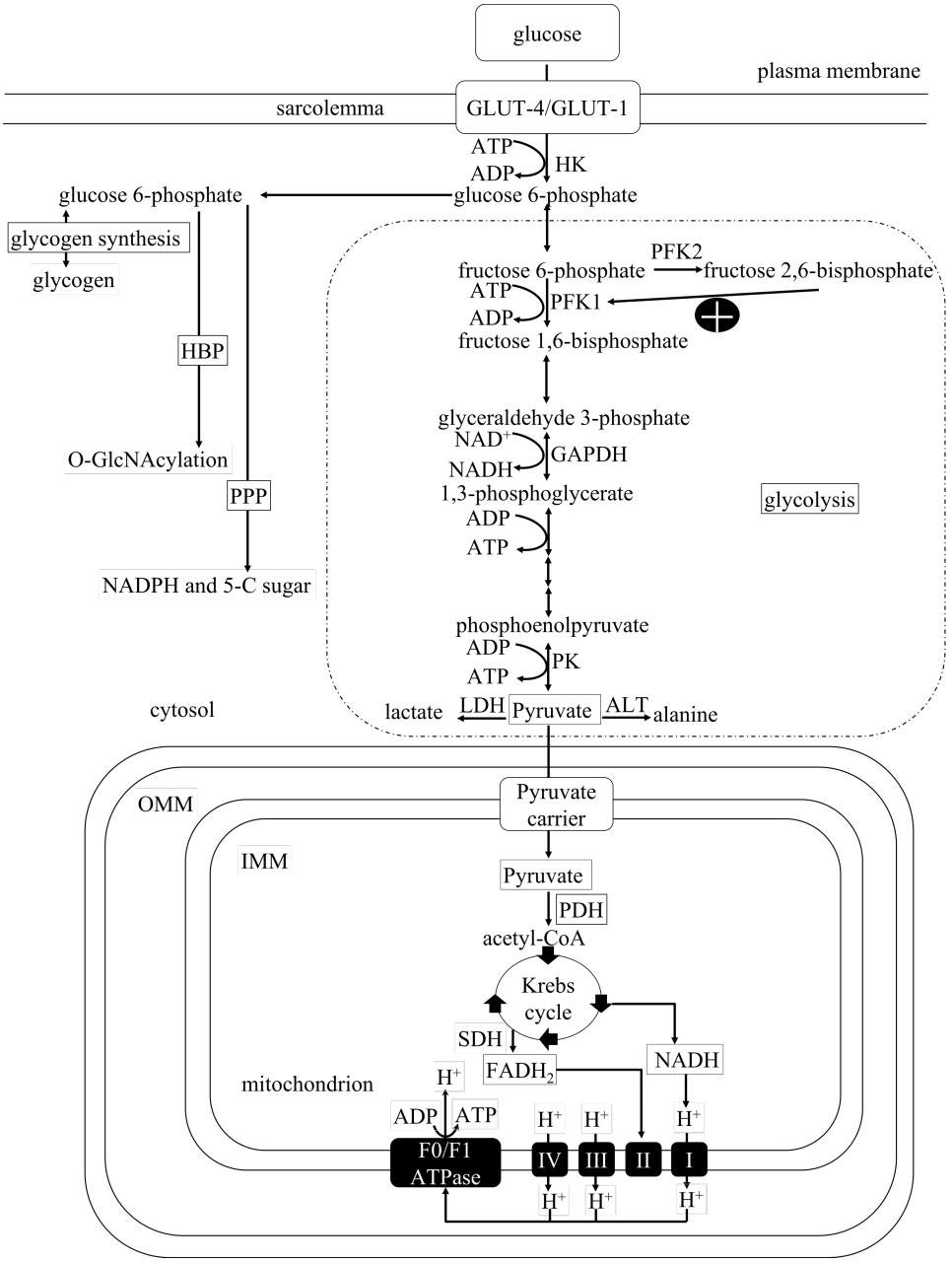


Figure 1.6 *Glucose metabolic pathways in the heart. Glucose is transported into cardiomyocytes through glucose transporter (GLUT)-4 or GLUT-1. Glucose is phosphorylated to glucose 6-phosphate by hexokinase (HK). In the cytosol, most glucose 6-phosphate is converted to pyruvate via glycolysis. Fructose 2,6-bisphosphate is a potent activator of PFK-1 for the production of fructose 1,6-bisphosphate. In the mitochondria, pyruvate is converted to acetyl-CoA for the Krebs cycle by pyruvate dehydrogenase (PDH). However, glucose 6-phosphate can also undergo multiple metabolic pathways, including glycogen synthesis, pentose phosphate pathway (PPP), or hexosamine biosynthetic pathway (HBP). ATP, adenosine triphosphate; ADP, adenosine diphosphate; PFK-1/-2, phosphofructokinase-1/-2; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrate; FADH, flavin adenine dinucleotide hydrate; PK, pyruvate kinase; ALT, alanine amino-transferase; GADPH, glyceraldehyde 3-phosphate; LDH, lactate dehydrogenase; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane, SDH, succinate dehydrogenase.*

Glycolysis and pyruvate oxidation is the predominant fate of glucose. However, glucose 6-phosphate derived from glucose can also feed through other metabolic pathways in the heart, including glycogen synthesis (196), the pentose phosphate pathway (PPP) (229), and the hexosamine biosynthetic pathway (HBP) (Fig. 1.6). Glucose 6-phosphate can be converted to glycogen by glycogen synthase (and a branching enzyme). Glycogen synthesis is important for normal heart development (230), and glycogen acts as an anaerobic energy source through glycogenolysis in anoxaemic emergencies (231, 232). Ribulose 5-phosphate derived from the non-oxidative (irreversible) reactions of PPP can be converted to fructose 6-phosphate, or used for purine synthesis. The oxidative PPP also may play a role in maintaining intracellular redox states and antioxidant potential through generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) (233). Fructose 6-phosphate is converted to glucosamine 6-phosphate by glutamine:fructose 6-phosphate amidotransferase (GFAT), thereby generating uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as the final product of HBP. HBP and O-GlcNAcylation play a role in sensing cellular stressors (transcription factors, forkhead box other-1, FOXO1) and nutrient levels (insulin and glucose levels) (234-236), and they have been implicated in the development of pathological cardiac hypertrophy (235, 237) and diabetes mellitus (238).

In the presence of oxygen, pyruvate enters mitochondria via a monocarboxylate carrier, which can be inhibited by α-cyano-4-hydroxycinnamate (CHC) (239). Pyruvate is produced either from glycolysis or from exogenous lactate. In the mitochondrial matrix, pyruvate is either decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex (PDC) or carboxylated to oxaloacetate and malate by pyruvate carboxylase and malic enzyme, respectively (anaplerosis) (46, 47, 240). Activation and inactivation of PDC regulates the oxidative decarboxylation of pyruvate to acetyl-CoA (241). PDC is a mitochondrial multienzyme complex, and is regulated by its substrates and products

including acetyl CoA, and by phosphorylation/dephosphorylation. Phosphorylation of PDH is catalysed by four specific PDH kinases: PDK1, PDK2, and PDK4. The increase in the level of ATP, NADH and acetyl-CoA activates PDH kinases. However, the activity of these kinases is inhibited when cellular energy charge is low, which is reflected by an increase in the level of pyruvate (196), ADP, NAD^+, CoASH (242-244). PDH phosphatases (PDP1 and PDP2) are stimulated by Ca^{2+} and Mg^{2+} (245, 246), thereby activating PDH by removing the phosphate groups (247). Hence, pyruvate that enters the mitochondria is predominantly oxidised in the Krebs cycle to form $FADH_2$ and NADH. $FADH_2$ is attached to succinate dehydrogenase, donating electrons via ubiquinone to complex III, then to complex IV. Oxidative phosphorylation of $FADH_2$ produces two molecules of ATP. In contrast, three molecules of ATP are formed from oxidation of one NADH molecule. Using non-integral P/O ratios (number of molecules of ATP synthesised per atom of oxygen) of 2.5 NADH and 1.5 $FADH_2$, oxidation of glucose yields some 31 molecules of ATP (12) (Fig. 1.6).

1.6 Amino acid metabolism in the healthy and diseased heart

The heart acts as a metabolic "omnivore" and is capable of utilising fatty acids, glucose, ketone bodies, pyruvate, lactate, and amino acids, in order of decreasing consumption. Protein from dietary intake in the fed state, or derived from muscle during starvation, can be degraded to amino acids for oxidative ATP production. Amino acids are a heterogenous group of acidic, neutral, and basic organic acids containing an amino (NH_2) group and a carboxyl (COOH) group. Amino acids are the building blocks of all myocardial protein. They are substrates of protein synthesis and products of protein degradation. The balance of this protein turnover is a major determinant for normal cardiac function. A failing heart is characterized by an increase in protein synthesis and a decrease in protein degradation (248).

Mammals are capable of synthesizing only 11 of the 20 amino acids that are coded for in protein synthesis. They are known as the non-essential amino acids. The other nine amino acids that cannot be synthesized are known as the essential amino acids. Amino acid transporters (AATs) are membrane-bound transport proteins that regulate the transfer of amino acids into and out of cells or cellular organelles. For instance, the large neutral amino acids transporter (NAA) solute carrier 7A6 (SLC7A6) is responsible for the transportation of L-glutamine in the heart by the sodium-dependent transport system (249). SLC transporters depend either on an electrochemical gradient (H^+) to facilitate the movement of the substrates across the membranes, or on ion gradients (Na^+, K^+) generated by ATP-dependent pumps to transport substrates against the concentration gradient (Table 1.2) (250-254).

Table 1.2 *Amino acids and their role in cardiac metabolism (Adapted from (250-252).*

	Amino Acid	Plasma Conc. (μM)	Metabolic Role in the Heart
Non-essential amino acid	Alanine	230–510	Alanine is converted to pyruvate via transamination.
	Arginine	13–64	Arginine is converted into 2-oxoglutarate.
	Asparagine	45–130	The amide in asparagine is easily hydrolysed. Asparagine is converted to oxaloacetate via transamination
	Aspartate	0–6	Aspartate is converted to oxaloacetate via transamination.
	Cysteine	30–65	Cysteine is converted to pyruvate
	Glutamate	18–98	Glutamate is converted to 2-oxoglutarate via transamination.
	Glutamine	390–650	Glutamine is the amide of glutamic acid, and its side chain is uncharged under all biological conditions. Glutamine is converted into glutamate. Glutamate and 2-oxoglutarate are readily interconvertible via transamination. 2-oxoglutarate can be oxidised through the Krebs cycle to yield NADH and guanosine-5'-triphosphate (GTP).
	Glycine	170–330	Glycine is converted to pyruvate. It acts as a precursor in many synthetic pathways.
	Proline	110–360	Proline is converted into 2-oxoglutarate. It is currently unknown what role it may play in cardiac metabolism.
	Serine	56–140	Serine is converted to pyruvate.
	Tyrosine	45–74	Tyrosine is converted to fumarate and acetyl-CoA. It is not metabolized by the heart.
Essential amino acids	Histidine	26–120	Histidine is converted to 2-oxoglutarate. Its extra-protein synthesis role in the heart is unknown.
	Isoleucine	42–100	It is converted to acetyl-CoA and succinyl-CoA (mixed glucogenic and ketogenic). It has a branched hydrocarbon side chain (branched-chain amino acids (BCAA).
	Leucine	66–170	Leucine has a branched hydrocarbon side chains (BCAA). It is converted to acetyl-CoA and acetoacetate (ketogenic amino acid).
	Lysine	150–220	Lysine is converted to acetyl-CoA.

Methionine	16–30	Methionine is converted to succinyl-CoA.
Phenyl-alanine	41–68	Phenylalanine is not metabolized by the heart.
Taurine	45–130	Taurine is an amino sulphonic acid, and is important in the heart. It has been shown to exert cardioprotective effects by reducing the severity and rate of arrhythmias and improving contractile function.
Threonine	92–240	Threonine is another hydroxyl-containing amino acid. Threonine can be converted to pyruvate
Tryptophan		Tryptophan is the largest of the amino acids. This amino acid is not metabolized by the heart.
Valine	150–310	Valine is a branched chain amino acid. Valine differs from threonine by replacement of the hydroxyl group with a methyl substitution. Valine can be converted to succinyl-CoA via branched-chain α-ketoacid dehydrogenase (glucogenic amino acid)

Glucose, fatty acids and other substrates require oxygen for energy production. Although some amino acids can be readily oxidized (notably the branched chain amino acids isoleucine, leucine, and valine) and synthesized (alanine, glutamine, glutamate, and aspartate), not all amino acids can be metabolized in the hearts (phenylalanine, tyrosine, and tryptophan). Transamination is a chemical reaction that transfers an amino group from the α-carbon of an amino acid to an α-ketoacid to form new amino acids. It is a first step in amino acid metabolism catalysed by abundant, reversible aminotransferases in the heart (255). Amino acids are mostly used to provide substrates for protein synthesis and anaplerosis. They can also act as a signalling molecules. For example, aspartate and leucine are sensors for the mammalian target of rapamycin (mTOR) signalling pathway. In addition, mTOR complex 1 (mTORC1) is responsible for cardiac adaptation to pressure overload and development of compensatory hypertrophy (256-259).

Amino acids are zwitterions, and become especially important during acute ischaemia for non-oxidative metabolism and their low contribution to cellular acidification due to their inherent buffering ability (260). Cardiac ischaemia occurs due to the partial or complete occlusion of a coronary artery, or during end-stage heart failure, and is associated with a decrease in intracellular levels of ATP, glutamate, aspartate, and glycogen, together with an increase in lactate production and alanine/glutamate ratio. Amino acid supplementation maybe cardioprotective by replenishing the Krebs cycle intermediates (anaplerosis), eliminating inhibition of succinate-CoA reductase by oxaloacetate and increased substrate-level phosphorylation of guanosine diphosphate (GDP) to GTP, providing high levels of NADH and $FADH_2$ for rapid oxidation in the presence of oxygen during reperfusion (162) and the production of reduced glutathione as an antioxidant (261). Aspartate, asparagine and the glucogenic branched chain amino acids (isoleucine and valine) are used to replenish intermediates in the Krebs cycle during cardiac ischaemia. (262-264). Glutamate and glutamine can be converted to 2-oxoglutarate to improve myocardial function for ischaemic and hypertrophied hearts by maintaining levels of NADH and GTP (265-268). Valine can act as an alternative substrate to glucose by providing high-energy phosphate in both cytoplasm and mitochondria to maintain action potential duration in the atrioventricular node (269). Heart is unable to metabolize the aromatic amino acids (phenylalanine, tryptophan and tyrosine) and their roles in cardiac metabolism remain to be elucidated (270-272).

Heart failure (HF) is associated with a deficiency of key nutrients, such as amino acids that are required for unimpeded energy transfer (173). Energy transfer in a beating heart is a process of interconnected cycles that receives major (lipid and carbohydrates) and subsidiary (amino acids) substrates through the circulation and which transfer energy from nutrients to ATP that can be used to support cyclic contractions. Amino acid derivatives,

such as taurine, creatine, carnitine, and glutathione have been shown to improve functional, structure, and haemodynamic parameters in HF patients by improving flux through the cycles of energy transfer in the heart (273). Thus, "subsidiary substrate" may provide therapeutic potential for patients with HF, including those associated with the provision of ATP (Coenzyme Q10 (CoQ10), L-carnitine, thiamine and the B vitamins), protein production (amino acids), intracellular calcium balance (taurine), and the reduction of oxidative stress (CoQ10 and taurine).

Amino acid metabolism is critical in cardiac ischaemia as the disease is associated with decreased levels of ATP, glycogen, glutamate and aspartate, and increased lactate production and alanine-to-glutamate ratios (250). Thus, amino acid supplementation can potentially provide a cardioprotective effect in several ways: 1) by replenishing the Krebs cycle intermediates (anaplerosis), 2) by increasing levels of the reducing equivalents NADH and NADPH for oxidative phosphorylation and 3) by ameliorating oxidative stress by the production of reduced glutathione (250, 261, 268).

1.7 Ketone bodies as fuel for the failing heart

The heart is capable of utilising fatty acids, glucose, ketone bodies, pyruvate, lactate, amino acids, in order of decreasing preference. Changes in substrate energy metabolism have been associated with cardiac diseases (12, 48, 274-281). Ketone body concentration is positively correlated with filling pressure in the setting of heart failure (29, 282-285). Metabolomic analysis in the human non-diabetic failing heart demonstrates an increased myocardial utilisation of βHB as indicated by upregulation of both serum βHB (0.145 mM) and key enzymes in the ketone oxidation pathway, including BDH1, BDH2 and SCOT (33). This study also found that mitochondrial fatty acid oxidation was downregulated, indicated by a decrease in long-chain acylcarnitine (33). Proteomic data demonstrates protein

expression of BDH1 are upregulated in a mouse model of HF, consistent with increased metabolites that reflect ketone body oxidation, including hydroxybutyrylcarnitine and acetylcarnitine, as well as succinate for the succinyl CoA-dependent SCOT reaction. Using the same quantitative mitochondrial proteomics, they found that fatty acid utilisation was downregulated in both mice with pressure-overload-induced hypertrophy and heart failure (32). These data provide strong evidence of increased reliance on ketone body utilisation in a mouse and human model of HF. Bedi and his colleagues postulated that increased myocardial ketone body utilisation is a late event in HF as the metabolite profile characteristic of ketone body oxidation is not observed in the stage of compensated hypertrophy (33). The elevation in serum levels of both fatty acids and ketone bodies is a consequence of enhanced lipolysis and hepatic ketogenesis, resulting in an increased βHB delivery to the failing heart. The failing heart has been linked to an energy-starved engine that has run out of fuel (286). Impaired oxidative phosphorylation, such as reduced activity of electron-transport chain complexes, ATP synthase capacity and phosphate acceptors ADP and creatine, can reduce cardiac function by providing an insufficient ATP supply to cardiac myocytes, leading to cardiac dysfunction and heart failure (138, 287-289). It remains unclear whether increased ketone body metabolism is a cause or consequences of heart failure, but the ketone body metabolism certainly occurs in the failing heart. This leads to the crucial question of whether exogenous ketones can act as an alternative method to raise blood βHB concentration and increase ATP production in the heart.

In the animal model of HF, ketogenic diet for 4 weeks can only increase circulating βHB up to ~1.3 mM (32). Apart from being unpalatable and difficult to prepare, the ketogenic diet can induce atherogenic risk by elevating serum levels of cholesterol and triacylglycerols (290). It has been postulated that a maladaptive effect of the ketogenic diet can affect endothelial vascular function and promote inflammation as well as atherosclerotic

lesions (291). FFA is increased in advanced, severe dilated cardiomyopathy (DCM) associated with impaired glucose uptake and reduced ATP levels (27). In failing human heart, circulating free fatty acid correlates positively with mitochondrial uncoupling protein, UCP2 and UCP3 (138). UCP can reduce cardiac efficiency by transporting fatty acid anion out of the mitochondrial matrix and dissipating the mitochondrial intermembrane proton gradient (140, 292-294). Alternatively, exogenous ketone, such as ketone ester, can elevate blood βHB more than 50% higher than exogenous ketone salt, despite having similar effects on lowering free fatty acids, triacylglycerols, and glucose (295). These findings are in agreement with that of ketone infusion decreased FFA levels under both fasting and fed conditions (77, 78, 296). Therefore, D-β-hydroxybutyrate from either ketone drinks or intravenous administration can improve cardiac efficiency in the failing heart by decreasing lipolysis and plasma fatty acids, mediated through adipocyte GPR109A signaling (74, 297) (Fig. 1.7).

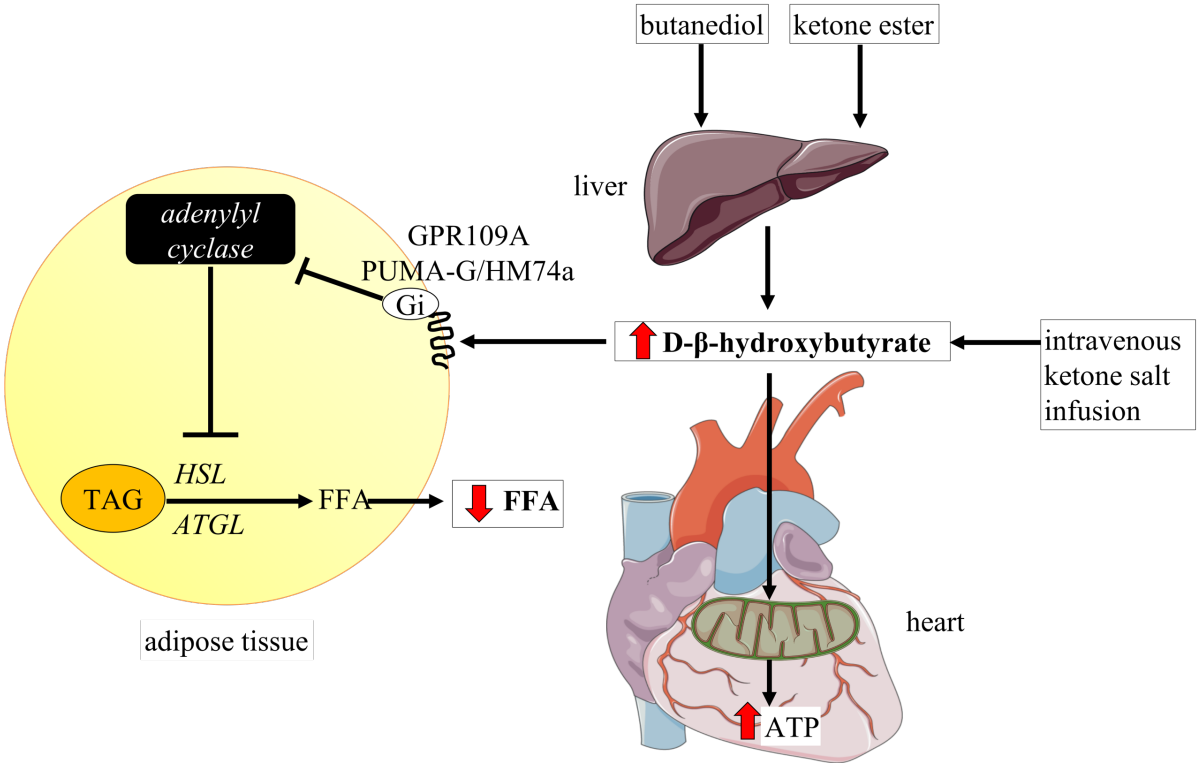


Figure 1.7 *Putative mechanism for inhibition of adipocyte lipolysis by D-β-hydroxybutyrate (βHB). βHB from either ketone drinks (e.g. Butanediol) or intravenous infusion activates protein upregulated in macrophages by interferon-gamma (mouse PUMA-G, human HM74) PUMA-G/HM74a through a G_i/G_o-dependent inhibition of adenylyl cyclase, thereby inhibiting adipocyte lipases, such as hormone-sensitive lipase (HSL) and adipocyte triglyceride lipase (ATGL). The suppression of the lipase reduces the amount of free (non-esterified) fatty acid (FFA). Oxidation of βHB increases the energy of (adenosine triphosphate) ATP hydrolysis (yielding more ATP per molecule of oxygen) by generating more NADH to be delivered to complex I within the electron transport chain. βHB, β-hydroxybutyrate; ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide hydrate; TAG, triacylglycerol.*

1.8 Thesis context and outline

D-β-hydroxybutyrate (βHB) is the reduced form of acetoacetate and is increased during fasting (298), ketogenic diet (299) and exercise (300). In normal adult hearts, fatty acid oxidation provides up to 70% of the ATP production, with the metabolism of glucose, lactate, amino acids and ketone bodies supplying the balance. Different substrates have different metabolic efficiencies, by virtue of their differing thermodynamics chemistry, but concepts of metabolic efficiency can be applied to energy yield or oxygen requirements (12, 43, 44, 301). Nevertheless, much research to date is limited to fatty acid and glucose

utilisation, and relatively limited knowledge is available concerning other substrates. There are growing interest about the possibility of using ketones to treat the failing heart (31, 302).

βHB can only enter the Krebs cycle as 2-carbon (2C) intermediates – acetyl-CoA. These carbons replace the two carbons lost as 2 × CO_2 molecules with each full turn of the cycle. βHB is unable to replenish Krebs cycle intermediates (anaplerosis) and these intermediates are constantly lost to other pathways (48, 49). Thus, βHB oxidation is cataplerotic, depleting Krebs cycle intermediates and impairing metabolic efficiency (49, 70). The cataplerotic effect of βHB oxidation must always be offset by anaplerotic substrates, such as circulating glucose, endogenous glycogen, and glucogenic amino acids via transamination (45, 47, 48, 72, 303, 304), providing pyruvate for carboxylation to oxaloacetate or malate by pyruvate carboxylase or malic enzyme, respectively. Pyruvate carboxylase is the major anaplerotic enzyme in heart (48, 71). Thus, the possibility arises that the interplay of substrate metabolism, possibly via cataplerosis-anaplerosis mechanisms, may be central to ketones metabolism in the heart. We hypothesise that βHB oxidation is limited by the amount of intermediates in the Krebs cycle. In the isolated rat hearts, the work in this Thesis aimed to determine the effects of:

1. Glycogen content on βHB oxidation
2. βHB oxidation on Krebs cycle intermediates using metabolomics
3. βHB oxidation on energetics using ^{31}P-nuclear magnetic resonance (NMR)
4. Glucogenic amino acids on βHB oxidation in the hearts lacking pyruvate precursors
5. Substrates and loading conditions on cardiac efficiency (the ratio between hydraulic work and oxygen consumption) in working rat heart.

Chapter 2: General methods

2.1 Animal model

All animal studies conformed to the United Kingdom Home Office guidelines under The Animal (Scientific Procedures) Act, 1986 and were approved by the University of Oxford local ethics committee. Male Wistar rats (starting body weight ~250 g, Harlan UK, Alconbury, United Kingdom) were housed at 20 ºC in a humidity-controlled environment on a 12-h light-dark photoperiod. The rats were fed *ad-libitum* on a standard rodent chow (Teklad Global Diets 2186) and were not fasted prior to experiments.

2.2 Isolated rat heart perfusion

Isolated rat hearts were perfused in the Langendorff mode (305) to determine the effects of:

1. Low and high glycogen content on βHB oxidation and glycolytic rates.
2. βHB oxidation on Krebs cycle intermediates.
3. βHB oxidation on cardiac energetics.
4. Key glucogenic amino acids on βHB oxidation in the hearts lacking pyruvate precursors.

Isolated rat hearts were perfused in the working mode (72) to determine the effects of substrates (glucose, fatty acids, βHB plus AcAc) and loading conditions on cardiac efficiency (the ratio between hydraulic work and oxygen consumption).

2.2.1 Measurement of energy metabolism in the Langendorff heart perfusion

The rats were terminally anaesthetized using an intraperitoneal injection of sodium pentobarbital (1 ml. kg^{-1} bodyweight; Euthatal). The hearts were excised for perfusion and rapidly arrested in ice-cold Krebs-Henseleit (KH) buffer containing NaCl 118 mM (Sigma-Aldrich, UK), KCl 4.7 mM (Fisher-Scientific, UK), $MgSO_4 7H_2O$ 1.2 mM (VWR Chemicals BDH, USA), $CaCl_2 2H_2O$ 1.75 mM (Fisher-Scientific, UK), Na_2EDTA 0.5 mM (VWR Chemicals BDH, USA), $NaHCO_3$ 25 mM (Sigma-Aldrich, UK). The buffer was gassed with

95% O_2:5% CO_2 (v:v) at 37˚C. Cardiac activity was maintained by perfusing the heart via coronary arteries using an aortic cannula inserted into the ascending aorta. Buffer was delivered to the heart in a retrograde manner via cannula (305). The first 50 ml of buffer was discarded to remove the blood and other contaminants, leaving a final circulating volume of 200 ml. Hearts were perfused at a constant pressure of 80 mmHg and an end-diastolic pressure of 4-8 mmHg. Cardiac contractile function was measured using a fluid-filled polyvinylidene chloride (PVC) balloon inserted into the left ventricle, which was attached to a bridge amplifier and PowerLab data acquisition system. Left ventricle developed pressure (LVDP) was determined as the difference between peak left ventricular systolic and left ventricular end-diastolic pressure. Rate-pressure product (RPP) was calculated as the product of LVDP and heart rate.

2.2.1.1 Glycogen manipulation

Isolated rat hearts were initially perfused in the Langendorff mode with non-recirculating buffer to either decrease or increase glycogen content. To deplete glycogen, hearts were perfused with KH buffer containing no glucose for 20 minutes (LG); to augment glycogen, hearts were perfused with KH buffer containing sodium pyruvate (4 mM, Sigma-Aldrich, UK), sodium L-lactate (1 mM, Sigma-Aldrich, UK), D-(+)-glucose (11 mM, Sigma-Aldrich, UK) and insulin (50 mU/ml, Novo nordisk, UK) for 60 minutes (HG). The hearts were freeze-clamped with liquid N_2-cooled Wollenberger tongs (306) at the end of perfusion for glycogen content (307).

2.2.1.2 Measurement of βHB oxidation, glycolytic, and glycogenolytic rates

Radioactive tracers are the determinants for metabolic flux rates. The principal behind the use of radioactive tracers in heart is that an atom in a chemical compound is replaced by a radioactive isotope. Although the radioactive isotope exists only in a low concentrations, radioactive decay releases a high amount of energy which can be detected by scintillation

counters (6). Quantitative $^{14}CO_2$ production from [^{14}C]-βHB (3-hydroxybutyric acid, DL-[1-^{14}C] sodium salt, American Radiolabeled Chemical, USA) was used to measure βHB oxidation rates. During oxidation of βHB, the heart releases CO_2 produced during passage of acetyl-CoA that derived from βHB through the Krebs cycle. Meanwhile, quantitative ^3H_2O production from [5-^{3}H]-glucose (glucose, D-[5-^{3}H(N)], PerkinElmer, USA) was used to measure glycolysis. ^3H_2O is released at the enolase steps of glycolysis. Glycolysis can be measured by collecting perfusate samples at time interval and separating ^3H_2O from [^{3}H]-glucose (90).

To determine the effect of low and high glycogen content on βHB oxidation and glycolytic rates, hearts were perfused with either buffer containing 11 mM glucose and 4 mM (R)-(-)-3-Hydroxybutyric acid sodium salt (Sigma-Aldrich, UK) plus 5 μCi ml^{-1} [^{14}C]-βHB (+βHB), or 11 mM glucose (D-(+)-Glucose, Sigma-Aldrich, UK) plus 0.2 μCi ml^{-1} [5-^{3}H]-glucose, respectively (90). To determine glycogenolysis in high glycogen hearts perfused with βHB (HG+βHB), hearts were perfused with [5-^{3}H]-glucose using a pulse-chase labelling technique.

To determine the effect of glucogenic amino acids at physiological concentration on βHB oxidation rates, myocardial glycogen content was depleted by pre-perfusion with KH buffer containing no glucose, before perfusing with buffer containing 2.5 mM glucose plus 4 mM [^{14}C]-βHB and either 0.13 mM L-Asparagine (Sigma-Aldrich, UK), 0.31 mM L-Valine (Sigma-Aldrich, UK) or 0.65 mM L-Glutamine (Sigma-Aldrich, UK) (250).

For the measurement of βHB oxidation rates, buffer was supplemented with [^{14}C]-βHB. Perfusate gas from the perfusion apparatus was drawn into a bubble trap containing 10 ml of a chemical CO_2 absorber (Carbosorb® E, Perkin Elmer, Massachusetts, USA) using a vacuum pump. The chemical solution was replaced every 10 min for 40 minutes. A volume of 5ml Carbosorb® E containing trapped gaseous $^{14}CO_2$ was aliquoted into glass vials

containing 10 ml of a scintillation cocktail (Permafluor® E, Perkin Elmer, Massachusetts, USA) specifically designed for counting $^{14}CO_2$ radioactivity. In addition, for the measurement of βHB oxidation rates, a volume of 1 ml perfusate samples were collected at 10-min intervals. Samples were placed in the central well of sealed Erlenmeyer flask containing 1 M of sulphuric acid (H_2SO_4, Sigma-Aldrich, UK), surrounded by 10 ml of Carbosorb® E. The samples were incubated overnight to trap $^{14}CO_2$ released from $H^{14}CO_3^-$ in solution within the perfusate. Carbosorb® E containing trapped $^{14}CO_2$ from the flask was then transferred into glass vial containing 10 ml of Permafluor® E and radioactivity was counted. $^{14}CO_2$ yield comprised the sum of the gaseous $^{14}CO_2$ and $H^{14}CO_3^-$ in the solution.

For the measurement of glycolytic rates, buffer was supplemented with [5-^{3}H]-glucose (90). Samples for ^3H_2O originating from perfusion of hearts with [5-^{3}H]-glucose were collected at 4-min intervals throughout the perfusion. A volume of 200 μl perfusate was added into glass columns containing Dowex 1-X 4 anion exchange resin (200 to 400 mesh) (Sigma-Aldrich, UK) and was left for 15 minutes. A volume of 1ml double distilled water was then added twice to the column. ^3H_2O was separated from [^{3}H]-glucose by use of glass columns (150 mm glass pasteur pipettes, VWR international, USA) containing Dowex 1-X 4 anion exchange resin (90). A volume of 10 ml scintillation cocktail (EcoLite, Fisher Scientific, UK) was added into collected ^3H_2O for radioactivity counting. For specific activity measurement, a volume of 200 μl sample was added directly into the glass vials containing 10 ml scintillation cocktail.

Quantitative ^3H_2O production was also used to measure glycogenolysis from [^{3}H]-labelled glycogen using pulse-chase labelling technique. Hearts were initially perfused with glucose-free buffer to deplete glycogen. Hearts were then perfused with (64 ul, 0.4 μCi [5-^{3}H]-glucose plus pyruvate (4 mM, Sigma-Aldrich, UK), lactate (1 mM, Sigma-Aldrich, UK), glucose (11 mM, Sigma-Aldrich, UK) and insulin (50 mU/min) in order to pulse-label

newly synthesized glycogen ([^{3}H]-glycogen). Glycogenolysis from labelled glycogen was subsequently determined during the "chase" perfusion period following the "pulse"-label. The production of ^3H_2O during the "chase' was collected at 4-min intervals throughout perfusion, and radioactivity was counted. The hearts were freeze-clamped at the end of perfusion for glycogen content and metabolomic analysis.

2.2.1.3 Glycogen assay

Hearts were freeze-clamped after glycogen manipulation and after perfusion with either glucose plus βHB or glucogenic amino acids plus βHB (+βHB) for determination of glycogen content. Myocardial glycogen content was determined in 0.3 g (dry weight) of ground myocardial tissue using a spectrophotometric enzymatic-coupled assay. The hearts were finely ground under liquid nitrogen. Heart tissue samples were extracted in 0.6 M of cold perchloric acid (PCA). After neutralization, amyloglucosidase (Sigma) was added to hydrolyse the glycogen into glucose. Hexokinase (Sigma®) was then added to convert the glucose into glucose 6-phosphate. Glucose-6-phosphate dehydrogenase (Sigma®) was added to convert glucose 6-phosphate into 6-phosphogluconolactone and NADPH. A_{340nm} was measured for 20 minutes; it monitors the formation of NADPH which was proportional to the amount of glucose present (308).

2.2.2 Measurement of efficiency in the working rat heart perfusion

The rats were terminally anaesthetized using an intraperitoneal injection of sodium pentobarbital (1 ml. kg^{-1} bodyweight; Euthatal). The hearts were excised for perfusion and rapidly arrested in ice-cold Krebs-Henseleit (KH) buffer containing NaCl 118 mM, KCl 4.7 mM, $MgSO_2$ 1.2 mM, $CaCl_2$ 1.75 mM, Na_2EDTA 0.5 mM, $NaHCO_3$ 25 mM. The buffer was gassed with 95% O_2:5% CO_2 (v:v) at 37˚C. The aorta and left atrium were individually cannulated enabling antegrade cardiac perfusion: warmed, oxygenated buffer entered the

left atrium, flowed into the left ventricle and was ejected through the aorta (and coronary arteries). For further details see Chapter 6.3.

2.2.2.1 Preparation of sodium acetoacetate and β-hydroxybutyrate

Acetoacetate is the simplest β-keto acid, which is unstable. Thus, sodium acetoacetate was synthesised to produce a stable acetatoacetate. A volume of 2.6 ml ethylacetoacetate (Sigma®) was added into 10.2 ml of 2 M NaOH and double distilled H_2O was added to make up to 20 ml. The solution was incubated overnight at room temperature before adding 1 M HCL to neutralise it. The solution was transferred into a round-bottom flask and placed on a rotary evaporator until the volume was reduced to 5ml. Na-acetoacetate was assayed to determine its concentration (309).

The concentration of AcAc was determined by a spectrophotometric assay before being diluted to a 0.2 M stock solution. β-Hydroxybutyrate dehydrogenase (βDH) (Sigma®) was added to the sample. The reaction was followed at 340nm using the kinetic programme on a UV-1700 PharmaSpec UV-visible spectrophotometer (Shimadzu, Japan). The decreases in extinction coefficient at 340 nm due to oxidation of NADH to NAD^+ is proportional to the amount of acetoacetate present (310).

$$\mathrm{D-\beta-hydroxybutyrate} + NAD^+ \underset{\mathrm{D-\beta-hydroxybutyrate\ dehydrogenase}}{\rightleftharpoons} acetoacetate + NADH + H^+$$

A concentration of 0.2 M stock solution is used to prepare a combination of 1 mM Na-acetoacetate and 4 mM βHB ((R)-(-)-3-hydroxybutyric acid sodium salt (Sigma®)) or 4 mM Na-acetoacetate and 1 mM βHB in 200ml of final KH buffer.

2.3 Metabolomic analysis

Metabolomic analysis was carried out in on all hearts perfused with βHB. This analysis was performed in collaboration with Dr David Hauton and Prof James McCullagh from

Department of Chemistry, University of Oxford.

2.3.1 Extraction of tissue metabolites for analysis by LC-MS

Hearts were freeze-clamped at the end of perfusion for metabolomic analyses. In brief, heart tissue (0.1g) was extracted in 80% methanol and homogenised using a Precellys homogenizer (311). The sample was centrifuged at 23,748 x g for 20 minutes. Each sample was diluted to create a final DNA concentration of 100ng/μl. The samples were analysed using up to three separate liquid chromatography coupled to mass spectrometry (LC-MS/MS) methods using two different LC systems (Thermo Scientific ICS-5000+ ion chromatography system and a Thermo Ultimate 3000). Three separate LC-MS methods were ion exchange chromatography-mass spectrometry (IC-MS/MS), C18 reversed phase (underivatized) and C18 reversed phase (derivatized). Each was coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Scientific, San Jose, CA). Raw data files were processed using ProgenesisQI (Waters, Elstree, UK). This process included alignment of retention times, peak picking by identification of the presence of natural abundance isotope peaks, characterising multiple adducts forms and identification of metabolites using our in house database. Retention times, accurate mass values, relative isotope abundances and fragmentation patterns were compared between authentic standards and the samples measured. Data are expressed in arbitrary units as the ratio of metabolite peak relative to the internal standard. Identifications were accepted only when the following criteria were met: <5ppm differences between measured and theoretical mass (based on chemical formula), <30 seconds differences between authentic standard and analyte retention times, isotope peak abundance measurements for analytes were >90% matched to the theoretical value generated from the chemical formula. Fragmentation patterns were matched to least the base peak and two additional peak matches in the MS/MS spectrum to within 12 parts per Million

(ppm). Untargetted metabolomic analysis was performed using MetaboAnalyst version 4.0, which consists of data processing and normalisation, statistical analysis, and high-level functional interpretation (312-314) (Appendix A.1).

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.1.1 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com. Significance was taken at $p < 0.05$ and all results are expressed as mean ± SEM, unless stated otherwise. If data were normally distributed and two means were compared, a student's t-test was used. Repeated measures one-way ANOVA were used to compare study conditions (3 or more means), or change in the level of a single variable, where data were normally distributed. Post-hoc Tukey corrections for multiple comparisons were performed as part of the ANOVA. Two-way repeated measures ANOVA was performed to compare the change in a given variable between conditions. Linear regressions were performed using Prism to give R square values for goodness of fit and correlations calculated separately for significance.

Chapter 3: Glycogen content and D-β-hydroxybutyrate (βHB) oxidation rates in the isolated rat heart

3.1 Abstract

In heart, the availability of glucose is important for anaplerotic flux and thereby the oxidation of D-beta-hydroxybutyrate (βHB). As a glucose store, glycogen availability may alter anaplerosis. Here, we determined the effects of glycogen content on βHB oxidation and glycolytic rates and downstream effects on energetics in the isolated rat heart. To raise glycogen (high glycogen: HG), hearts were perfused with buffer containing pyruvate, lactate, glucose and insulin. To lower glycogen, hearts were perfused with glucose-free buffer (low glycogen: LG). Following glycogen manipulation, hearts were perfused with buffer containing 11 mM glucose plus [^{14}C]-βHB or [5-^{3}H]-glucose to measure βHB oxidation or glycolytic rates, respectively. Hearts were freeze-clamped for glycogen and metabolomic analyses. The free cytosolic [NAD^+]/[NADH] and the mitochondrial [Q^+]/[QH_2] ratios were estimated using the lactate dehydrogenase and succinate dehydrogenase reactions, respectively. Substrate manipulation decreased myocardial glycogen from 43.5 to 5.3 μmol glycosyl units.gww^{-1}. βHB oxidation rates were 49% lower in LG than in HG hearts, with βHB oxidation directly related to glycogen content. βHB oxidation decreased glycolysis in all hearts. Glycogenolysis in HG hearts perfused with βHB alone was 3-fold higher than in hearts perfused with βHB and glucose. Hearts perfused with βHB and glucose had significantly higher levels of the glycolytic intermediates, fructose 1,6-bisphosphate and 3-phosphoglycerate, and higher free cytosolic [NAD^+]/[NADH]. βHB oxidation was associated with increased levels of the Krebs cycle intermediates, citrate, 2-oxoglutarate and succinate, the total nicotinamide adenine dinucleotide phosphate (NADP/H) pool and decreased the mitochondrial [Q^+]/[QH_2]. We conclude that glycogen is needed for βHB oxidation. Although βHB oxidation inhibited glycolysis, glycolytic intermediates were not depleted, and cytosolic free NAD remained oxidised. βHB oxidation was associated with increased Krebs cycle intermediates and reduced mitochondrial Q.

3.2 Introduction

Unlike a ketogenic diet, exogenous ketones delivered by intravenous infusion (315, 316) or in ketone drinks (317, 318) can raise blood D-beta-hydroxybutyrate (βHB) concentrations in the presence of normal, or even high, glucose levels. In the glycogen replete (fed) state, βHB oxidation following a ketone ester drink may account for ~10-18% of total oxygen consumption during exercise in humans (60), although βHB oxidation in cardiac muscle is unknown. As captured in the aphorism 'fat burns in the fire of carbohydrate' (145), carbohydrate is required to facilitate fat oxidation, so may also be required to oxidise ketones (45). Glucose and βHB are both metabolized to 2-carbon acetyl CoA, which requires 4-carbon oxaloacetate to form 6-carbon citrate in the Krebs (tricarboxylic acid) cycle (Fig. 3.1). However, glucose, via pyruvate and pyruvate carboxylase, can form oxaloacetate, and oxaloacetate enables acetyl CoA from βHB to enter the Krebs cycle to maintain Krebs cycle intermediate levels. This process is termed anaplerosis (45-47, 304) (Fig. 3.1).

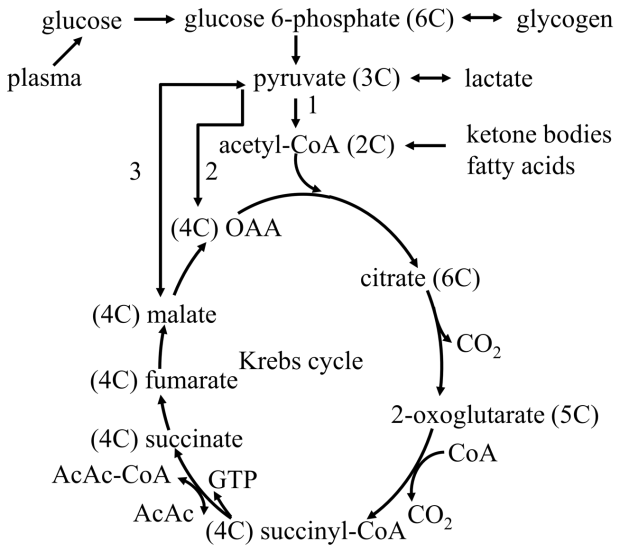


Figure 3.1 *Glucose and glycogen metabolism for anaplerosis. Numbers refer to the enzymes catalysing the reaction. Pyruvate derived from glucose or endogenous glycogen can be decarboxylated to acetyl-CoA by pyruvate dehydrogenase (1). Pyruvate can be carboxylated to oxaloacetate by pyruvate carboxylase (2). Pyruvate can be converted to malate by malic enzyme (3). OAA, oxaloacetate; AcAc, acetoacetate.*

Glucose is phosphorylated to glucose 6-phosphate by hexokinase for glycolysis and glycogen synthesis. For glycogen synthesis, glucose 6-phosphate is converted to glucose 1-phosphate, which reacts with uridine triphosphate (UTP) to form uridine diphosphate glucose (UDP-glucose). A primer in the form of $\alpha(1\rightarrow4)$-glucose oligosaccharide is attached to glycogenin. Glycogenin catalyses the initial step in glycogen synthesis by polymerizing the first few glucose monomers (proglycogen) (319). Activated protein kinase B (PKB) from insulin also leads to phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3), which activates glycogen synthase, converting glycogen synthase *b* (inactive form) to glycogen synthase *a* (active form) (145, 320-322). Glycogen synthase transfers uridine diphosphate-glucose residues onto this polymeric glycogen strand via α-1,4-glycosidic linkage (bonds), which releases UDP (323, 324). After 10-14 glucose residues (323), the glycogen-branching enzyme attaches a strand of seven glucose residue via α-1,6-glycosidic linkage (325, 326). Glycogen synthase extends this chain until an additional branch point is added. This reaction continues and results in a highly branched glycogen structure (319) (Fig. 3.2).

The incorporation rate of exogenous glucose into glycogen *in vivo* (327) is significantly greater than observed in experiments *ex vivo* (328) as a result of competing substrates (lactate, pyruvate, and free fatty acids), availability of hormones (insulin, glucagon, or epinephrine), and mechanical activity (through changes in cystolic Ca^{2+} concentration), which also controls the net flux of glucose 1-phosphate out of the glycogen stores (Fig. 3.2) (196, 329-335). The muscle's storage capacity for glycogen is relatively limited as it is stored with hydration water (219) as both large α particles (300 nm in diameter) and small β particles (50 nm in diameter) (225, 335, 336).

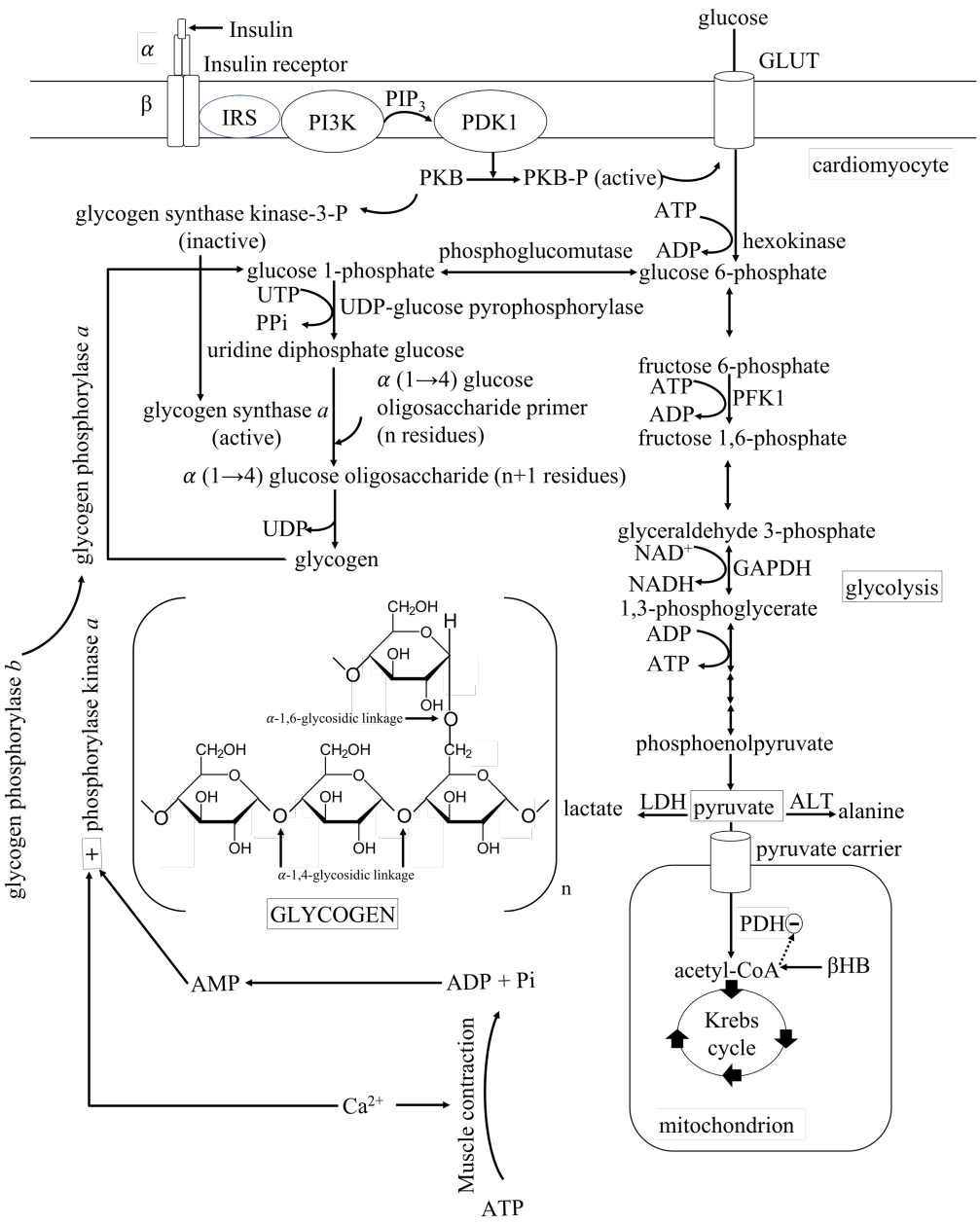


Figure 3.2 *Glucose and glycogen metabolism in heart. Insulin stimulates glucose transport for glycogen synthesis via protein kinase B (PKB, also known as Akt) signalling. Activated PKB increases glucose transport across cell membrane via glucose transporter 4 (GLUT4). Glucose is phosphorylated to glucose 6-phosphate via hexokinase, which can be used for glycogen synthesis and glycolysis. Glycogen synthesis and breakdown are catalyzed by glycogen synthase and phosphorylase, respectively. D-β-hydroxybutyrate (βHB) potentially increases glycogen synthesis by inhibiting glycolysis at pyruvate dehydrogenase (PDH), glyceraldehyde dehydrogenase (GAPDH), and phosphofructokinase (PFK). GLUT, glucose transporter; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate UDP, uridine diphosphate; UTP, uridine triphosphate; PPi, pyrophosphate; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; PDH, pyruvate dehydrogenase, IRSs, insulin receptor substrates; PI3K, phosphatidylinositol-3-kinase;PIP3, phosphatidylinositol (3', 4', 5')-triphosphate; PDK1, 3'-phosphoinositide dependent kinase-1; PKB, protein kinase B, Pi, inorganic phosphate.*

Glycogen occupies about 2 % of the cell volume in the adult cardiac myocyte and more than 30% of the cell volume in the foetal and newborn cardiomyocyte (337). Glucose is not liberated from glycogen during normal activity, but is reserved to serve as an energy 'buffer' to maintain glucose availability (231, 232). During brief low-flow ischaemia when glycogen is not fully depleted, glycogen prevents myocardial injury by maintaining a sufficient glycolysis to outweigh any detrimental effect of increased Na^+-H^+ exchange. However, during prolonged ischaemia when glycogen is fully depleted, myocardial injury occurs due to ATP depletion leading to low pH and high intracellular [Na^+] (307, 335). The breakdown of glycogen to glucose (glycogenolysis) is catalysed by glycogen phosphorylase (328, 338-340). Glycogenolysis is random (stochastic) (328, 335, 341) and the rate of glycogenolysis in hearts perfused with glucose-free buffer is slower than in the presence of epinephrine (226) and during hypoxia (339). Conversion of glycogen phosphorylase *b* (dephosphorylated, inactive form) to *a* (phosphorylated, active form) is the major mechanism for glycogenolysis, either by cyclic adenosine monophosphate (cAMP)-dependent protein kinase or by Ca^{2+}-activated phosphorylase kinase (339, 342) (339) (Fig. 3.2). Glycogen phosphorylase acts on glycogen to release glucose 1-phosphate, which is converted to glucose 6-phosphate (331, 338-340). Glucose 6-phosphate can be utilised for anaplerosis, via glycolysis, to pyruvate, and hence oxaloacetate by pyruvate carboxylase or malate by malic enzyme (47, 155, 304) (Fig. 3.1). Thus, glycogen may contribute 3-carbon intermediary compounds required for the efficient oxidation of βHB (45-47, 304). Here, we determined the effects of glycogen content on βHB oxidation and glycolytic rates, together with glycolytic and Krebs cycle intermediates, in the isolated perfused rat heart.

3.3 Methods

3.3.1 Measurement of myocardial D-β-hydroxybutyrate oxidation, glycolysis and glycogenolysis

Isolated hearts from fed male Wistar rats (Chapter 2.1) were perfused in the Langendorff mode (Fig. 3.3; Chapter 2.2.1). Hearts were initially perfused with oxygenated non-recirculating Krebs-Henseleit (KH) buffer to either decrease or increase the glycogen content. To deplete glycogen, hearts were perfused with glucose-free KH buffer for 20 minutes (low glycogen: LG) (226, 307); and to augment glycogen, hearts were perfused with KH buffer containing pyruvate (4 mM), lactate (1 mM), glucose (11 mM) and insulin (50 mU/min) for 60 minutes (high glycogen: HG) (307). In initial experiments, hearts were freeze-clamped in order to determine myocardial glycogen (Fig. 3.4; Chapter 2.2.1.3).

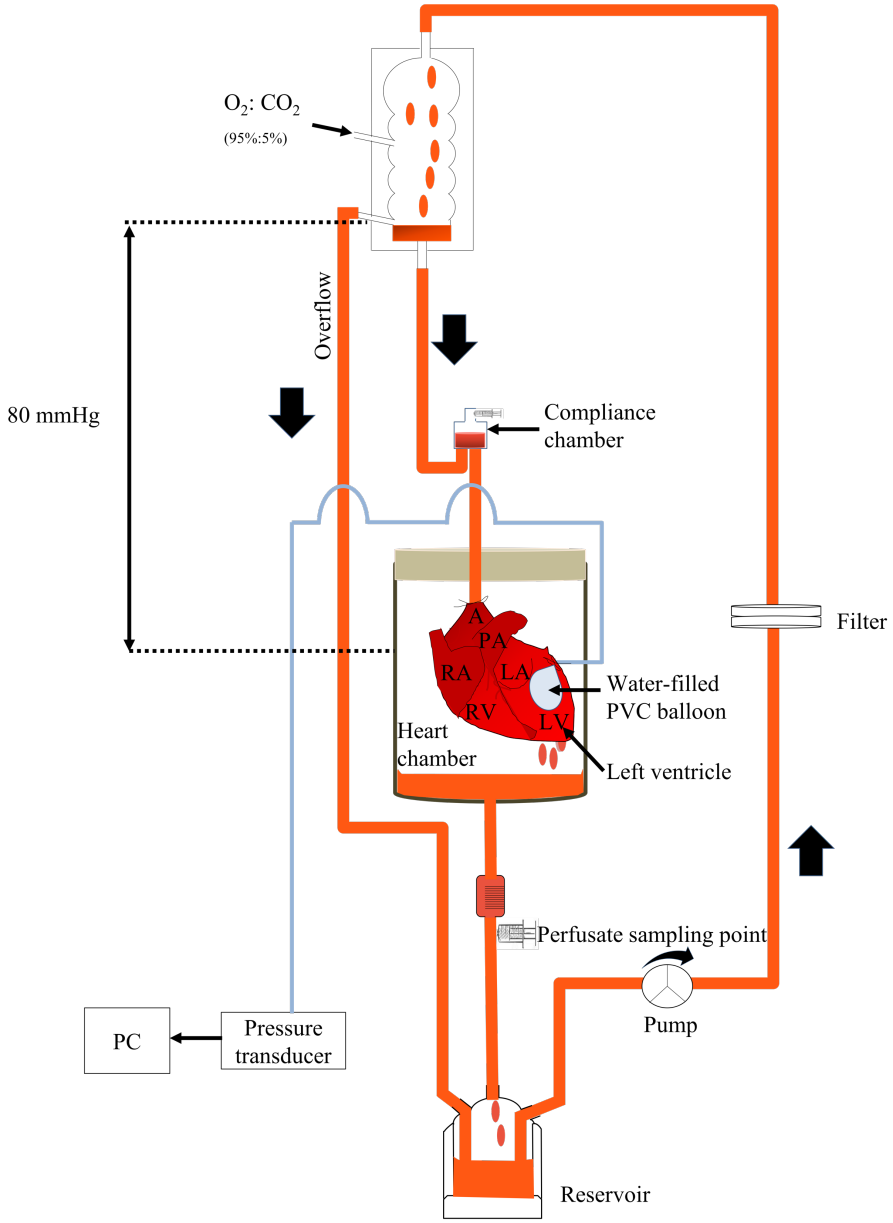


Figure 3.3 *Langendorff heart perfusion. The heart was excised and the aorta was cannulated for retrograde perfusion (Chapter 2.2.1); buffer entered through the aorta. The first 50 ml of buffer was discarded to remove the blood and other contaminants, leaving a final circulating volume of 200 ml. Hearts were perfused at a constant pressure of 80 mmHg and left-ventricular end-diastolic pressure of 4-8 mmHg. Cardiac contractile function was measured using a fluid-filled polyvinylidene chloride (PVC) balloon inserted into the left ventricle, which was attached to a bridge amplifier and PowerLab data acquisition system. Perfusate was collected to determine glycolysis and βHB oxidation. The perfusate was gassed with 95%: 5% (v:v) O_2 and CO_2 and maintained at 37℃. A=aorta; PA=pulmonary artery; LA=left atrium; LV=left ventricle; RA=right atrium; RV=right ventricle.*

For the measurement of βHB oxidation rates, hearts were perfused with buffer containing 11 mM glucose and 5 μCi D-[1-^{14}C] 3-hydroxybutyric acid (sodium salt), American Radiolabelled Chemicals, USA) in the presence (LG+βHB; HG+βHB) of 4 mM D-3-hydroxybutyric acid (sodium salt) (Sigma®, UK). βHB oxidation rates were determined by the production of $^{14}CO_2$ collected at 10 minute intervals for 40 minutes (Fig. 3.4; Chapter 2.2.1.2). In experiments for the measurement of glycolytic rates, buffer was supplemented with 0.2 μCi [5-^{3}H]-glucose plus 11 mM glucose (Sigma®, UK), in the presence or absence of 4 mM βHB (LG/HG ±βHB) (90). The rates of glycolysis were determined by the production of $^{3}H_2O$ collected at 4 minute intervals for 40 minutes (90) (Fig. 3.4; Chapter 2.2.1.2). Figure 3.5 depicts the schematic diagram of measurement of glycolysis and βHB oxidation.

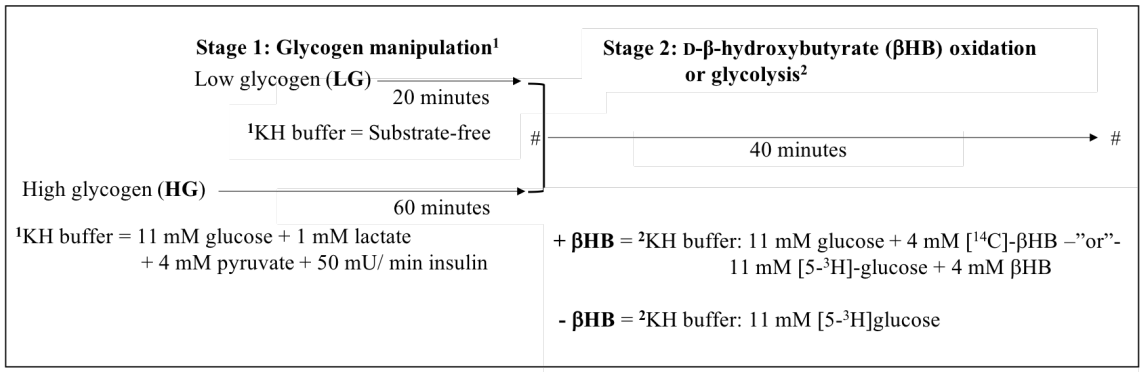


Figure 3.4 *Perfusion protocol during measurement of myocardial glycolysis and βHB oxidation. To raise glycogen (high glycogen; HG), hearts were perfused with buffer containing pyruvate, lactate, glucose and insulin. To lower glycogen, hearts were perfused with glucose-free buffer (low glycogen; LG). Following glycogen manipulation, hearts were perfused with buffer containing 11 mM glucose plus [^{14}C]-βHB or [5-^{3}H]-glucose to measure βHB oxidation or glycolytic rates, respectively. Hearts were freeze-clamped for glycogen and metabolomic analyses. (n=39). #, hearts freeze-clamped for subsequent analysis; KH, Krebs-Henseleit saline buffer; βHB, D-β-hydroxybutyrate; [1], stage 1; [2], stage 2.*

In this study, it was hypothesized that glycogen can be utilised for anaplerosis, via glycolysis to pyruvate and oxaloacetate, to increase the heart's ability to oxidise βHB. Direct measurement of oxaloacetate from glycogen is, however, not feasible. Oxaloacetate could

not be measured using this current technique as it is present in minuscule concentrations (5-10 nmol/g) and its turnover rate is very rapid, up to 800 times per minute (48), making it difficult to quantify (181). Therefore, a modified perfusion protocol was performed to determine glycogenolysis in high glycogen content (HG) hearts perfused with βHB, and anaplerosis was estimated indirectly from labelled glycogen using a pulse-chase labelling technique (Fig. 3.6).

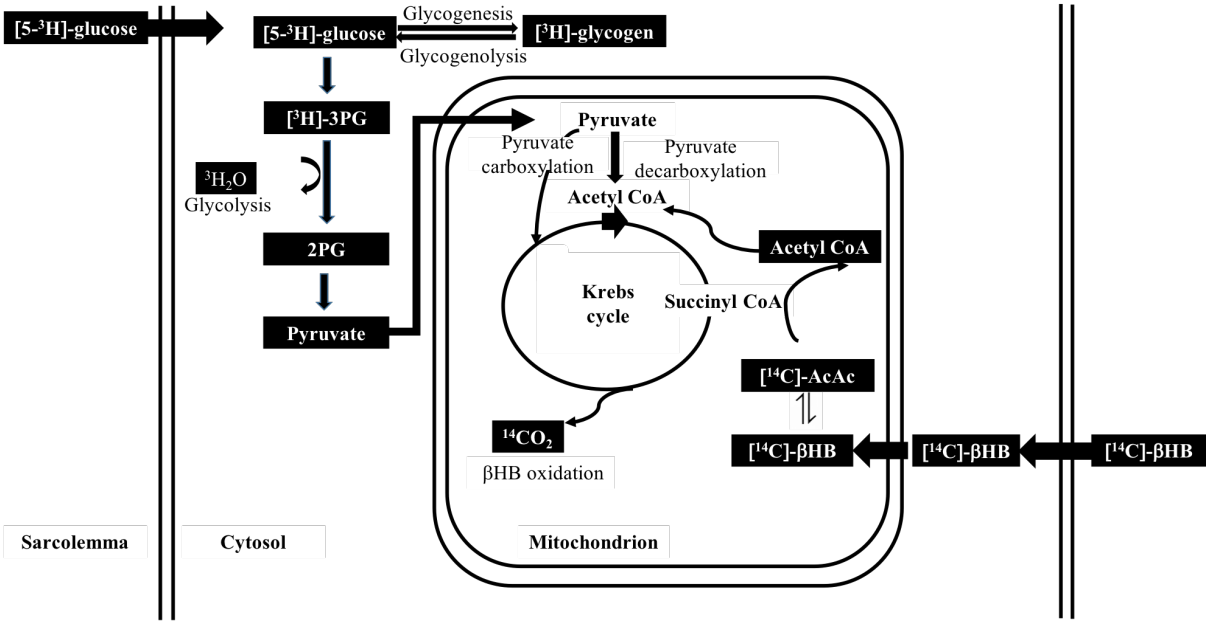


Figure 3.5 *Metabolic fates of myocardial [5-^{3}H]-glucose, [^{3}H]-labelled glycogen and [^{14}C]-βHB. The rates of glycolysis and glycogenolysis were determined by the production of ^3H_2O. The rates of βHB oxidation were determined by the production of $^{14}CO_2$. PG, phosphoglycerate; βHB, D-β-hydroxybutyrate; AcAc, acetoacetate.*

The hearts were initially perfused with glucose-free KH buffer for 20 minutes to deplete glycogen (226, 307). For the "pulse" experiment, the hearts were then perfused with KH buffer containing [5-^{3}H]-glucose, pyruvate (4 mM), lactate (1 mM), glucose (11 mM), insulin (50 mU/min), and βHB (4 mM) for 60 minutes to increase glycogen content and hence enabling the incorporation of [5-^{3}H]-glucose into ^{3}H-glycogen (pulse)(high glycogen). Glycogenolysis from [^{3}H]-labelled glycogen in HG hearts was then determined during the "chase" that contained either glucose-free buffer alone (-Glucose - βHB), or 11

mM glucose alone (+Glucose - βHB), or 4 mM βHB alone (-Glucose + βHB), or 11 mM glucose plus 4 mM βHB (+Glucose + βHB). The production of ^3H_2O, collected at 4-min intervals, was used to determine the rate of glycogenolysis from ^{3}[H]-glycogen (Fig 3.6). Figure 3.5 depicts the schematic diagram for the measurement of glycogenolysis. Hearts were freeze-clamped in liquid N_2-cooled Wollenberger (306) tongs at the end of each stage for glycogen (Chapter 2.2.1.3) and metabolomic (Chapter 2.3 and Appendix A.1) analyses.

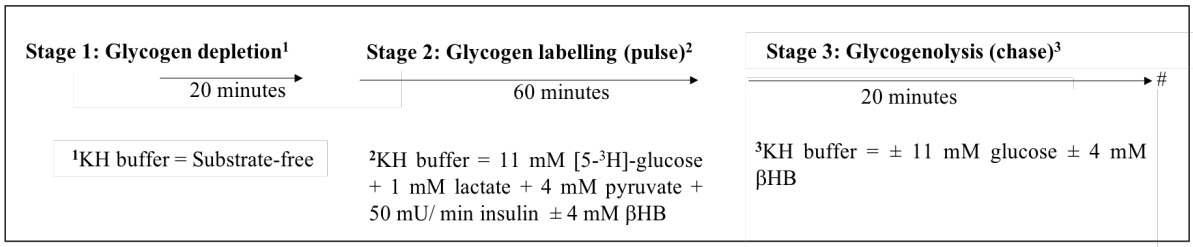


Figure 3.6 *Perfusion protocol (pulse-chase labelling technique) for measurement of myocardial glycogenolysis. Hearts were initially perfused with [5-^{3}H]-glucose to enable the incorporation of [5-^{3}H]-glucose into ^{3}H-glycogen (pulse). Glycogenolysis from [^{3}H]-labelled glycogen was determined by quantitative ^3H_2O production (chase) (n=17). #, hearts freeze-clamped for analysis; KH, Krebs-Henseleit; βHB, D-β-hydroxybutyrate; [1], stage 1; [2], stage 2; [3], stage 3.*

3.3.2 Glycogen content

The hearts were freeze-clamped in liquid N_2-cooled Wollenberger tongs (306) at the end of the perfusion. Myocardial glycogen content was determined in 0.3 g (dry weight) of ground myocardial tissue using a spectrophotometric enzymatic-coupled assay (Chapter 2.2.1.3).

3.3.3 Metabolomic analysis of cardiac muscle

Heart tissue (0.1g) was diluted to create a final DNA concentration of 100ng/μl before analysis using liquid chromatography coupled to mass spectrometry (LC-MS/MS) as described in Chapter 2.3.

3.3.4 Cytosolic redox state

The free cytosolic [NAD^+]/[NADH] ratio was estimated from the measured components of the lactate dehydrogenase reaction. The substrates of lactate dehydrogenase is likely to be in equilibrium with free NAD^+ and NADH, and the ratio of the free dinucleotides can be calculated from the measured concentrations of the substrates (44, 62, 343):

$$\frac{[NAD^+]}{[NADH]} = \frac{[Lactate]}{[Pyruvate]}$$

where lactate and pyruvate were measured by metabolomic analysis (Chapter 3.3.3).

3.3.5 Mitochondrial Q

The free mitochondrial [Q^+]/[QH_2] ratio was estimated from the measured components of the succinate dehydrogenase reaction. The substrates of succinate dehydrogenase is likely to be in equilibrium with free Q^+ and QH_2, and the ratio of the free mitochondrial [Q^+]/[QH_2] can be calculated from the measured concentrations of the substrates (44, 62, 343):

$$\frac{[Q^+]}{[QH_2]} = \frac{[Fumarate]}{[Succinate]}$$

where fumarate and succinate were measured by metabolomic analysis (Chapter 3.3.3).

3.3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism as described in Chapter 2.4. Net glycogenesis rates were calculated from the change in glycogen content over the 40-minute perfusion period. The effect of substrate manipulation on left ventricular function and the effect of myocardial glycogen content on βHB oxidation rates were compared by two-way

analysis of variance (ANOVA), with glycogen content and βHB as factors. The effects of βHB oxidation on glucose utilisation and Krebs cycle intermediates were compared using two-way ANOVA. Tukey *post hoc* corrections were made for multiple comparisons to identify specific significant differences. Results, presented as means ± SEM unless otherwise specified, were considered significant at $p < 0.05$. Statistical analysis of metabolomic results is described in Appendix A.1

3.4 Results

3.4.1 The effect of differing substrate on left ventricular function

The perfusion of hearts with KH buffer containing either pyruvate, lactate, glucose and insulin to increase glycogen levels (high glycogen), or without substrate to deplete glycogen (low glycogen), did not alter the rate-pressure product (RPP), recorded during the protocol as an index of left ventricular function. This effect was similar when the hearts were subsequently perfused with glucose alone, or perfusion with glucose plus βHB (Table 3.1).

Table 3.1 *Left ventricular function in isolated perfused rat hearts. The effect of differing substrate on left ventricular function was determined after glycogen manipulation, after perfusion with glucose alone, or perfusion with glucose plus βHB (n=42). Left ventricular function, recorded during the entire period is expressed as rate pressure product (RPP), calculated as heart rate multiplied by left ventricular developed pressure. Values are mean ± SEM. βHB, D-β-hydroxybutyrate.*

Group	**Rate pressure product** (mmHg.min^{-1} x 10^3)	
	Low glycogen (LG)	**High glycogen (HG)**
After glycogen manipulation	21 ± 2	24 ± 2
After perfusion with glucose alone	23 ± 1	21 ± 4
After perfusion with glucose plus βHB	23 ± 2	19 ± 2

3.4.2 The effect of myocardial glycogen content on myocardial βHB oxidation.

Substrate manipulation decreased myocardial glycogen from 43.5 to 5.3 μmol glycosyl units.gww^{-1} (Fig. 2). In low glycogen (LG) hearts, subsequent perfusion with glucose alone (-βHB) or glucose plus βHB (+βHB) increased the glycogen content from 5.3 to 18.4 or 5.3 to 37.1 μmol glycosyl units.gww^{-1}, respectively (Fig. 3.7A). In high glycogen (HG) hearts, addition of βHB did not further increase the glycogen content (Fig. 3.7A). βHB oxidation in LG hearts was 49% lower than in HG hearts (0.72 ± 0.14 vs. 1.41 ± 0.25 μmol. gww^{-1}.min^{-1}; $p < 0.05$) (Fig. 3.7B). There was a positive correlation between βHB oxidation rates and total myocardial glycogen content at the end of perfusion ($R^2 = 0.51$, $p < 0.02$) (Fig. 3.7C).

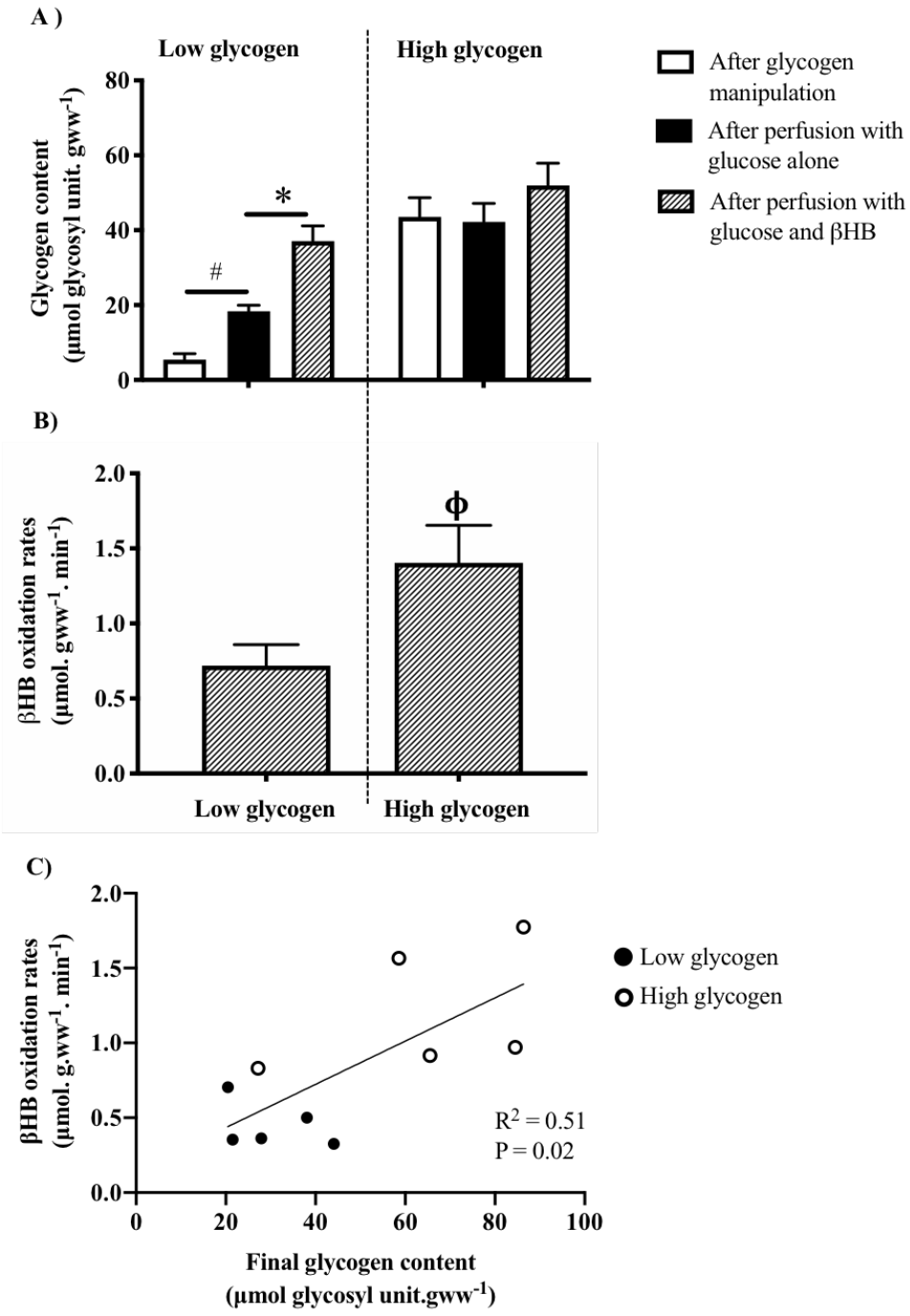


Figure 3.7 *Glycogen content and D-β-hydroxybutyrate (βHB) oxidation rates in the isolated rat heart. (A) Myocardial glycogen content was measured after glycogen manipulation (the hearts were perfused either with glucose-free buffer to decrease glycogen content or buffer containing 11 mM glucose, 1 mM lactate, 4 mM pyruvate, and 50 mU.min^{-1} insulin to increase glycogen content), after subsequent perfusion with glucose alone, or after subsequent perfusion with glucose and βHB (n=42). (B) βHB oxidation rates in low glycogen and high glycogen hearts after subsequent perfusion with glucose plus βHB. (C) Individual myocardial βHB oxidation rates plotted against the final myocardial glycogen content in all hearts after subsequent perfusion with glucose plus βHB. Values are means ± SEM. Effect of glucose is indicated as: # P<0.05. Effect of βHB is indicated as: * P<0.05. Effect of glycogen content is indicated as: ϕ P<0.05. Significance was taken at p < 0.05.*

3.4.3 The effect of βHB oxidation on glucose utilisation

Net glycogenesis was calculated from the change in glycogen content over the 40 minute perfusion period (Fig. 3.8A). In LG hearts, subsequent perfusion with glucose only (-βHB) or glucose plus βHB increased net glycogenesis from 0.3 to 0.8 μmol glycosyl units.gww^{-1}.min^{-1}. In HG hearts, switching from perfusion with glucose alone to perfusion with glucose and βHB did not increase glycogenesis (Fig. 3.8A). Glycolysis from exogenous glucose in HG hearts perfused with glucose alone was significantly higher than that of the LG hearts (0.77 ± 0.03 vs. 0.50 ± 0.08 μmol. $gww^{-1}.min^{-1}$, $p<0.05$). βHB decreased glycolysis to 0.2 μmol. $gww^{-1}.min^{-1}$ in all hearts (Fig. 3.8B).

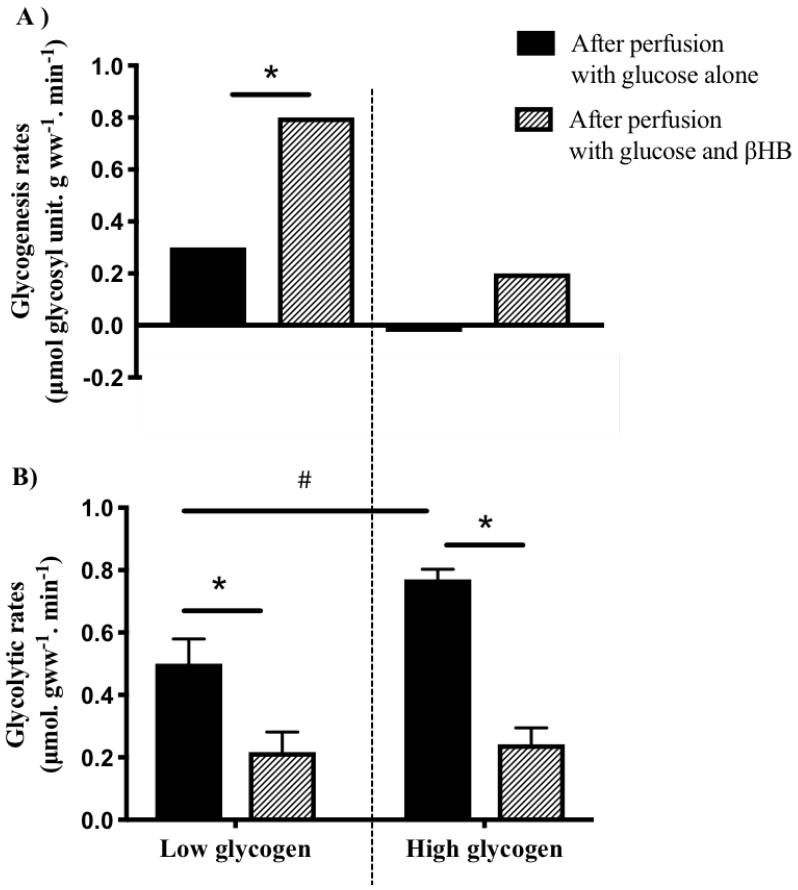


Figure 3.8 *Net glycogenesis and glycolysis rates in the isolated rat heart. Net glycogenesis and glycolysis rates were determined in low glycogen (LG) and high glycogen (HG) hearts after perfusion with glucose alone or glucose and βHB. (A) Net glycogenesis was calculated from the change in glycogen content over the 40-minute perfusion period. (B) Glycolysis was estimated by perfusing hearts with ^{3}H-glucose (n=28). Values are means ± SEM. Effect of glycogen is indicated as : # P<0.05. Effect of βHB is indicated as: * P<0.05. Significance was taken at p<0.05.*

3.4.4 Summary of the effects of βHB oxidation on glucose utilisation in all hearts

Rates of exogenous glucose utilisation were calculated as the sum of total glycolytic and glycogenesis rates. In high glycogen (HG) hearts, high rates of βHB oxidation (~1.44 μmol.gww^{-1}. min^{-1}) decreased rates of exogenous glucose utilisation (~0.44 μmol.gww^{-1}.min^{-1}). In low glycogen (LG) hearts, low rates of βHB oxidation (~0.72 μmol.gww^{-1}. min^{-1}) increased rates of exogenous glucose utilisation (~1.0 μmol.gww^{-1}.min^{-1}). The effects of βHB oxidation rates on exogenous glucose utilisation in low and high glycogen hearts are summarised in Table 3.2.

Table 3.2 *Rates of βHB oxidation, glycolytic and glycogenesis in low (LG) and high glycogen (HG) hearts. Hearts were perfused with buffer containing either 11 mM glucose alone (LG/HG-βHB), or 11 mM glucose plus 4 mM βHB (LG/HG+βHB). Values are mean ± SEM. βHB, D-β-hydroxybutyrate.*

	Low glycogen		**High glycogen**	
	-βHB	**+βHB**	**-βHB**	**+βHB**
βHB oxidation (μmol.gww^{-1}. min^{-1})	-	0.72 ± 0.14	-	1.41 ± 0.25
Glycolysis (μmol.gww^{-1}.min^{-1})	0.50 ± 0.08	0.22 ± 0.06	0.77 ± 0.03	0.24 ± 0.05
Net glycogenesis (μmol.gww^{-1}.min^{-1})	0.32	0.80	0.00	0.20

3.4.5 Myocardial glycogenesis and glycogenolysis from [^{3}H]-labelled glycogen in HG hearts

Glycogen may provide anaplerotic substrates, via pyruvate, to increase the heart's ability to oxidise βHB. Direct measurement of anaplerosis from glycogen is, however, problematic as oxaloacetate is present in very low concentrations (5-10 nmol/g) (48). Therefore, a modified perfusion protocol was performed in order to determine glycogenolysis in high glycogen (HG) hearts perfused with βHB alone, and hence indirectly estimate anaplerosis from labelled glycogen using a pulse-chase labelling technique. In brief, hearts were initially perfused with glucose-free buffer to deplete glycogen. Hearts were then perfused with buffer containing glucose, lactate, pyruvate, insulin and [5-^{3}H]-glucose in order to pulse-label newly synthesized glycogen ([^{3}H]-glycogen), thereby containing replete, labelled, high glycogen (HG hearts). Following this "pulse", glycogenolysis from [^{3}H]-labelled glycogen in HG hearts perfused with βHB was determined during the "chase". Whilst this technique assumes that βHB oxidation rates were the same, it does not permit differentiation between metabolic fates of the mobilised glycogen (i.e. oxidation via acetyl-CoA and the Krebs cycle versus incorporation of 3C pyruvate units into the Krebs cycle through anaplerosis), but it provides an estimate of the potential anaplerotic flux in the absence of substrate-limiting conditions.

In the absence of βHB, glucose (+Glucose -βHB) significantly decreased myocardial glycogenolysis by ~80% (0.10 ± 0.01 vs. 0.02 ± 0.01 μmol. $gww^{-1}.min^{-1}$, $p<0.05$) (Fig. 3.9A), which increased myocardial glycogen by ~66 % (36.9 ± 7.1 vs. 61.1 ± 2.3 μmol glycosyl units.gww^{-1}) (Fig. 3.9B).

In the presence of βHB, as expected, glycogenolysis in hearts perfused with βHB alone (-Glucose +βHB) was 3-fold higher than in HG hearts perfused with glucose and βHB

(+Glucose +βHB) (0.03 ± 0.01 vs. 0.01 ± 0.01 μmol. $gww^{-1}.min^{-1}$, $p<0.05$) (Fig. 3.9A), which decreased glycogen by ~26 % (49.8 ± 4.4 vs. 62.7 ± 3.3 μmol glycosyl units.gww^{-1}) (Fig. 3.9B).

In the absence of glucose, βHB (-Glucose +βHB) significantly decreased myocardial glycogenolysis by ~ 70% (0.10 ± 0.01 vs. 0.03 ± 0.01 μmol. $gww^{-1}.min^{-1}$, $p<0.05$) with the same myocardial glycogen content (Fig. 3.9B). In the presence of glucose, βHB (+Glucose +βHB) significantly decreased myocardial glycogenolysis by ~ 50% (0.02 ± 0.01 vs. 0.01 ± 0.01 μmol. $gww^{-1}.min^{-1}$, $p<0.05$) (Fig. 3.9A), but did not the increase glycogen content (Fig. 3.9B).

This result also suggests that glycogenolysis was maximal in the absence of glucose and βHB (0.08 μmol. $gww^{-1}.min^{-1}$). Meanwhile, glycogenolysis was minimal in the presence of glucose plus βHB (0.01 ± 0.01 μmol. $gww^{-1}.min^{-1}$) Thus, the net rate of glycogenolysis (in this case an indirect measurement of anaplerosis) in hearts perfused with βHB alone was 0.01 ± 0.01 μmol. $gww^{-1}.min^{-1}$) (Fig. 3.9A).

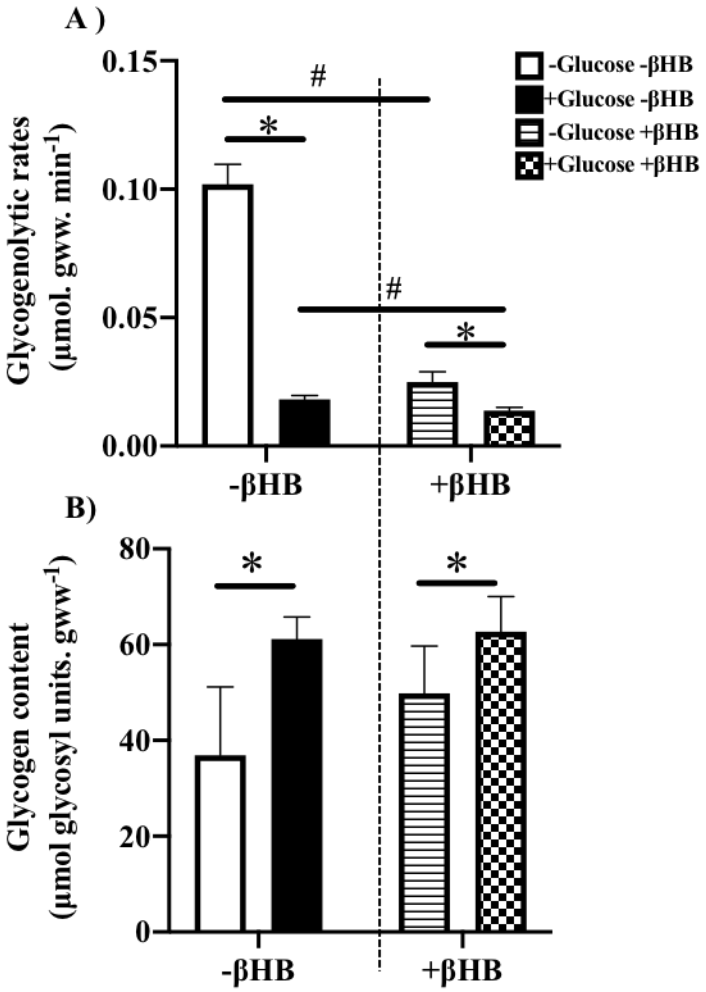


Figure 3.9 *Rate of glycogenolysis and glycogen content in hearts with [^{3}H]-labelled glycogen. Glycogenolytic rate and glycogen content were measured in high glycogen (HG) hearts that were perfused with buffer containing either glucose-free buffer alone (-Glucose -βHB), or 11 mM glucose alone (+Glucose -βHB), or 4 mM βHB alone (-Glucose +βHB), or 11 mM glucose plus 4 mM βHB (+Glucose +βHB) during the chase experiment (n=4-5). For further details text. Values are means ± SEM. Significance was taken at p < 0.05. Effect of glucose is indicated as: * P<0.05. Effect of βHB is indicated as: # P<0.05. βHB, D-β-hydroxybutyrate.*

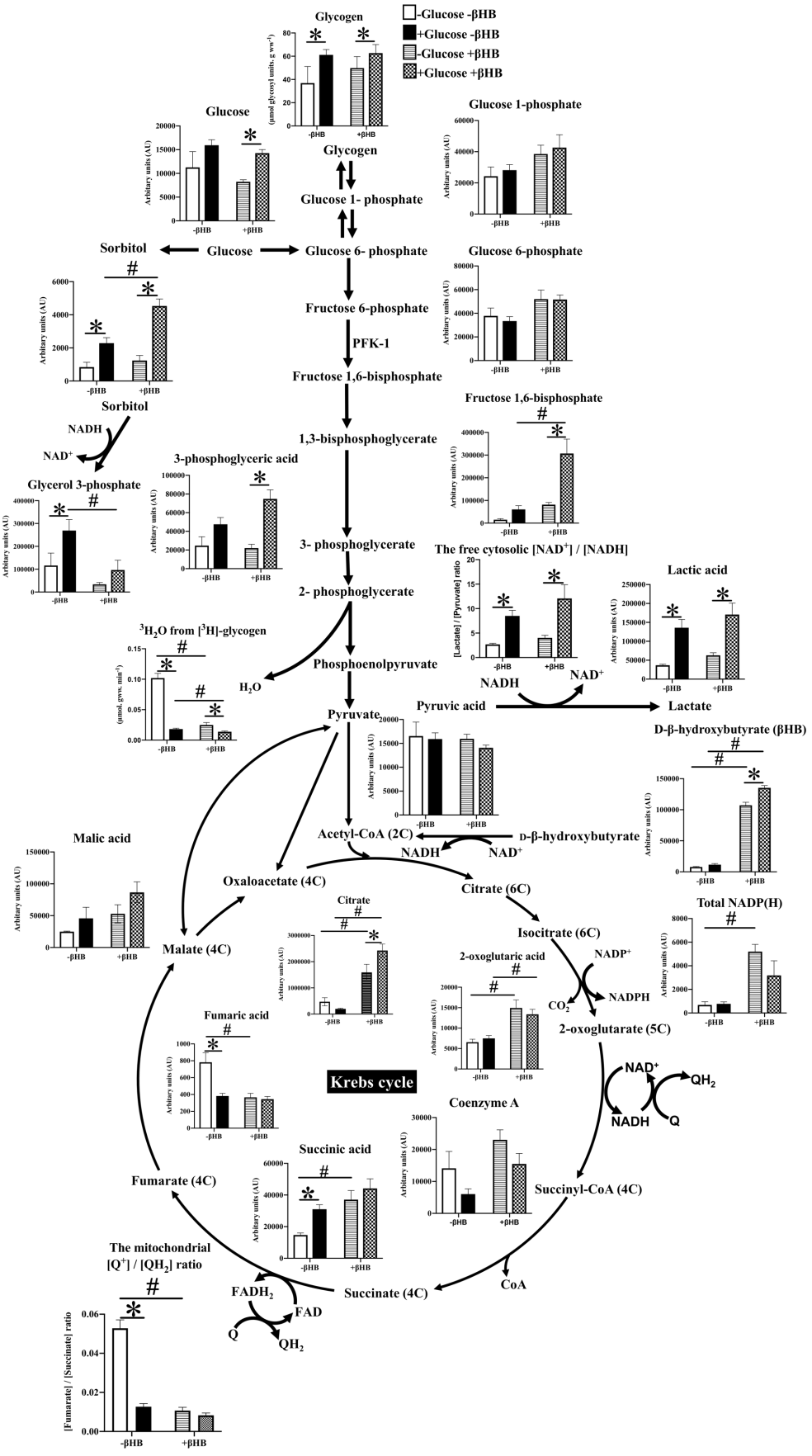
3.4.6 The effect of βHB oxidation on Krebs cycle intermediates using targetted metabolomics

Targetted metabolomic analysis, a method that prioritises compound identification and quantification, was used to assess the influence of βHB oxidation on Krebs cycle intermediates (Fig. 3.10). High glycogen (HG) hearts were perfused with KH buffer containing either no substrate (-Glucose -βHB), or 11 mM glucose alone (+Glucose -βHB), or 4 mM βHB alone (-Glucose +βHB), or 11 mM glucose plus 4 mM βHB (+Glucose +βHB). Intermediates were measured by metabolomic analysis (Chapter 2.3 and Appendix A.1).

In the heart perfused with glucose alone (+Glucose -βHB), myocardial sorbitol, glycerol 3-phosphate, lactate, and the free cytosolic $[NAD^+]/[NADH]$ calculated from the [lactate]/[pyruvate] ratio (44, 62, 343) were significantly higher than in the heart perfused with no substrate (-Glucose -βHB) (Fig. 3.10). In the heart perfused with glucose alone (+Glucose -βHB), the free mitochondrial $[Q^+]/[QH_2]$, calculated from the [fumarate][succinate] ratio (44, 62, 343) was significantly lower than in the heart perfused without glucose (-Glucose -βHB).

In the heart perfused with glucose and βHB (+Glucose +βHB), glucose, fructose 1,6-bisphosphate, 3-phosphoglycerate, the free cytosolic $[NAD^+]/[NADH]$, lactate, βHB and citrate were significantly higher than in the heart perfused with βHB alone (-Glucose +βHB).

Generally, in the heart perfused with βHB, citrate, 2-oxoglutarate and the total nicotinamide adenine dinucleotide phosphate (NADP/H) pool were significantly higher than in the heart perfused without βHB. In the heart perfused with βHB, the free mitochondrial $[Q^+]/[QH_2]$ ratio was significantly lower than in the heart perfused without βHB (Fig. 3.10).



(legend on next page)

Figure 3.10 *Levels of glycolytic and Krebs cycle intermediates in high glycogen (HG) hearts. Hearts previously loaded with glycogen (HG hearts) were perfused with buffer containing glucose-free buffer alone (-Glucose -βHB), or 11 mM glucose alone (+Glucose -βHB), or 4 mM βHB alone (-Glucose +βHB), or 11 mM glucose plus 4 mM (+Glucose +βHB) over a 40-minute perfusion period (n=4-5). Glycogen levels were measured by enzymatic analysis (Chapter 2.2.1.3). Glycogenolysis was measured from the production of production of* $^{3}H_2O$ *from* ^{3}*[H]-glycogen (Chapter 2.2.1.2). Values are means ± SEM. Significance was taken at p < 0.05. Significant effect of glucose is indicated as:* ***** *P<0.05. Effect of βHB is indicated as: # P<0.05. βHB, D-β-hydroxybutyrate; ADP, adenosine diphosphate; GDP, guanosine diphosphate; PFK-1, phosphofructokinase-1; PGK, 3-phosphoglycerate kinase; NADPH, reduced nicotinamide adenine dinucleotide phosphate.*

3.4.7 Multivariate statistical analysis (untargetted metabolomic analysis)

Untargetted metabolomic analysis (a method that does not make compound identification a priority) was performed to provide information that contributes to a systems-level understanding of biological context and subsequently a molecular phenotype, such as variation between experimental conditions and also metabolic pathways, which can be subjected for future works (312, 314). Data processing and normalization were performed using MetaboAnalyst (Appendix 7.1.5 and 7.1.6) prior to multivariate statistical analysis, which are principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA). PCA is an unsupervised clustering method, and it is performed based on four parameters, including retention times, accurate mass values, relative isotope abundances and fragmentation patterns (Appendix A.1). PCA summarises data into a few components (principal components 1 and 2), which explains most of the data variance. Principal components maximise abundance variation in dataset, which generally decrease as the principle components increase. PCA scores plot showed discrimination among the groups when 68 identified compounds were selected (18.8 % vs. 10.8 % for the main principal component). No obvious outlier could be detected (Fig. 3.11A).

Furthermore, PLS-DA is a supervised clustering method, and it was performed based on the three parameters identified as most important. PLS-DA was performed since there were good separation patterns n in the PCA scores plot. The discrimination between the groups from PLS-DA (17.1 % vs. 11.7 % for the main principal component) was significant as sum of squares captured by the model (R^2) value was closer to 1 and higher than cross-validated R^2 (Q^2) value (Fig. 3.11B) (Appendix A.1).

Sparse PLS-DA (sPLS-DA) was also performed based on the most discriminative features. The results showed that there was still a separation among the groups (11.8% vs. 10% for the main principal component) (Fig. 3.11C). Significance of analysis microarray

(SAM) uses permutation of data to determine the expression of metabolites. SAM showed that there was a significant difference between HG hearts perfused with βHB ('case') and HG hearts perfused without βHB ('control') as the expected proportion of "discoveries" to be false is very small (false discovery rate (FDR) < 0.05) (Appendix A.1). SAM revealed a total of 95 metabolites were differentially expressed among identified metabolites as the green circles exceed the specified threshold (Fig. 3.11D).

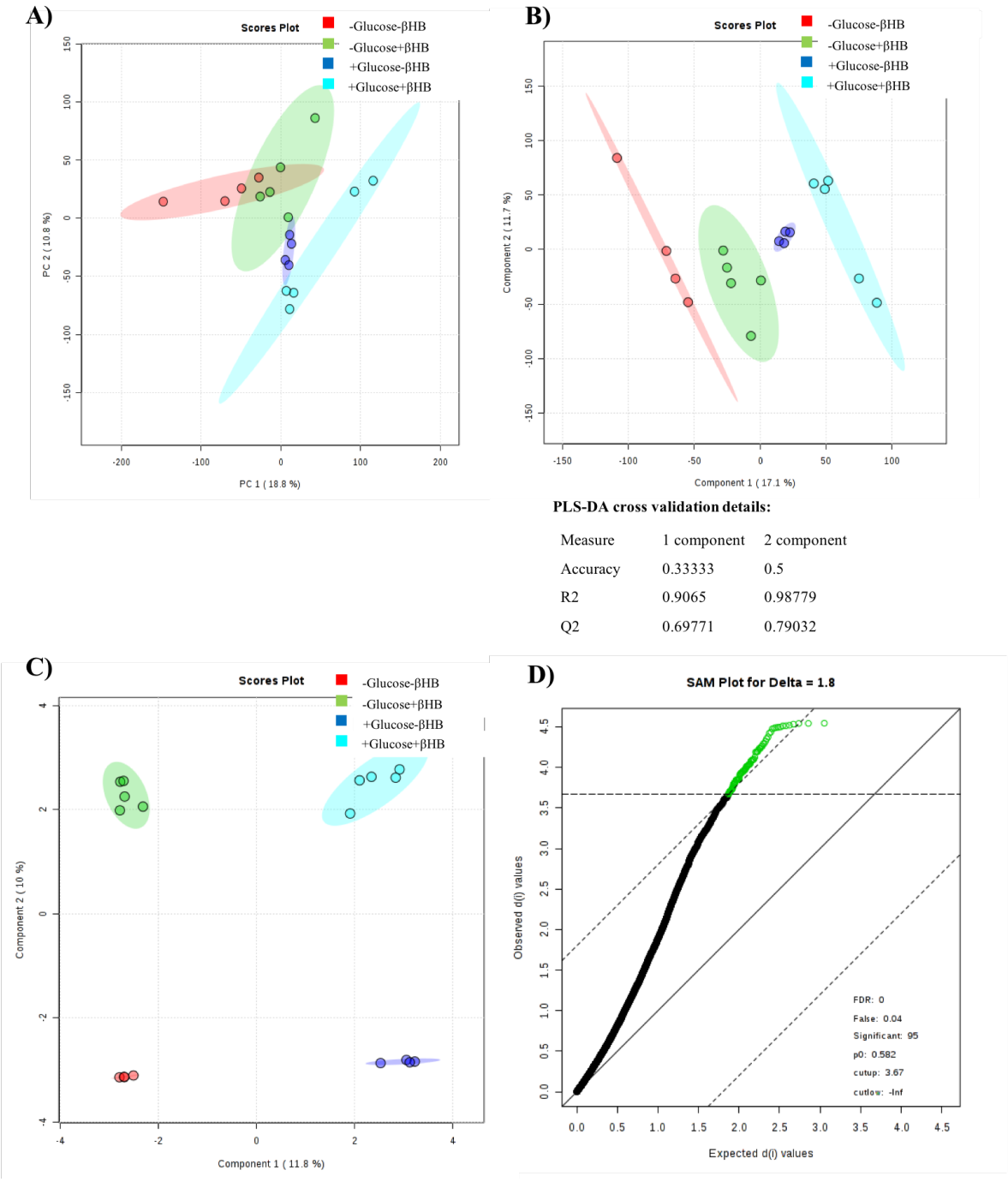


Figure 3.11 *Multivariate data analysis of the metabolome in high glycogen (HG) hearts. Hearts previously loaded with glycogen (HG hearts) were perfused with chase buffer containing either glucose-free buffer alone (-Glucose - βHB), or 11 mM glucose alone (+Glucose -βHB), or 4 mM βHB alone (-Glucose +βHB), or 11 mM glucose plus 4 mM βHB (+Glucose +βHB) (n=4-5). (A) Principal component analysis (PCA) score plot. (B) Partial least squares-discriminant analysis (PLS-DA) score plots were assessed by sum of squares captured by the model (R^2), cross-validated R^2 (Q^2), prediction accuracies through cross-validation (accuracy). (C) Sparse partial least squares-discriminant analysis score plot. (D) Significant features identified by significance analysis of microarray (SAM). The green circles represent features that exceed the specified threshold. βHB, D-β-hydroxybutyrate.*

3.4.8 A clustering-based approach to the metabolome (untargetted analysis)

A clustering-based approach was performed to identify a similar feature of the metabolome to a known biomarker based on peak intensity. The feature was reorganized from a non-hierarchical (Fig. 3.12A) to a hierarchical clustering heat-map (Fig. 3.12B) for better visualisation. A hierarchical clustering heat-map showed a distinctive fold change (the ratio between final concentration and initial concentration) among the groups. Generally, and expectedly, the effect of glucose caused a very distinctive fold change as observed between HG hearts perfused with glucose-free buffer only (-Glucose -βHB) and those hearts with glucose alone (+Glucose -βHB). The effect of βHB also caused a distinctive fold change as observed between HG hearts perfused with βHB alone (-Glucose +βHB) and those hearts with glucose plus βHB (Fig. 3.12A and B). The dendrogram showed that metabolome was clustered in the groups accordingly (Fig. 3.12C).

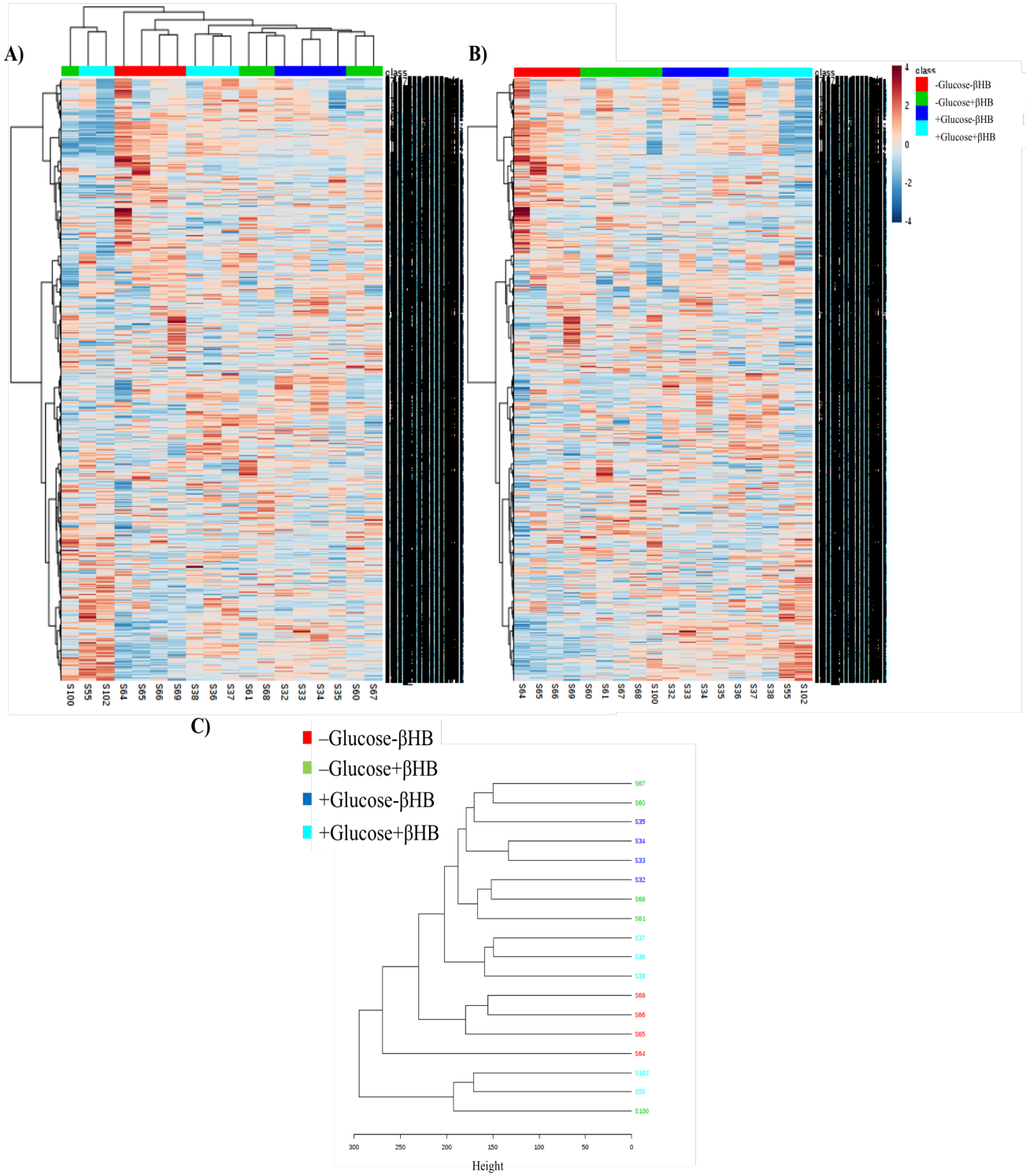


Figure 3.12 *A cluster-based approach of the metabolome in high glycogen (HG) hearts. Hearts previously loaded with glycogen (HG hearts) were perfused with buffer containing either glucose-free buffer alone (-Glucose -βHB), or 11 mM glucose alone (+Glucose -βHB), or glucose-free buffer plus 4 mM βHB (-Glucose +βHB), or 11 mM glucose plus 4 mM βHB (+Glucose +βHB) (n=4-5). (A) A non-hierarchical clustering heat-map shown as fold change regardless of the group. (B) A hierarchical clustering heat-map shown as fold change according to group. A fold-change threshold of 1.5 is used to determine significance. (C) A dendrogram depicting hierarchical clustering results of samples from A and B. βHB, D-β-hydroxybutyrate.*

3.4.9 A hierarchical clustering heat-map, Venn diagram, and permutation tests by two-way analysis of variance (ANOVA)

A hierarchical clustering heat-map and Venn diagram were analysed by two-way ANOVA, with glucose and βHB as factors. These factors caused a very distinctive fold change in HG hearts (Fig 3.13A). A two-way ANOVA was conducted to determine which factors (glucose, βHB, their interaction) caused the variation of metabolites in HG hearts. Among the metabolites, the abundance of 14 metabolites were simultaneously affected by glucose, βHB, and their interactions (Fig. 3.13B). A permutation test was performed using 100 or more permutations with a separation distance (B/W) test statistic to avoid overfitting of the model. This test involves randomly reassigning the class labels and performing PLS-DA on the newly relabelled data set. The performance measures were plotted on a histogram for visual assessment. The permutation result showed that the addition of glucose and βHB significantly altered the metabolome, which was based on separation distance test statistic indicated by a red arrow (P-value <0.05). The further to the right of the distribution, the more significant is the separation between two groups (Fig 3.13C).

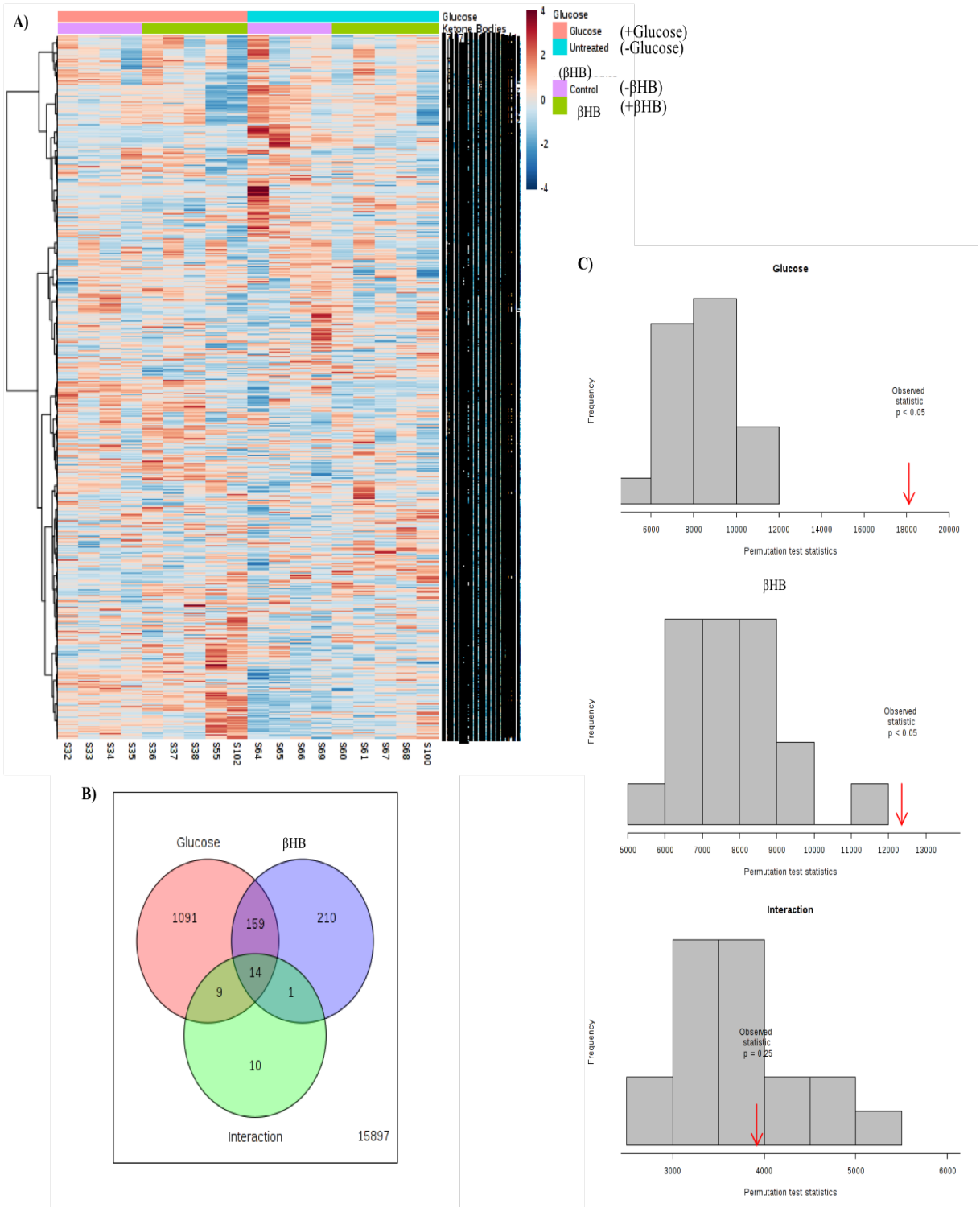
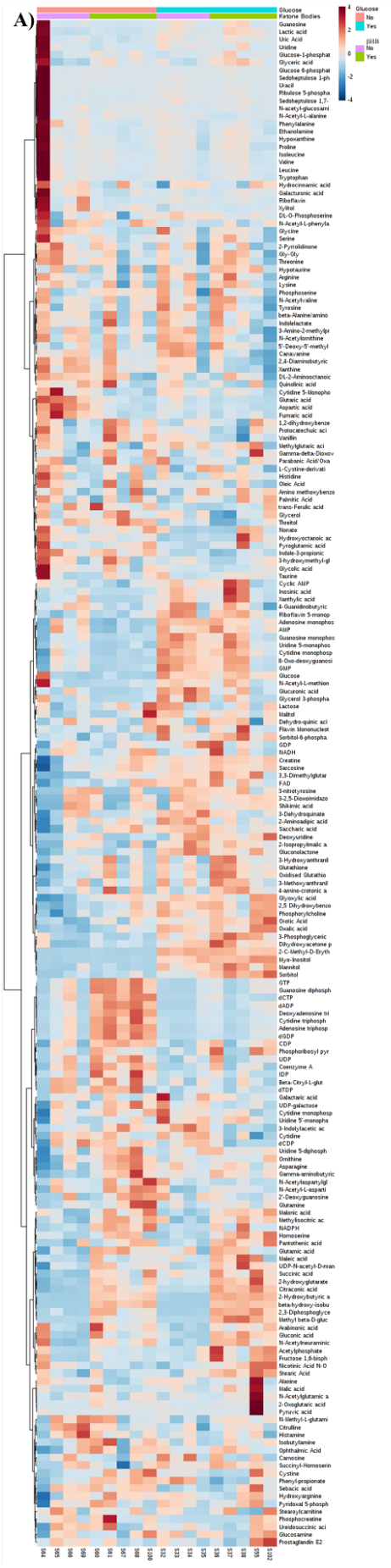
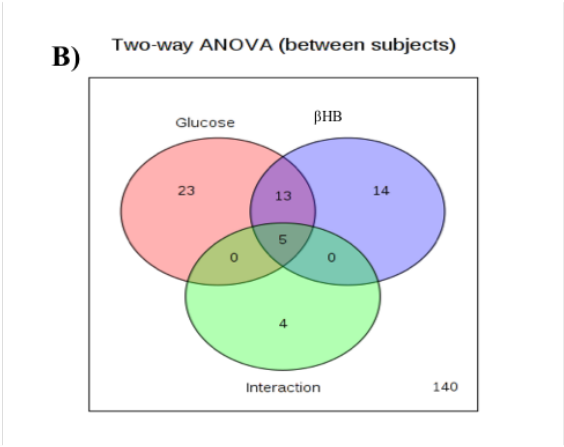


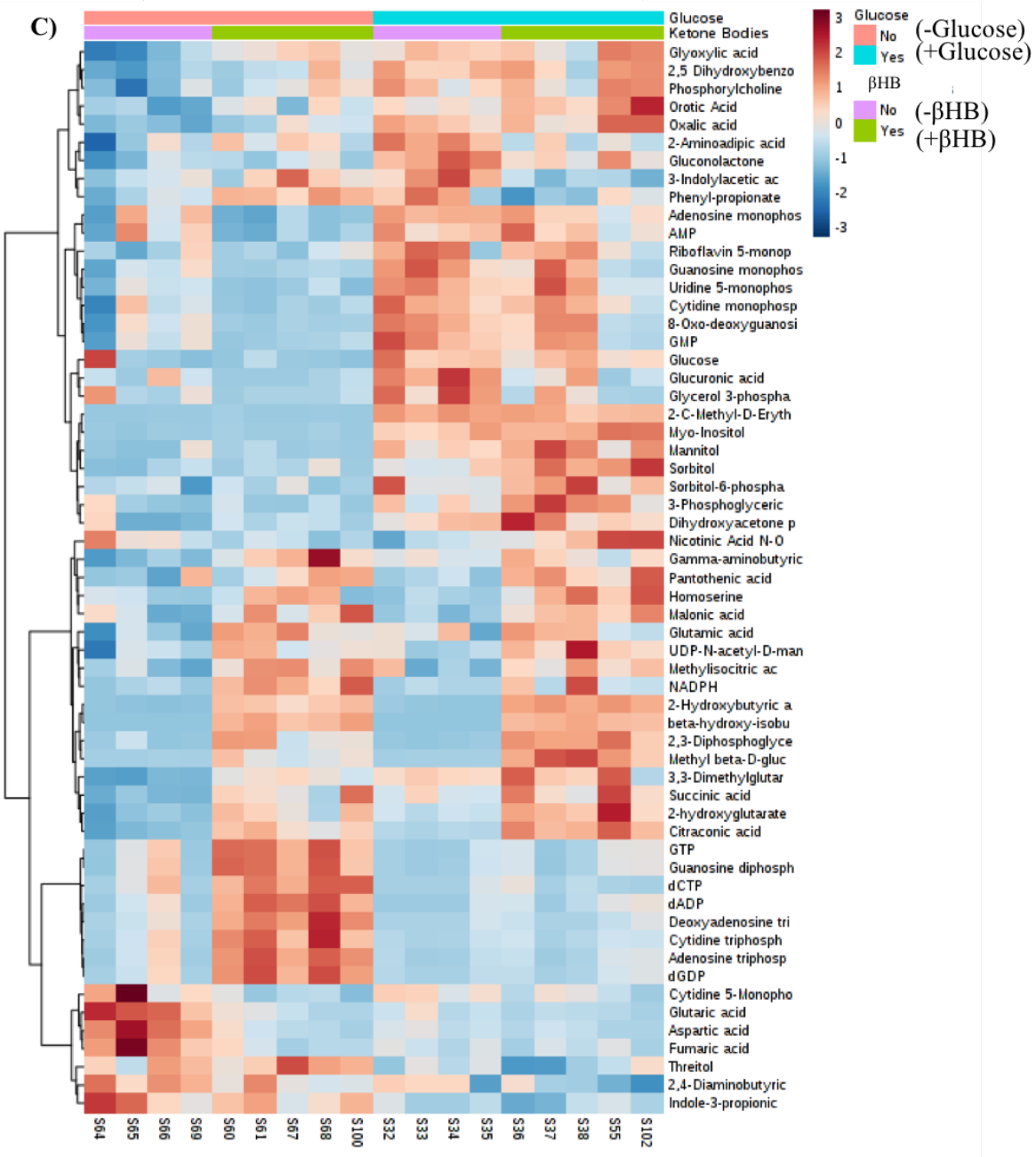
Figure 3.13 *A hierarchical clustering heat-map, Venn diagram and permutation tests by two-ways analysis of variance (ANOVA) in high glycogen (HG) hearts. Hearts previously loaded with glycogen (HG hearts) were perfused with buffer containing either glucose-free buffer alone (-Glucose -βHB), or 11 mM glucose alone (+Glucose -βHB), or glucose-free buffer plus 4 mM βHB (-Glucose +βHB), or 11 mM glucose plus 4 mM βHB (+Glucose +βHB) (n=4-5). (A) A hierarchical clustering heat-map by two-way ANOVA. A fold-change threshold of 1.5 is used to determine significance. (B) Venn diagram by two-way ANOVA. (C) The results of permutation tests summarized by a histogram. βHB, D-β-hydroxybutyrate.*

3.4.10 List of identified compounds and variable importance in projection (VIP) scores by two-way ANOVA

A hierarchical clustering heat-map analysis by two-way ANOVA showed fold changes of metabolites in all groups (Fig. 3.14A). Among the identified metabolites, the abundance of 5 metabolites were simultaneously affected by glucose, βHB, and their interactions (Fig. 3.14B). A hierarchical clustering heat-map analysis by two-way ANOVA was then generated from the Venn diagram (Fig. 3.14C). Generally, and expectedly, 2-hydroxybutyric acid (βHB) was identified as the main compound by the variable importance in projection (VIP) scores using mean decrease in accuracy. The identification of methyl beta-D-glucopyranoside, sorbitol, and myo-inositol indicates the metabolism of glucose. Meanwhile, biosynthesis of purine was determined by the identification of deoxyguanosine diphosphate (dGDP), guanosine-5'-triphosphate (GTP), 2-C-methyl-D-erythritol 4-phosphate, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), sedoheptulose 1-phosphate, 8-oxo-deoxyguanosine, and cytidine triphosphate (Fig. 3.14D).


A)
Glucose
Ketone Bodies
Guanosine
Lactic acid
Uric Acid
Uridine
Glucose-1-phosphat
Glyceric acid
Glucose 6-phosphat
Sedoheptulose 1-ph
Uracil
Ribulose 5-phospha
Sedoheptulose 1,7-
N-acetyl-glucosami
N-Acetyl-L-alanine
Phenylalanine
Ethanolamine
Hypoxanthine
Proline
Isoleucine
Valine
Leucine
Tryptophan
Hydrocinnamic acid
Galacturonic acid
Riboflavin
Xylitol
DL-O-Phosphoserine
N-Acetyl-L-phenyla
Glycine
Serine
2-Pyrrolidinone
Gly-Gly
Threonine
Hypotaurine
Arginine
Lysine
Phosphoserine
N-Acetylvaline
Tyrosine
beta-Alanine/amino
Indolelactate
3-Amino-2-methylpr
N-Acetylornithine
5'-Deoxy-5'-methyl
Canavanine
2,4-Diaminobutyric
Xanthine
DL-2-Aminooctanoic
Quinolinic acid
Cytidine 5-Monopho
Glutaric acid
Aspartic acid
Fumaric acid
1,2-Dihydroxybenze
Protocatechuic aci
Vanillin
Methylglutaric aci
Gamma-delta-Dioxov
Parabanic Acid/ Oxa
L-Cystine-derivati
Histidine
Oleic Acid
Amino methoxybenzo
Palmitic Acid
trans-Ferulic acid
Glycerol
Threitol
Nonate
Hydroxyoctanoic ac
Pyroglutamic acid
Indole-3-propionic
3-hydroxymethyl-gl
Glycolic acid
Taurine
Cyclic AMP
Inosinic acid
Xanthylic acid
4-Guanidinobutyric
Riboflavin 5-monop
Adenosine monophos
AMP
Guanosine monophos
Uridine 5-monophos
Cytidine monophosp
8-Oxo-deoxyguanosi
GMP
Glucose
N-Acetyl-L-methion
Glucuronic acid
Glycerol 3-phospha
Lactose
Maltol
Dehydro-quinic aci
Flavin Mononucleot
Sorbitol-6-phospha
GDP
NADH
Creatine
Sarcosine
3,3-Dimethylglutar
FAD
3-nitrotyrosine
3-2,5-Dioxoimidazo
Shikimic acid
3-Dehydroquinate
2-Aminoadipic acid
Saccharic acid
Deoxyuridine
2-Isopropylmalic a
Gluconolactone
3-Hydroxyanthranil
Glutathione
Oxidised Glutathio
3-Methoxyanthranil
4-amino-crotonic a
Glyoxylic acid
2,5 Dihydroxybenzo
Phosphorylcholine
Orotic Acid
Oxalic acid
3-Phosphoglyceric
Dihydroxyacetone p
2-C-Methyl-D-Eryth
Myo-Inositol
Mannitol
Sorbitol
GTP
Guanosine diphosph
dCTP
dADP
Deoxyadenosine tri
Cytidine triphosph
Adenosine triphosp
dGDP
CDP
Phosphoribosyl pyr
UDP
Coenzyme A
IDP
Beta-Citryl-L-glut
dTDP
Galactaric acid
UDP-galactose
Cytidine monophosp
Uridine 5'-monopho
3-Indolylacetic ac
Cytidine
dCDP
Uridine 5-diphosph
Ornithine
Asparagine
Gamma-aminobutyric
N-Acetylaspartylgl
N-Acetyl-L-asparti
2'-Deoxyguanosine
Glutamine
Malonic acid
Methylisocitric ac
NADPH
Homoserine
Pantothenic acid
Glutamic acid
Maleic acid
UDP-N-acetyl-D-man
Succinic acid
2-hydroxyglutarate
Citraconic acid
2-Hydroxybutyric a
beta-hydroxy-isobu
2,3-Diphosphoglyce
Methyl beta-D-gluc
Arabinonic acid
Gluconic acid
N-Acetylneuraminic
Acetylphosphate
Fructose 1,6-bisph
Nicotinic Acid N-O
Stearic Acid
Alanine
Malic acid
N-Acetylglutamic a
2-Oxoglutaric acid
Pyruvic acid
N-Methyl-L-glutami
Citrulline
Histamine
Isobutylamine
Ophthalmic Acid
Carnosine
Succinyl-Homoserin
Cystine
Phenylpropionate
Sebacic acid
Hydroxyarginine
Pyridoxal 5-phosph
Stearoylcarnitine
Phosphocreatine
Ureidosuccinic aci
Glucosamine
Prostaglandin E2
S64
S65
S66
S69
S60
S61
S67
S68
S100
S92
S93
S94
S95
S96
S97
S98
S99
S102
Glucose
No
Yes
PHB
No
Yes
4
2
0
-2
-4


B)
Two-way ANOVA (between subjects)
Glucose
βHB
23
13
14
5
0
0
4
Interaction
140


C)
Glucose
Ketone Bodies
Glyoxylic acid
2,5 Dihydroxybenzo
Phosphorylcholine
Orotic Acid
Oxalic acid
2-Aminoadipic acid
Gluconolactone
3-Indolylacetic ac
Phenyl-propionate
Adenosine monophos
AMP
Riboflavin 5-monop
Guanosine monophos
Uridine 5-monophos
Cytidine monophosp
8-Oxo-deoxyguanosi
GMP
Glucose
Glucuronic acid
Glycerol 3-phospha
2-C-Methyl-D-Eryth
Myo-Inositol
Mannitol
Sorbitol
Sorbitol-6-phospha
3-Phosphoglyceric
Dihydroxyacetone p
Nicotinic Acid N-O
Gamma-aminobutyric
Pantothenic acid
Homoserine
Malonic acid
Glutamic acid
UDP-N-acetyl-D-man
Methylisocitric ac
NADPH
2-Hydroxybutyric a
beta-hydroxy-isobu
2,3-Diphosphoglyce
Methyl beta-D-gluc
3,3-Dimethylglutar
Succinic acid
2-hydroxyglutarate
Citraconic acid
GTP
Guanosine diphosph
dCTP
dADP
Deoxyadenosine tri
Cytidine triphosph
Adenosine triphosp
dGDP
Cytidine 5-Monopho
Glutaric acid
Aspartic acid
Fumaric acid
Threitol
2,4-Diaminobutyric
Indole-3-propionic
S64
S65
S66
S69
S60
S61
S67
S68
S100
S32
S33
S34
S35
S36
S37
S38
S55
S102
3
2
1
0
-1
-2
-3
Glucose
No
Yes
(-Glucose)
(+Glucose)
βHB
No
Yes
(-βHB)
(+βHB)

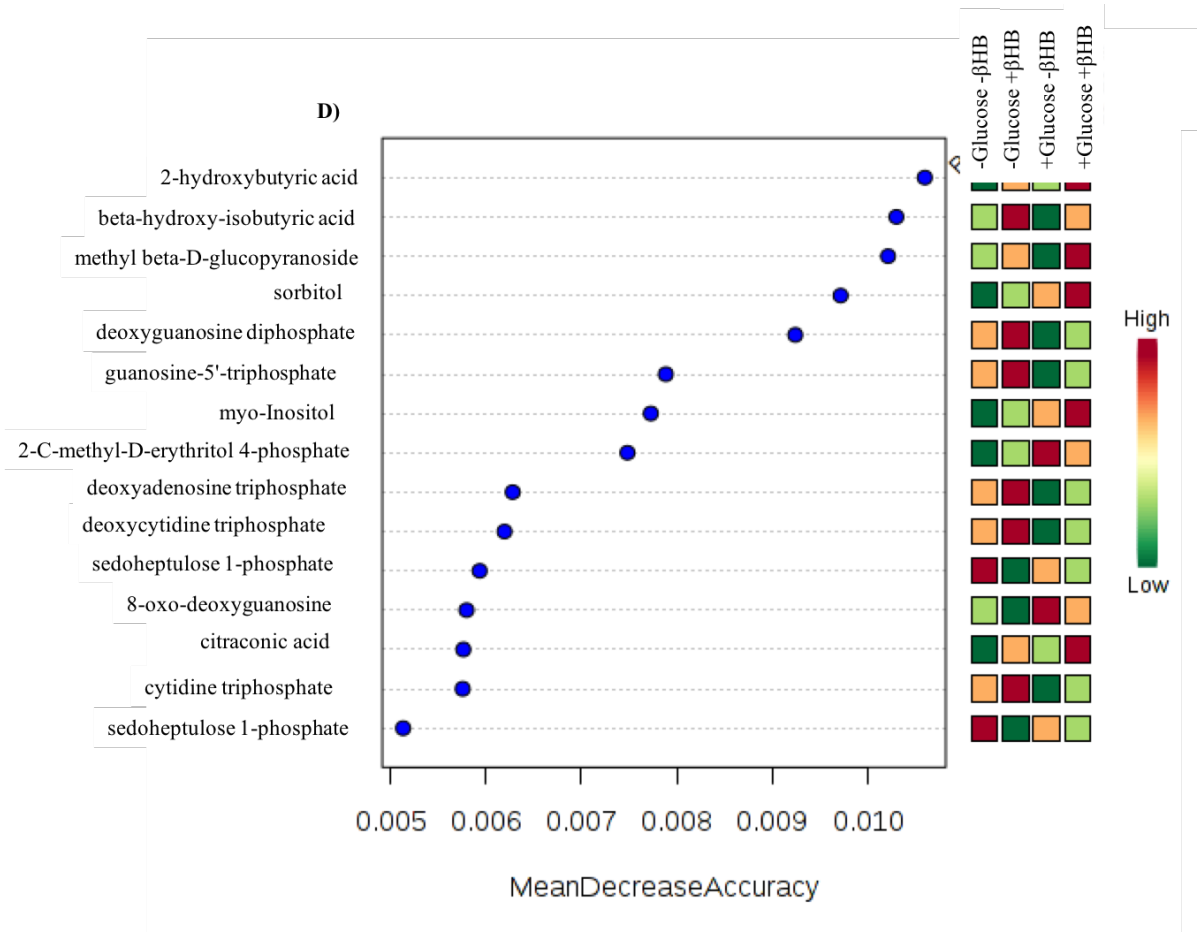


Figure 3.14 *List of identified compounds via two-way analysis of variance (ANOVA) in high glycogen (HG) hearts. Hearts previously loaded with glycogen (HG hearts) were perfused with buffer containing either glucose-free buffer alone (-Glucose -βHB), or 11 mM glucose alone (+Glucose -βHB), or glucose-free buffer plus 4 mM βHB (-Glucose +βHB), or 11 mM glucose plus 4 mM βHB (+Glucose +βHB) (n=4-5). (A) A hierarchical clustering heat-map shown as fold change for all identified compounds via two-way ANOVA. A fold-change threshold of 1.5 was used to determine significance. (B) Venn diagram via two-way ANOVA. (C) A hierarchical clustering heat-map shown as fold change for significantly different compound via two-way ANOVA. A fold-change threshold of 1.5 was used to determine significance. (D) The top 15 compounds ranked by variable importance in projection (VIP) scores based on mean decrease in accuracy (MDA), variance between experimental groups. Red colour represents an expression level above mean, green colour represents an expression lower than the mean. βHB, D-β-hydroxybutyrate.*

3.5 Discussion

This study was designed to determine the effect of myocardial glycogen levels on βHB oxidation and glycolytic rates. The results showed that βHB oxidation increased with higher myocardial glycogen. βHB oxidation decreased exogenous glucose utilisation in all hearts, redirecting glucose into glycogen. This study hypothesised that myocardial glycogen can act as an anaplerotic substrate, via glycogenolysis and glycolysis, to pyruvate and possibly oxaloacetate in order to support the heart's ability to oxidise βHB. Therefore, a rigorous pulse-chase experiment was performed to measure anaplerosis indirectly from ^{3}[H]-labelled glycogen in HG hearts perfused with βHB. As expected, glycogenolysis from HG hearts with ^{3}[H]-labelled glycogen plus βHB alone was 3-fold higher than those hearts perfused with glucose plus βHB. Metabolomic analysis showed that the increase in glycogenolysis significantly increased the utilisation of βHB, via fructose 1,6-bisphosphate and 3-phosphoglycerate. Thus, the increase in βHB oxidation was associated with increased citrate, 2-oxoglutarate and succinate, and total NADP (H) pool. Importantly, βHB oxidation reduced mitochondrial Q as reported by Sato and his colleagues (44). This study suggest that high myocardial glycogen could significantly increase βHB oxidation rates.

3.5.1 Myocardial glycogen content significantly altered βHB oxidation and βHB oxidation altered glycolysis in all hearts

Perfusion of HG hearts with glucose alone did not further increase glycogen, possibly due to the upper limit of glycogen content having been reached (~40 μmol glycosyl units.gww^{-1}) (307). Nevertheless, perfusion with βHB plus glucose significantly increased glycogen in LG hearts, suggesting that βHB oxidation increased glycogen synthesis in severely glycogen-depleted myocardium potentially by inhibiting glycolysis through pyruvate dehydrogenase (PDH) (68, 243, 344), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (345) or phosphofructokinase (PFK) (68, 243, 344), which was associated with

increased the free cytosolic [NAD^+]/[NADH] ratio and glycogen (334). It is postulated that βHB metabolism inhibits the activity of pyruvate dehydrogenase, via activation of pyruvate dehydrogenase kinase activity (68, 346, 347). Such metabolic effects of βHB on glycogen synthesis were also found in dog and working perfused rat hearts (334, 348).

Glycolysis from exogenous glucose in LG hearts perfused with glucose alone was significantly lower than in HG hearts perfused with glucose as some of the exogenous glucose was preferentially used to replenish glycogen. This suggests that glucose utilisation from exogenous [5-^{3}H]-glucose must be interpreted as the sum of net glycogenesis and glycolytic rates (assuming no other major metabolic fates of exogenous glucose; [eg. the pentose phosphate pathway, which accounts for some exogenous glucose, but was not measured in the present study, as its activity is relatively low in heart (229)). The lower glycolysis in LG hearts perfused with βHB was also due to some exogenous glucose being used to replenish glycogen following the preceding glycogen depletion, as indicated by the increase in net glycogenesis rate from 0.3 to 0.8 μmol glycosyl units.gww^{-1}. min^{-1}.

As expected, addition of βHB decreased glycolysis in all hearts, as βHB inhibits the activity of PDH, (68, 243, 344), GAPDH (345) or PFK (68, 243, 344). βHB oxidation rates in HG hearts were 2-fold higher than in LG hearts. High βHB oxidation rates significantly decreased exogenous glucose utilisation (0.44 vs. 1.0 μmol.gww^{-1}.min^{-1}), potentially due to inhibition of glucose uptake (68, 346, 347).

It has been reported that catabolism of leucine to ketone bodies inhibits translocation of GLUT4-containing vesicles via acetylation (349), with βHB providing significant energy. In contrast, βHB inhibited glycolysis, but did not decrease glucose uptake (Fig. 3.10) (334). The inhibition on glucose uptake affected by βHB was restudied here. Moreover, the expression of GLUT-4 could be determined by Western blots (although the time course of this experiment may too rapid to detect changes).

3.5.2 Glycogen mobilised for anaplerosis via fructose 1,6-bisphosphate and 3-phosphoglycerate

High glycogen augmented βHB oxidation, seemingly via glycolysis to pyruvate and anaplerosis to oxaloacetate. Therefore, an attempt was made to indirectly estimate anaplerosis from labelled glycogen (glycogenolysis) by perfusing HG hearts with βHB alone. Glycogenolysis in HG hearts with [^{3}H]-labelled glycogen perfused with βHB alone was 3-fold higher than in hearts perfused with glucose and βHB, thereby decreasing glycogen by ~26%. The net rate of glycogenolysis, or potential anaplerotic flux from ^{3}[H]-labelled glycogen, was 0.01 ± 0.01 μmol.gww^{-1}.min^{-1}. After glycogen repletion, exogenous glucose significantly increased myocardial sorbitol and lactate. It was reported that exogenous glucose was converted to lactate via lactate dehydrogenase (241). Aldose reductase catalyses nicotinamide adenosine dinucleotide phosphate (NADP)-dependent reduction of glucose to sorbitol (polyol pathway). Hence, sorbitol is converted to glycerol 3-phosphate, which was associated with the increased the free cytosolic [NAD^+]/[NADH] ratio (350). Glycerol 3-phosphate is involved in glycerolipid biosynthetic pathway (350), which was not measured in this study. Moreover, glycogenolysis in HG hearts perfused with glucose-free buffer alone was significantly higher than those hearts perfused with glucose alone. The labelled glycogen was preferentially oxidised in the absence of substrates, which was associated with decreased glycogen content (226).

3.5.3 The increase in βHB oxidation was associated with increased Krebs cycle intermediates

The increase in βHB oxidation was associated with increased Krebs cycle intermediates, citrate, 2-oxoglurate and succinate, and total NADP(H) pool measured by metabolomics. During the fed state, NADPH is primarily produced from glucose metabolism by the pentose phosphate pathway (351). However, NADPH can also be produced from metabolism of βHB

in the Krebs cycle (44, 352). During ketosis, mitochondrial SIRT3 deacetylates and activates the NADP-dependent isocitrate dehydrogenase IDH2 (353), which increased 2-oxoglutarate and succinate measured using metabolomics. It was reported that βHB inhibited class I and IIa histone deacetylases (HDACs), which increased genes encoding oxidative stress resistance factors FOXO3a and MT2 (354). FOXO3a induces the expression of a cytoplasmic form of NADP-dependent isocitrate dehydrogenase IDH1 (355). The citrate or isocitrate from βHB metabolism in mitochondria can be exported to by the citrate-isocitrate carrier to the cytoplasm for the production of NADPH by IDH1. This leads to increased NADPH production, which maintains levels of reduced glutathione (353). NADPH provides the electrons for the reduction of molecular antioxidants (glutathione disulphide, glutathione, vitamin C, and vitamin E), which decreases reactive oxygen species or reactive nitrogen species through glutathione peroxidase (351).

The increase in βHB oxidation was associated with increased succinate from succinyl-CoA coupled with substrate-level phosphorylation of guanosine diphosphate (GDP) to GTP catalysed by succinyl-CoA synthase (SCS) (356). Moreover, SCS can replenishes succinyl-CoA for βHB metabolism, where CoA is transferred from succinyl-CoA to form acetoacetyl-CoA in the presence of 3-ketoacyl-CoA transferase (357). The increase in succinate was associated with decreased fumarate catalysed by the reversible succinate dehydrogenase reaction (44, 62, 343). Thus, βHB oxidation decreased the free mitochondrial $[Q^+]/[QH_2]$ ratio (44, 358).

Targetted metabolomic analysis showed that the increase in βHB oxidation was associated with increased Krebs cycle intermediates. Untargetted metabolomic analysis was performed to provide further information about the effect of glycogen or βHB oxidation on specific metabolic pathways. Targetted metabolomic analysis showed that the increase in βHB oxidation was associated with increased Krebs cycle intermediates and the reduction

of mitochondrial Q. This is in agreement with the untargetted metabolomic analysis, which identified compounds that are involved in muscle energy production via purine nucleotide cycle, as GDP, GTP, and ATP. This leads to a very important question whether the increase in βHB oxidation can increase cardiac energetics. Untargetted metabolomic analysis also successfully identified βHB (2-hydroxybutyric acid) and glucose (methyl beta-D-glucopyranoside, sorbitol, and myo-inositol) as top compounds without prioritising compound identification, corroborating the methodology. Importantly, both targetted and untargetted metabolomic analyses showed that glucose and glycogen were required for maximal βHB oxidation.

One caveat of this study is that the perfusion buffer did not contain fatty acids, a major fuel of the heart. Fatty acids and ketone bodies are 2-carbon units, hence are cataplerotic. The presence of different substrates induces substrate competition, making it more difficult to determine the net contribution of each substrate.

In conclusion, glycolytic flux from glycogen increased the heart's ability to oxidise βHB. Glucose and glycogen were required for maximal βHB oxidation. The increase in βHB oxidation was associated with increased Krebs cycle intermediates, which may affect cardiac energetics.

Chapter 4: Glycogen needed for D-β-hydroxybutyrate (βHB) to increase energetic parameters in the isolated rat heart: A ^{31}P-NMR spectroscopy study

4.1 Abstract

The work described in Chapter 3 showed that, in hearts with a high glycogen content, the increase in βHB oxidation was associated with increased Krebs cycle intermediates, but unknown is whether this alters in cardiac energetics. Here, the effect of βHB oxidation on energetics was determined in low and high glycogen content. To lower glycogen, hearts were perfused with glucose-free buffer (low glycogen; LG). To increase glycogen (high glycogen; HG), hearts were pre-perfused with buffer containing pyruvate, lactate, glucose and insulin. Following changes in glycogen, hearts were subsequently perfused with 11 mM glucose before perfusing with 4 mM βHB plus 11 mM glucose. ATP, phosphocreatine (PCr), inorganic phosphate (Pi) and cytosolic pH were measured using ^{31}P-NMR spectroscopy. It was found that the concentration of ATP was similar in all hearts. In high glycogen hearts, βHB perfusion increased the free energy of ATP hydrolysis (ΔG_{ATP}) by increasing creatine kinase activity (the ratio ([PCr]/[ATP]), and the phosphorylation potential (the ratio ([ATP]/[ADP][Pi])). βHB was unable to alter the already low energy level in low glycogen hearts. In conclusion, glucose and glycogen were required for maximal βHB oxidation, which increased the free energy of ATP hydrolysis.

4.2 Introduction

Heart, being composed principally of striated red muscle fibres, has a high energy demand and so high oxygen consumption, but having limited endogenous energy reserves. For instance, the heart has a relatively low ATP content (5 μmol. g wet wt^{-1}) and a high rate of ATP hydrolysis (~30 μmol. g wet wt^{-1}. min^{-1} at rest), thereby a complete turnover of the myocardial ATP pool occurs approximately every 10 s under normal conditions. Therefore, human heart synthesises ~6.6 kg of ATP per day (12). However, the rate of energy turnover, not the tissue content of ATP, is determinant of energy metabolism in the heart (359). Consequently, the heart is adapted to use any available fuel substrate to maintain a high ATP concentration in order to maintain cardiac performance (278, 286, 360). The work in Chapter 3 showed that βHB oxidation could increase Krebs cycle intermediates in high glycogen hearts, but the effect on energetics were not measured.

Human heart synthesises ATP through oxidative phosphorylation. Respiratory chain complexes I to IV transfer electrons from NADH to oxygen, with proton extrusion across the inner mitochondrial membrane (IMM), thereby creating a proton electrochemical gradient across the IMM, producing NAD^+ and water. The proton gradient drives the F_1, F_o -ATP synthase, which phosphorylates ADP to produce the "high-energy phosphate" compound ATP (5, 145, 361). Substrate level phosphorylation (e.g. glycolysis) and phosphocreatine provide limited supplemental sources of ATP (362).

The heart converts chemical energy in the form of ATP into mechanical energy. The creatine kinase (CK) system is the final step, which provides a direct link between ATP-producing (oxidative phosphorylation in the mitochondria and glycolysis in the cytosol) and ATP-utilising pathways (actomyosin ATPase in the myofibril, the Ca^{2+}-ATPase in the sarcoplasmic reticulum, and the Na^+/K^+-ATPase in the sarcolemma) (360, 363-365). For example, ATP is transferred out of the mitochondrion to be consumed by myofibrillar

ATPase and other ATP-consuming reactions, including sarcolemmal and sarcoplasmic reticulum ion pumps (286, 363, 366). The CK reaction is the primary energy reserve of the heart, reversibly exchanging a high-energy phosphate group between ATP and PCr in the reaction:

$$PCr + MgADP^{-} + H^{+} \leftrightarrow MgATP^{2-} + \text{creatine} \quad (367)$$

In normal heart, the creatine kinase reaction catalyses the phosphorylation of approximately two-thirds of the total creatine pool to form phosphocreatine (363). Figure 4.1 depicts the creatine kinase system in cardiomyocytes (286, 368).

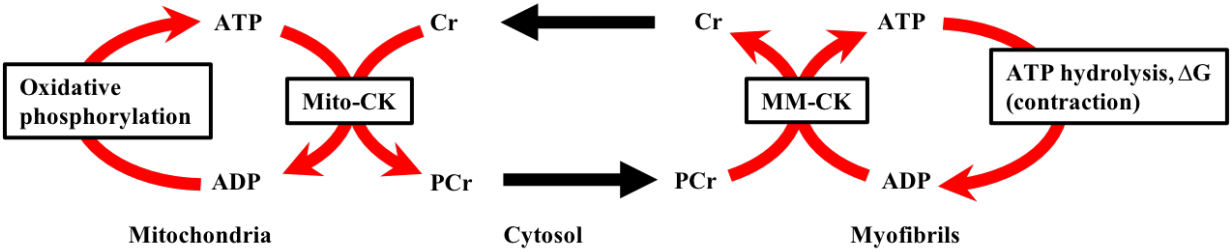


Figure 4.1 *The creatine kinase energy shuttle. Mitochondrial creatine kinase (Mito-CK) catalyses the transfer of a phosphoryl group from ATP to creatine (Cr) to form phosphocreatine (PCr) and ADP. Phosphocreatine is a smaller molecule than ATP, and able rapidly to diffuse into the myofibrils to regenerate ATP and creatine via transferring a phosphoryl group or inorganic phosphate (Pi) to ADP catalyzed by myofibrillar creatine kinase (MM-CK).*

The work in Chapter 3 showed that the increase in βHB oxidation was associated with increased Krebs cycle intermediates. Therefore, this study was designed to determine the effect of βHB oxidation on PCr, ATP, Pi, and cytosolic pH using ^{31}P-NMR spectroscopy, in isolated rat hearts with low and high glycogen contents.

4.3 Methods

4.3.1 Cardiac energetics measured using nuclear magnetic resonance (NMR) spectroscopy

Isolated rat hearts from fed male Wistar rats (Chapter 2.1) were perfused in the Langendorff mode (Chapter 2.2.1). Isolated rat hearts were initially perfused with oxygenated non-recirculating buffer to either decrease (LG) or increase (HG) glycogen content. To deplete glycogen, hearts were perfused with glucose-free Krebs-Henseleit (KH) buffer for 20 minutes (low glycogen: LG) (226, 307); and to augment glycogen, hearts were perfused with KH buffer containing pyruvate (4 mM), lactate (1 mM), glucose (11 mM) and insulin (50 mU/min) for 60 minutes (high glycogen: HG) (307). The hearts were then perfused with 11 mM glucose for 10 minutes before perfusing with 11 mM glucose plus 4 mM βHB for 40 minutes (Fig. 4.2 A).

Two independent recirculating perfusion buffer circuits (B and C) were used. Each circuit had a 1 L upper reservoir, which was kept filled to a fixed volume of 800 ml from a 3 L reservoir at the base of the perfusion rig. A central 3-way tap allowed immediate changes in the perfusion buffer to the heart (Fig. 4.2 B and C). The aorta was cannulated at the end of the umbilical cord tip, and surrounded by a 20 mm radius glass NMR tube. Hearts were perfused at a constant pressure of 100 mmHg and a left-ventricular end-diastolic pressure of 4-8 mmHg. Cardiac contractile function was measured using a fluid-filled polyvinylidene chloride (PVC) balloon inserted into the left ventricle, which was attached to a bridge amplifier and PowerLab data acquisition system (Fig. 4.2 D).

The heart was lowered into the isocentre of the magnet bore and perfusion buffer was supplied using 1.2 m ‘umbilical cord’, with buffer maintaining the temperature of the heart at 37 ℃. Fully relaxed ^{31}P-NMR spectra were acquired every 10 minutes throughout perfusion (Fig. 4.2).

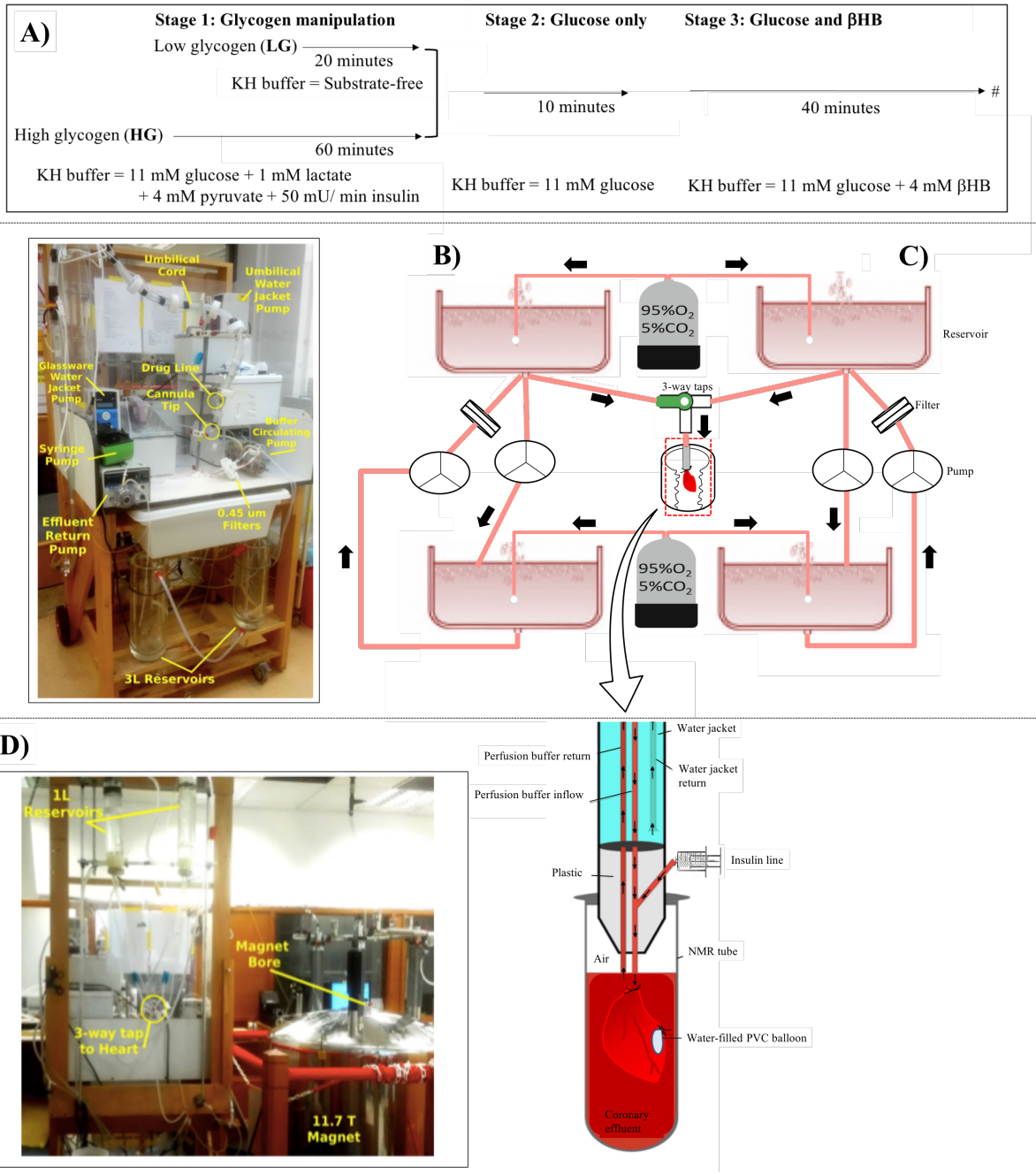


Figure 4.2 *Perfusion system used to measure cardiac energetics using ^{31}P-NMR spectroscopy. Following changes in glycogen, the hearts were perfused with 11 mM glucose (-KB) before perfusing with 11 mM glucose plus 4 mM βHB (+KB) (n=10) (A). The aorta was cannulated on a specially constructed, MR-compatible umbilical perfusion cord, and surrounded by a glass NMR tube. Two independent recirculating perfusion buffer circuits were maintained (B and C). The perfusion buffer inflow and outflow are indicated as red lines. The umbilical water jacket inflow and outflow are indicated as blue lines. Coronary effluent was pumped out of the NMR tube to waste. The hearts were positioned in the isocentre of the magnet bore (verified using ^{1}H MRI scout images) (D). #, hearts freeze-clamped for analysis; KH, Krebs-Henseleit saline buffer; βHB, D-β-hydroxybutyrate.*

^{31}P-NMR spectroscopy measures metabolites by detecting signals from the phosphorus nuclei of ATP (with three phosphates, α, β, and γ, at different chemical shifts), phosphocreatine (PCr), and inorganic phosphate (Pi) in the beating heart (369). All NMR spectroscopic methods were performed using a 500 MHz (11.7 T) MR System, comprising an 11.7 T vertical-bore magnet (Magnex Scientific, Oxford, UK) and a Bruker Avance console (Bruker Medical, Ettlingen, Germany). A dual-tuned birdcage coil with a 20 mm inner-diameter (Rapid Biomedical, Wurzburg, Germany) was used as the transmitting and receiving RF coil.

NMR spectra were analysed in the time domain using the AMARES algorithm in the jMRUI 5.2 software package (MRUI Consortium, Universitat Autònoma de Barcelona). Fits were performed for ATP (α, β, and γ,), phosphocreatine and inorganic phosphate (Pi) peaks. AMARES prior knowledge was acquired from a previous rat heart spectrum on the same NMR system and specified the J-coupling patterns of the three ATP peaks (α, β, and γ,), the peak positions, and the use of Lorentzian line shapes. The same commands were reproduced as illustrated in Figure 4.3.

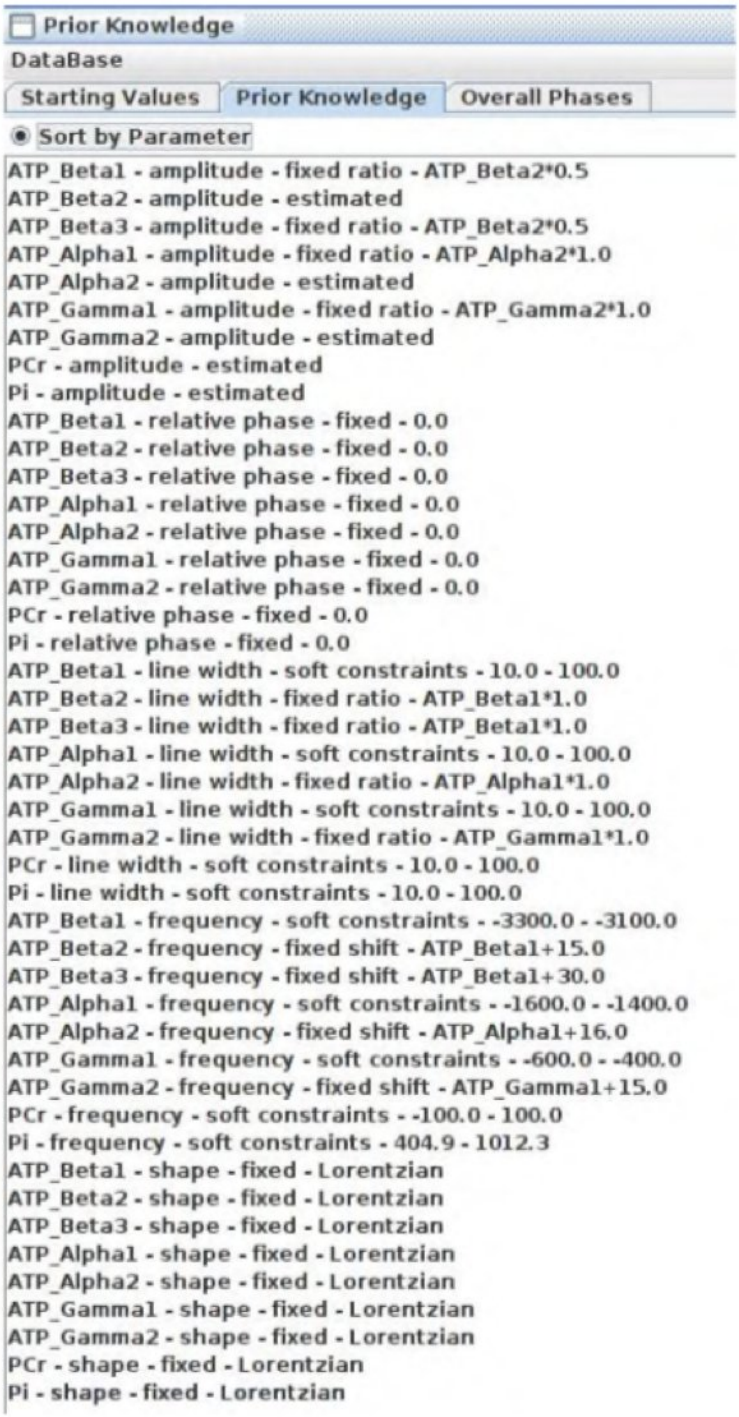


Figure 4.3 *AMARES prior knowledge. Commands were grouped by parameters.*

PCr and ATP were converted to metabolite concentrations using a two-point scale, with the average amplitude of the γ-ATP peaks recorded during perfusion with glucose plus βHB set as the previously established ATP concentration in rat heart, 10.6 ± 0.8 mM (369). The free creatine concentration was calculated from the PCr concentration, assuming that total creatine ([Cr] + [PCr]) was 27.9 mM (370). Cytosolic pH was measured using the chemical shift of Pi relative to PCr in ^{31}P-NMR spectra (44, 369).

The concentration of cytosolic free adenosine diphosphate [ADP] was calculated from:

$$[ADP]_{free} = \frac{[ATP][Cr]}{[PCr][H^+]K_{eq}} \qquad \textit{the creatine kinase reaction:}$$

where free [Mg^{2+}] = 1 mM, K_{eq} = 1.66 x 10^9 M^{-1} (371) (Eq. 1)

The concentration of free cytosolic adenosine monophosphate [AMP] was calculated from:

$$[AMP]_{free} = \frac{[ADP]^2_{free} \times K_{eq}}{[ATP]} \qquad \textit{the adenylate kinase reaction:}$$

where free [Mg^{2+}] = 1 mM, K_{eq} = 1.05 (371) (Eq. 2)

The cytosolic phosphorylation potential was calculated from:

$$\frac{[ATP]}{[Pi][ADP]_{free}} = \frac{[PCr][H^+]\,K_{eq}}{[Cr][Pi]}$$

where free [Mg^{2+}] = 1 mM, K_{eq} = 1.66 x 10^9 M^{-1} (371) (Eq. 3)

The free energy of ATP hydrolysis (ΔG_{ATP}) was calculated from

$$\Delta G_{ATP} = \Delta G^{\circ}_{ATP} + RT \times \ln \frac{[Cr][Pi]}{[PCr][H^{+}]K_{eq}}$$

where R = 8.314 J K^{-1} $mole^{-1}$, T = 310 K, ΔG^{o}_{ATP} = -30.5 kJ. mol^{-1} (84), K_{eq} = 1.66 x 10^{9} M^{-1} (371) (Eq. 4).

4.3.2 Statistical analysis

All energetic parameters (free adenosine diphosphate ([ADP]), adenosine monophosphate ([AMP]), phosphocreatine ([PCr]), cytosolic phosphorylation potential, and free energy of ATP hydrolysis (ΔG_{ATP})) were analysed by two-way analysis of variance (ANOVA), with glucose and βHB as factors. Tukey *post hoc* corrections were made for multiple comparisons to identify specific significant differences. Results, presented as means ± SEM, were considered significant at $p < 0.05$.

4.4 Results

4.4.1 The effect of βHB oxidation on energetics using ^{31}P-NMR spectroscopy

The height and integrated areas of the ATP (γ-ATP, β-ATP and α-ATP) resonance peaks were consistent in all spectra. In high glycogen hearts, addition of βHB (+βHB) increased the phosphocreatine (PCr) concentrations, although this did not occur in low glycogen hearts perfused with βHB (+βHB) (Fig. 4.4 and Fig. 4.5).

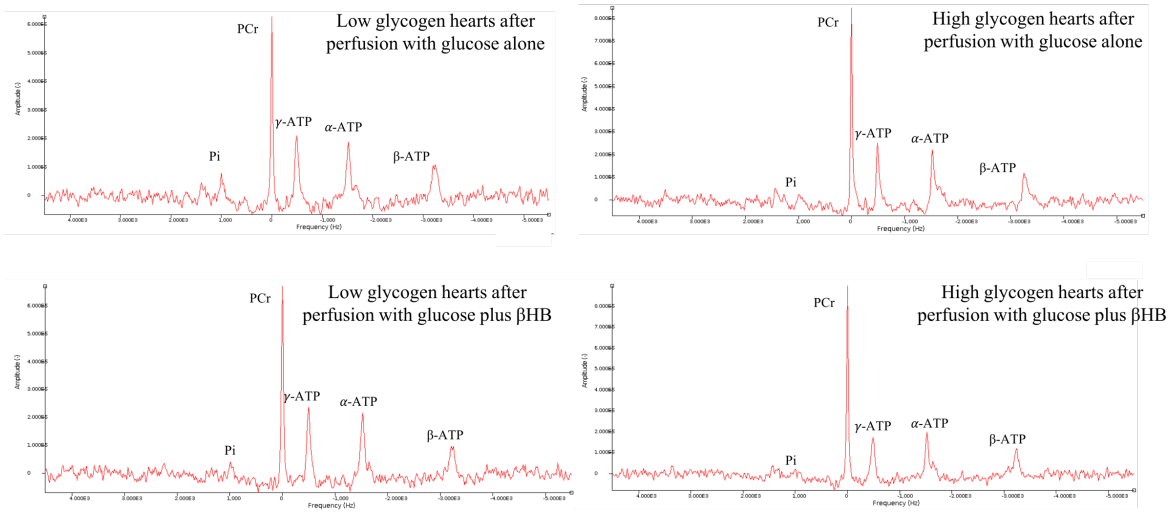


Figure 4.4 *Example spectra acquired in hearts with low and high glycogen content. Following changes in glycogen content, the hearts were perfused with glucose alone (- βHB) or glucose plus βHB (+ βHB) (n=10). βHB, D-β-hydroxybutyrate.*

4.4.2 The effect of glycogen content and β-hydroxybutyrate (βHB) on energy parameters

As expected, concentrations of adenosine triphosphate ([ATP]) and the cytosolic pH were similar in all hearts. In high glycogen hearts, βHB increased the free energy of ATP hydrolysis (ΔG_{ATP}) by increasing creatine kinase activity (the ratio ([PCr]/[ATP]) and the cytosolic phosphorylation potential (the ratio ([ATP]/[ADP][Pi])), and decreasing concentrations of adenosine diphosphate ([ADP]), and adenosine monophosphate ([AMP]). This did not occur in low glycogen hearts (Fig. 4.5).

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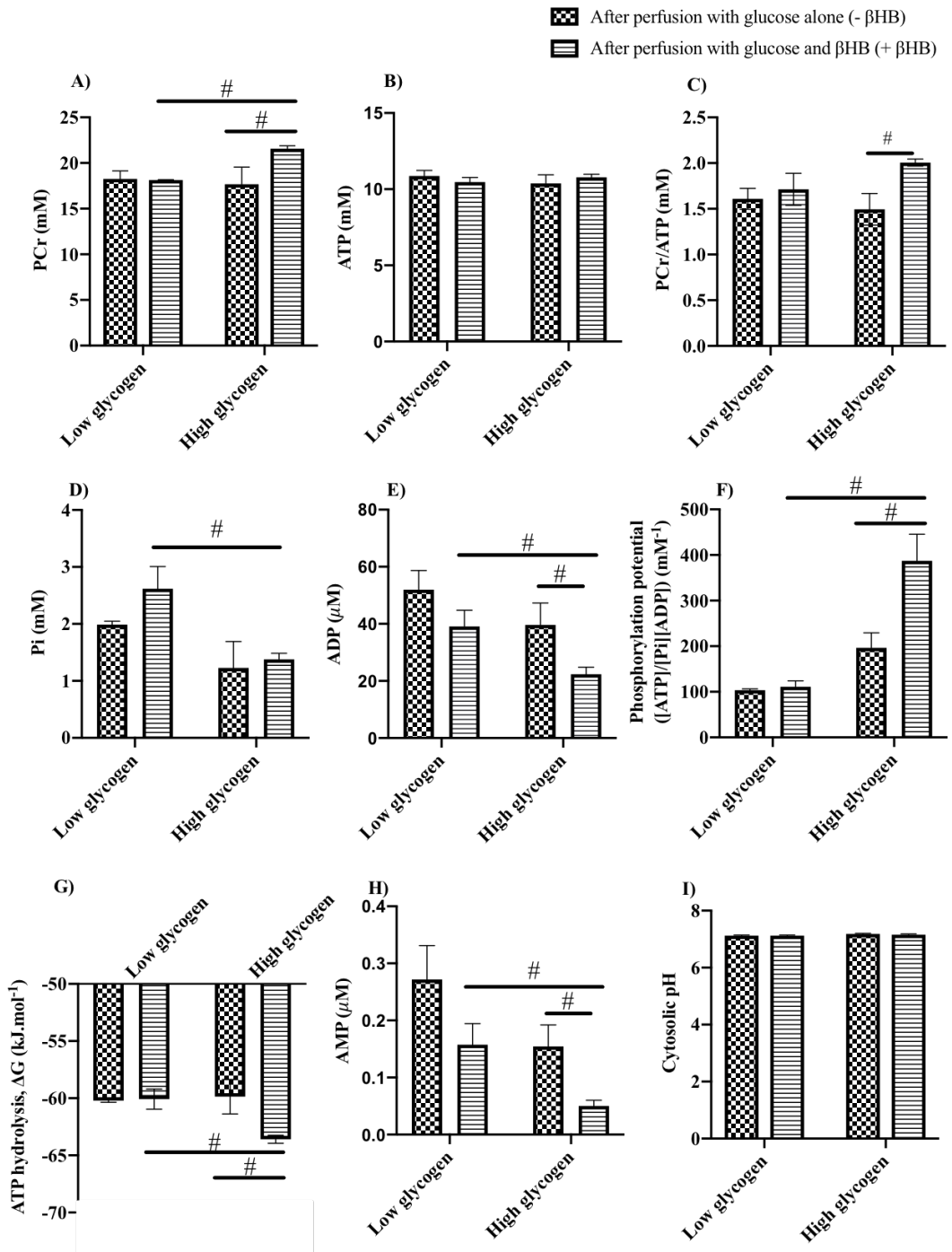


Figure 4.5. *The effect of βHB on energy parameters in low and high glycogen hearts. Following changes in glycogen content, the hearts were perfused with glucose alone (- βHB) or glucose plus βHB (+ βHB) (n=10). Phosphocreatine (A), adenosine triphosphate (B), [PCr]/[ATP] ratio (C), inorganic phosphate (D), adenosine diphosphate (E), phosphorylation potential (F), free energy of ATP hydrolysis (G), adenosine diphosphate (H), and cytosolic pH (I) were measured using ^{31}P-NMR spectroscopy. Values are means ± SEM. Effect of βHB on energy parameters is indicated as: # P<0.05. βHB, D-β-hydroxybutyrate.*

4.5 Discussion

In hearts with high glycogen, the increase in βHB oxidation was associated with increased Krebs cycle intermediates. Here, βHB increased the free energy of ATP hydrolysis by increasing [PCr] and the cytosolic phosphorylation potential, but only in high glycogen hearts. In the heart, oxidation of βHB decreased the free mitochondrial $[Q^+]/[QH_2]$ ratio, making more energy available for the synthesis of ATP (44, 348) (Fig. 4.6). Moreover, mitochondrial creatine kinase catalyses the transfer of a phosphoryl group from ATP to form PCr. PCr is a transport molecule with the reverse reaction catalysed by myofibrillar creatine kinase to liberate ATP, which increased [PCr]/[ATP] ratio and phosphorylation potential. The increase in phosphorylation potential increases the free energy of ATP hydrolysis (286, 301, 363, 364). The measured absolute values of [PCr] is consistent with those previously reported (307, 367). Thus, βHB oxidation reduced mitochondrial Q, ultimately increasing the free energy of ATP hydrolysis (ΔG_{ATP}) (Fig. 4.6).

In high glycogen hearts, βHB decreased [ADP] and [AMP], potentially because maximal ATP may have been reached. As expected, [ATP] was similar in all hearts at ~10.6 mM (372-374). The concentration of ATP is maintained by the enzyme adenylate kinase by transferring phosphoryl groups between adenine nucleotides: $2ADP \leftrightarrow ATP + AMP$ (363). The heart can oxidise a variety of carbon-based fuels for ATP synthesis to ensure that [ATP] remains relatively constant despite varying ATP turnover rates due to changes in work load (363). βHB oxidation also maintained a low [ADP] and [Pi] in order to increase the phosphorylation potential via the creatine kinase reaction (375). In low glycogen hearts, βHB oxidation was too low to increase the free energy of ATP hydrolysis due to low glycolytic flux from pyruvate.

In conclusion, myocardial βHB oxidation reduced mitochondrial Q (Chapter 3), which increased [PCr]/[Cr] ratio and phosphorylation potential, ultimately increasing free energy of ATP hydrolysis.

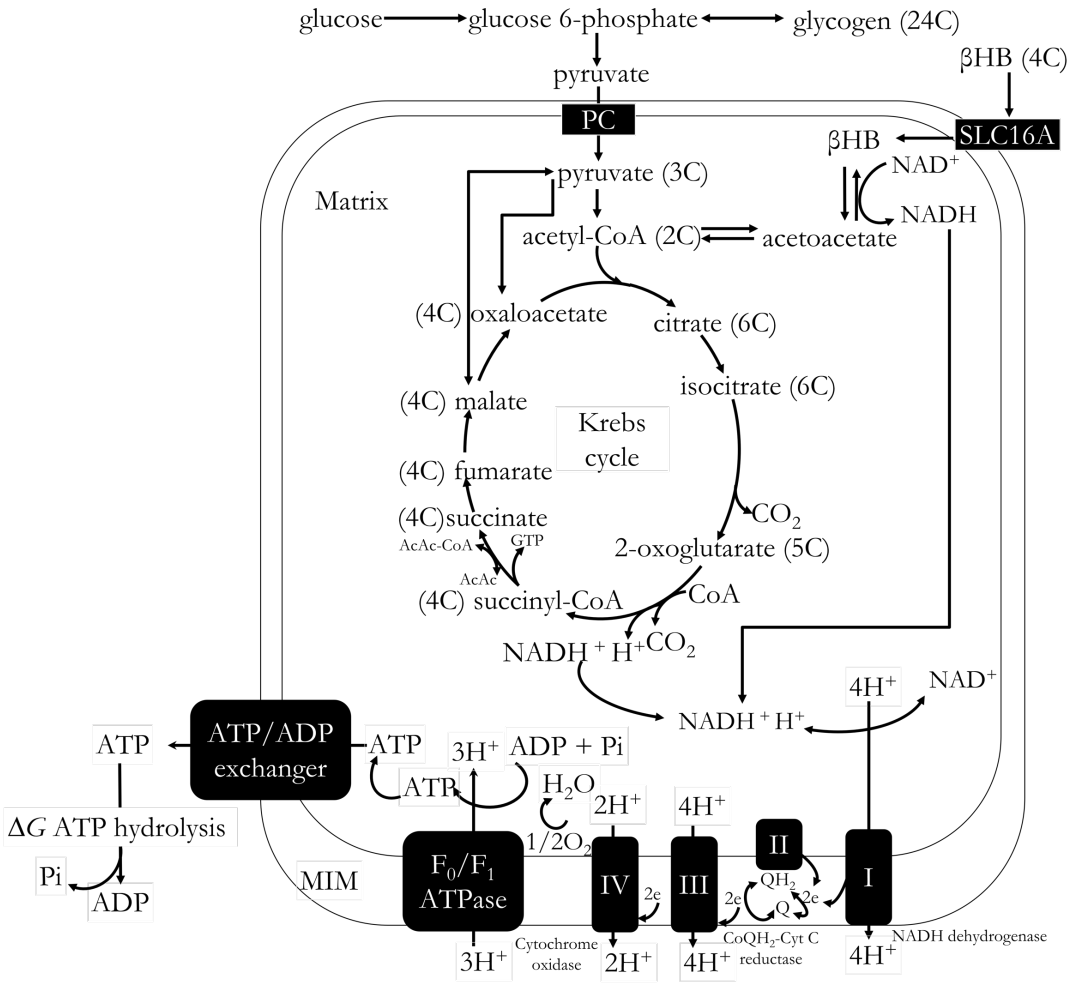


Figure 4.6 *The pathway of βHB oxidation increases cardiac energetics. βHB reduces mitochondrial Q, which increases [PCr]/[ATP] ratio, thereby increasing the free energy of ATP hydrolysis. CoA, coenzyme A; NAD+(H), nicotinamide adenine dinucleotide oxidized (reduced); Pi, inorganic phosphate; ADP, adenosine diphosphate; MIM, mitochondrial inner membrane; Cyt, cytochrome C; e-, electron; H+, hydrogen ion, F_o/F_1 ATPase, ATP synthase; Q, ubiquinone; QH_2, ubiquinol; I, II, III, IV, complexes I-IV of the electron transport chain, respectively. βHB, D-β-hydroxybutyrate*

Chapter 5: Glucogenic amino acids and D-β-hydroxybutyrate (βHB) oxidation in the isolated rat heart

5.1 Abstract

Low glycolytic flux may be accompanied by decreased carboxylation of pyruvate, which would decrease the availability of oxaloacetate required for the oxidation of D-β-hydroxybutyrate (βHB) in the Krebs cycle. In such circumstances, the availability of glucogenic amino acids as alternative sources of ≥ 3-carbon compounds may impact the heart's ability to oxidise βHB. The amino acids that can be metabolised into Krebs cycle intermediates are asparagine (into oxaloacetate), valine (into succinyl-CoA) and glutamine (into 2-oxoglutarate). Here, asparagine, valine and glutamine were tested for their ability to support βHB oxidation in the isolated perfused rat heart. To limit carbon flux through pyruvate and oxaloacetate, the glycogen content of isolated rat hearts was depleted by pre-perfusion with glucose-free Krebs-Henseleit (KH) buffer. Hearts were then perfused with 2.5 mM glucose plus 4 mM [^{14}C]- βHB, and one of 0.13 mM asparagine, 0.31 mM valine, or 0.65 mM glutamine, or with no additional substrates. Hearts were freeze-clamped for subsequent glycogen and metabolomic analyses. In glycogen-depleted hearts, perfusion with 2.5 mM glucose resulted in 0.2 μmol. gww^{-1}. min^{-1} βHB oxidation rates. Asparagine also increased βHB oxidation rates 2-fold, via increased intermediary metabolites involved in the malate-aspartate shuttle and purine nucleotide cycle (aspartate, guanosine triphosphate, and fumarate), but did not alter total NAD(H) and total NADP(H) pool. Neither valine nor glutamine increased βHB oxidation rates. Glucose was preferentially oxidised following glycogen depletion. In conclusion, anaplerosis of oxaloacetate via asparagine increased βHB oxidation, via the malate-aspartate shuttle and the purine cycle, but did not alter total NAD(H) pool. Anaplerosis of 2-oxoglutarate via glutamine or succinyl-Coa via valine did not augment βHB oxidation.

5.2 Introduction

The first study in Chapter 3 demonstrated that decreasing glycolysis from glycogen decreased myocardial βHB oxidation rates, potentially via lower anaplerotic flux from pyruvate to oxaloacetate. In liver, glucogenic amino acids can be converted to glucose under such circumstances, but their role as anaplerotic substrates in the heart is not well understood.

In the fed state, any amino acid surpluses can be metabolized following deamination to non-nitrogenous energy substrates, including glucose or fatty acids and stored prior to metabolism. Amino acid deamination produces ammonia, which is then converted to non-toxic urea for urinary excretion (urea cycle) (376, 377). The survival of fasting man is dependent upon glucogenic amino acids (e.g. glutamine, asparagine, and valine), ketogenic amino acids (isoleucine and leucine) plus non-esterified fatty acids (NEFA) and glycerol (glucogenic precursor) derived from stored triacylglycerol (40, 378-384) (Fig. 5.1).

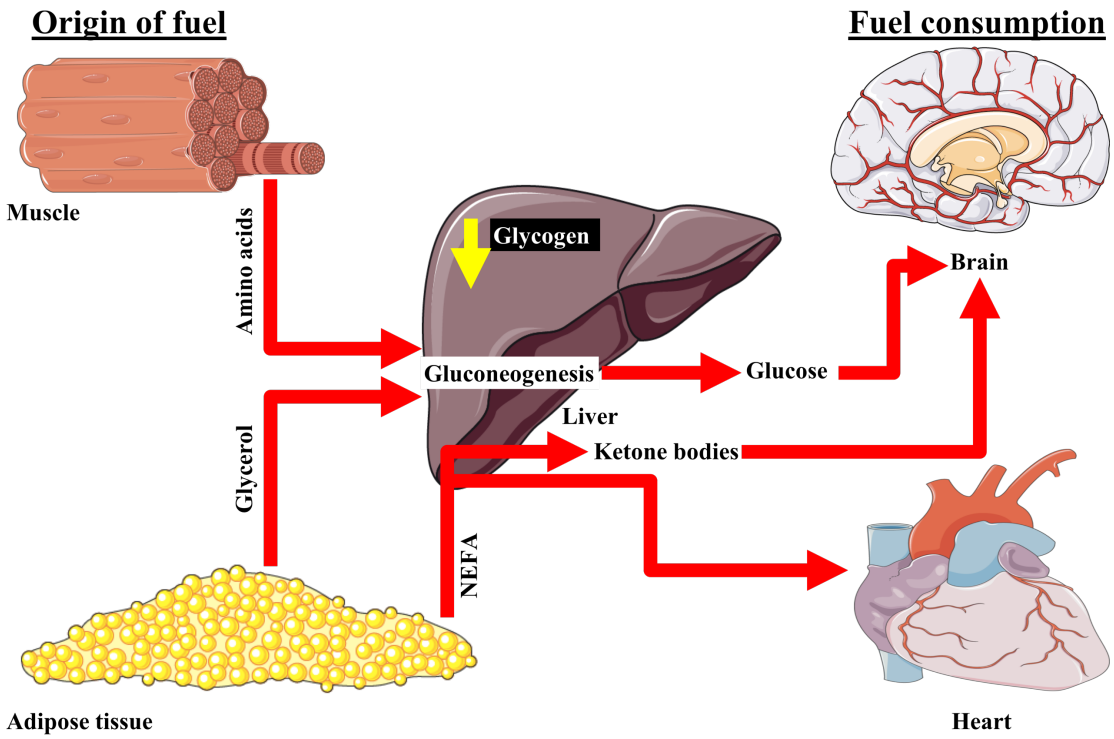


Figure 5.1 *Overall scheme of fuel metabolism in starvation. In starvation, hepatic glycogen content is low, and glucogenic amino acids from muscle are utilised by liver for gluconeogenesis. Glycerol from adipose tissue enters the hepatic glucogenic pathway at triose phosphate. The heart can also utilise glycerol via glycerol kinase. In fasting, the liver oxidises non-esterified fatty acids (NEFA) to ketone bodies (D-β-hydroxybutyrate and acetoacetate). Amino acids can also be converted to ketone bodies (ketogenic amino acids). Brain utilises both ketone bodies and glucose. Heart utilises non-esterified fatty acids, but the utilisation of amino acids directly is uncertain.*

Together with alanine, glutamine is the principal amino acid exported from muscle undergoing proteolysis in the fasted state. Glutamine is the most abundant amino acid in many tissues, including the myocardium (385). In the heart, glutamine has several sources: 1) uptake from the circulation (386), 2) proteolysis of myocardial proteins, and 3) synthesis by glutamine synthetase (387-390). Glutaminase catalyzes hydrolysis and release of the amide (NH_2) nitrogen of glutamine as ammonia (NH_4^+) to form glutamate (385, 391, 392). Glutamate can be amidated to transport amine groups out of the cell (250). Glutamate dehydrogenase uses NAD^+ (or $NADP^+$) as a coenzyme to catalyze the formation of 2-oxoglutarate from glutamate (249, 385) (Fig. 5.2).

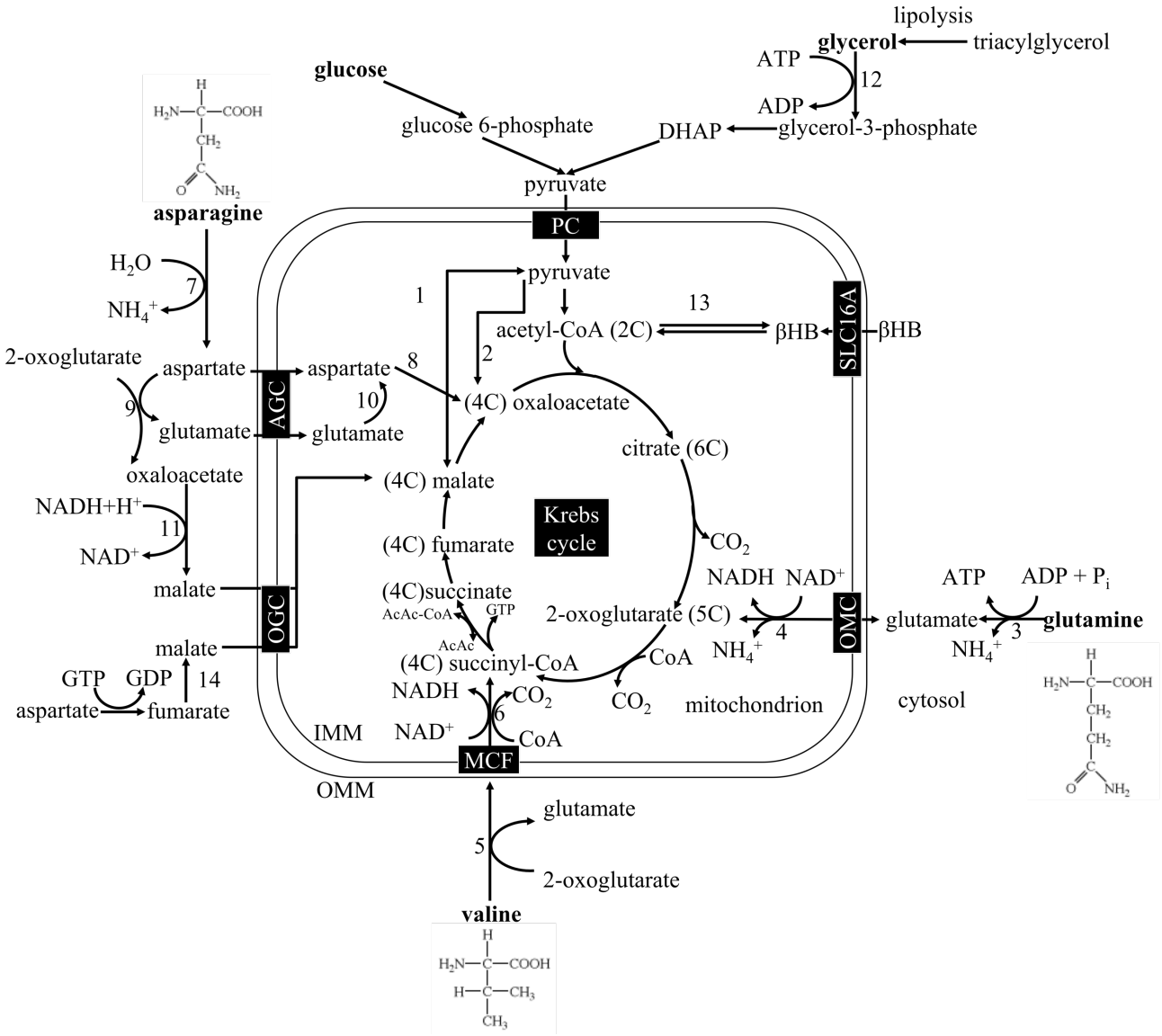


Figure 5.2. *Anaplerotic pathways of glucose, amino acids, and glycerol. Numbers refer to the enzymes catalysing the following reactions that will be referred to in the text: 1, malic enzyme; 2, pyruvate carboxylase; 3, glutaminase; 4, glutamate dehydrogenase; 5, branched-chain aminotransferase; 6, branched-chain α-ketoacid dehydrogenase (BCKDH); 7, asparaginase; 8, aspartate aminotransferase; 9, 10, glutamate oxaloacetate transaminase; 11, malate dehydrogenase; 12, glycerol kinase; 13, D-β-hydroxybutyrate dehydrogenase; 14, fumarase. PC, pyruvate carrier; OMC, oxoglutarate-malate carrier; MCF, mitochondrial carrier family; OGC, oxoglutarate carrier; AGC, aspartate/glutamate carrier; SLC16A, solute carrier 16A; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; NAD+(H), nicotinamide adenine dinucleotide oxidised (reduced); AcAc, acetoacetate; NH4+, ammonium; GDP, guanosine diphosphate; GTP, guanosine triphosphate, adenosine diphosphate, ADP; adenosine triphosphate, ATP; CoASH, coenzyme; AcAc, acetoacetate; AcAc-CoA, acetoacetyl-CoA; DHAP, dihydroxyacetone phosphate; OAA, oxaloacetate; βHB, D-β-hydroxybutyrate*

Glutamine at concentration of 2.5 mM can prevent a postischaemic decrease in cardiac function and restore myocardial metabolites to normal in the working rat heart (391). Glutamine at ~0.6 mM concentration delays (by 38s) the onset time of ST-segment depression in patients with chronic stable angina (386).

The plasma concentration of asparagine is 0.13 mM (250). In heart, asparagine can be converted into aspartate, via asparaginase (393), for the malate-aspartate shuttle, which balances reducing equivalents between cytosol and mitochondria (394) (Fig. 5.2). Aspartate can be converted into oxaloacetate via aspartate aminotransferase or glutamate oxaloacetate aminotransferase (32, 71, 221, 240). The heart can metabolise most of the amino acids through different pathways, making their involvement in metabolism difficult to quantify (250). For instance, aspartate from asparagine can also be converted into fumarate to replenish the Krebs cycle via the purine nucleotide cycle (395), and fumarate can be converted to malate by fumarase (396) (Fig. 5.2). A decrease in endogenous myocardial aspartate has been reported in a rat ischaemia-reperfusion injury model (397) and in patients during coronary artery surgery (398). Increasing aspartate transporter expression and activity (system X_{ag}-transporter, excitatory amino acid transporter EAAT3; SLC1A1: sodium-dependent transport system) facilitated aspartate entry into hypertrophied cardiomyocytes, thereby providing myocardial protection by maintaining ATP levels, and reducing lactate levels (399). Exogenous aspartate and glutamate provide myocardial protection after ischaemia and reperfusion injury by maintaining ATP, GDP and phosphocreatine levels (400, 401). Interestingly, inhibition of fatty acid uptake with the sarcolemmal fatty acid translocase (FAT/CD36) inhibitor, sulfo-N-succinimidyl oleate (SSO) increased fumarate and oxaloacetate potentially to replenish Krebs cycle intermediate in diabetic hearts (402).

The branched chain amino acids (BCAAs) include leucine (ketogenic), isoleucine (mixed glucogenic and ketogenic) and valine (glucogenic). Uniquely, their metabolism is sited in muscle rather than liver, and they undergo extensive oxidation in skeletal muscle. Valine is an essential amino acid. In muscle, valine is converted to its corresponding branched-chain alpha-ketoacid (BCKAs), α-ketoisovaleric acid, by a branched-chain amino-transferase (BCAT) which catalyses a reversible reaction, followed by an irreversible decarboxylation by branched-chain α-ketoacid dehydrogenase (BCKDH) complex to isobutyryl-CoA, then propionyl-CoA, and ultimately metabolized to succinyl-CoA for oxidation in the Krebs cycle (403) (Fig. 4.2). Defective BCAA metabolism is associated with abnormal cardiac development (404), poor glucose metabolism, and increased ischaemia-reperfusion injury (405). Succinyl-CoA from valine can be used to synthesise haem via δ-aminolevulinic acid synthase (ALAS; a potentially cataplerotic pathway), and protects ischaemia–reperfusion in rat hearts (406).

The availability of fatty acids for respiration is increased in starvation and diabetes (67, 346, 347, 407, 408) due to increased adipose tissue lipolysis, which is associated with an increase in the plasma level of glycerol (0.15 mM) (409, 410) (Fig. 5.1). Glycerol kinase is present in liver and is also expressed in rat heart to a significant extent (411, 412). This enzyme is absent from white adipose tissue, thus glycerol cannot be metabolized further in fat (413, 414). The tissue-specific glycerol kinase enables phosphorylation of glycerol to glycerol phosphate, which can be converted to pyruvate for anaplerosis (181) (Fig 5.2).

Pyruvate carboxylase is the major anaplerotic enzyme in the heart (46, 48). The work in Chapter 3 showed that anaplerosis of oxaloacetate from glycogen, via pyruvate carboxylase increased βHB oxidation in high glycogen hearts. Thus, βHB oxidation may be limited by low anaplerotic flux from pyruvate to oxaloacetate. Oxaloacetate, being unstable, cannot reliably be measured using current methodology. Indeed, oxaloacetate is only present

in small concentrations in the mitochondrion (5-10 nmol/g), and its turnover rate is very rapid, up to 800 times per minute (48). Oxaloacetate can be used for condensation with acetyl-CoA derived from βHB to form citrate, thereby facilitating the heart's ability to oxidise βHB. Hence the availability of glucogenic amino acids as alternative sources of ≥ 3-carbon compounds may affect the heart's ability to oxidise βHB (Fig. 5.2). The amino acids that can be metabolised into Krebs cycle intermediates are asparagine (into oxaloacetate), valine (into succinyl-CoA) and glutamine (into 2-oxoglutarate). The work in this Chapter aimed to investigate the facilitatory (anaplerotic) effect of key glucogenic amino acids at their physiological concentrations on myocardial βHB oxidation rates.

5.3 Methods

5.3.1 Isolated heart perfusion

Rat hearts were perfused at a constant aortic pressure of 80 mmHg and an end-diastolic pressure of 4-8 mmHg in the Langendorff mode (Fig. 3.3; Chapter 2.2.1) with non-recirculating Krebs-Henseleit (KH) buffer containing no glucose for 20 minutes in order to decrease the glycogen content (226, 307). This glucose-free buffer was aerated with 95% O_2: 5% CO_2 (v:v) at 37˚C. Hearts were subsequently perfused with KH buffer containing 2.5 or 11 mM glucose, 5 μCi [^{14}C]-βHB (55 mCi.mmol^{-1}) (Sigma®), 4 mM βHB, plus either: 1) a glucogenic amino acid (0.65 mM L-glutamine, or 0.31 mM L-valine, or 0.13 mM L-asparagine) or 2) 0.16 mM glycerol or 3) no additional substrate (Fig. 5.3). βHB oxidation was estimated by collecting the $^{14}CO_2$ evolved from the [^{14}C]-βHB in the perfusate and radioactivity was counted as described in Chapter 2.2.1.2. The hearts were subsequently freeze-clamped in liquid N_2-cooled Wollenberger (306) tongs at the end of the perfusion for glycogen (Chapter 2.2.1.3) and metabolomic analysis (Chapter 2.3 and Appendix A.1) (Fig. 5.3).

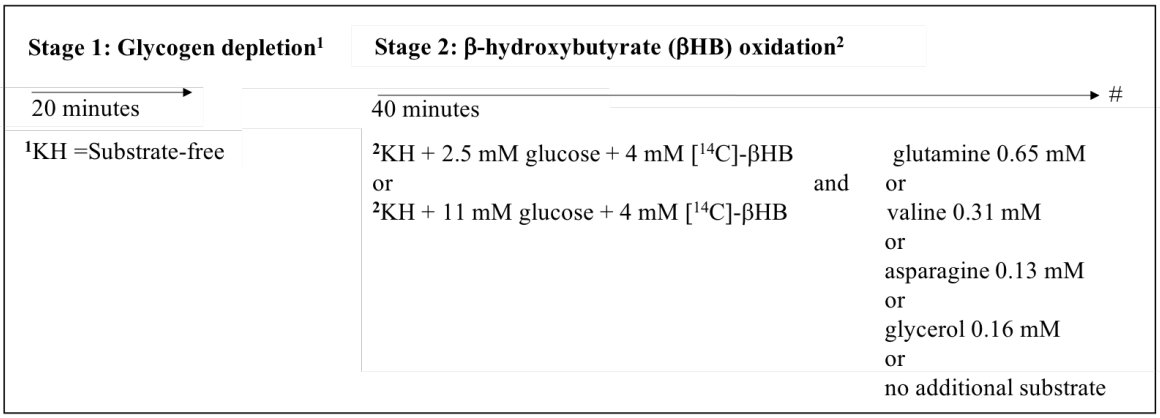


Figure 5.3 *Perfusion protocol for measurement of myocardial βHB oxidation in the presence of key glucogenic amino acids or glycerol. #, hearts freeze-clamped for subsequent analysis; KH, Krebs-Henseleit; βHB, D-3-hydroxybutyrate; [1], stage 1; [2], stage 2. βHB, D-β-hydroxybutyrate.*

5.3.2 Statistical analysis

Left ventricular function, myocardial glycogen and βHB oxidation rates were compared by two-way analysis of variance (ANOVA), with glucose and glucogenic amino acids as factors. Tukey *post hoc* corrections were made for multiple comparisons to identify specific significant differences. Levels of glycolytic and Krebs cycle intermediates were analysed by one-way ANOVA. Results, presented as means ± SEM unless otherwise specified, were considered significant at $p<0.05$. Statistical analysis of metabolomics is described in the text and Appendix A.1.

5.4 Results

5.4.1 The effect of glucogenic amino acids on left ventricular function

Rate-pressure product (RPP) was calculated as the product of left ventricular (LV) developed pressure (LVDP: peak LV pressure - end-diastolic pressure) and heart rate. RPP was recorded throughout the perfusion protocol. RPP was used to estimate left ventricular systolic function. In glycogen-depleted hearts perfused with 2.5 mM or 11 mM glucose plus βHB, addition of glucogenic amino acids did not significantly alter left ventricular function (Fig. 5.4).

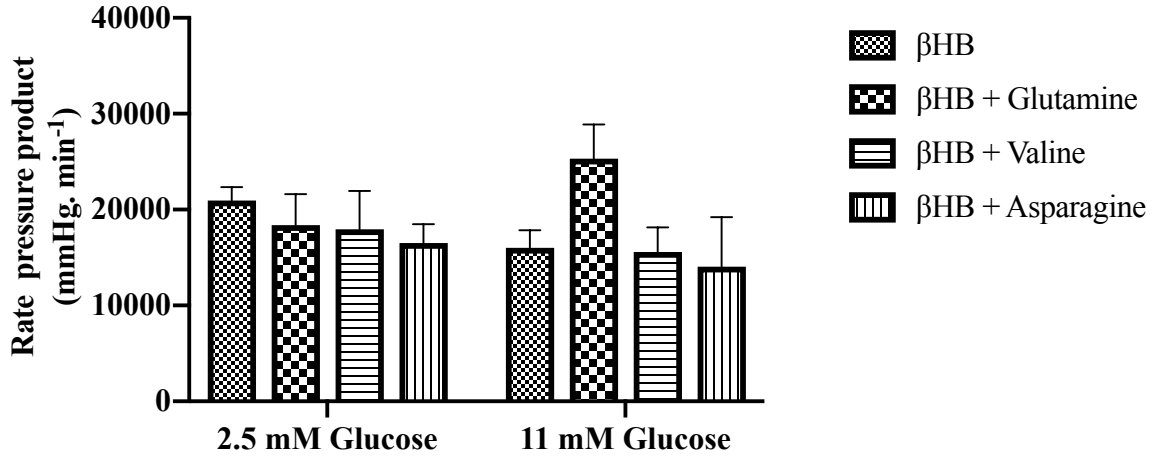


Figure 5.4 *The effect of glucogenic amino acids on left ventricular function. The hearts were perfused with Krebs-Henseleit buffer containing 2.5 mM glucose or 11 mM glucose plus either 4 mM βHB alone (βHB), or 4 mM βHB and 0.65 mM glutamine (βHB + Glutamine), or 4 mM βHB and 0.31 mM valine (βHB + Valine), or 4 mM βHB and 0.13 mM asparagine (βHB + Asparagine) (n=3-4). Values are means ± SEM. βHB, D-β-hydroxybutyrate*

5.4.2 The effect of glucogenic amino acids on myocardial glycogen content

Glycogen content was measured in frozen whole heart tissue (Chapter 2.2.1.3) at the end of the perfusion protocol. A subsequent perfusion of glycogen-depleted hearts (5.3 μmol glycosyl units. gww^{-1}, Fig. 3.7) with 2.5 mM glucose plus βHB did not increase the low glycogen content (9.6 μmol glycosyl units. gww^{-1}) (Fig. 5.5). In such circumstances, addition of glucogenic amino acids increased the glycogen content 2- to 3- fold, which was statistically significant in the case of glutamine and asparagine (Fig. 5.5). However, the glycogen content was lower than normal glycogen content (~40 μmol.glycosyl units.gww^{-1}, Fig. 3.7).

Generally, and expectedly, the glycogen content in hearts perfused with 11 mM glucose plus βHB was 3-fold higher than in hearts perfused with 2.5 mM glucose plus βHB (9.6 vs. 31.8 μmol glycosyl units. g ww^{-1}). In glycogen-depleted hearts perfused with 11 mM glucose plus βHB, addition of glucogenic amino acids did not increase glycogen content further, suggesting that glucose was preferentially oxidised following glycogen depletion. The glycogen content in hearts perfused with 11 mM glucose plus βHB and asparagine was 2-fold higher than in hearts perfused with 2.5 mM glucose plus βHB and asparagine (25.5 vs. 49.5 μmol.gww^{-1}.min^{-1}), achieving a normal glycogen content (~40 μmol.glycosyl units.gww^{-1}, Fig. 3.7). This effect did not achieve statistically significant in hearts perfused with either glutamine or valine (Fig 5.5).

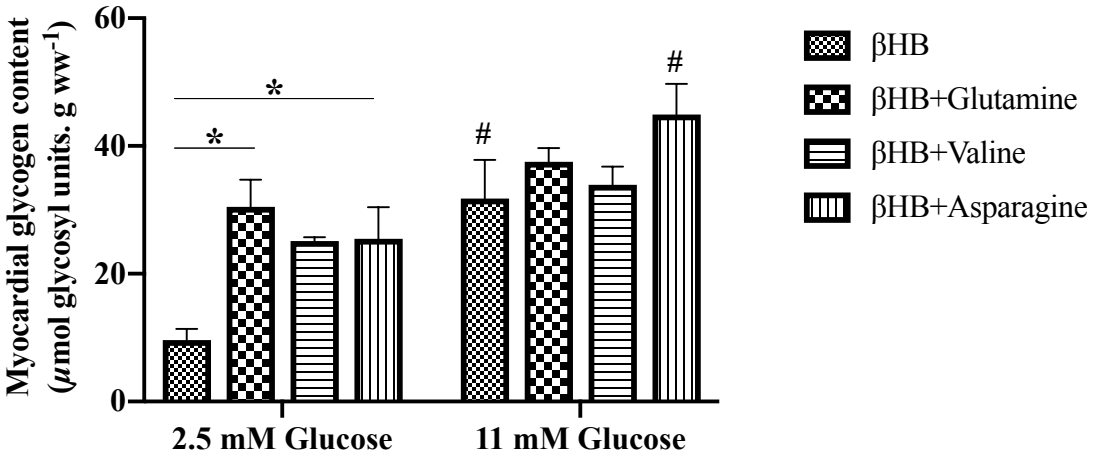


Figure 5.5 *Glycogen content of rat hearts perfused with glucogenic amino acids at different glucose concentrations. Myocardial glycogen content was initially depleted before perfusing with Krebs-Henseleit buffer containing 2.5 mM glucose or 11 mM glucose plus either 4 mM βHB alone (βHB), or 4 mM βHB and 0.65 mM glutamine (βHB+Glutamine), or 4 mM βHB and 0.31 mM valine (βHB+Valine), or 4 mM βHB and 0.13 mM asparagine (βHB+Asparagine) (n=3-4). Values are means ± SEM. Significant effect of glucogenic amino acids is indicated as: * P<0.05. Effect of glucose concentration (2.5 mM vs. 11 mM glucose) is indicated as: # P<0.05. Significance was taken at p < 0.05. βHB, D-β-hydroxybutyrate*

5.4.3 The effect of glucogenic amino acids and glycerol on myocardial β-hydroxybutyrate (βHB) oxidation rates

In glycogen-depleted hearts, addition of 2.5 mM glucose resulted in 0.2 μmol. min^{-1}. gww^{-1} βHB oxidation rates (Fig. 5.6). The addition of valine, glutamine, or glycerol did not alter βHB oxidation rates. However, asparagine increased βHB oxidation rates 2-fold to 0.4 μmol. min^{-1}. gww^{-1} (Fig. 3.7B), although the rate of βHB oxidation was lower than those in high glycogen (Fig. 3.7) . In glycogen-depleted hearts, addition of 11 mM glucose did not alter the low βHB oxidation rates, nor did perfusion with the glucogenic amino acids, suggesting that glucose was preferentially oxidised following glycogen depletion (Fig 5.6).

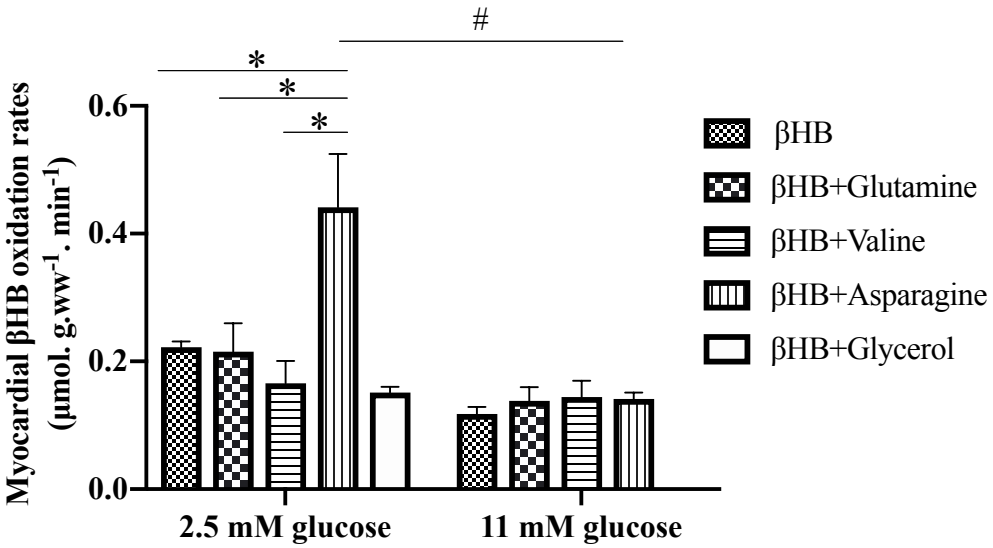


Figure 5.6 *βHB oxidation rates in rat hearts perfused with glucogenic amino acids at different glucose concentrations. Myocardial glycogen content was initially depleted before perfusing with Krebs-Henseleit buffer containing 2.5 mM glucose or 11 mM glucose, plus either 4 mM βHB alone (βHB), or 4 mM βHB and 0.65 mM glutamine (βHB + Glutamine), or 4 mM βHB and 0.31 mM valine (βHB + Valine), or 4 mM βHB and 0.13 mM asparagine (βHB + Asparagine). Additional hearts were perfused with 4 mM βHB and 0.16 mM glycerol at low (2.5 mM) glucose (n=3-4). Values are means ± SEM. Effect of glucogenic amino acids on myocardial βHB oxidation rates is indicated as: * P<0.05. Effect of glucose concentration on myocardial βHB oxidation rates is indicated as: # P<0.05. Significance was taken at p < 0.05. βHB, D-β-hydroxybutyrate.*

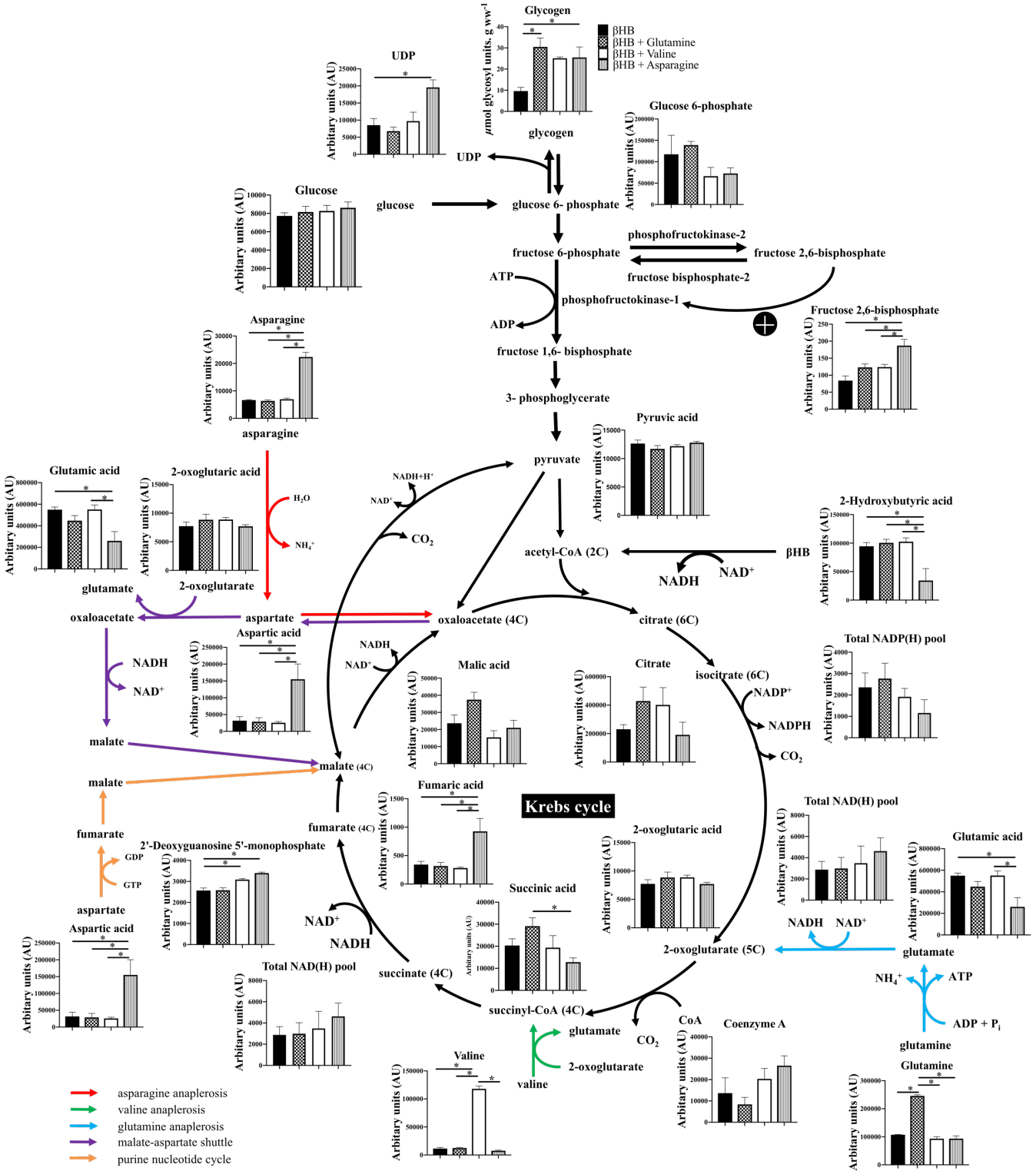
5.4.4 The effect of βHB oxidation on Krebs cycle intermediates in glycogen-depleted hearts perfused with glucogenic amino acids (targetted analysis)

Targetted metabolomic analysis was employed in order to determine the effect of key glucogenic amino acids and βHB oxidation on glycolytic and Krebs cycle intermediates in hearts lacking pyruvate precursors. Thus, glycogen-depleted hearts were perfused with Krebs-Henseleit buffer containing 2.5 mM glucose plus either 4 mM βHB alone (βHB), or 4 mM βHB and 0.65 mM glutamine (βHB + Glutamine), or 4 mM βHB and 0.31 mM valine (βHB + Valine), or 4 mM βHB and 0.13 mM asparagine (βHB + Asparagine). Concentrations of myocardial glycolytic and Krebs cycle intermediates were measured using metabolomic analysis (Chapter 2.3 and Appendix A.1).

Metabolomic analysis showed that the addition of glutamine, valine, and asparagine successfully increased myocardial levels of glutamine, valine, and asparagine, respectively, validating the methodology (Fig. 5.7). Myocardial levels of glucose and pyruvate were the same for all hearts.

Glutamine increased the glycogen content, but did not significantly alter myocardial levels of glutamate, total NAD(H) and NADP(H) pools or any Krebs cycle intermediates. Valine did not significantly alter any Krebs cycle intermediate nor free CoA levels, but increased 2'-deoxyguanosine 5'-monophosphate (a metabolomic derivative of GTP).

Addition of asparagine increased the glycogen content, which was associated with a significant increase in uridine diphosphate (UDP) and fructose 2,6-bisphosphate (a positive regulator of glycolysis). Only asparagine increased oxidation of 2-hydroxybutyric acid (βHB), via increased intermediary metabolites involved in the malate-aspartate shuttle and purine nucleotide cycle (aspartate, 2'-deoxyguanosine 5'-monophosphate, and fumarate), but did not alter others Krebs cycle intermediates, total NAD(H) and NADP(H) pools (Fig. 5.7).



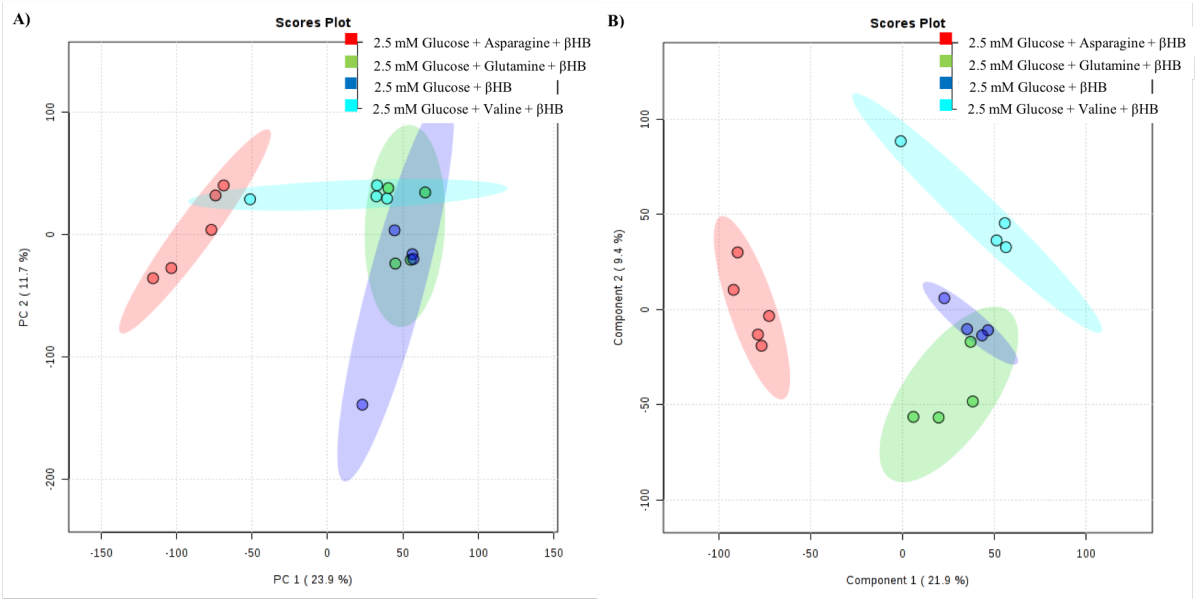
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Figure 5.7 *Myocardial levels of glycolytic and Krebs cycle intermediates in glycogen-depleted rat hearts perfused with 2.5 mM glucose plus βHB and glucogenic amino acids. The intermediates were measured using metabolomic analysis of tissue from hearts that had been perfused with Krebs-Henseleit buffer containing 2.5 mM glucose plus 4 mM βHB alone (βHB), or 4 mM βHB and 0.65 mM glutamine (βHB + Glutamine), or 4 mM βHB and 0.31 mM valine (βHB + Valine), or 4 mM βHB and 0.13 mM asparagine (βHB + Asparagine) (n=17). Values are means ± SEM. Significant effect of glucogenic amino acids is indicated as *P<0.05. Glycogen levels were measured by enzymatic analysis (Chapter 2.2.1.3). For clarity the malate-aspartate shuttle is shown in simplified diagrammatic form without mitochondrial transport mechanisms. OAA, oxaloacetate; βHB, D-β-hydroxybutyrate; CoA, coenzyme-A; UDP, uridine diphosphate.*

5.4.5 Multivariate statistical analysis (untargetted analysis)

Besides the targetted metabolomic analysis employed and predicted on the effect of glucogenic amino acids on the Krebs cycle (Chapter 5.4.4), untargetted metabolomic analysis was also performed in order to investigate other, unanticipated effects of glucogenic amino acids on βHB oxidation in glycogen-depleted hearts (312, 314). Data processing and normalization were performed using MetaboAnalyst (Appendices A.1.5 and A.1.6) prior to multivariate statistical analysis (principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA)). The PCA scores plot showed significantly greater discrimination between asparagine and other glucogenic amino acids (clusters of glutamine and valine appeared to overlap each other) when 68 identified compounds were selected (23.9 % vs. 11.7 % for the main principal component). No obvious outlier could be detected (Fig. 5.8 A). The discrimination between the groups from PLS-DA (21.9 % vs. 9.4 % for the main principal component) was significant as sum of squares captured by the model (R^2) value was closer to 1 and higher than cross-validated R^2 (Q^2) value (Chapter 8.6.1) (Fig. 5.8 B). Sparse PLS-DA showed a better separation between the cluster of glucose and the glucogenic amino acids (21.9 % vs. 7.8 %). A cluster of glutamine still appeared to overlap with a cluster of valine. However, there was still a separation between the cluster of asparagine and glucose (Fig. 5.8 C).

Significance analysis of microarray (SAM) showed that there was a significant difference between glycogen-depleted hearts perfused with βHB plus glucogenic amino acids (‘case’) and glycogen-depleted hearts perfused with βHB alone (‘control’) as the expected proportion of “discoveries” to be false is very small (false discovery rate (FDR) < 0.05, Appendix A.1.5) (Fig. 5.8 D).



PLS-DA cross validation details:

Measure	1 component	2 component
Accuracy	0.29412	0.29412
R2	0.67806	0.94679
Q2	0.34048	0.39296

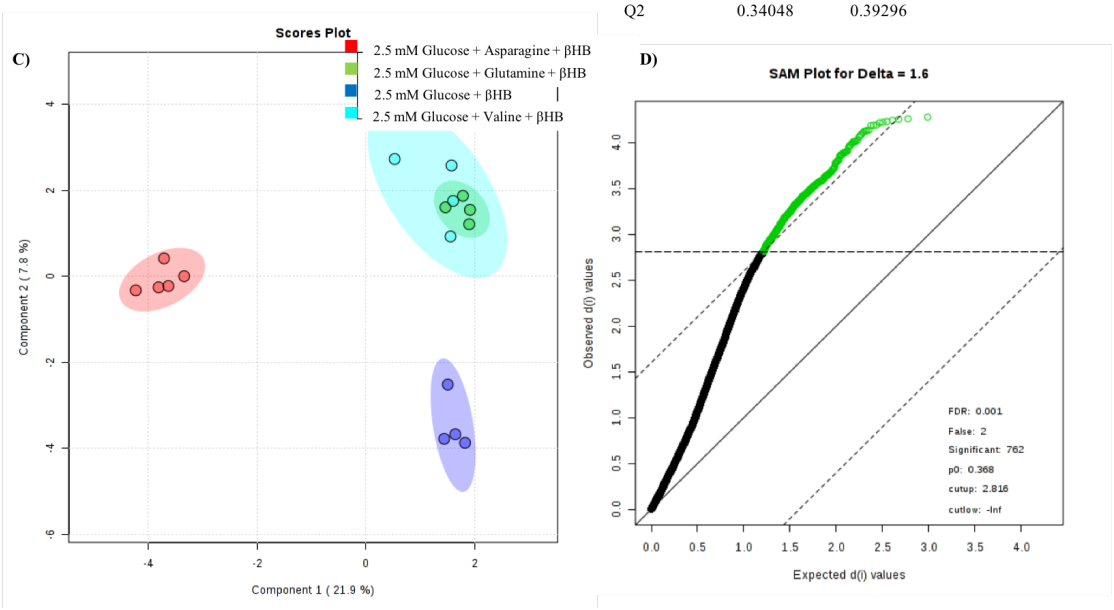


Figure 5.8 *Multivariate data analysis of the metabolome in glycogen-depleted rat hearts perfused with 2.5 mM glucose plus βHB and glucogenic amino acids. The hearts were perfused with Krebs-Henseleit buffer containing 2.5 mM glucose plus either 4 mM βHB alone (2.5 mM Glucose + βHB), or 4 mM βHB and 0.65 mM glutamine (2.5 mM Glucose + Glutamine + βHB), or 4 mM βHB and 0.31 mM valine (2.5 mM Glucose + Valine + βHB), or 4 mM βHB and 0.13 mM asparagine (2.5 mM Glucose + Asparagine + βHB). (A) Principle component analysis (PCA) score plot. (B) Partial least squares-discriminant analysis (PLS-DA) score plots was assessed by sum of squares captured by the model (R^2), cross-validated R^2 (Q^2), prediction accuracies through cross-validation (accuracy). (C) Sparse partial least squares-discriminant analysis score plot. (D) Significance analysis of microarray (SAM) plot. βHB, D-β-hydroxybutyrate.*

5.4.6 A clustering-based approach to the metabolome (untargetted analysis)

The fold change of the metabolome affected by the addition of glucogenic amino acids was reorganized from a non-hierarchical (Fig. 5.9 A) to a hierarchical clustering heat-map (Fig. 5.9 B) for better visualisation. A distinctive change of the metabolome was observed in glycogen-depleted hearts perfused with asparagine, but not in the hearts perfused with glutamine or valine (Fig. 5.9 B). The hierarchical clustering dendrogram indicates the similarities between the samples. Most of hearts perfused with glutamine or valine grouped with samples treated with glucose alone, but not with asparagine (Fig. 5.9 C).

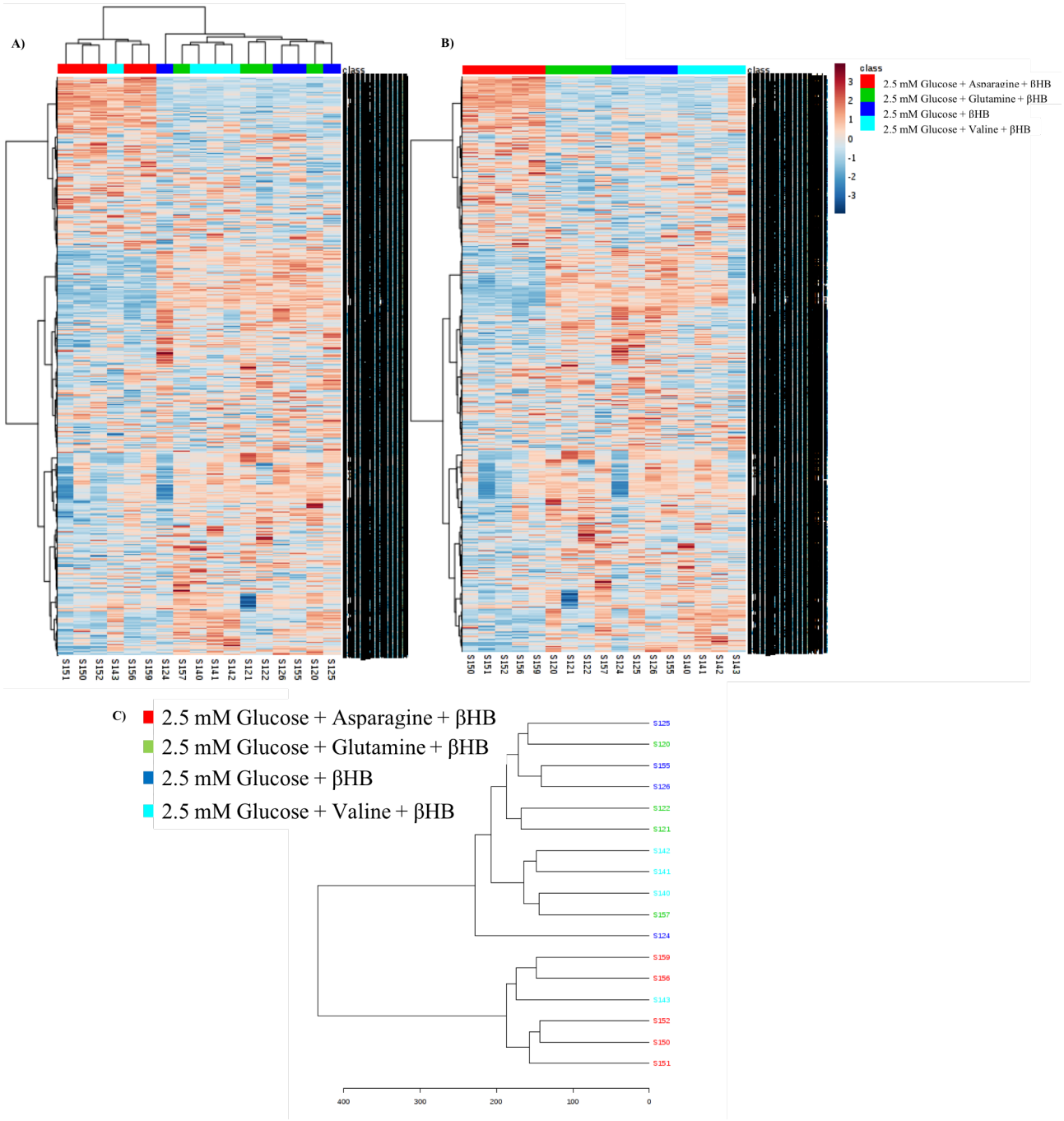
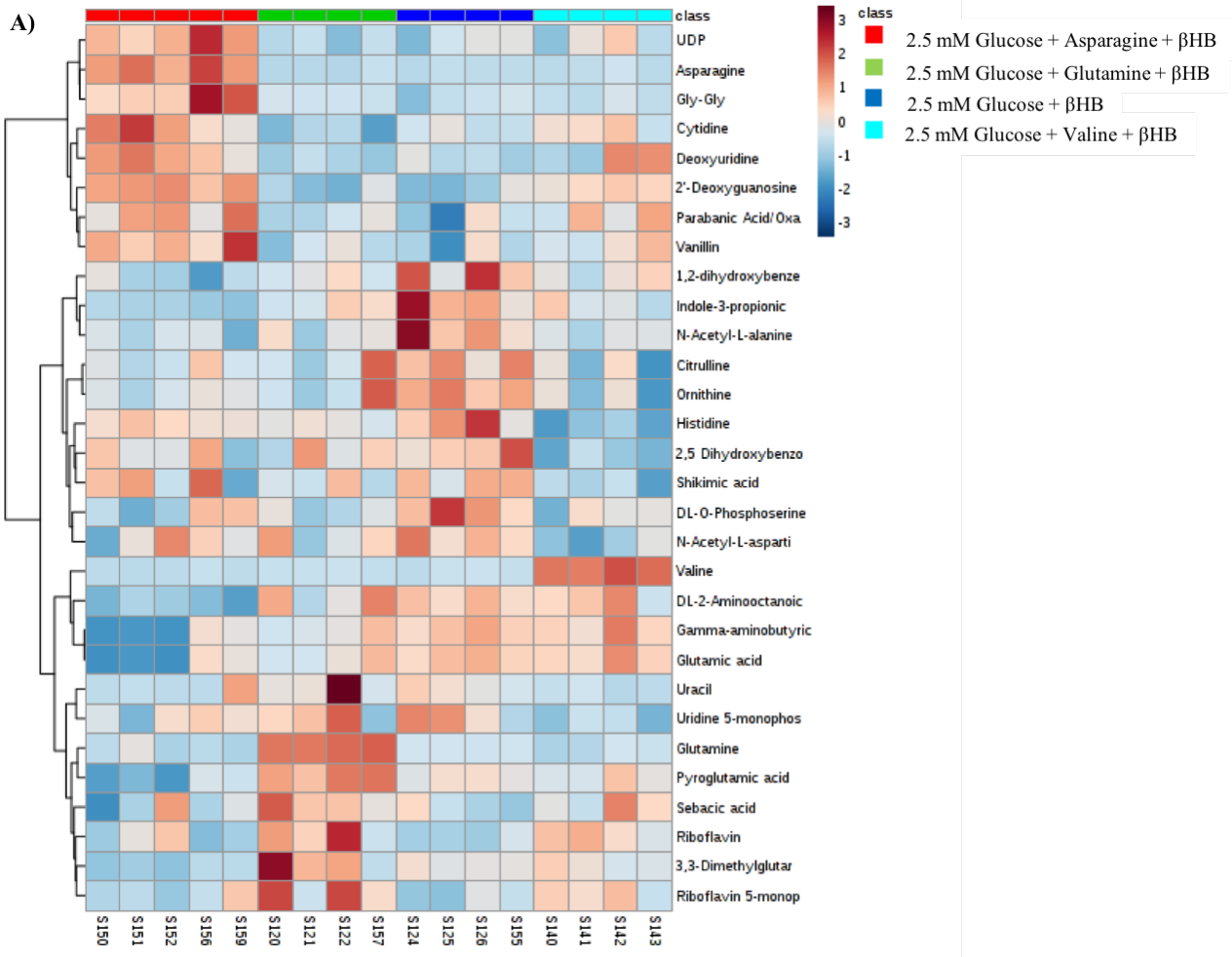


Figure 5.9 *A cluster-based approach of the metabolome in glycogen-depleted rat hearts perfused with 2.5 mM glucose plus βHB and glucogenic amino acids. The hearts were perfused with Krebs-Henseleit buffer containing 2.5 mM glucose plus either 4 mM βHB alone (2.5 mM Glucose + βHB), or 4 mM βHB and 0.65 mM glutamine (2.5 mM Glucose + Glutamine + βHB), or 4 mM βHB and 0.31 mM valine (2.5 mM Glucose + Valine + βHB), or 4 mM βHB and 0.13 mM asparagine (2.5 mM Glucose + Asparagine + βHB). (A) A non-hierarchical clustering heat-map shown as fold change regardless of the group. Red colour represents an expression level above mean, blue colour represents an expression lower than the mean. (B) A hierarchical clustering heat-map shown as fold change according to group. (C) A dendrogram depicting hierarchical clustering results of samples from A and B. βHB, D-β-hydroxybutyrate.*

5.4.7 The effect of glutamine, valine, or asparagine on the specific compounds in glycogen-depleted hearts perfused with 2.5 mM glucose plus βHB

The effect of glutamine, valine, or asparagine on the specific compounds was listed in a hierarchical clustering heat-map (Fig. 5.10 A). The variable importance in projection (VIP) showed a list of the top 15 identified compounds affected by the addition of glucogenic amino acids, which are indole-3-propionic acid, 3,3-dimethylglutaric acid, cytidine, 2'-deoxyguanosine 5'-monophosphate, glycylglycine, asparagine, histidine, DL-2-aminooctanoic, pyroglutamic acid, valine, glutamic acid, arginine, creatine, N-Acetyl-L-alanine, and fumaric acid, in order of decreasing number (mean decrease in accuracy; the decrease in model accuracy from important feature) (Fig. 5.10 B).



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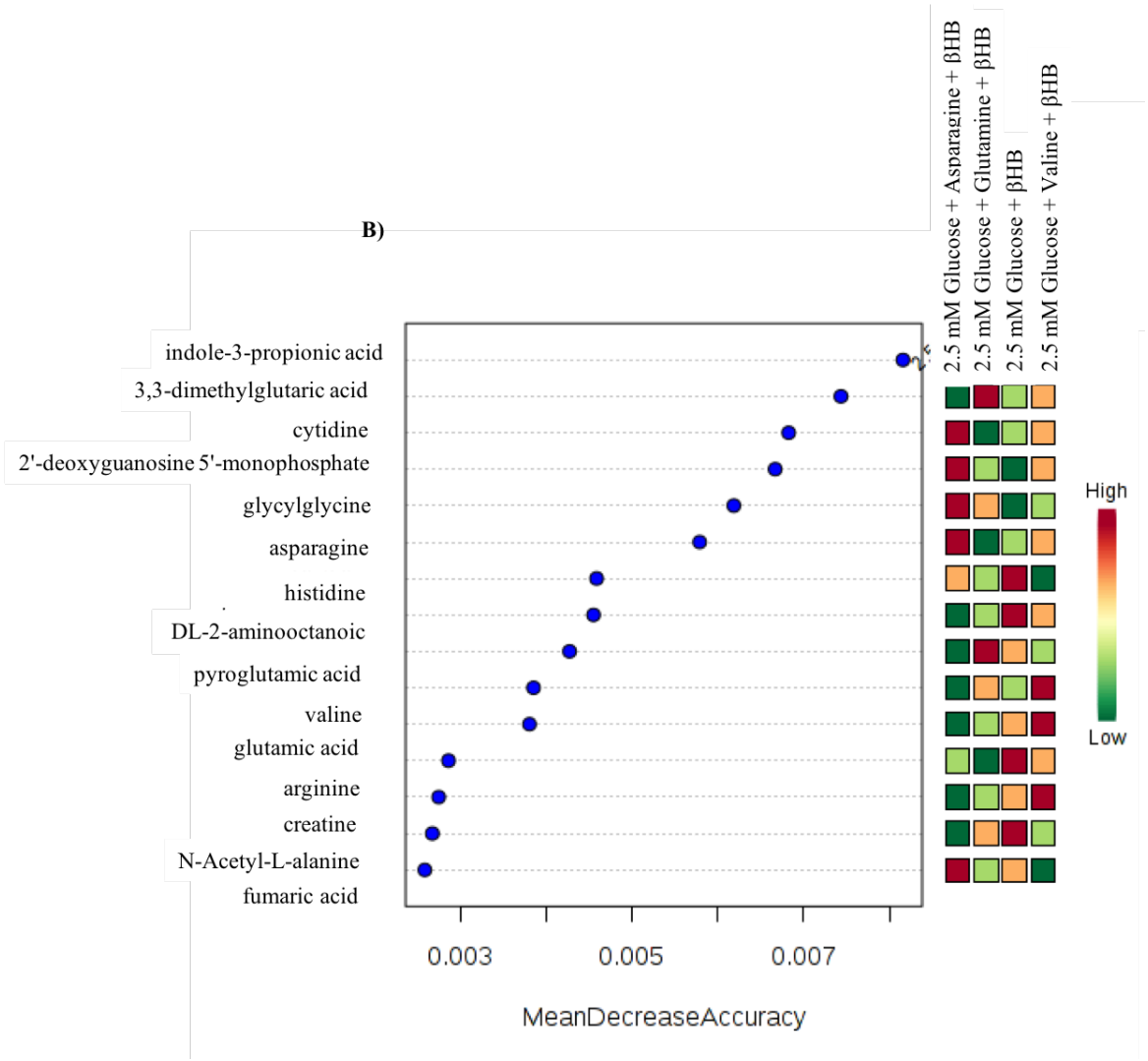


Figure 5.10 *The effect of glutamine, valine, or asparagine on the specific compounds in glycogen-depleted hearts perfused with 2.5 mM glucose plus βHB. The hearts were perfused with Krebs-Henseleit buffer containing 2.5 mM glucose plus either 4 mM βHB alone (2.5 mM Glucose + βHB), or 4 mM βHB and 0.65 mM glutamine (2.5 mM Glucose + Glutamine + βHB), or 4 mM βHB and 0.31 mM valine (2.5 mM Glucose + Valine + βHB), or 4 mM βHB and 0.13 mM asparagine (2.5 mM Glucose + Asparagine + βHB). A cluster-based approach was used to identify specific compounds in glycogen-depleted rat hearts perfused with 2.5 mM glucose plus βHB and glucogenic amino acids. (A) A hierarchical clustering heat-map shown as fold change of identified compounds that are only significantly different. A fold-change threshold of 1.5 is used to determine significance. (B) The top 15 compounds ranked by variable importance in projection (VIP) scores based on mean decrease in accuracy (MDA). Red colour represents an expression level above mean, blue colour represents an expression lower than the mean. βHB, D-β-hydroxybutyrate.*

5.4.8 Metabolic pathway analysis of identified compounds in glycogen-depleted hearts perfused with asparagine (untargetted analysis).

Metabolic pathway analysis bypasses the typical bottleneck of compound or metabolite identification by linking compound features with metabolic pathway. Untargetted analysis (PCA, PLS-DA, sPLS-DA) showed that the cluster of asparagine was separated from the cluster of glucose. The addition of asparagine caused a significant fold change in the levels of identified compounds. Thus, metabolomic pathway analysis (high-level functional interpretation) was performed to improve pathway interpretation from untargetted mass spectrometry (MS) data. Asparagine significantly altered metabolic pathways ($p<0.05$) that also had high impact values (pathway impact), which include glutamate and glutamine metabolism, alanine, aspartate and glutamate metabolism, pyrimidine metabolism, purine metabolism, purine metabolism, arginine and proline metabolism, pentose phosphate pathway, and galactose metabolism (Fig. 5.11 A). Furthermore, the prediction of pathway activity from a ranked untargetted mass spectral data using the *mummichog* (*Fundulus heteroclitus*, a small Atlantic killifish) algorithm showed that asparagine significantly altered pathways ($p<0.05$) that also had high impact values (enrichment factor), which include glutamine and glutamate metabolism, arginine and ornithine metabolism, valine, leucine and isoleucine biosynthesis, glycine, serine and threonine metabolism, propionate metabolism, amino-acyl-tRNA biosynthesis, and unsaturated fatty acid synthesis (Fig. 5.11 B).

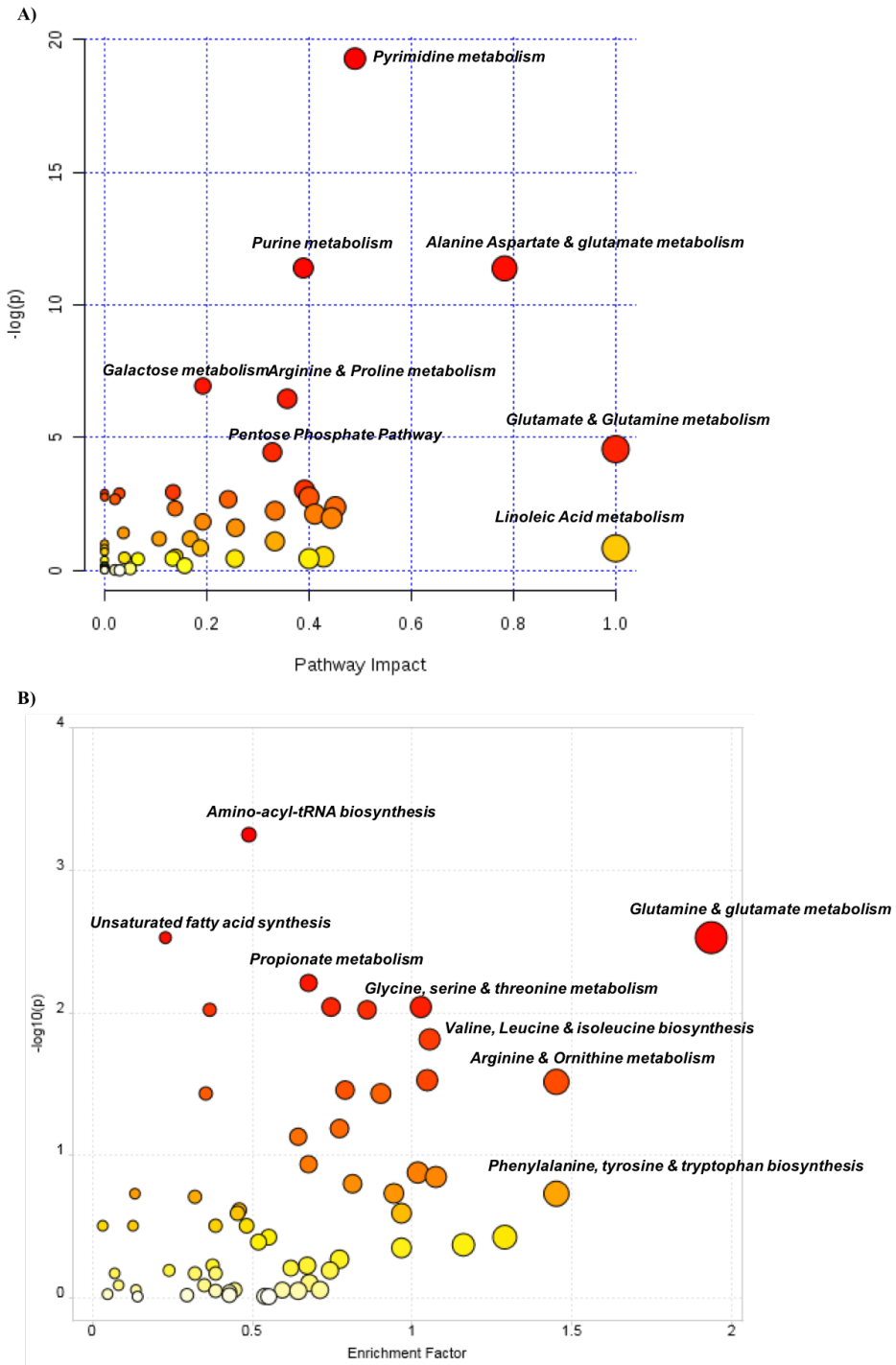


Figure 5.11 *Metabolic pathway analysis of identified compounds in glycogen-depleted rat hearts perfused with 2.5 mM glucose plus βHB and asparagine. (A) The 'metabolome view' showing all metabolic pathways arranged according to the scores (p value, colour) from pathway enrichment analysis (y-axis) and pathway impact values from topology analysis (x-axis, circle). (B) Prediction of pathway by mummichog algorithm. βHB, D-β-hydroxybutyrate.*

5.5 Discussion

The work in Chapter 3 showed that glycogen, an anaplerotic source of oxaloacetate, increased myocardial βHB oxidation, although oxaloacetate is unstable, and hence cannot reliably be measured by the current methodology. Thus, this study was developed in order to be able to define anaplerosis by decreasing pyruvate precursors with subsequent selective perfusion with alternative anaplerotic substrates. The main source of pyruvate is glucose, either directly from the circulation/perfusate, or through glycogen (alanine is also transaminated to pyruvate, but was not investigated here since glucose and glycogen as pyruvate precursors had already been studied (Chapter 3). Limiting glucose availability decreased the glycogen content to very low levels (Chapter 3). Thus, the remaining glycogen from exogenous 2.5 mM glucose was preferentially oxidised after glycogen depletion, which resulted in low βHB oxidation rates of 0.2 μmol. min^{-1}. gww^{-1}. Asparagine increased βHB oxidation rates 2-fold, via intermediary metabolites involved in the malate-aspartate shuttle and purine nucleotide cycle (aspartate, 2'-deoxyguanosine 5'-monophosphate, and fumarate). However, the rate of βHB oxidation was still lower than those with high glycogen (Fig. 3.7). Thus, βHB oxidation did not alter total NAD(H) and total NADP(H) pool. Neither glutamine nor valine (both also potential anaplerotic agents, but entering the Krebs cycle at different points) increased βHB oxidation rates under such conditions. On metabolomic analysis, the cluster of asparagine had much better separation than that of glutamine or valine determined by PCA and PLS-DA (untargetted analysis). Clusters of glutamine and valine appeared to overlap, indicating a similar, potentially anaplerotic effect of glutamine and valine on βHB oxidation rates. In glycogen-depleted hearts perfused with 2.5 mM glucose and βHB, asparagine decreased glycogenolysis, via increased the positive regulator of glycolysis, fructose 2,6-bisphosphate, via stimulating 6-phosphofructo-1-kinase (PFK-1). Asparagine also increased the level of UDP, which increased glycogenesis. The glycogen is

was still lower than in normal glycogen (~25 vs ~40 μmol glycosyl units.gww^{-1}). However, all glucogenic amino acids did not further increase glycogen content in hearts perfused with 11 mM glucose and βHB. This could potentially be explained by glycogen having reached an upper limit (~40 μmol glycosyl units.gww^{-1}). Importantly, glycogen synthesis was prioritised over βHB oxidation following glycogen depletion. Pyruvate carboxylase is the major anaplerotic enzyme and glycogen significantly increased for βHB oxidation in high glycogen hearts (Chapter 3). In conclusion, anaplerosis of oxaloacetate from asparagine increased βHB oxidation in glycogen-depleted hearts via increased intermediary metabolites involved in the malate-aspartate shuttle and purine nucleotide cycle, but did not alter total NAD(H) pool. The anaplerotic potential of succinyl-CoA from valine and 2-oxoglutarate from glutamine did not augment myocardial βHB oxidation. Glucose was preferentially oxidised following glycogen depletion. However, asparagine could provide synergistic effect on βHB oxidation in the heart if glycogen is successfully maintained.

5.5.1 Glucogenic amino acids increased the myocardial glycogen content

Following glycogen depletion, increasing glucose concentration to 11 mm plus βHB restored the glycogen content. There was no significant difference in glycogen content between hearts perfused with glucose-free buffer (5.3 μmol glycosyl units. g ww^{-1}, after glycogen depletion, Chapter 3) and glycogen-depleted hearts perfused with 2.5 mM glucose plus βHB alone (9.6 μmol glycosyl units. g ww^{-1}). This indicates that the glycogen content was low at the start of the low glucose plus βHB alone perfusion. Exogenous (perfusate) glucose from subsequent perfusion of hearts with 2.5 mM glucose plus βHB was likely required for anaplerosis to facilitate even a relatively low rate of myocardial βHB oxidation rates (0.2 μmol. min^{-1}. g.ww^{-1}) and there was inadequate exogenous glucose to replenish glycogen content, resulting in the low glycogen content observed. This result confirms that 2.5 mM glucose is a

limitingly-low concentration and an appropriate "dose" to 'force' the heart into using alternative substrates.

The metabolomic analysis showed that the addition of glutamine, valine, or asparagine successfully increased myocardial levels of glutamine, valine, or asparagine, respectively, validating the protocol. These compounds were also among the top 15 compounds identified by metabolomic analysis. Provision of glucogenic amino acids in the perfusate does permit storage of some of the exogenous glucose into glycogen. Exogenous glucose can be either oxidised or used for anaplerosis, but can also be used to replenish glycogen. Addition of glutamine, valine and asparagine increased glycogen content in glycogen-depleted hearts perfused with 2.5 mM glucose. Asparagine specifically increased glycogen synthesis by increasing UDP level measured by metabolomics. During glycogen synthesis, glycogen synthase transfers uridine diphosphate-glucose residues onto this polymeric glycogen strand via α-1,4-glycosidic linkage (bonds), which releases UDP (323, 324). However, the glycogen content was still lower than in those in normal heart (~25 vs ~40 μmol.glycosyl units.gww^{-1}). Addition of 11 mM glucose plus βHB and asparagine increased glycogen content to ~40 μmol. glycosyl units.gww^{-1}) (307). This data suggests that glycogen synthesis is prioritised over others metabolic pathway and glucose is preferentially oxidised in the hearts lacking pyruvate precursors (328). Glycogen prevents myocardial injury by maintaining sufficient glycolytic ATP for the Na^+ pump to keep intracellular Na^+ low, despite increased Na^+-H^+ exchange (307, 335). Therefore, it is important to maintain the normal glycogen content in order to provide anaplerotic substrate for βHB oxidation in the heart (Chapter 3).

5.5.2 The effect of glucogenic amino acids on glycolytic intermediates

Interestingly, in low (2.5 mM) exogenous glucose conditions, the addition of asparagine significantly increased the positive regulator of glycolysis, fructose 2,6-bisphosphate. The

increased fructose 2,6-bisphosphate was also associated with significantly increased glycogen (2-fold, measured by enzymatic analysis). This could be explained by the increased fructose 2,6-bisphosphate activating PFK-1, which catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate for glycolysis, thereby decreasing glycogenolysis (415). However, the upper limit of glycogen could only be achieved by perfusing hearts with 11 mM glucose plus βHB and asparagine (although the glycolytic and Krebs cycle intermediates were not measured using metabolomic analysis). The work in Chapter 3 showed that βHB decreased glycolysis in all hearts at baseline 0.2 μmol. $gww^{-1}.min^{-1}$ (Fig. 3.8). Thus, βHB oxidation in hearts perfused asparagine decreased glycolysis at baseline, but, glycolytic intermediates are not depleted by maintaining fructose 2,6-bisphosphate (68).

In glycogen-depleted hearts perfused with 11 mM glucose plus βHB, the addition of various glucogenic amino acids did not further increase glycogen. This could potentially be explained by glycogen having reached an upper limit, as this glycogen content (~40 μmol glycosyl units.gww^{-1}) was approximately similar to in high glycogen hearts (Chapter 3.4.2). It has been reported that glutamine increased myocardial glycogen resynthesis during postischaemic reperfusion, potentially via increasing cytosolic citrate levels (267): a potent inhibitor of glycolysis at the site of phosphofructokinase-1 (416). However, the addition of valine did not significantly increase glycogen. Moreover, the level of glucose and pyruvate remained unchanged with the addition of glucogenic amino acids, thereby potentially maintaining the Krebs cycle activity as the cardiac function (measured by rate-pressure product, RPP) was unchanged during low glycolytic flux. Endogenous triacylglycerol could be utilised to maintain cardiac function under such condition (72). A similar cardiac function was also observed in glycogen-depleted hearts perfused with high (11 mM) glucose. The work in Chapter 4 showed that ATP was similar in all hearts measured by ^{31}P-NMR spectroscopy, as reported by many studies (370, 372-374). The heart can oxidise a variety

of carbon-based fuels for ATP synthesis to ensure that [ATP] remains relatively constant despite varying ATP turnover rates (278).

Interestingly, under conditions of carbohydrate sufficiency (11 mM glucose), myocardial βHB oxidation rates were not increased, despite the hearts having, under these conditions, adequate anaplerotic precursors, including pyruvate sources. This study suggests that glycogen synthesis is prioritised over βHB oxidation. Thus, the heart is preferentially deriving most of its energy from glucose oxidation (although this was not directly measured in these experiments) following glycogen depletion.

5.5.3 The effect of glucogenic amino acids on Krebs cycle intermediates and myocardial βHB oxidation rates

The metabolomic analysis showed that addition of exogenous 4-carbon asparagine increased the myocardial level of asparagine. The increased asparagine was associated with an increase in aspartate and decrease in glutamate levels- intermediary metabolites that are involved in the malate-aspartate shuttle through the action of asparaginase and glutamate oxaloacetate transaminase (45). In the cytosol, asparaginase catalyses the hydrolysis of asparagine to aspartate (417). Aspartate aminotransferase catalyses the conversion of aspartate and 2-oxoglutarate to oxaloacetate and glutamate (418). Cytosolic NADH is oxidised by the reduction of oxaloacetate to malate by malate dehydrogenase. The reducing equivalents and malate are transported into the mitochondrial matrix via the malate/aspartate shuttle (419). Malate is converted back to oxaloacetate via malate dehydrogenase. In the Krebs cycle, the 2-carbon acetyl group of acetyl-CoA derived from βHB is completely oxidised to two CO_2, with no net change in any of the intermediates. Addition of asparagine significantly increased the βHB oxidation rates, determined by quantification of $^{14}CO_2$ (gaseous $^{14}CO_2$ and $H^{14}CO_3^-$) production. Metabolomic analysis also confirmed the utilisation of 2-hydroxybutyric acid (βHB). It was reported that perfusion of hearts with

acetoacetate (AcAc) (45) or βHB (420) was associated with a decrease in aspartate. It was reported that the addition of 2 mM asparagine increased acetoacetate oxidation (45). It has previously been postulated that oxaloacetate limits acetoacetate oxidation (45). Thus, the addition of asparagine significantly increased myocardial βHB oxidation rates, potentially via replenishing oxaloacetate through the malate-aspartate shuttle (45).

The increase in myocardial βHB oxidation rates and βHB utilisation following the addition of exogenous asparagine was associated with an increase in the levels of 2'-deoxyguanosine 5'-monophosphate (a metabolomic derivative of GTP) and fumarate: intermediary metabolites that are involved in the purine nucleotide cycle (395). During muscle contraction, the decrease in ATP concentration increases adenosine monophosphate (AMP). AMP can generate inosine monophosphate (IMP), which couples with guanine triphosphate (GTP) to generate fumarate from aspartate (421). Fumarate can be converted to malate by fumarase (396). Malate is transported into the mitochondrion, where it can be used to produce oxaloacetate for condensation with acetyl-CoA from βHB. Thus, oxaloacetate is the Krebs cycle intermediate required for condensation with acetyl-CoA to form citrate, other Krebs cycle intermediates having to be converted to oxaloacetate before citrate synthesis can occur, and its availability is key to Krebs cycle activity and hence rate. Addition of asparagine as a precursor for oxaloacetate therefore critically determined the Krebs flux (48), which increased βHB oxidation. However, the rate of βHB oxidation was lower than those in high glycogen hearts (Fig. 3.7). Thus, βHB oxidation was unable to alter total NAD(H), total NADP(H) pool, or any Krebs cycle intermediates, which could not increase cardiac energetic (although it was not measured in this study). Glycogen, via the major anaplerotic enzyme, pyruvate carboxylase (46, 48) significantly increased βHB oxidation, which increased ATP hydrolysis (Chapters 3 and 4). However, asparagine could

alternate with glucose to provide anaplerotic substrate for βHB oxidation only when glycogen is successfully maintained (422).

Exogenous glutamine increased myocardial levels of glutamine measured by metabolomic analysis. However, glutamine did not significantly increase the myocardial level of glutamate, perhaps explained by the level of glutaminase activity being high in the heart (268). It has been reported that glutaminase activity depends upon the levels of ATP (392, 423), whereby ATP was presumably low in glycogen-depleted hearts (preserving the RPP; Fig. 5.4). Both metabolomic analysis and radioactive ([^{14}C]-βHB) labelling studies showed that glutamine did not increase βHB oxidation rates, which was associated with similar total NAD(H) pool, 2-oxoglutarate, succinate, malate and citrate. Although Taegtmeyer and his colleagues postulated that additional anaplerotic substrates, such as glucose and lactate reversed the contractile failure in hearts perfused with acetoacetate due to inhibition of the enzyme 2-oxoglutarate dehydrogenase (45), they also found that the beneficial effect was not associated with improved flux through 2-oxoglutarate dehydrogenase (47). One study showed that the effect of glutamine was not mediated by anaplerosis, but most likely via the hexosamine biosynthesis pathway (HBP) (424), a branch of glucose metabolism that is responsible for the production of key substrates for protein glycosylation (234, 425). Lauzier et al. 2013 also reported that no definitive data exist demonstrating that glutamine acts as an anaplerotic substrate, as only 5% of 2-oxoglutarate was labelled when hearts were perfused with [U-$^{13}C_5$]-glutamate under normoxia and low-flow ischaemia (426). In cancer studies, the contribution of glutamine to the Krebs cycle is minor under physiological conditions *in vivo* (427, 428). The synthesis of glutamate from the deamidation of glutamine in the isolated working rat heart could not be detected (429).

Metabolomic analysis showed that the addition of physiological concentrations of valine (0.31 mM) (250) successfully increased myocardial levels of valine. However, valine

did not significantly increase either myocardial levels of 2-hydroxybutyric acid (βHB) nor βHB oxidation rates. Ketone bodies are readily oxidised by most body tissues (68) including heart. βHB is oxidised to acetoacetate (AcAc), then AcAc is activated to acetoacetyl-CoA by CoA transferase [succinyl-CoA:3-oxoacid-CoA transferase (SCOT)] (68). Provision of succinyl-CoA from valine (430) is required for the conversion of acetoacetate to acetyl-CoA (68) although this is not strictly an anaplerotic mechanism. It was therefore expected that valine would facilitate βHB oxidation rates. However, the metabolomic analysis showed that addition of valine did not increase levels of any Krebs cycle intermediate nor CoA. One early study showed that perfusion of hearts with 3-carbon propionate (0.5 mM), another precursor of succinyl-CoA, did not alter acetoacetate oxidation (45). It was reported that propionate shifted the redox state of the mitochondrial NAD^+/NADH redox couple in the direction of βHB production (although ketone bodies had not reached equilibrium with the mitochondrial NAD couple by the end of the perfusion) (45). This present study showed that valine did not alter total NAD(H) pool. It was argued that perfusion of hearts with acetoacetate alone was associated with a decrease in free coenzyme A caused by the formation of CoA-thioesters, acetoacetyl-CoA and acetyl-CoA (45, 431), hence inhibiting the 2-oxoglutarate dehydrogenase as well as limiting acetoacetate oxidation (432). However, the changes in these metabolites are mainly based on steady-state Krebs cycle intermediates concentrations, which depend on numerous factors, such as enzyme activities, concentration of reactants, compartmentation and turnover rates (48). Although the decrease in total coenzyme A level was due to a decrease in mitochondrial coenzyme A (45), the inhibitory effect of acetoacetate in the 2-oxoglutarate dehydrogenase is still unclear, as 2-oxoglutarate dehydrogenase was not found to be regulated by acetoacetate, nor acetoacetyl-CoA, nor acetyl-CoA (45), potentially explaining why succinyl-CoA from valine did not facilitate the ability of the heart to oxidise βHB. The addition of glucose or lactate reversed contractile

dysfunction in hearts perfused with acetoacetate, but did not improve flux through 2-oxoglutarate dehydrogenase (45, 47). Additionally, valine was used as an alternative to glucose to maintain action potential duration (269). A study of oxoacid analogues of valine showed branched-chain 2-oxoacids were predominantly decarboxylated in the heart, but are transaminated in the brain (433). Metabolomic analysis showed that clusters of glutamine and valine appeared to overlap. This is in agreement with our previous radioactive labelling studies, which showed that βHB oxidation were similar in both groups.

Besides glucogenic amino acids acting as anaplerotic candidates, glycerol is also an attractive candidate as an anaplerotic substrate since it is released, with non-esterified fatty acids (NEFA), during adipose tissue lipolysis and could provide the means to permit oxidation of the accompanying fatty acid in those tissues that utilise FAs, but which can also phosphorylate glycerol: in this scheme, adipose tissue would provide both (2 carbon equivalent) oxidative fuel (fatty acid) and the anaplerotic means to metabolise it (glycerol). Therefore, hearts were only perfused with glycerol at low (2.5 mM) glucose. During starvation, the activity of glycerol kinase in rat liver is 117 μmol of L-3-glycerophosphate formed/g. fresh wt. of tissue/hr (434). In rat heart, the activity of glycerol kinase is 0.65 μmol/g fresh wt of tissue/h (411, 412). Although glycerol from lipolysis can be converted to glucose 3-phosphate via glycerol kinase to generate pyruvate for anaplerosis, the present study showed that glycerol did not increase βHB oxidation rates at low glucose concentrations. This could potentially be explained by the contribution of glycerol being relatively minor with the increase in fatty acids in fasting and increased energy demand in the heart (435). For all these reasons, glycogen and metabolites were not measured in the heart perfused with glycerol plus 2.5 mM glucose.

Increasing glucose concentrations did not increase myocardial βHB oxidation rates. One explanation for this is that 11 mM glucose may hold preference over 4 mM βHB and

different glucogenic amino acids following glycogen depletion: a condition when the healthy heart is stressed. Glycogen synthesis is prioritised over other metabolic pathway following glycogen depletion (435). Ingestion of carbohydrates decreased the excretion of ketoacids and ammonium in starving human (40, 436).

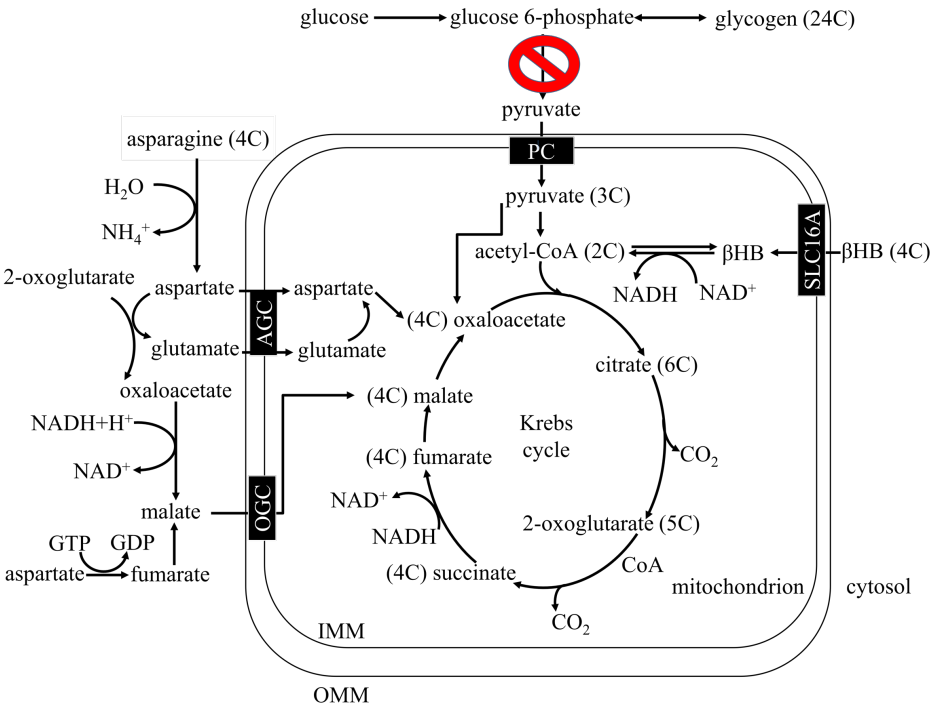


Figure 5.12 *Putative mechanism through which asparagine increased βHB oxidation, via the malate-aspartate shuttle and purine nucleotide cycle. Consequently, βHB oxidation increased mitochondrial redox potential in hearts lacking pyruvate precursors. PC, pyruvate carrier; OGC, oxoglutarate carrier; AGC, aspartate/glutamate carrier; SLC16A, solute carrier 16A; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; NAD^+(H), nicotinamide adenine dinucleotide oxidised (reduced); AcAc, acetoacetate; NH_4^+, ammonium; GDP, guanosine diphosphate; GTP, guanosine triphosphate, adenosine diphosphate, ADP; adenosine triphosphate, ATP; CoA, coenzyme; βHB, D-β-hydroxybutyrate.*

In summary, myocardial βHB oxidation was low in hearts lacking pyruvate precursors (low glycogen, low glucose). The addition of asparagine increased βHB oxidation under such conditions, potentially via the malate-aspartate shuttle and purine nucleotide cycle, but was unable to alter total NAD(H) or any Krebs cycle intermediates. Anaplerosis via succinyl-CoA from valine and 2-oxoglutarate from glutamine did not augment βHB oxidation.

Chapter 6: Substrates, loading conditions and efficiency in the isolated working rat heart

6.1 Abstract

High fat feeding decreases rat cardiac efficiency (the ratio between hydraulic work and oxygen consumption) by increasing oxygen consumption (293), whereas ketone bodies, D-β-hydroxybutyrate (βHB) plus acetoacetate (AcAc) increases cardiac efficiency by increasing hydraulic work (44). During increased workloads, the healthy heart can increase left ventricle contractile power and myocardial oxygen consumption, which requires a proportional increase in the generation of NADH and $FADH_2$ from energy fuel substrates. This study was designed to determine the effects of D-β-hydroxybutyrate plus acetoacetate (the effect of redox state), fatty acids, and loading conditions on cardiac efficiency. Here, the efficiency of working rat hearts perfused with fatty acids or βHB plus AcAc, was determined at low and high pre- and afterloads. Hearts were perfused with 11 mM glucose alone, or with 0.4 mM palmitate, or with βHB plus AcAc at low and high pre- and afterloads. Hydraulic work was calculated using the aortic and coronary flow rates together with systolic pressures. Oxygen consumption was calculated using arterio-venous differences and coronary flow rates. Cardiac efficiency was calculated as the ratio between hydraulic work and oxygen consumption. At low and high pre- and afterloads, fatty acids decreased cardiac efficiency by increasing oxygen consumption and decreasing hydraulic work. At high pre- and afterloads, βHB plus AcAc increased efficiency which was on average ~17 % by increasing hydraulic work. This did not occur at low pre- and afterloads since hydraulic work was the same. The redox state of βHB and AcAc did not affect cardiac efficiency. In conclusion, ketone bodies increased cardiac efficiency more than glucose alone or with fatty acids at high pre- and afterloads.

6.2 Introduction

The mechanical activity of the heart can be increased by adrenaline (epinephrine) stimulation (10) or transition from a Langendorff retrogradely-perfused non-working heart to an arterially perfused pumping ("working") preparation (221, 437). During increased heart work, carbohydrate are preferentially oxidised with similar exogenous fatty acid oxidation (103). High fat feeding decreases rat cardiac efficiency by increasing oxygen consumption (293) whereas D-β-hydroxybutyrate (βHB) increases cardiac efficiency by increasing hydraulic work (44). Neither fatty acids (72) nor ketone bodies (45) can serve as the sole substrate for the heart due to Krebs cycle intermediate depletion (cataplerosis) as an anaplerotic source (e.g. from glucose) is also required. During increased workload, left ventricle contractile power and myocardial oxygen consumption are higher than during the baseline state, which requires a proportional increase in NADH and $FADH_2$ generation from energy substrate oxidation. D-β-hydroxybutyrate can reduce mitochondrial Q, which may increase cardiac efficiency (the ratio between hydraulic work and oxygen consumption).

The P/O ratio of oxidative phosphorylation represents the number of moles of ATP synthesized per mole of oxygen used by the mitochondrial electron transport chain (22). The ratio varies according to the energy substrate used for generation of NADH and $FADH_2$ (361, 438). Stoichiometrically, complete oxidation of palmitate generates 129 molecules of ATP and consumes 50 atoms of oxygen (439), whereas the complete oxidation of D-β-hydroxybutyrate generates 21 molecules of ATP and consumes 8 atoms of oxygen (22, 361). Although the use of fatty acids, which are highly reduced, can generate the greater amount of ATP per molecule, this comes at the expense of a greater oxygen requirement than the use of the more oxidised β-hydroxybutyrate. Thus, the relative P/O ratio of palmitate is less than that of β-hydroxybutyrate, indicating a less "oxygen-efficient" energy substrate for ATP production. Metabolism of D-β-hydroxybutyrate and acetoacetate are more "oxygen-

efficient" than fatty acids (palmitate) and pyruvate, but less "oxygen-efficient" than glucose (12, 440) (Table 6.1). The reduction of acetoacetate to βHB by β-hydroxybutyrate dehydrogenase (BDH) involves oxidation of NADH, generating NAD^+ (62, 310); oxidation of βHB to acetoacetate by BDH involves a reduction of the free mitochondrial NAD^+, generating NADH for more ATP production (62, 310).

Table 6.1 *Metabolic oxygen efficiency and heat of combustion of different energy substrates.*

Substrates	Molecules of ATP produced (P)	Atoms of oxygen reduced (O)	Metabolic oxygen efficiency (P/O ratio)	Heat of combustion (ΔH^o kcal/mol C_2 units)
Palmitate ($C_{16}H_{32}O_2$)	129	50	2.6	-298.0
Glucose ($C_6H_{12}O_6$)	36	12	3.0	-223.6
D-β-hydroxybutyrate ($C_4H_8O_3$)	21	8	2.6	-243.6
Ethyl acetoacetate ($C_6H_{12}O_3$)	20	7	2.8	-225.2
Pyruvate ($C_3H_4O_3$)	14	6	2.3	-185.7

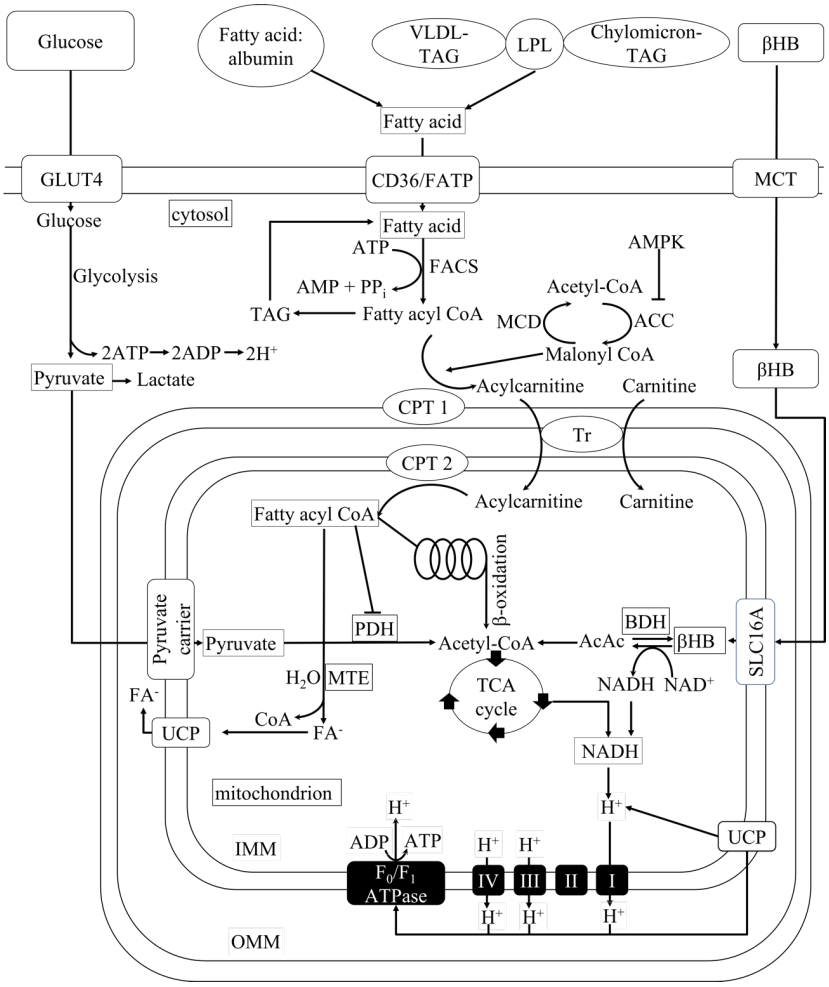


Figure 6.1 *Overview of glucose, fatty acid and ketone body metabolism in the heart. Increased fatty acid oxidation can decrease cardiac efficiency via activation of uncoupling protein (UCPs). UCPs dissipate the high proton concentration in the mitochondrial intermembrane space by transferring the proton back into the mitochondrial matrix. When the supply of fatty acyl-CoA exceeds the rate of fatty acid β-oxidation, excess fatty acyl-CoA can be hydrolyzed to yield free CoA and a fatty acid anion (FA^-) by mitochondrial thiesterases (MTE). FA^- may leave the mitochondrial matrix via UCP; hence oxygen consumption occurs without ADP phosphorylation ('uncoupling') lowering P/O ratio and metabolic inefficiency results. Increased fatty acid oxidation can also decrease cardiac efficiency by inhibiting pyruvate dehydrogenase (PDH) for glucose oxidation and increasing glycolysis that can cause intracellular acidosis. Oxidation of D-β-hydroxybutyrate (βHB) can increase redox energy by generating nicotinamide adenine dinucleotide (NADH) for adenosine triphosphate (ATP) production. BDH, D-β-hydroxybutyrate dehydrogenase; AcAc, acetoacetate; SLC16A, solute carrier 16A; GLUT4, glucose transporter 4; βHB, D-β-hydroxybutyrate; MCT, monocarboxylate transporter; LPL, lipoprotein lipase. PDH, pyruvate dehydrogenase; VLDL, very-low-density lipoprotein; TAG, triacylglycerol; FACS, fatty acyl-CoA synthase; CPT, carnitine palmitoyltransferase; Tr, carnitine-acylcarnitine translocase; ACC, acetyl CoA carboxylase; MCD, malonyl-CoA decarboxylase; AMPK, AMP-activated protein kinase; MTE, mitochondrial thiesterases; CoA, coenzyme A; ADP, adenosine diphosphate; F_o/F_1 ATPase, ATP synthase; H^+, hydrogen ion; I, II, III, IV, complexes I-IV of the electron transport chain, respectively.*

Moreover, if glucose were burned in a bomb calorimeter, it would liberate 224 kcal/mole of C_2 units, whilst the combustion of D-β-hydroxybutyrate would liberate 244 kcal/mole of C_2 units, or 9% more calories per C_2 unit than glucose (301). However, the combustion of D-β-hydroxybutyrate produces less energy per two carbon moiety than long-chain fatty acids (palmitate). Thus, palmitate is mechanical "energetically" more efficient than D-β-hydroxybutyrate (298 vs. 244 kcal/mole of C_2 units) (301, 440) (Table 6.1). Notably, fatty acid β-oxidation produces only two carbon units: acetyl-CoA, thereby the reaction decreases Krebs cycle intermediates (cataplerosis). Fatty acid oxidation is less efficient with regard to ATP synthesis. During oxidation of fatty acids, the reducing equivalents are released in the respiratory chain at NADH dehydrogenase. $FADH_2$ from fatty acids oxidation donates electrons to electron transfer flavoprotein (ETF) for the reduction of ubiquinone to ubiquinole, which does not pump protons (12, 301, 440).

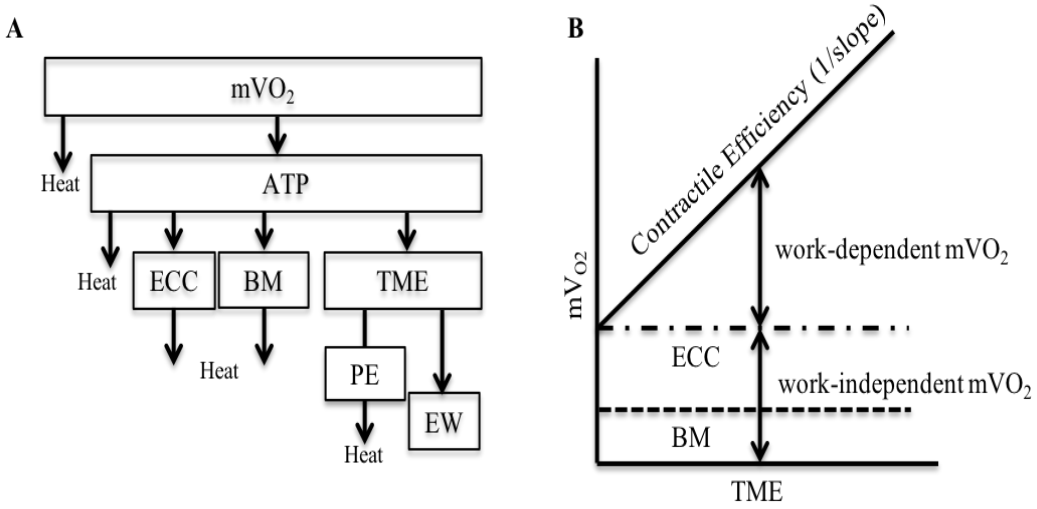


Figure 6.2 *Cardiac mechanical efficiency. The heart converts the chemical energy of ATP into heat and mechanical energy. ATP is used for non-mechanical activity, such as excitation-contraction coupling (ECC) and basal metabolism (BM), and for total mechanical energy (TME), including potential energy (PE) and external work (EW). At each level, part of the energy is dissipated as heat (**A**). A linear relationship between TME and mVO_2 (**B**). The Y-intercept of work - mVO_2 relationship indicates the oxygen cost for non-mechanical processes. The inverse of the slope indicates cardiac mechanical efficiency (441).*

The heart is highly dependent on optimal substrate metabolism with efficient energy production and utilisation to sustain its continuous pumping activity (i.e. the efficiency of energy production by metabolism is closely related to the efficiency of cardiac mechanical function) (6, 362). Cardiac efficiency is expressed as the ratio between hydraulic work (external work) and myocardial oxygen consumption (mV_{O2}) (442). Energy is required for both mechanical and non-mechanical processes in the heart (Fig. 6.2) (441).

The heart uses ATP for non-mechanical work, which includes excitation-contraction coupling (ECC; such as calcium pumping in cardiac sarcoplasmic reticulum), and basal metabolism (BM; such as oxygen utilisation for maintaining mitochondrial membrane potential against proton leakage, for generating ATP required for protein synthesis, and for maintaining transmembrane Na^+ and Ca^{2+} gradients (443)).

The beating heart uses energy for total contraction or total mechanical energy (TME) (Fig. 6.2), in which ATP is used to generate force by cross-bridge cycling of actin and myosin filaments. TME includes the generation of myocardial tension in the ventricular wall (potential energy, PE) and pressure in the left ventricle for ejection of blood against an afterload pressure (external work, EW). Under normal conditions, EW accounts for 20-25% of total oxygen consumption (444). Each component of mechanical and non-mechanical work determines the overall cardiac efficiency (441). In heart failure, decreased expression and activity of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) can increase sarcolemmal Na^+-Ca^{2+} exchange activity, which decreases Ca^{2+} transport during ECC, thereby decreasing cardiac efficiency (441).

During increased workloads, the healthy heart can increase left ventricle contractile power and myocardial oxygen consumption up to 6-fold of the resting values, which requires a proportional increase in the generation of NADH and $FADH_2$ from substrates, including fatty acid and ketone body oxidation. However, the question as to whether ketone bodies

can hold hierarchical preference over fatty acids under such conditions has not yet been examined (for example, how would cardiac efficiency be affected by increased workload-during ketotic states). Fatty acids (72) and ketone bodies (45) are both cataplerotic, hence an anaplerotic source from glucose is required (72, 304, 445). Here, the effects of substrates and loading conditions on cardiac efficiency (the ratio between hydraulic work and oxygen consumption) in working rat heart were determined in the presence of glucose.

6.3 Methods

6.3.1 Measurement of efficiency in the working rat heart

All animals studies are described in Chapter 2.1. Hearts from anaesthetised fed rats were excised and rapidly arrested in ice-cold Krebs-Henseleit (KH) buffer. The aorta and left atrium were individually cannulated enabling antegrade cardiac perfusion: warmed, oxygenated buffer entered the left atrium, flowed into the left ventricle and was ejected through the aorta (and coronary arteries). The first 50 ml of buffer was discarded to remove the blood and other contaminants, leaving a final circulating volume of 200 ml. During stabilization, the initial left-ventricular filling pressure (preload) was set to 15 cm H_2O (11 mmHg), whereas the afterload was set to a height of 80 cm H_2O (58 mmHg). Hearts were then perfused with KH buffer containing either 11 mM glucose alone, or 11 mM glucose plus 0.4 mM palmitate (pre-bound to 1% (w:v) albumin), or 11 mM glucose plus a mixture of either 4 mM (R)-(-)-3-hydroxybutyric acid sodium salt (βHB) (Sigma®) and 1 mM sodium acetoacetate (AcAc), or mixture of 1 mM βHB and 4 mM AcAc, under varying loading conditions. The aim of using different mixture of ketone bodies was to distinguish the effect of substrate availability and redox state since βHB can reduce mitochondrial Q. Preparation of sodium acetoacetate and βHB were described in Chapter 2.2.2.1. For low pre- and afterloads, hearts were switched to working mode with a 15 cm H_2O preload (left ventricular filling pressure) and 60 cm H_2O afterload (aortic pressure) for 15 minutes (221). For high pre- and afterloads, hearts were switched to working mode with a 25 cm H_2O preload and 100 cm H_2O afterload for 15 minutes (44, 221, 293). The hearts were subsequently freeze-clamped in liquid N_2-cooled Wollenberger tongs (306) at the end of the perfusion (Fig. 6.3).

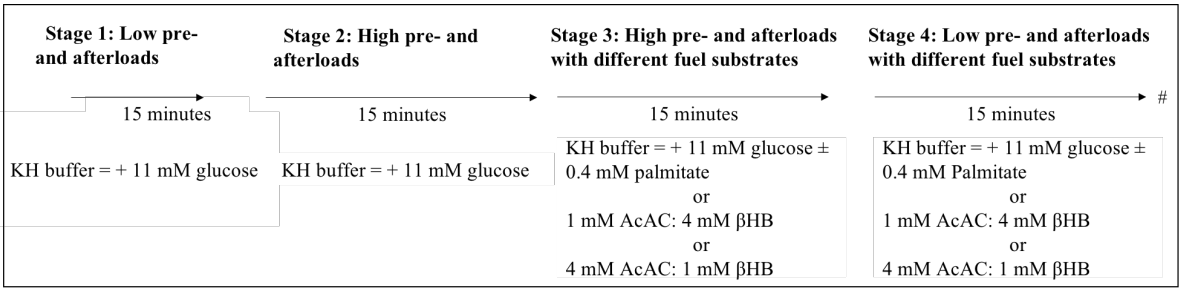


Figure 6.3 *Perfusion protocol for measurement of cardiac efficiency at low and high pre- and afterloads. The hearts were initially perfused with 11 mM glucose at low pre- (15 cm H_2O) and afterloads (60 cm H_2O) for 15 minutes and switched to high pre- (25 cm H_2O) and afterloads (100 cm H_2O) for another 15 minutes. At the high pre- and afterloads, the hearts were then perfused with either 0.4 mM palmitate (fatty acid, FA) or a mixture of 4 mM acetoacetate (AcAc) and 1 mM D-β-hydroxybutyrate (βHB) or a mixture of 4 mM β-hydroxybutyrate and 1 mM acetoacetate for another 15 minutes and switched to low pre- and afterloads for 15 minutes (n=24). # , hearts freeze-clamped for analysis; KH, Krebs-Henseleit.*

6.3.2 Physiological measurement

Aortic and coronary outflow rates were measured volumetrically ($ml.min^{-1}$). Aortic flow rates were calculated by measuring the time taken for the heart to eject 10 ml of perfusate. Coronary flow rates were calculated from the time taken to collect 10 ml of perfusate leaving the heart through the coronary circulation. Cardiac output was the sum of aortic and coronary flow rates. Systolic and diastolic pressure, and heart rate were measured using a pressure transducer attached to a bridge amplifier and PowerLab data acquisition system (AD Instruments, United Kingdom). Oxygen extraction was determined by measuring real-time arterial and venous oxygen content using Microx TX2-AOT flow-through cell-housed oxygen microsensors (Presens, Precision Sensing-GmbH, Germany) (Fig. 6.4). The system was calibrated prior to use with 0% O_2 (using the reducing agent 4% w/v sodium hydrosulphite) and 95% O_2 gassed (95% O_2 : 5% CO_2) water (446). One oxygen sensor probe was placed in the aortic inflow arterial line. Another oxygen sensor probe was placed in the coronary venous line at the (enclosed) heart chamber (Fig. 6.4). The oxygen content of arterial and venous perfusate was recorded as percentage air saturation. Bunsen's

solubility coefficient (22.7 uL O_2/mL at 1 atm) is the volume of gas, measured at 0°C, 760 mmHg, dissolved in a volume of liquid saturated with the gas under 760 mmHg pressure (447, 448), the coefficient used to calculate the oxygen content of the perfusate.

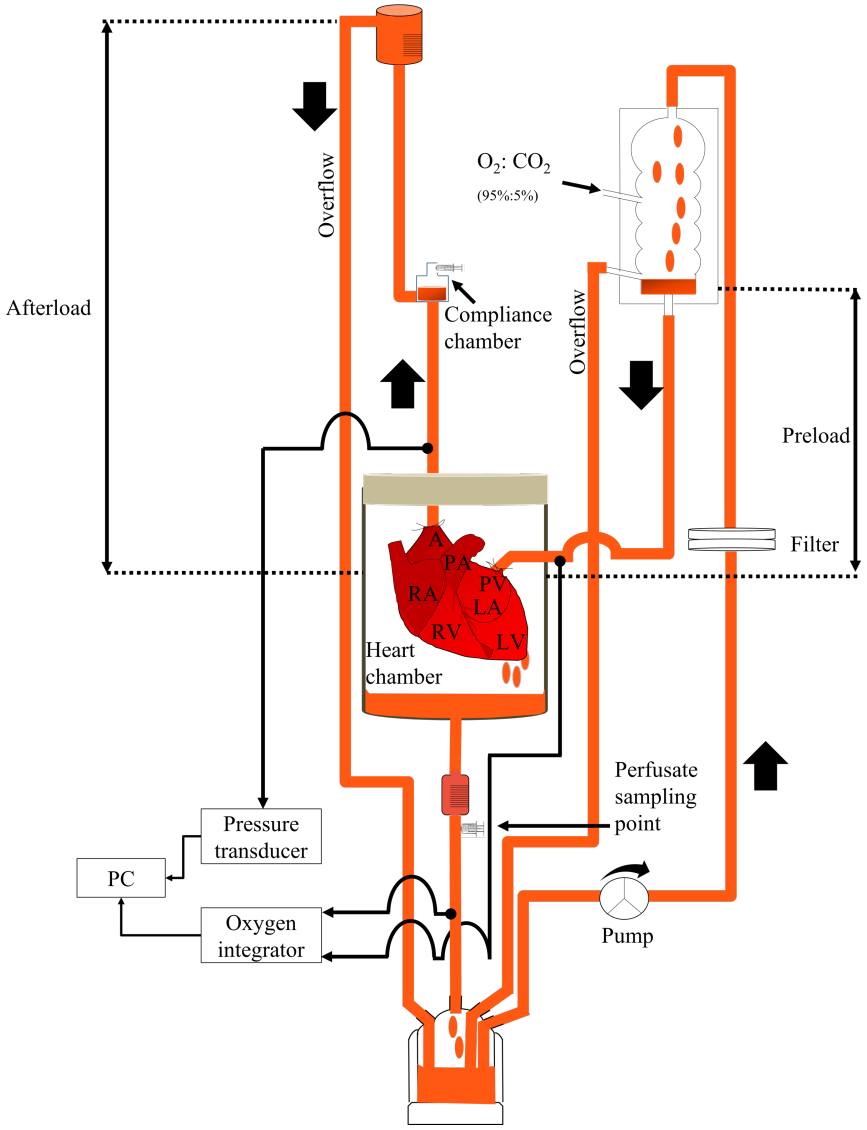


Figure 6.4 *Isolated working heart apparatus. Perfusion medium reached the heart directly from the preload line via a left-atrial cannula. The heart pumped perfusate out of the aorta up to the overflow chamber, as indicated, by the afterload line. Aortic flow rate was then calculated after measuring the time taken for the heart to eject 10 ml of perfusate in the overflow chamber. Coronary flow rates were calculated from the time taken to collect 10 ml of perfusate leaving the heart through the coronary circulation. Coronary flow rates were collected at the bottom of the perfusion chamber and the coronary venous effluent was fed back to the reservoir to be recirculated. A, aorta; RA, right atrium; RV, right ventricle; PA, pulmonary artery; PV, pulmonary vein; LA, left atrium; LV, left ventricle; PC, computer.*

Hydraulic work was calculated as:

Cardiac hydraulic work ($J.min^{-1}.\ g^{-1}$) =

$$\frac{[aortic + coronary\ flow\ rates\ (ml.min^{-1})] \times\ 10^{-6}(m^3.ml^{-1}) \times systolic\ pressure\ (mmHg)}{heart\ dry\ weight\ (g)} \times \frac{1010{,}325(Nm^{-2})}{760\ (mmHg)}$$

(Eq. 1)

Oxygen consumption (μmol.min^{-1}. g^{-1}) was calculated as:

$$\frac{[atrial\ (O_2\%) - venous\ (O_2\%)] \times\ a_{O_2}\ \times coronary\ flow\ rates\ (ml.min^{-1})}{heart\ dry\ weight\ (g)}$$

a_{O_2} is the solubility of oxygen (i.e. the Bunsen solubility coefficient), taken to be 0.0227 ml O_2/mL plasma (447, 448)

(Eq. 2)

Cardiac efficiency (%) was calculated as:

$$\frac{hydraulic\ work\ (J.min^{-1}.g^{-1}) \times 100}{oxygen\ consumption\ (\mu mol.min^{-1}.g^{-1})\ \times\ respiratory\ chain\ energy\ (J.\mu mol^{-1})}$$

where the respiratory chain energy was taken to be 0.421 J.μmol^{-1} (44)

(Eq. 3)

6.3.3 Statistical analysis

Physiological parameters (coronary flow rate, cardiac output, cardiac hydraulic work, and myocardial oxygen consumption) and cardiac efficiency were compared by two-way analysis of variance (ANOVA), with substrates and loading condition as factors. Tukey *post hoc* corrections were made for multiple comparisons to identify specific significant differences. The effect of βHB plus AcAc on the efficiency at high pre- and afterloads was determined using a Student's paired t-test. Results, presented as mean ± SEM, were considered significant at $p < 0.05$.

6.4 Results

6.4.1 The effect of fatty acids on physiological parameters and cardiac efficiency

In the working rat heart, fatty acids decreased cardiac efficiency by ~37 % (17.9 ± 0.7 vs 28.6 ± 1.7 %) and ~28 % (17.1 ± 1.0 vs 25.8 ± 2.3 %) at low and high pre- and afterloads, respectively, via decreased cardiac output and hydraulic work, and increased oxygen consumption (Fig. 6.5). As expected, in all hearts, increasing workload by increasing preload and afterload from low to high increased coronary flow rates, oxygen consumption, and hydraulic work. However, glucose alone maintained efficiency from low to high pre- and afterloads, validating the protocol.

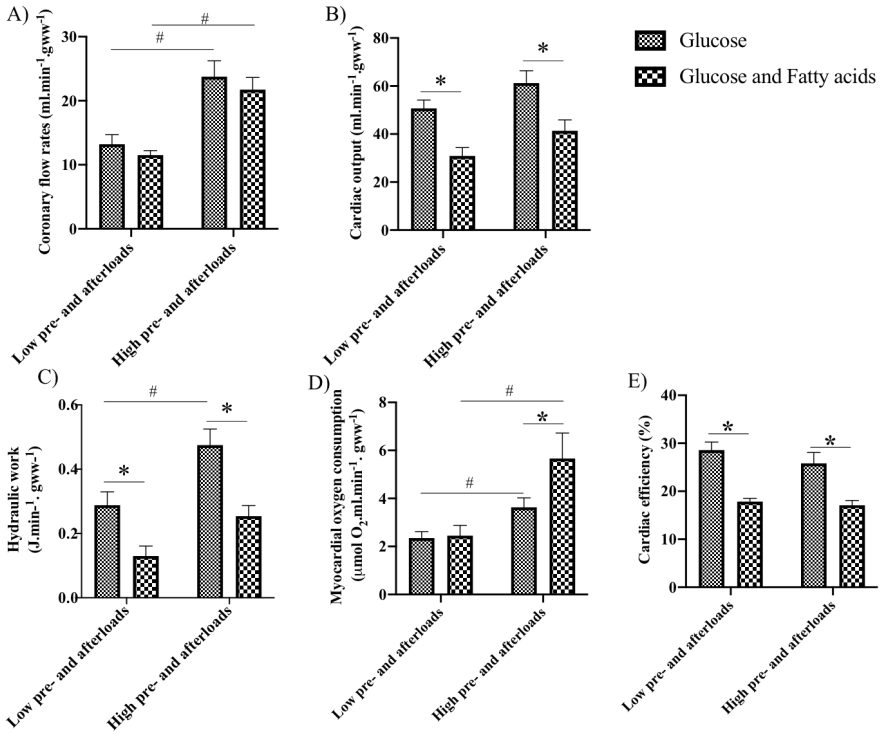


Figure 6.5. *The effect of fatty acids on physiological parameters and efficiency. Coronary flow rate (A), cardiac output (B), hydraulic work (C), myocardial oxygen consumption (D), and cardiac efficiency (E) in the working rat hearts. The hearts were perfused at low and high pre- and afterloads with Krebs-Henseleit buffer containing 11 mM glucose only (Glucose), or with the addition of 0.4 mM fatty acids (Glucose and Fatty acids). Values are means ± SEM (n=24). Effect of fatty acids on physiological parameters and efficiency at low and high pre- and afterloads is indicated as: * P<0.05. Effects of loading on physiological parameters and efficiency is indicated:* $^{\#}$ *P < 0.05.*

6.4.2 The effect of βHB plus AcAc on physiological parameters and efficiency of the working rat hearts

At high pre- and afterloads, a mixture of 4 mM AcAc and 1 mM βHB or a a mixture of 4 mM βHB and 1 mM AcAc increased cardiac efficiency by ~18 % (19.7 ± 0.8 vs. 16.6 ± 1.4 %) and ~17 % (19.4 ± 0.9 vs. 16.6 ± 1.4 %), respectively, by increasing hydraulic work. This did not occur at low pre- and afterloads since the hydraulic work was the same.

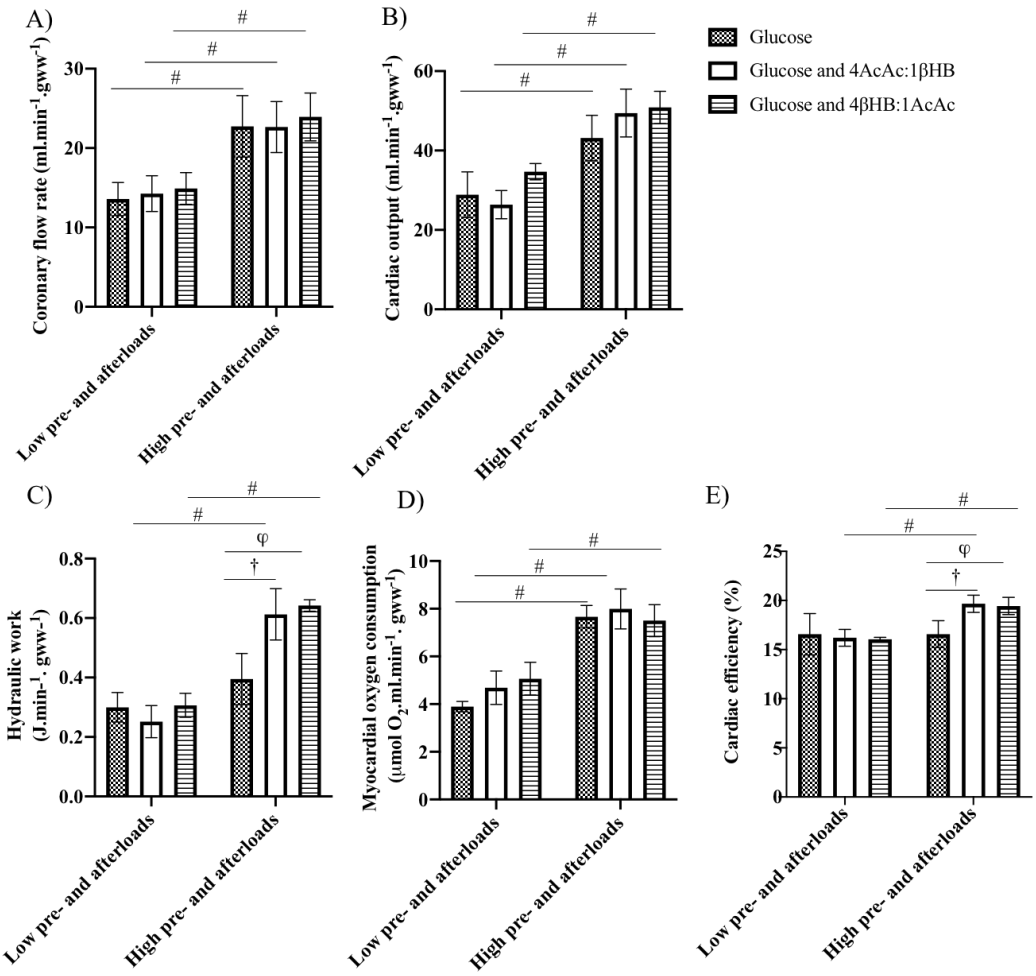


Figure 6.6. *The effect of βHB plus AcAc on physiological parameters and efficiency. Coronary flow rate (A), cardiac output (B), hydraulic work (C), myocardial oxygen consumption (D), and cardiac efficiency (E) in the working rat hearts. The hearts were perfused at low and high pre- and afterloads with Krebs-Henseleit buffer containing 11 mM glucose only (Glucose), or with the addition of a mixture of 4 mM AcAc and 1 mM βHB. Values are means ± SEM (n=24). Effect of βHB plus AcAc on physiological parameters and efficiency at low and high pre- and afterloads is indicated as: * P<0.05. Effects of loading on physiological parameters and efficiency is indicated:* $^{\#}$ *P < 0.05. βHB, D-β-hydroxybutyrate; AcAc, acetoacetate.*

6.5 Discussion

This study was designed to determine the effects of D-β-hydroxybutyrate plus acetoacetate (the effect of redox state), fatty acids, and loading conditions on efficiency in working rat heart. Fatty acids decreased efficiency at low and high pre- and afterloads by increasing oxygen consumption and decreasing hydraulic work. At high pre- and afterloads, βHB plus AcAc increased efficiency which was on average ~17 % by increasing hydraulic work. This effect did not occur at low pre- and afterloads since hydraulic work was the same. The redox state of βHB and AcAc did not influence cardiac efficiency. Thus, ketone bodies increased cardiac efficiency more than glucose alone or with fatty acids at high pre- and afterloads.

6.5.1 Glucose successfully maintained efficiency in all hearts

Glucose successfully maintained efficiencies from at high pre- and afterloads in all hearts, confirming the validity of this model as a means to assess efficiency. Increasing loading conditions increased workload, thereby increased ATP turnover. Thus, adenosine diffuses out of the cell and enters the interstitial fluid where it can induce arteriolar dilation, thereby increasing coronary blood flow at a given coronary perfusion pressure to increase oxygen consumption (449, 450). Adenosine is a potent coronary vasodilator, which causes relaxation of the vascular smooth muscle by interacting with a specific receptor on the coronary myocyte (451). Thus, it can act as a messenger to couple oxygen requirements of the myocardium to vasomotor tone of the coronary resistance vessel, optimising delivery of arterial blood to the myocardium (452). These results confirmed that the coronary vasculature during low workload conditions in this isolated, crystalloid fluid-perfused heart preparation was not maximally dilated, but retained the ability to dilate in order to match oxygen delivery to oxygen requirement, confirming both the integrity of this preparation and that oxygen availability was not limited in the Krebs-Henseleit (KH, non-blood)

perfused model. This data suggests that the protocol of changing loading conditions from low to high pre- and afterloads was successful that glucose successfully maintained cardiac efficiency in all hearts.

6.5.2 Fatty acids decreased cardiac efficiency at low and high pre- and afterloads

Fatty acids decreased cardiac efficiency at low and high pre- and afterloads (i.e. at both low and high workloads) by 37% and 28%, respectively, by increasing oxygen consumption and decreasing hydraulic work. The relative P/O ratio of fatty acids is less than that of β-hydroxybutyrate, indicating a less "oxygen-efficient" energy substrate for ATP production. (Table 6.1). Although fatty acid has even more inherent chemical energy available during combustion than βHB, fatty acid β-oxidation is less efficient with regards to ATP synthesis since only half of the reducing equivalents released enter the respiratory chain at NADH dehydrogenase and the other half enter at a flavoprotein site (453). This result in the loss of the synthesis of about 1 of the 6 possible molecule of ATP (a loss of 5 % in efficiency) (301). The limitation of oxygen provision during increased workload could also lead to fatty acids accumulation. An excess of fatty acyl-CoA can be hydrolysed to yield free CoA and a fatty acid anion (FA^-). FA^- may leave the mitochondrial matrix via UCP; hence oxygen consumption occurs without adenosine diphosphate (ADP) phosphorylation ('uncoupling'), resulting in metabolic inefficiency (Fig. 6.1) (12). Cole and his colleagues reported that high fat feeding decreased rat cardiac efficiency by increasing oxygen consumption and mitochondrial uncoupling protein 3 (UCP3). Fatty acids can also divert glucose from oxidation (454) to glycolysis (72), resulting in accumulation of protons, thereby causing intracellular acidosis (140). Intracellular acidosis can decrease the responsiveness of myofilaments to calcium, thereby decreasing contractile force (12).

This present study also showed that fatty acids were unable to increase cardiac efficiency from low to high pre- and afterloads. It was reported that free fatty acid uptake

(221) and exogenous fatty acid oxidation (455) were similar during increased workloads (220, 456). This could also be explained that carbohydrates are more "oxygen efficient" than fatty acids during increased workload, such as exercise in humans (189, 457-459), β-adrenergic stimulation, increased afterload in perfused rat hearts (455, 460-464) and increased afterload in large animals (462-464). Thus, fatty acids are less efficient than glucose (103, 301, 440, 465). Carbohydrates are preferentially oxidised to deal with increased energy demand (102, 103, 219, 226, 328, 435, 460, 461) (Table 6.1). Additionally, glucose oxidation from glycogenolysis (in the presence of fatty acids at physiological concentration) provided an extra energy source for the heart during increased workload (103, 460). However, the net rate of total substrates utilisation were not measured in this study. Indeed, lactate can replace all other respiratory fuels for the heart in the metabolic state of exercise (102, 466), emphasizing the utility of carbohydrate-derived energy in conditions of increased cardiac workload and potentially limited oxygen provision (12).

6.5.3 βHB plus AcAc increased cardiac efficiency at high pre- and afterloads

At high pre- and afterloads βHB plus AcAc increased efficiency, which was on average ~17 % by increasing hydraulic work. Our data are in agreement with those of Sato et al. (44), who indicated that ketones increased cardiac efficiency. In humans during exercise in the glycogen replete state, ketone body oxidation from ketone ester drinks may account for ~10 % to ~18 % of the total oxygen consumption (60). During increased heart work, Opie and colleagues reported that the decrease in non-esterified fatty acids was associated with decreased myocardial production of βHB and AcAc, potentially explaining the utilisation of βHB and AcAc (221).

The combustion of βHB provides more energy per two carbon moiety than pyruvate. The greater energy inherent in βHB relative to pyruvate is due to higher ratio of H-C in βHB compared to pyruvate (2 H per C vs. 1.3 H per C) (44, 301, 440, 467) (Table 6.1). The

reduction of acetoacetate to βHB by β-hydroxybutyrate dehydrogenase (BDH) involves oxidation of NADH, generating NAD^+ (62, 310); oxidation of βHB to acetoacetate by BDH involves a reduction of the free mitochondrial NAD^+, generating NADH for more ATP production (62, 310). However, this ex vivo study suggests that the redox state of βHB and AcAc might not measurably influence cardiac efficiency, at least to the precision possible with present techniques. It is important to note that the oxidation of βHB to acetoacetate is reversible, which may not influence the redox state. However, in vivo studies showed that both βHB and AcAc were increased during prolonged starvation, but the magnitude of this increment was greatest for βHB, generating NADH for more ATP production (468, 469)

The work in Chapters 3 and 4 clearly showed that the oxidation of βHB increases mitochondrial redox state, which generates more ATP by chemiosmosis (44, 301, 348). Thus, βHB and AcAc increased the free energy of ATP hydrolysis to drive ATPase reactions, which increased the efficiency during increased heart work.

In conclusion, fatty acids decreased cardiac efficiency at low and high pre- and afterloads. At high pre- and afterloads, ketone bodies increased cardiac efficiency more than glucose alone or with fatty acids at high pre- and afterloads.

Chapter 7: General discussion

7.1 Main findings

The work in this thesis was inspired by the aphorism 'fat burns in the flame of carbohydrates' (145). The primary work of this thesis was to determine the importance of glycogen in cardiac βHB metabolism and the interaction between glucose and βHB metabolism. The main findings were:

1. Glycogen was essential for maximal myocardial βHB oxidation rates. Although βHB oxidation inhibited glycolysis, glycolytic intermediates were not depleted and free cytosolic NAD remained oxidised. βHB alone or with glucose was associated with increased the Krebs cycle intermediates, citrate, 2-oxoglutarate and succinate, the total nicotinamide adenine dinucleotide phosphate (NADP/H) pool and reduced mitochondrial Q (Chapter 3).
2. In HG hearts, βHB oxidation increased free energy of ATP hydrolysis by increasing [PCr] and phosphorylation potential (the ratio ([ATP]/[ADP][Pi])). However, βHB was unable to increase the low free energy of ATP hydrolysis in LG hearts due to limited glycolytic flux (Chapter 4). Thus, glycogen was required to allow βHB oxidation to increase the free energy of ATP hydrolysis.
3. In low glycogen hearts, anaplerosis of succinyl-CoA from valine or 2-oxoglutarate from glutamine did not alter Krebs cycle intermediates or βHB oxidation rates. Asparagine increased βHB oxidation rates, via increased intermediary metabolites involved in the malate-aspartate shuttle and purine nucleotide cycle. However, the βHB oxidation was lower than in high glycogen hearts, and βHB oxidation did not increase total NAD(H) and NADP(H) pools because glycogen synthesis was prioritised over others metabolic pathways in low glycogen hearts. Thus, glycogen and glucose are required for βHB oxidation in the heart.

4. βHB plus AcAc increased cardiac efficiency more than glucose alone or with fatty acids at high pre- and afterloads by increasing hydraulic work.

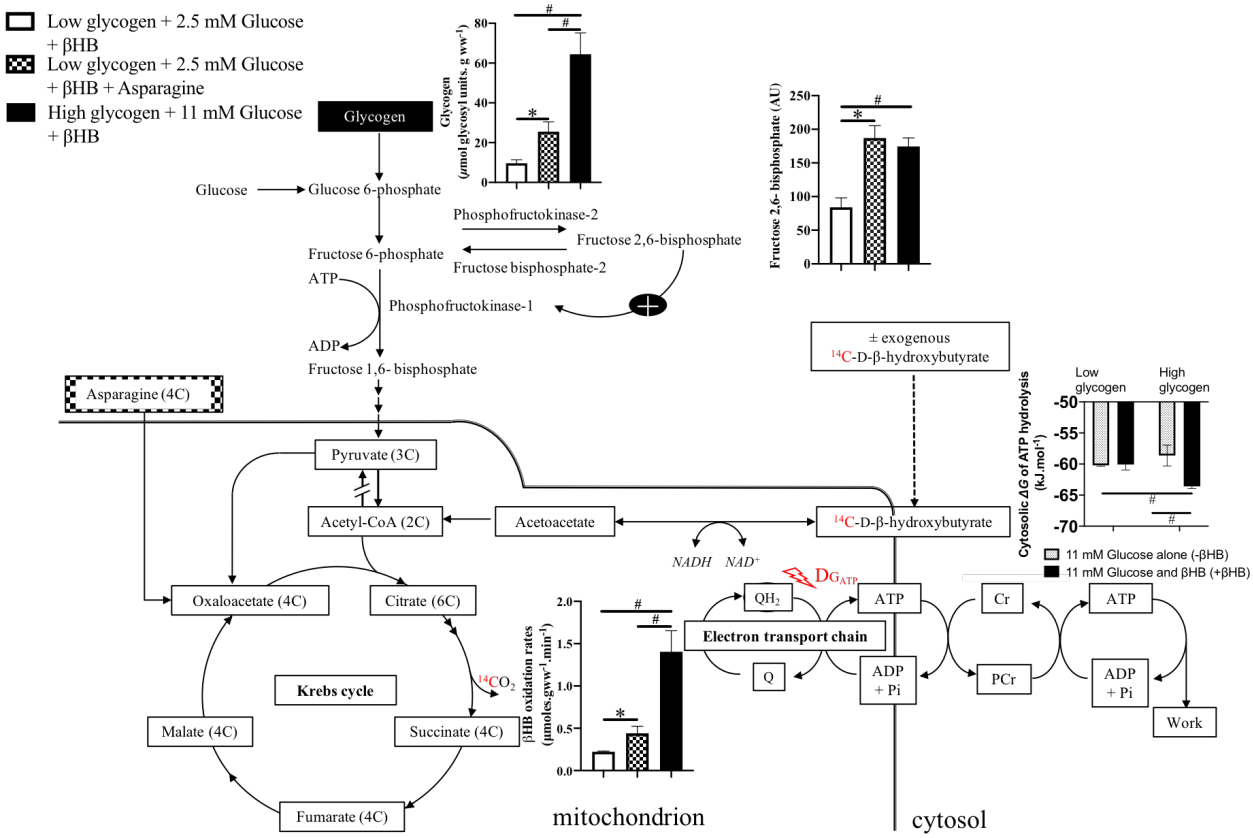


Figure 7.1 *The effect of glycogen and asparagine on βHB oxidation in the heart. Glycogen increased βHB oxidation, which increased the* ΔG_{ATP} *to drive ATPase reactions, thereby increased cardiac efficiency. Minimal glycolysis was successfully maintained by increasing fructose 2,6-bisphosphate. Asparagine indicated that flux through oxaloacetate is required for βHB oxidation. Thus, glycogen and glucose, via pyruvate carboxylase are essential for maximal βHB oxidation.*

7.2 Glycogen, via pyruvate carboxylase is required for ketones oxidation

Pyruvate carboxylation prevents the decline in contractile function of rat hearts oxidizing AcAc (46). βHB is the reduced form of acetoacetate. βHB and AcAc are increased during prolonged starvation, but the magnitude of this increment was greatest for βHB (468). The work in Chapter 3 concludes that glycogen and glucose are required for myocardial βHB oxidation, thereby reducing mitochondrial Q, increasing the free energy of ATP hydrolysis (Chapter 4) and cardiac efficiency (Chapter 6). This did not occur in glycogen-depleted

hearts perfused with asparagine and βHB. Asparagine can replace glucose, but not glycogen. Moreover, anaplerosis of oxaloacetate from glycogen and asparagine increased βHB oxidation, but glycolytic intermediates was not depleted by maintaining positive regulator for glycolysis, fructose 2,6-bisphosphate. Thus, glycolysis at baseline 0.2 μmol. $gww^{-1}.min^{-1}$ measured by quantitative ^3H_2O production from [5-^{3}H]-glucose (Fig. 3.8) was successfully maintained (Fig. 7.1). This study showed that asparagine indicated that flux from through oxaloacetate was required for βHB oxidation. The work in this thesis supports the hypothesis that pyruvate carboxylase is the major anaplerotic enzyme in the heart (46, 48, 155). Thus, glycogen and glucose, via pyruvate carboxylase are essential for maximal βHB oxidation.

7.3 Asparagine can replace glucose, but not glycogen

The work in this thesis concludes that glucose and glycogen are essential for maximal βHB oxidation in the heart. This leads to an important question whether asparagine can replace glucose when cardiac glycogen is low. In the liver, asparagine can serve as a substrate for endogenous glucose production during starvation (383). In glycogen-depleted heart, asparagine increased βHB oxidation, but glycolysis at baseline 0.2 μmol. $gww^{-1}.min^{-1}$ (Fig. 3.8) was successfully maintained by stimulating fructose 2,6-bisphosphate (216, 217, 415), which was also observed in high glycogen hearts (Fig. 7.1). However, βHB oxidation in low glycogen hearts perfused with asparagine was lower than in high glycogen hearts perfused with glucose (0.4 vs 1.4 $\mu moles.gww^{-1}.min^{-1}$) (Fig. 7.1). The glycogen in hearts perfused with asparagine was also lower than in those in normal heart (~25 vs ~40 μmol.glycosyl $units.gww^{-1}$) (307) (Fig. 7.1). Only addition of 11 mM glucose increased glycogen to ~40 μmol. glycosyl $units.gww^{-1}$ (307) (Fig. 5.5). Thus, asparagine can replace glucose, but not glycogen. Glucose is preferentially converted to glycogen when glycogen is low (328). During brief, low-flow ischaemia, glycogen prevents myocardial injury by maintaining sufficient glycolytic ATP for the Na^+ pump to keep intracellular Na^+ low, despite increased

Na^+-H^+ exchange. However, during prolonged ischaemia when glycogen is fully depleted, myocardial injury occurs due to ATP depletion leading to low pH and high intracellular [Na^+] (307, 335). Thus, asparagine can replace glucose, but not glycogen. It is possible that asparagine can alternate with glucose only when glycogen is successfully maintained. Glycogen and glucose are essential for maximal βHB oxidation.

7.4 Ketones as metabolic signals

The first recognition that ketones act as metabolic signals, as well as energetic substrates, came with the observation that they can regulate glycolysis and inhibit adipose tissue lipolysis (thereby limiting their own production in a feed-back inhibition mechanism) (68). Ketones can inhibit glycolysis by inhibiting the activity of PDH, (68, 243, 344), GAPDH (345) or PFK (68, 243, 344), but glycolysis at baseline is successfully maintained by increasing fructose 2,6-bisphoshate (216, 415). Ketone-mediated mechanisms affecting a wide variety of cellular functions (470-474).

βHB is a ligand for G protein-coupled receptors (GPCRs), including GPR109A (74) and GPR41 (475). GPR109A is also the niacin (nicotinic acid) receptor, and the action of βHB as the endogenous ligand of this receptor in adipose tissue mediates its anti-lipolytic activity (74, 297). Other actions have also been assigned to this receptor, including vasodilatation (which offloads a failing heart) and reverse cholesterol transport, with effects on inflammation and atherogenesis that may be relevant to a signalling role for βHB in diverse aspects of cardiovascular biology and disease (GPR109A agonism of immune cells in mice inhibits atherogenesis (476); see (470, 474)). βHB may also decrease sympathetic nervous system activity (with resulting decreased heart rate and resting energy expenditure) by modulating short chain fatty acid signalling through GPR41 (475) though the physiological significance of this effect is unclear.

Ketones signalling also involves dinucleotide function – hence an extended role for NAD in ketones biology is now emerging. The role of NAD as an electron carrier coenzyme in energy homeostasis is well established and is the basis for ketone oxidation modulation of NAD redox state and hence mitochondrial bioenergetics (62), but its role as a substrate for signalling enzymes involved in the response to energy and oxidative stress has only recently been appreciated. NAD acts a substrate for sirtuins (SIRT) – mono-ADP-ribosyltransferases and NAD-dependent deacetylases; βHB has been found to inhibit class I histone deacetylases (HDAC)(354), increasing histone acetylation at the oxidative stress resistance factors *Foxo3a* and *Mt2* promoters and hence increasing expression of genes ameliorating oxidative stress (354). Furthermore, βHB has the ability to reduce $NADP^+$, the resulting NADPH enhancing antioxidant capacity by increasing reduced glutathione (292). NAD-dependent SIRT can increase hepatic 3-hydroxymethylglutaryl-CoA synthase 2 (HMGCS2) to increase serum βHB levels (73), ketones again regulating ketosis. Furthermore, βHB can regulate gene expression by β-hydroxybutyrylation of histone lysine residues (477). It is still unknown how NAD and ketone bodies are functionally inter-related, but the signalling ability of βHB may positively influence some of the deleterious effects of dysregulated metabolism and heart disease, and NAD status is likely critical in cardiac disease - decreased myocardial NAD levels have been shown in both ischaemic and non-ischaemic heart disease (478), and increasing NAD precursor via phosphorylation of nicotinamide riboside improved left ventricular function and remodelling in a murine heart failure (HF)/dilated cardiomyopathy model (479).

7.5 Future work

7.5.1 D-β-hydroxybutyrate (βHB) could ameliorate oxidative stress through the NADPH system

Results from Chapter 3 showed that βHB oxidation increased the total NADP(H) pool mainly through the action of NADP-dependent isocitrate dehydrogenase (IDH) (351, 353, 354, 471, 480, 481), which was associated with increased 2-oxoglutarate and succinate measured using metabolomics. During fasting, mitochondrial SIRT3 deacetylates and activates IDH2, which increased NADPH production, thereby increasing the reduced glutathione in mitochondria (353). βHB oxidation increased NADH and ATP, which may inhibit IDH2 in mitochondria (351). However, citrate or isocitrate from βHB oxidation can also be exported by the citrate-isocitrate carrier to the cytosol in order to synthesis NADPH catalysed by IDH1 (351). This results in increased 2-oxoglutarate and succinate in the Krebs cycle (Fig. 7.2). IDH1 expression is induced by forkhead box(O) family (FOXO1 and FOXO3a) (355). Thus, NADPH provides the electrons for the reduction of molecular antioxidants (glutathione disulphide, glutathione, vitamin C, and vitamin E), which decreases reactive oxygen species or reactive nitrogen species (351, 353, 354, 471, 480, 481) (Fig. 7.2).

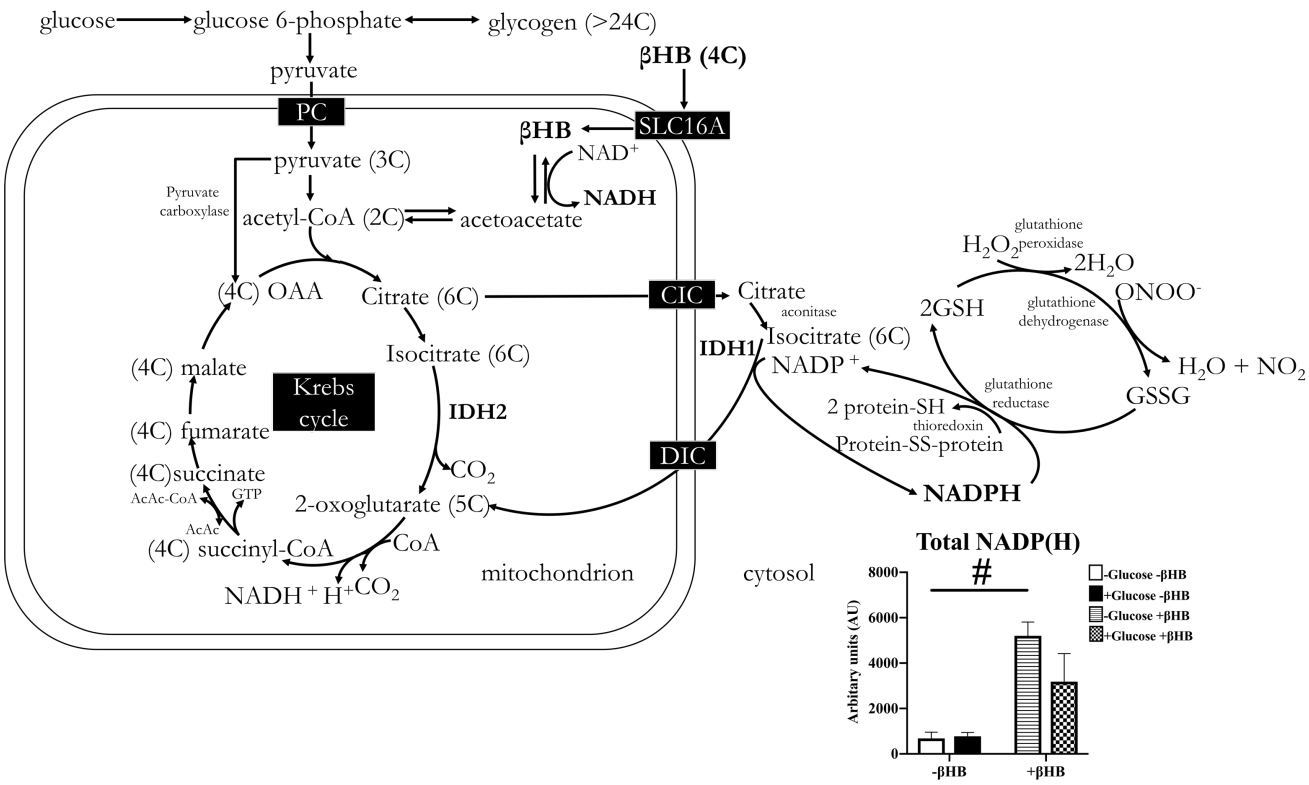


Figure 7.2 *Proposed model for regulation of oxidative stress by βHB through the NADPH system. βHB oxidation increases the total NADP(H) pool mainly through the action of NADP-dependent isocitrate dehydrogenase (IDH). NADPH provides the electrons for the reduction of molecular antioxidants (glutathione disulphide and glutathione), which decreases reactive oxygen species or reactive nitrogen species.*

NADPH oxidase-4 (Nox4) maintained cardiac energetics in hypertrophied heart by increasing fatty acid utilisation (482). However, the work in Chapter 6 showed that βHB is more efficient than fatty acids during increased workload. Ketones may also unveil new therapeutic strategy to treat the failing heart (31, 302). Therefore, the preliminary data in this thesis suggests that βHB could ameliorate oxidative stress through the NADPH system, raising several questions for future directions:

1. The interaction between Nox4 and βHB oxidation could be explored in heart failure.
2. Glycogen is required for βHB oxidation.
3. NAPDH oxidase-2 (Nox2) is a major producer of reactive oxygen species. Thus, βHB could provide a cardioprotective effect against oxidative stress in cardiomyocyte-targeted Nox2.

7.6 Conclusion and implications

Glycogen and glucose are required for maximal ketones oxidation. Asparagine indicated that flux through oxaloacetate was required for ketones oxidation. Thus, this study supports the hypothesis that pyruvate carboxylase is the major anaplerotic enzyme in the heart. In the glycogen replete (fed) state humans, ketone body oxidation from ketone ester drinks may account for ~10%-18% of total oxygen consumption during exercise (60). Therefore, in the availability of carbohydrate (glucose and glycogen), ketosis can also be achieved by ingesting exogenous ketones or their precursors, which can result in high blood ketone concentrations, as captured in the aphorism 'fat burns in the fire of carbohydrates'. Since endogenous carbohydrate is not depleted, and glycogen stores are replete, endogenous metabolism is largely unaffected, with glucose profile is successfully maintained. Therefore, exogenous ketones or their precursors ketone drink can maintain ketosis, allowing a normal meal pattern, providing an alternative dietary method to achieve ketosis and lowering fatty acids and glucose in the management of heart failure and diabetic cardiomyopathy.

Appendix A

A.1 Metabolomic analysis: Analysis of cell metabolite extracts by liquid chromatography–mass spectrometry (LC-MS))

Hearts samples perfused with various substrate combinations were analysed using up to three separate LC-MS/MS methods using two different LC systems (Thermo Scientific ICS-5000+ ion chromatography system and a Thermo Ultimate 3000). Each was coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Scientific, San Jose, CA).

A.1.1 Ion exchange chromatography-mass spectrometry (IC-MS/MS)

Ion exchange chromatography was performed using a ICS-5000+ HPLC system incorporating an electrolytic anion generator (KOH) which was programmed to produce a OH^- gradient over 37 min. An inline electrolytic suppressor removed OH^- ions and cations from the post-column eluent stream prior to MS analysis (Thermo Scientific Dionex AERS 500). A 10 µL partial loop injection was used for all analyses and the chromatographic separation was performed using a Thermo Scientific Dionex IonPac AS11-HC 2 × 250 mm, 4 µm particle size column with a Dionex Ionpac AG11-HC 4 µm 2x50 guard column inline. The IC flow rate was 0.250 mL/min. The total run time was 37 mins and the hydroxide ion gradient comprised as follows: 0mins, 0mM; 1min, 0mM; 15mins, 60mM; 25mins, 100mM; 30mins, 100mM; 30.1mins, 0mM; 37mins, 0mM. Analysis was performed in negative ion mode using a scan-range from *m/z* 60-900 and resolution set to 70,000. The tune file source parameters were set as follows: Sheath gas flow 60 mL/min; Aux gas flow 20 mL/min; Spray voltage 3.6v; Capillary temperature 320 ºC; S-lens RF value 70; Heater temperature 350ºC. AGC target was set to 1e6v ions and the Max IT value was 250ms. The column temperature was kept at 30°C throughout the experiment. Full scan data were acquired in continuum mode.

A.1.2 C18 reversed phase (underivatized)

C18 reversed-phase analysis of underivatised samples was performed using a Thermo Ultimate 3000 UHPLC system with a gradient elution programme coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer. A 5 µL partial loop injection was used for all analyses with pre-and post-injection wash program. A Waters CORTECS UPLC T3 1.6µm (2.1x100mm) column was used with a flow rate of 0.4mL/min. The total run time was 18 mins. Mobile phase A comprised milli-Q water with 0.1% formic acid and mobile phase B was 100% methanol with 0.1% formic acid. The gradient elution program was as follows: 0mins, 5%B; 4min, 50%B; 12min, 99%B; 15mins, 99%B; 15.1min, 5%B; 18min, 5%B. The column temperature was kept at 40°C throughout the experiment. Mass spectrometry analysis was performed in positive and negative ion mode separately using a scan-range from *m/z* 60-900 and resolution set to 70,000. The tune file source parameters were set as follows: Sheath gas flow 60 mL/min; Aux gas flow 20 mL/min; Spray voltage 3.6v; Capillary temperature 320 ^{o}C; S-lens RF value 70; Heater temperature 350°C. Full MS setting were AGC target 5e6 ions and the Max IT value was 120ms. Full scan data were acquired in continuum mode. A data directed tandem mass spectrometry method was utilised (ddMS^2) with no inclusion list. The orbitrap detector and HCD setting for ddMS^2 were as follows: Microscans 2, resolution 17,500, AGC target 5e4 ions, maximum IT 80ms, loop count 10 and NCE 35.

A.1.3 C18 reversed phase (derivatized)

The third LC-MS method used a sample derivatisation protocol followed by analysis based on a modified version of the Waters AccQ-Tag method (Salazar et al., 2011). C18 reversed-phase analysis of derivatised samples was also performed using the Thermo Ultimate 3000 UHPLC system coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer. A 5 µL partial loop injection was used for all analyses with pre and post

injection wash programme. A Waters AccQ-Tag column (2.1x100mm) was used with a flow rate of 0.5mL/min. The total run time was 9.5 mins. Mobile phase A and B comprised commercially available AccQ-Tag reagents prepared as recommended by Waters (Waters PLC, Elstree, UK). The gradient elution program was modified from the published AccQ-Tag method as follows: 0mins, 0.1%B; 0.54min, 9.1%B; 5.74min, 21.2%B; 7.74mins, 59.6%B; 8.04min, 90%B; 8.05min, 90%B; 8.64min, 0%B; 9.5min, 0.1%B. The column temperature was kept at 40°C throughout the experiment. Mass spectrometry analysis was performed in positive ion mode separately using a scan-range from *m/z* 70-1050 and resolution set to 70,000. The tune file source parameters were set as follows: Sheath gas flow 60 mL/min; Aux gas flow 20 mL/min; Spray voltage 3.6v; Capillary temperature 320 ^{o}C; S-lens RF value 70; Heater temperature 350°C. Full MS setting were AGC target 3e6 ions and the Max IT value was 200ms. Full scan data were acquired in continuum mode.

A.1.4 Data processing

Raw data files were processed using ProgenesisQI (Waters, Elstree, UK). This process included alignment of retention times, peak picking by identification of the presence of natural abundance isotope peaks, characterising multiple adducts forms and identification of metabolites using our in house database. Retention times, accurate mass values, relative isotope abundances and fragmentation patterns were compared between authentic standards and the samples measured. Identifications were accepted only when the following criteria were met: <5ppm differences between measured and theoretical mass (based on chemical formula), <30 seconds differences between authentic standard and analyte retention times, isotope peak abundance measurements for analytes were >90% matched to the theoretical value generated from the chemical formula. Fragmentation patterns were matched to least the base peak and two additional peak matches in the MS/MS spectrum to within 12ppm.

The top 10 data directed fragmentation method was not always able to provide fragment ions for all ions measured in the MS 1 spectrum.

A.1.5 Data analysis and statistics

MetaboAnalyst is an online tool that can be used to analyse a wide range of metabolomics datasets (312): Univariate statistical analysis includes determining fold-change and t-tests between experimental groups for compound features and combined in volcano plots (false discovery rate (FDR)-adjusted p-values should be reported). A fold-change threshold of 1.5 and a FDR adjusted p-value cut-off of 0.05 (occasionally an FDR adjusted p-value <0.1) is used to determine significance. Unsupervised multivariate statistical analysis commonly includes Principle Component Analysis (PCA) used to visualize global metabolic profiles and determine sample outliers. Supervised multivariate statistical analysis using Partial Least Squares Discriminant Analysis (PLS-DA) is used to model important compound features when the number of samples per experimental group is sufficient (even if samples are differentiated by a model it is unlikely to be validated (by permutation test) without sufficient samples per group; usually >9) . Feature loadings and variable importance in the projection (VIP) scores can be ranked for validated models to determine important metabolite features. Models ware assessed by cross validation (using R2, Q2 and Accuracy; R2 should be higher than Q2 which should be >0.4 and within 0.3 if the R2 values, the closer to 1 the R2 and Q2 the strong the model). To avoid overfitting of the model a permutation test is performed using 100 or more permutations with a separation distance (B/W) test statistic. Hierarchical clustering can be visualised using heat-maps with a Euclidean distance measure and all compound features. Pathways Analysis is performed to aid functional interpretation of the dataset and interpret within a biological context. MetaboAnalyst is used for this analysis in addition to pathways heat-maps using an in-house Excel macro that mapped identified compound features onto (Kyoto encyclopaedia of genes and genomes)

KEGG metabolic pathways relevant to known metabolic pathways active in Homo sapiens (https://www.kegg.jp/kegg/atlas/?01100). The pathway Enrichment Analysis uses a global Test statistic and Relative Betweeness Centrality for topology analysis.

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Published work

Cardiac ketone body metabolism

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Abstract

The ketone bodies, D-β-hydroxybutyrate and acetoacetate, are soluble 4-carbon compounds derived principally from fatty acids, that can be metabolised by many oxidative tissues, including heart, in carbohydrate-depleted conditions as glucose-sparing energy substrates. They also have important signalling functions, acting through G-protein coupled receptors and histone deacetylases to regulate metabolism and gene expression including that associated with anti-oxidant activity. Their concentration, and hence availability, increases in diabetes mellitus and heart failure. Whilst known to be substrates for ATP production, especially in starvation, their role(s) in the heart, and in heart disease, is uncertain. Recent evidence, reviewed here, indicates that increased ketone body metabolism is a feature of heart failure, and is accompanied by other changes in substrate selection. Whether the change in myocardial ketone body metabolism is adaptive or maladaptive is unknown, but it offers the possibility of using exogenous ketones to treat the failing heart.

***Key words*:**

ketone body; acetoacetate; β-hydroxybutyrate; ketone ester; heart; metabolism.

***Abbreviations*:**

AcAc: acetoacetate; BCAA: branched chain amino acid; BCKD: branched chain ketoacid dehydrogenase; BD: butanediol; βHB: D-β-hydroxybutyrate; BDH1: β-hydroxybutyrate dehydrogenase; BNP: B-type natriuretic peptide; CoA-SH: free coenzyme A; CPT: carnitine palmitoyltransferase; DCM: dilated cardiomyopathy; FA: fatty acid; FSTL1: follistatin-like protein; GPCR: G-protein coupled receptor; HDAC: histone deacetylase; HF: heart failure; HMGCL: 3-hydroxymethylglutaryl-CoA lyase; HMG-CoA: 3-hydroxymethylglutaryl-CoA; HMGCS2: 3-hydroxymethylglutaryl-CoA synthase; IGF: insulin-like growth factor; IMM: inner mitochondrial membrane; KB: ketone body; KD: ketone diet; KO: knock-out; LCHF: low-carbohydrate high fat; MCT: medium chain triglyceride; MCTD: medium chain triglyceride diet; MEE: myocardial external energy efficiency; mThiolase: mitochondrial thiolase; mTORC: mammalian target of rapamycin complex; NAD^+(H): nicotinamide adenine dinucleotide oxidized (reduced); OMM: outer mitochondrial membrane; PPARα: peroxisome proliferator activated receptor-α; ROS: reactive oxygen species; SGLT2: sodium-glucose co-transporter 2; SIRT: silent information regulation 2 (sirtuin); SLC: solute ligand carrier protein; SCOT: succinyl-CoA:3-oxoacid-CoA transferase; TAG: triacylglycerol; UCP: uncoupling protein.

Since the nineteenth century, when they were first found in high concentrations in the urine of gravely ill diabetic patients, ketone bodies (KB) were regarded by clinicians with fear and apprehension, apparently representing toxic markers of severe disease. And indeed, clinicians of today still view ketosis with trepidation, a feature of life-threatening diabetic ketoacidosis and inadequate nutrition. However, the concept of KBs being waste products of metabolism was revised in the twentieth century when it became apparent that they are vital energy substrates, representing an alternative fuel source, especially for the brain, in starvation and carbohydrate shortage. Indeed, as the brain oxidises glucose, but not fatty acids (FA), the brain's ability to utilise FA-derived ketone bodies for ATP production represents a major glucose-sparing mechanism (Cahill, 2006b). From an evolutionary view point, animals that have developed a ketogenic pathway can afford to store most of their energy as highly energy-dense triacylglycerol (TAG), and little as energetically inefficient glycogen. This model of ketones as energy substrates was further revised in the 1970's when an additional role, that of metabolic signals, was ascribed to them (Robinson and Williamson, 1980b). It also become apparent that, in addition to their critical use as glucose-sparing substrates for the brain, ketone bodies may supply energy to other oxidative tissues, including muscle and heart. Furthermore, their role in the myocardium now appears to extend beyond energy provision in the healthy heart to a crucial role in heart disease, both as metabolic substrate and signal, raising the possibility of using KBs to treat cardiac dysfunction. However, despite these new insights, it is still not clear whether the role of ketones is essentially adaptive or maladaptive in cardiac disease.

Ketone body metabolism

The ketone bodies, D-β-hydroxybutyrate (βHB) and acetoacetate (AcAc), are water-soluble 4-carbon compounds produced, principally in liver mitochondria, from plasma non-esterified fatty acids derived from lipolysis of TAG in adipose tissue. A further ketone body

is the 3-carbon acetone, produced by spontaneous decarboxylation of acetoacetate, but its role in metabolism is controversial and will not be discussed further. βHB is the reduced form of acetoacetate and is not (chemically) a ketone, but the biochemical nomenclature ("ketones") is well established. KBs are used by non-hepatic oxidative tissues, such as brain, heart, kidney cortex and skeletal muscle, for terminal oxidation as a metabolic response to physiological states characterized by limited carbohydrate availability (fasting/starvation), post-exercise, or impaired insulin signalling (Fukao et al., 2004b). Falling insulin:glucagon ratios increase the activities of the adipose lipase enzymes, hormone-sensitive lipase and adipose triglyceride lipase, to increase plasma "free" fatty acid delivery to the liver and other tissues. Adipose tissue lipolysis also occurs with increased sympathetic nervous system activity, mediated by the catecholamines, epinephrine and norepinephrine, a situation that occurs in cardiac disease.

Ketogenesis:

KB synthesis occurs principally in liver mitochondria (hepatic ketogenesis) from plasma fatty acids. Non-hepatic tissues, including astrocytes (Guzman and Blazquez, 2001), may undertake ketogenesis, but evidence for this is based in part on the reversible kinetics of the ketone utilisation pathway (Forder, 1996), so it will not be discussed further. Some ketogenesis also derives from the branched chain amino acid, leucine. In hepatocytes, fatty acids are converted to long-chain acyl-CoA and undergo β-oxidation in mitochondria, producing acetyl-CoA (Ac-CoA). In carbohydrate-limited, catabolic conditions with increased gluconeogenesis, insufficient oxaloacetate is available for combination with the acetyl-CoA, which instead is converted to acetoacetate, then reduced to βHB in an NAD^+/NADH coupled equilibrium reaction catalysed by mitochondrial βHB dehydrogenase 1 (BDH1; Figure 1). BDH1 modulates mitochondrial redox potential in liver and heart (Williamson et al., 1967b). Finally ketone bodies are released by the liver into the aqueous

plasma via solute carrier 16A (SLC 16A), the monocarboxylate transporter family member 1, 6, and 7 (Halestrap and Wilson, 2012a)) and circulate to extrahepatic tissue for terminal oxidation (Puchalska and Crawford, 2017). Hence KBs essentially represent a transport form of acetyl-CoA, usable by any tissue, such as the heart, which has the machinery of ketolysis (conversion of KB back to acetyl-CoA) and the ability to oxidise acetyl-CoA in the Krebs cycle. For obvious reasons, the liver lacks ketolytic enzymes. The main plasma KB is βHB, which is typically present in about five times AcAc concentrations. The distinction between βHB and AcAc is important because 1. Many studies have measured only one KB or the other, yet the total (βHB + AcAc) represents the true KB delivery (Williamson et al., 1962a) 2. The proportion can change in different (patho)physiological states, and 3. The ratio is a determinant of the mitochondrial redox ratio (Williamson et al., 1967b, Krebs et al., 1969) and hence will influence cellular bioenergetics.

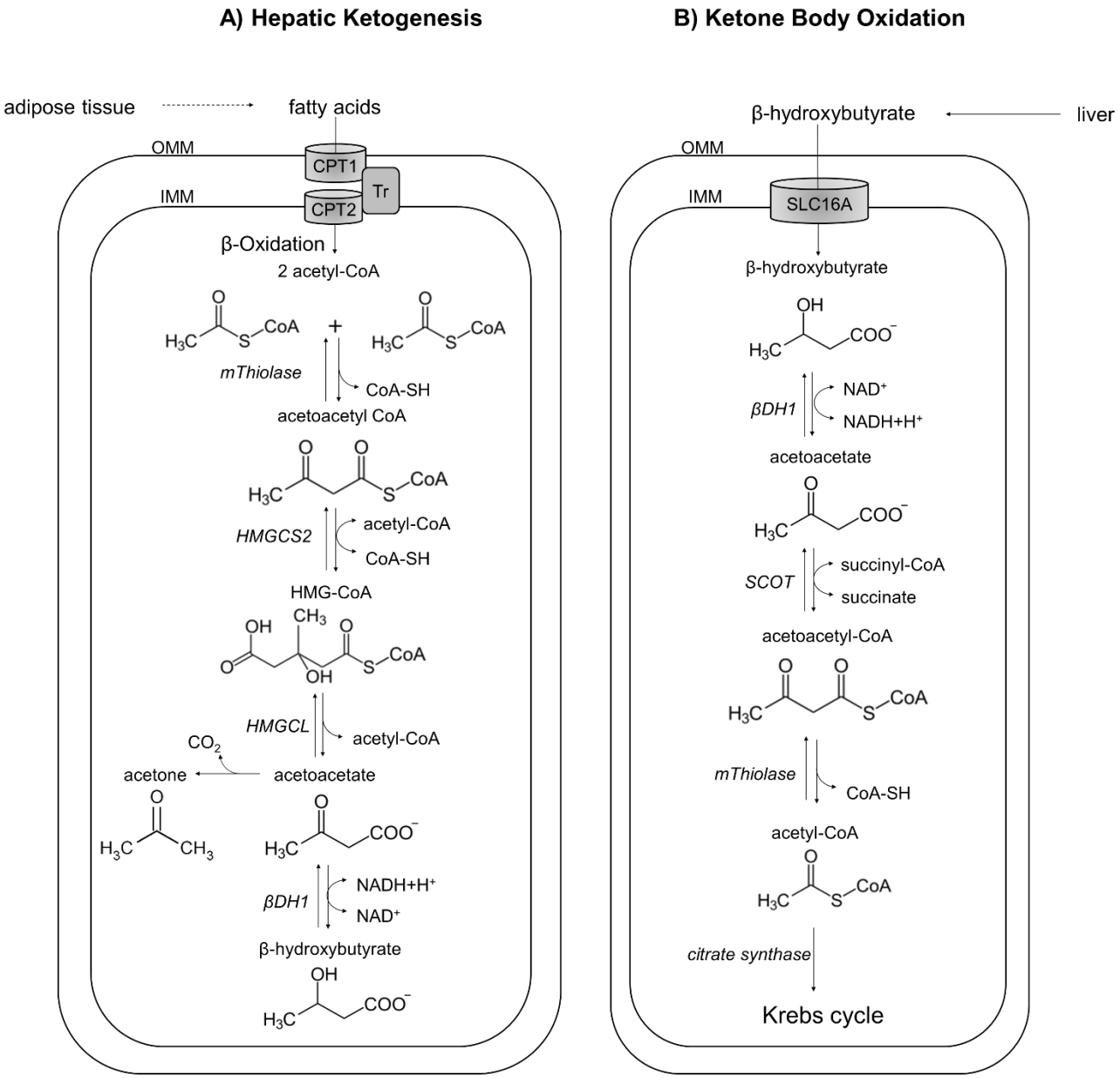


Figure 1. Ketone body metabolism. *(A) Ketone body synthesis in liver mitochondria. (B) Ketone body oxidation in extrahepatic tissue mitochondria via β-hydroxybutyrate dehydrogenase (BDH1) and succinyl-CoA:3-oxoacid-CoA transferase (SCOT). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; CPT, carnitine palmitoyltransferase; Tr, translocase; CoA-SH, free coenzyme A; HMGCS2, 3-hydroxymethylglutaryl-CoA synthase 2; HMG-CoA, 3-hydroxymethylglutaryl-CoA; HMGCL, HMG-CoA lyase; NAD$^+$(H), nicotinamide adenine dinucleotide oxidized (reduced); solute ligand carrier protein (SLC) 16A (SLC16A); mThiolase, mitochondrial thiolase.*

Ketolysis and ketone body oxidation:

The NAD^+/NADH-linked oxidation of βHB to AcAc is catalysed by BDH1 in the mitochondria of extrahepatic tissue. AcAc is activated to acetoacetyl-CoA by 3-oxoacid-CoA transferase 1 (OXCT1), also called succinyl-CoA:3-oxoacid-CoA transferase (SCOT). SCOT is expressed in all oxidative tissues, except liver (Fukao et al., 1997b). SCOT catalyses a near-equilibrium reaction that exchanges coenzyme A between succinate and AcAc (Stern et al., 1956b), a mechanism linking ketolysis with the 2nd part of the Krebs cycle. A reversible mitochondrial acetoacetyl-CoA thiolase (mThiolase) reaction yields two molecules of acetyl-CoA that enter the Krebs cycle (Fukao et al., 2004b, Garland et al., 1962, Robinson and Williamson, 1980b, Williamson et al., 1971b). Cardiac SCOT can undergo post-translational tyrosine nitration in diabetes (Turko et al., 2001, Wang et al., 2010a) or following endotoxin exposure (Marcondes et al., 2001), which can impair the enzyme's activity, whilst nitration of SCOT tryptophan residues in aged rat hearts increases enzyme activity (Rebrin et al., 2007).

Since the product of ketolysis is solely a 2-carbon unit (Ac-CoA), terminal oxidation of ketones (like fatty acid oxidation) is a cataplerotic process – ketone bodies (i.e. Ac-CoA equivalents) are not capable of replenishing Krebs cycle intermediates, and efficient utilisation of KBs requires an anaplerotic source, such as carbohydrate (including glycogen) or glucogenic amino acids, such as arginine (Taegtmeyer, 1983b, Taegtmeyer et al., 1980a).

Ketone bodies as energy substrates:

Plasma total KB concentration varies from about 100 μM in the fed state to about 1 mM following a 24 h fast; comparable levels are seen following prolonged exercise. Levels rise to 5-7 mM in prolonged fasting, but can reach > 20 mM in untreated, insulin-deficient diabetes (Cahill, 2006b, Robinson and Williamson, 1980b, Koeslag et al., 1980b, Puchalska and Crawford, 2017). The human liver can produce 300 g of KBs per day (Balasse and Fery,

1989). In the fed state, ketones provide about 5% of the body's total energy requirement, rising to about 20% in fasting (Balasse et al., 1978, Cox et al., 2016b) and, in a prolonged fast, KBs can provide more than two thirds of the brain's energy requirement (Owen et al., 1967a). Ketone body flux occurs down a concentration gradient and tissue utilisation of KBs is concentration-dependent and probably saturable (Edmond et al., 1987, Balasse and Fery, 1989). Ketone bodies are readily oxidised by brain, skeletal muscle and cardiac muscle (Balasse and Fery, 1989, Owen et al., 1967a, Sultan, 1988).

Ketone body efficiency:

The energetic and thermodynamic efficiency of ketone bodies, relative to other major energy sources, continues to be debated. In purely chemical terms, fatty acids are more reduced than ketone bodies, which are in turn more reduced than glucose. Consequently, it can be predicted that the molar yield of ATP per mole of substrate occurs in the order FA>KB>glucose. Biochemically, however, the concept of metabolic efficiency includes ATP production per mole of oxygen consumed (essentially the P/O ratio) or "oxygen efficiency" of the oxidation of these fuels. This is a particularly important concept in the heart because arterial-coronary sinus oxygen content measurements indicate that heart has the highest oxygen extraction of any tissue, reflecting a high metabolic demand together with the anomaly that most myocardial blood flow occurs only during diastole. Myocardial O_2 delivery is compromised in heart disease, especially hypertrophy. This is a complex issue in metabolism, capturing the bioenergetics of the relevant metabolic pathway, as well as the integrity of oxidative phosphorylation and the electron transport chain. Hence, FAs require initial energy input to generate fatty acyl-CoA, but also induce mitochondrial uncoupling proteins (UCP) (Thompson and Kim, 2004), which dissipate the mitochondrial proton gradient and therefore diminish metabolic efficiency, compared to ketone bodies. Veech and colleagues reported that more KB-derived reducing equivalents are delivered to

the electron transport chain than those from FA β-oxidation (Veech, 2004a). Ketone bodies keep mitochondrial ubiquinone oxidised, maintaining an increased redox span between complexes I and III of the electron transport chain, and ultimately increasing the free energy of ATP hydrolysis ($\Delta G'_{ATP}$) relative to FA oxidation – effectively increasing the P/O ratio of KB oxidation (and augmenting ionic gradients) (Veech, 2004a, Sato et al., 1995b, Kashiwaya et al., 1994a). In addition to upregulating UCP expression, FA oxidation also increases reactive oxygen species (ROS) generation, potentially further blunting metabolic efficiency. Kashiwaya *et al* (Kashiwaya et al., 1997a) reported an increase in metabolic (oxygen) efficiency of 28% in isolated glucose-perfused working rat hearts following addition of insulin (ascribed to decreased mitochondrial free NAD^+/NADH and oxidation of ubiquinone (CoQ)/ubiquinol ($CoQH_2$) ratios, thereby doubling the phosphorylation ratio (ATP/ADP.Pi); these positive energetic effects were reproduced by perfusion with 4 mM βHB + 1 mM AcAc (Kashiwaya et al., 1997a, Sato et al., 1995b, Veech et al., 2001). Hence KBs have been attributed greater metabolic efficiency (and hence ATP production) than FAs or, indeed, glucose (Cahill and Veech, 2003), although some researchers still consider P/O ratios for cardiac glucose oxidation to be greater than those for KBs, even if the P/O ratio for ketone oxidation is higher than for fatty acids (Lopaschuk and Verma, 2016a). Furthermore, because glucose is anaplerotic, it may be considered more metabolically efficient than the cataplerotic KBs and FAs, which always require carbohydrate and/or glucogenic amino acid anaplerotic input to maintain metabolic efficiency (see below.).

Ketone bodies as metabolic signals:

The first recognition that ketone bodies act as metabolic signals, as well as energetic substrates, came with the observation that they can modulate the action of insulin and inhibit adipose tissue lipolysis (thereby limiting their own production in a feed-back inhibition mechanism) (Robinson and Williamson, 1980b). Recent research has highlighted at least

two major ketone-mediated mechanisms affecting a wide variety of cellular functions (Puchalska and Crawford, 2017, Newman and Verdin, 2014b, Newman and Verdin, 2014a, Rojas-Morales et al., 2016, Cotter et al., 2013).

βHB is a ligand for G protein-coupled receptors (GPCRs), including GPR109A (Taggart et al., 2005a) and GPR41 (Kimura et al., 2011b). GPR109A is also the niacin (nicotinic acid) receptor, and the action of βHB as the endogenous ligand of this receptor in adipose tissue mediates its anti-lipolytic activity (Taggart et al., 2005a, Tunaru et al., 2003a). Other actions have also been assigned to this receptor, including vasodilatation (which offloads a failing heart) and reverse cholesterol transport, with effects on inflammation and atherogenesis that may be relevant to a signalling role for βHB in diverse aspects of cardiovascular biology and disease (GPR109A agonism of immune cells in mice inhibits atherogenesis (Lukasova et al., 2011); see (Cotter et al., 2013, Puchalska and Crawford, 2017)). βHB may also decrease sympathetic nervous system activity (with resulting decreased heart rate and resting energy expenditure) by modulating short chain fatty acid signalling through GPR41 (Kimura et al., 2011b) though the physiological significance of this effect is unclear.

KB signalling also involves dinucleotide function – hence an extended role for NAD in KB biology is now emerging. The role of NAD as an electron carrier coenzyme in energy homeostasis is well established and is the basis for ketone oxidation modulation of NAD redox state and hence mitochondrial bioenergetics (Williamson et al., 1967b), but its role as a substrate for signalling enzymes involved in the response to energy and oxidative stress has only recently been appreciated. NAD acts a substrate for sirtuins (SIRT) – mono-ADP-ribosyltransferases and NAD-dependent deacetylases; βHB has been found to inhibit class I histone deacetylases (HDAC)(Shimazu et al., 2013), increasing histone acetylation at the oxidative stress resistance factors *Foxo3a* and *Mt2* promoters and hence increasing

expression of genes ameliorating oxidative stress (Shimazu et al., 2013). Furthermore, βHB has the ability to reduce $NADP^+$, the resulting NADPH enhancing antioxidant capacity by increasing reduced glutathione (Veech et al., 2001). NAD-dependent SIRT can increase hepatic 3-hydroxymethylglutaryl-CoA synthase 2 (HMGCS2) to increase serum βHB levels (Grimsrud et al., 2012b), KBs again regulating ketosis. Furthermore, βHB can regulate gene expression by β-hydroxybutyrylation of histone lysine residues (Xie et al., 2016). It is still unknown how NAD and ketone bodies are functionally inter-related, but the signalling ability of βHB may positively influence some of the deleterious effects of dysregulated metabolism and heart disease, and NAD status is likely critical in cardiac disease - decreased myocardial NAD levels have been shown in both ischaemic and non-ischaemic heart disease (Klein et al., 1981), and increasing NAD precursor via phosphorylation of nicotinamide riboside improved left ventricular function and remodelling in a murine heart failure (HF)/dilated cardiomyopathy model (Diguet et al., 2018).

B. Role of ketone bodies in cardiac metabolism

Ketone body utilisation by the healthy heart:

The foetal heart is essentially glycolytic, with limited oxidative capacity, owing to hypoxic conditions *in utero* (Lopaschuk et al., 1992); myocardial ketone body utilisation increases gradually post-partum (Bartelds et al., 1998). The adult heart, however, is a highly oxidative organ, consuming more energy – about 400 kcal/kg/day – than any other tissue (Ashrafian et al., 2007b, Wang et al., 2011, Wang et al., 2010b), albeit having a limited energy reserve comprising some TAG within lipid droplets (Evans and Hauton, 2016) together with glycogen (Allard et al., 1997). The heart can utilize all energy substrates, but fatty acids (both in non-esterified and esterified (TAG) form) and carbohydrates (glucose and lactate) are its key energy source: together their oxidation accounts for approximately

90% of cardiac ATP production, with metabolism of KBs and branched-chain amino acids (BCAA) supplying the balance (Bing, 1954, Wisneski et al., 1987a, Lopaschuk and Gamble, 1994, van der Vusse et al., 2000a, Evans and Clarke, 2012a). FAs and glucose are broken down by β-oxidation and glycolysis, respectively, KBs and BCAAs generate Ac-CoA via BDH1 and branched-chain α-ketoacid dehydrogenase (BCKD), respectively (Figure 2)(Bing, 1954, Wisneski et al., 1987a, Lopaschuk and Gamble, 1994, van der Vusse et al., 2000a, Evans and Clarke, 2012a). Glucose, FAs, KBs and BCAAs all compete as sources of acetyl-CoA for the Krebs cycle (and, specifically, βHB inhibits myocardial fatty acid oxidation, an effect independent of malonyl-CoA (Stanley et al., 2003c)) by the Randle cycle (Randle et al., 1963b, Wisneski et al., 1985b, Taegtmeyer et al., 2008b, Taegtmeyer et al., 2016b, Forsey et al., 1987). Hence the heart is a metabolic omnivore and, in health, possesses the metabolic flexibility to rapidly alter substrate utilisation according to substrate availability.

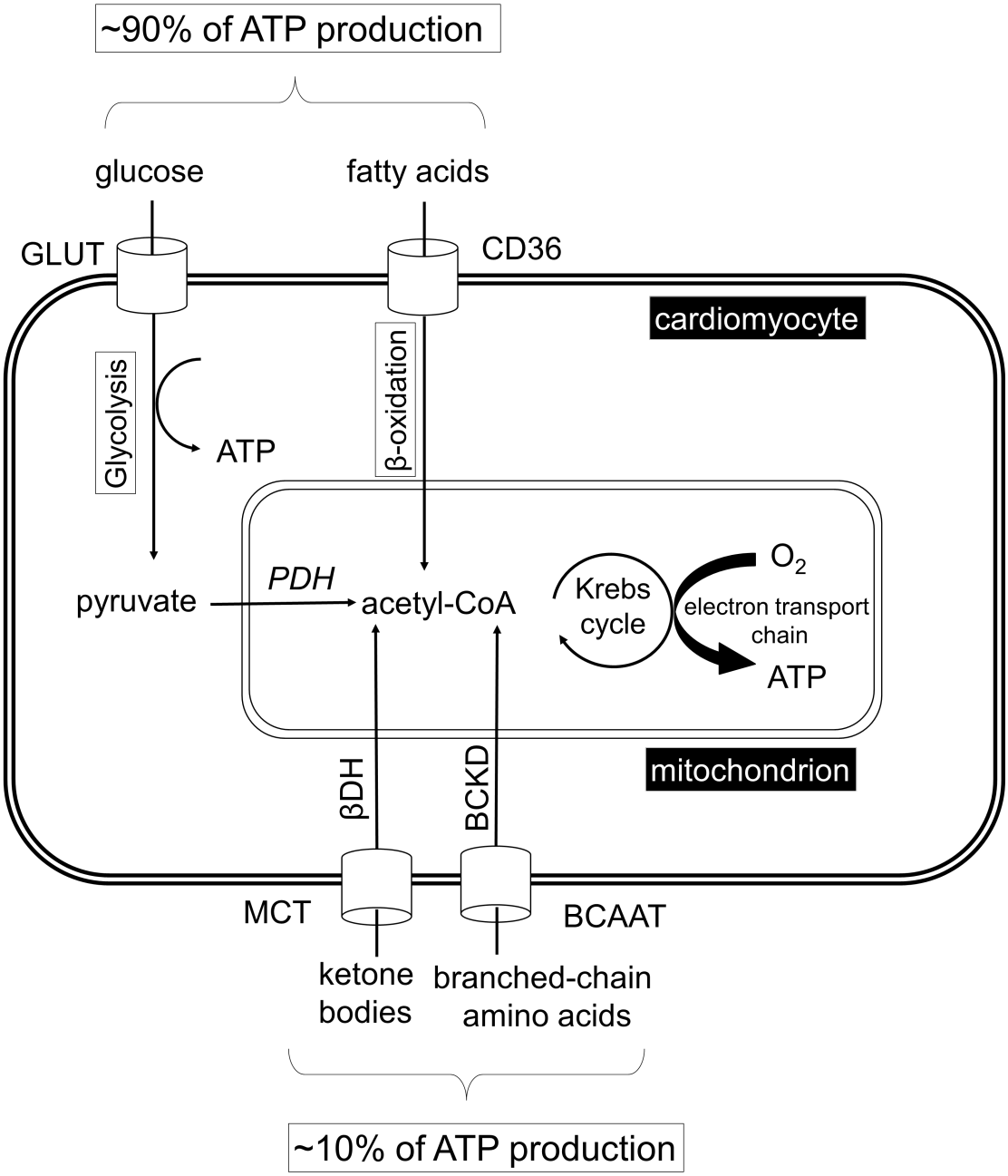


Figure 2. Cardiac metabolism. *The heart is capable of oxidizing a wide variety of substrates for ATP production. Energy substrates compete for mitochondrial oxidative energy metabolism via generation of acetyl-CoA for the Krebs cycle. Pyruvate from glucose can generate acetyl-CoA via pyruvate dehydrogenase (PDH). Fatty acids can generate acetyl-CoA via β-oxidation. Fatty acids and carbohydrate contribute ~90% of oxidative energy metabolism. Branched-chain amino acids (BCAAs) and ketone bodies can generate acetyl-CoA via branched-chain α-ketoacid dehydrogenase (BCKD) and β-hydroxybutyrate dehydrogenase (βDH), respectively. Both BCAA and ketone bodies contribute ~10% of oxidative energy metabolism. BCAAT, branched chain amino acid transporter; CD36, cluster of differentiation/fatty acid translocase; GLUT, glucose transporter; MCT, monocarboxylate transporter.*

The heart is the most avid consumer of KBs on a weight basis (Bing, 1954, Fukao et al., 2004b, Lopaschuk et al., 2010b, Taegtmeyer et al., 2002, Young et al., 2002). However, KBs (and FAs and the ketogenic BCAA, leucine) are cataplerotic, causing depletion of Krebs cycle intermediates. Perfusion of rat heart with AcAc alone decreased contractile function due to Krebs cycle inhibition by sequestering coenzyme A and by inhibition of the Krebs cycle at the level 2-oxoglutarate (α-ketoglutarate) dehydrogenase (Taegtmeyer, 1983b, Taegtmeyer et al., 1980a). KBs can only enter the Krebs cycle as 2-carbon (2C) intermediates – acetyl-CoA; these carbons replace the two carbons lost as 2 x CO_2 molecules with each full turn of the cycle. KBs are not able to replenish Krebs cycle intermediates (anaplerosis) and these intermediates are constantly lost to other pathways (Des Rosiers et al., 2011b, Owen et al., 2002a). Thus, ketone body oxidation is cataplerotic, depleting Krebs cycle intermediates and impairing metabolic efficiency (Koeslag et al., 1980b, Owen et al., 2002a). The cataplerotic effect of ketone body oxidation must always be offset by anaplerotic substrates, such as circulating glucose, endogenous glycogen, and glucogenic amino acids via transamination (Des Rosiers et al., 2011b, Gibala et al., 2000a, Russell and Taegtmeyer, 1991b, Russell and Taegtmeyer, 1991f, Russell and Taegtmeyer, 1992b, Taegtmeyer et al., 1980a, Taegtmeyer, 1983b) providing pyruvate for carboxylation to oxaloacetate or malate by pyruvate carboxylase or malic enzyme, respectively. Pyruvate carboxylase is the major anaplerotic enzyme in heart (Des Rosiers et al., 2011b, Owen et al., 2002a). Myocardial βHB oxidation is proportional to glycogen content in rat hearts perfused without other anaplerotic sources [Kadir, unpublished data].

Cardiac ketone body utilisation in heart disease:

Whilst the adult heart is oxidative and metabolically flexible, cardiac disease is associated with a loss of metabolic flexibility and alterations in cardiac substrate utilisation that correlate with changes in mechanical function (Ashrafian et al., 2007b, Neubauer,

2007b, Lopaschuk and Gamble, 1994, Lopaschuk et al., 2010b, Abel and Doenst, 2011a, Boudina and Abel, 2010b, van der Vusse et al., 2000a, Ingwall and Weiss, 2004b, Jaswal et al., 2011b, Kolwicz and Tian, 2011, Karwi et al., 2018b) failing heart has been compared to an engine that has run of fuel (Neubauer, 2007b): inefficient fuel selection and impaired oxidative phosphorylation, including reduced activity of electron-transport chain complexes, ATP synthase and the phosphate acceptors, ADP and creatine, can impair cardiac function by providing insufficient ATP to cardiomyocytes, leading to heart failure (Quigley et al., 2000a, Marin-Garcia et al., 2001a, Marin-Garcia et al., 2001b, Casademont and Miro, 2002, Murray et al., 2008, Murray et al., 2004b).

The changes in myocardial metabolism that accompany heart disease have been difficult to characterise and remain controversial. The current consensus is that in hypertrophy and ischaemia-induced HF, there is a shift away from predominantly FA oxidation towards glucose utilisation (with the presumption that the failing heart reverts to the more oxygen-efficient carbohydrate for energy). Besides impaired FA oxidation, some studies report increased glucose oxidation whereas others decreased glucose oxidation (Lopaschuk and Ussher, 2016a). Glycolytic rates are generally increased, and this is associated with a reversion to a foetal isoenzyme gene expression programme in the heart (Razeghi et al., 2001). By contrast, increased FA oxidation is found in diabetes (Mazumder et al., 2004), which has been blamed for the aetiology of diabetic cardiomyopathy as well as the worsened ischaemia-reperfusion characteristics of the diabetic myocardium (Boudina and Abel, 2010b, An and Rodrigues, 2006, Rodrigues et al., 1998). Furthermore, glucose intolerance is a common feature of heart failure, and the non-diabetic failing heart may itself be insulin resistant (Nikolaidis et al., 2004); the implications of this for myocardial ketone body utilisation in non-diabetic heart failure are not understood. The conundrum of which comes first, cardiac disease or metabolic derangement (Carley et al., 2014), has proved

difficult to resolve. Genetically-induced disruption of cardiac metabolism characteristically impairs cardiac mechanical function, cardiomyopathy and HF (Antozzi and Zeviani, 1997, Ashrafian et al., 2003, Frey et al., 2012), and the notion that inappropriate fuel utilisation by the heart impairs cardiac function has driven attempts to alter cardiac metabolism in order to treat heart failure and improve cardiac function. These efforts have focussed on FA and glucose metabolism, but a role for ketone metabolism in heart disease has recently become apparent (Kolwicz et al., 2016, Nakamura and Sadoshima, 2019, Lopaschuk and Ussher, 2016a). Ketone body metabolism is clearly present in the failing heart, but it is unclear whether enhanced cardiac ketone body metabolism causes, is caused by or is unrelated to cardiac dysfunction. This leads to the crucial question of whether ketosis and increased KB availability can provide a method to increase ATP production, and hence function, in the heart.

It is established that plasma ketone body concentration is increased in HF, and is positively correlated with filling pressure (Kupari et al., 1995a, Lommi et al., 1996a, Lommi et al., 1997a, Neely et al., 1972a). Indeed NMR-metabolomic analysis of plasma from heart failure patients revealed a positive correlation between elevation of myocardial energy expenditure and βHB and acetone levels, leading the authors to conclude that these could be used as biomarkers in HF patients (Du et al., 2014). In 1973, a study reported altered ketone body metabolism in patients with cardiomyopathy (Rudolph and Schinz, 1973). Recently, metabolomic analysis of human non-diabetic, end-stage failing hearts demonstrated decreased concentrations of long chain fatty acyl-carnitines, suggesting decreased cardiac FA oxidation, despite increased myocardial Ac-CoA levels, together with increased β-hydroxybutyryl-CoA and increased myocardial utilisation of βHB, as indicated by upregulation of both serum βHB (0.145 mM) and key enzymes in the ketone oxidation pathway, including BDH1 and SCOT (Bedi et al., 2016a). Interestingly, anaplerotic

propionyl-CoA levels were decreased (Bedi et al., 2016a). The finding of increased KB utilisation in the failing hearts was interpreted as ketones providing an alternative fuel for energy provision - a metabolic adaptation in the context of severe HF. By contrast, in a study using arterio-coronary sinus blood sampling, A-V differences and extraction of ketone bodies were not altered in severely failing hearts, although myocardial A-V oxygen difference was increased (Janardhan et al., 2011). However, mitochondrial proteomic analysis in a cardiac pressure overload-hypertrophy/failure mouse heart model (Aubert et al., 2016a) supported the findings of increased KB oxidation in human HF (Bedi et al., 2016a). In humans, failing hypertrophied heart had FA oxidation proteins downregulated, but increased BDH1, a key enzyme in ketolysis and hence KB oxidation (Figure 1). *Ex vivo* hypertrophied failing hearts had increased rates of ^{13}C-βHB oxidation, together with increased metabolites of KB oxidation, including hydroxybutyryl-carnitine and acetyl-carnitine as well as succinate for the succinyl-CoA-dependent SCOT reaction (Aubert et al., 2016a). Together, these data provide evidence of increased reliance on ketone body utilisation in human and mouse models of HF, with increased myocardial KB utilisation in end-stage HF a consequence of increased systemic availability due to increased ketogenesis resulting from sympathetic overactivity and increased lipolysis that accompanies HF/low cardiac output states, and despite potentially competitive raised FA levels (Kolwicz et al., 2016). Bedi and colleagues postulated that increased ketone body utilisation is a late event in HF as the metabolite profile characteristic of ketone body oxidation is not observed in the earlier stage of compensated hypertrophy (Bedi et al., 2016a).

The importance of myocardial ketone body oxidation has been demonstrated by cardiac dysfunction in the cardiac-specific Cre-lox BDH1-KO mouse hearts, which lack functional BDH1 and hence the ability to oxidise βHB (though not acetoacetate) (Horton et al., 2019a). The BDH1-KO mouse hearts have increased fasting stress and worse

pathological cardiac remodeling following pressure overload/ischaemic damage (transverse aortic constriction and myocardial infarction) compared to BDH1-expressing hearts capable of utilizing βHB. Acetoacetate metabolism, via the reaction catalyzed by intact SCOT, could not fully substitute for BDH to meet cardiac demands in the BDH1-deficient hearts. Increased plasma βHB ameliorated pathological remodeling and cardiac dysfunction, both in a mouse model of transverse aortic constriction (using a ketogenic diet to induce ketosis), and in pacing-induced HF dogs (using βHB infusion); notably, the decreased myocardial O_2 consumption associated with cardiac failure was prevented by βHB infusion, consistent with increased cardiac hydraulic work, and in conjunction with increased myocardial βHB uptake (Horton et al., 2019a). βHB also enhanced the thermodynamics of isolated cardiac mitochondria, a mechanism ascribed to effects on fatty acids – mitochondria superfused with βHB were able to maintain a greater (more negative) membrane potential, $\Delta\psi$, and improved respiratory efficiency, an effect that depended on BDH1 activity (Horton et al., 2019a). KB uptake across pacing-induced failing canine hearts was nearly twice that of control hearts (together with a higher glucose oxidation and lower fatty acid oxidation rates), whereas infusion of FSTL1 (follistatin-like protein, a novel cardiokine/myokine that is upregulated in HF) decreased KB uptake (and increased glucose oxidation) in both failing and control hearts (Seki et al., 2018). FSTL1 was cardioprotective in animal models of HF, but this study does not support a protective role for KBs.

Ketones also have a role in pathological remodeling and free radical homeostasis. Cardiomyocyte-specific *Oxct-1* (SCOT) knockout mice have a normal phenotype, tolerate fasting and ketogenic diet ketosis normally, but have increased cardiac FA oxidation (Schugar et al., 2014a). However, aortic constriction-induced pressure overloaded hearts had increased myocardial ROS in the absence of SCOT, with mitochondrial and myofibrillar disruption, such that left ventricular mass was increased and ejection fraction decreased in

SCOT-KO hearts, but not controls after 8 weeks pressure overload (Schugar et al., 2014a). The authors concluded that inability to metabolise ketone bodies accelerates adverse ventricular remodeling, and ketone body metabolism is critical to the adaptive response to pressure overload, potentially by free radical homeostasis. In support of these findings, isolated perfused hearts from cardiac-specific *Bdh1*-transgenic mice over-expressing βHB dehydrogenase-1 showed increased ketone body oxidation and no other phenotype; however, following aortic constriction and pressure overload, these hearts were resistant to fibrosis, contractile dysfunction and oxidative damage (Uchihashi et al., 2017a) – the increased ketone body oxidation was associated with decreased oxidative stress and mitochondrial ROS production, and reduced apoptosis.

Ketosis is a key feature of diabetes mellitus; increased plasma ketone body concentrations increase tissue (including cardiac) availability and should increase myocardial KB uptake and utilisation. However, plasma fatty acids are also increased in diabetes as a result of increased adipose lipolysis. Given the competition between these substrates (Ferrannini et al., 1983, Stanley et al., 2003c) it is not clear which predominates in diabetic cardiac metabolism. Considerable evidence exists that fatty acid utilisation is increased in the diabetic heart, at the expense of glucose uptake (Boudina and Abel, 2010b, Boudina and Abel, 2007, Fillmore et al., 2014, Chong et al., 2017, Rodrigues et al., 1995, Lopaschuk et al., 2010b), and indeed myocardial fatty acid utilisation has been estimated at > 90% of ATP requirement in diabetic myocardium (Heather and Clarke, 2011, Jia et al., 2018, Evans and Clarke, 2012a). This is a feature of the metabolic inflexibility of the diabetic heart (Rodrigues et al., 1998, An and Rodrigues, 2006, Larsen and Aasum, 2008), in which metabolic efficiency is compromised (Hafstad et al., 2007, How et al., 2006, Boardman et al., 2009) and steatosis and increased oxidative stress results (McGavock et al., 2007, Levelt et al., 2016). It has been considered the principal aetiological factor in the

diastolic dysfunction seen in diabetes – diabetic cardiomyopathy. However, the role of KB metabolism in the diabetic heart is poorly understood. Arterio-venous measurements across human diabetic hearts indicate that glucose and lactate extraction are halved, whereas total ketone body, β-hydroxybutyrate, and acetoacetate extraction are doubled (Mizuno et al., 2017) (interestingly in this study, no difference was noted in fatty acid uptake). This increased cardiac KB utilisation may represent an adaptive mechanism to optimize metabolic efficiency and cardiac function (Stanley et al., 2003c, Cahill and Veech, 2003, Mudaliar et al., 2016a). The same study (Mizuno et al., 2017) noted a positive correlation between plasma brain/B-type natriuretic peptide (BNP) levels and myocardial KB uptake, supporting the notion that cardiac KB utilisation increases with worsening LV function (Fillmore et al., 2014). Recently, increased expression of ketogenic enzymes, especially the rate-limiting HMGCS2, both at mRNA (Shukla et al., 2017, Cook et al., 2017) and protein expression level (Cook et al., 2017)) in the type-1 diabetic heart has been demonstrated. This is intriguing as adult heart is a consumer of ketone bodies and is not considered to be a significant source of KBs; the effect may be part of a general foetal gene expression programme typically seen in heart failure or may reflect an adaptive response by the diabetic heart to synthesise ketone bodies in order to "dispose" of excess acetyl-CoA derived from the increased FA β-oxidation typically occurring in the diabetic myocardium. Whilst it is still uncertain whether increased ketone body metabolism in the failing heart is a beneficial, adaptive response, or a maladaptive feature of HF, these results indicate that enhanced KB metabolism is a key event in cardiac failure and pathological remodeling.

C. Manipulation of ketone body metabolism to treat cardiac disease

In cardiac disease, ketone bodies potentially affect cardiac function either directly or indirectly. Directly, by supplying energy for cardiac metabolism and, in doing so, potentially competing with and displacing other substrates, and/or as a cardiac signal by

altering cellular redox potential, and/or as an antioxidant to decrease myocardial ROS and hence oxidative damage. Indirectly, by altering the general metabolic state of the body through non-cardiac effects. Both may result in an altered cardiac substrate utilisation profile. Induced ketosis offers the potential for treating heart disease and improving cardiac function, but studies of systemic ketosis on cardiac metabolism and function in heart disease must consider that any effects observed may be due to KBs themselves, or the influence that ketosis, or its generation, may have on general metabolic profile (e.g. fatty acid and glucose levels).

1. Endogenous ketosis and heart disease:

Ketosis can be achieved by manipulation of dietary intake in order to stimulate endogenous ketogenesis (Table 1)(Fery and Balasse, 1983b, Cahill, 1970c). Physiological endogenous ketosis is generated by carbohydrate depletion and low insulin, with decreased blood glucose and elevated fatty acid concentrations, and depleted glycogen. The raised fatty acid levels increase hepatic ketogenesis. However, the βHB stimulates the nicotinic acid receptor GPR109A, limiting lipolysis and further ketogenesis (Taggart et al., 2005a). KBs also lower blood glucose by limiting glucose appearance (Mikkelsen et al., 2015a), and are insulinotropic (Biden and Taylor, 1983), stimulating insulin secretion from pancreatic β-cells (Goberna et al., 1974) and INS-1 cells (MacDonald et al., 2008) by an unknown mechanism but likely related to glucose-FA-KB interplay. Increased proteolysis mobilises both the ketogenic (leucine) and glucogenic, amino acids (e.g. arginine). Therefore the effects of carbohydrate depletion are profound and not limited solely to ketogenesis; glycogen depletion, in particular, may have metabolic implications for the anaplerosis necessary for cataplerotic fuels such as fatty acids and KBs. This makes interpretation of the effects of calorie restriction/carbohydrate depletion difficult, so data on the effects of endogenous ketosis on cardiac function is limited.

Table 1. Human plasma βHB concentrations during endogenous and exogenous ketosis. *BD, butanediol; βHB, β-hydroxybutyric acid; LCHF, low-carbohydrate high-fat; MCT, medium chain triglyceride.*

	Condition	βHB (mM)	Reference
Endogenous ketosis	Fed	0.05 - 0.1	[1]
	Fasting (24 h)	0.3 - 0.5	[128]
	Starvation (10 d)	3 - 6	[1]
	Starvation (4 wk)	5 - 8	[129]
	LCHF diet	2 - 4	[133]
Exogenous ketosis	*(R,S)*-1,3-butanediol	0.2	[183]
	MCT	0.5-2	[162]
	Ketone salt	1 - 3	[198]
	Ketone ester	2 - 6	[24]

i. Fasting/calorie restriction ketosis:

Increased ketosis is a key feature of carbohydrate-depleted states such as fasting, together with low insulin and blood glucose, and increased plasma fatty acids . βHB and AcAc are readily utilised by non-hepatic oxidative tissue, including the heart. Ketogenesis increases with prolonged starvation (Cahill, 2006b). Accompanying proteolysis will also increase BCAA availability. However, calorie-restricted dietary regimes are difficult to sustain in order to achieve long-term ketosis. Prior to the discovery of insulin, carbohydrate restriction was the only therapeutic strategy for managing diabetes mellitus; restriction of carbohydrate intake is still practiced, but raises the possibility of ketoacidosis, glycogen depletion, hypoglycaemia and dyslipidaemia (Seckold et al., 2019).

ii. Ketogenic diet:

Manipulation of the macronutrient composition of the diet, such as low-carbohydrate high-fat diets (LCHF, also known as ketogenic diets (KD) – typically about 88% fat, 10% protein, 2% carbohydrate) can be used to promote endogenous ketogenesis without fasting. Ketogenic diet has been used as a therapy for clinical conditions (Gilbert et al., 2000b), being

easier to sustain than calorie restriction, but the diet can cause side effects, including dehydration, constipation, acidosis, and lethargy (Branco et al., 2016). Apart from being unpalatable, it is difficult to maintain ketosis because moderate protein or carbohydrate can immediately inhibit endogenous ketone production (Amari et al., 1995). The ketogenic diet may also induce hypoglycaemic symptoms, as it may take weeks to achieve ketosis (Zhang et al., 2013). Despite ketosis, the metabolic profile is again high FA levels, together with low insulin and glucose. KBs induced by ketogenic diets act as substrates, but also as signalling molecules via extracellular receptors and as endogenous inhibitors of HDACs, conferring anti-oxidant potential and also inhibiting pro-hypertrophic transcription (Newman and Verdin, 2014b, Newman and Verdin, 2014a, Shimazu et al., 2013). In addition KDs have broad effects on gene expression (Badman et al., 2007).

KDs can lower body weight (Bueno et al., 2013) and blood glucose concentrations in type 2 diabetes mellitus (Feinman et al., 2015). Beneficial effects of the diet have been reported for patients with Parkinson's disease (Vanitallie et al., 2005) and epilepsy (decreased seizures) (Kossoff et al., 2003), paediatric oncology (Nebeling et al., 1995) in which tumour growth is an example of an unregulated/metabolically inflexible tissue, and specific genetic disorders of fatty acid metabolism, such as acyl-CoA dehydrogenase deficiency (Van Hove et al., 2003). In athletes, adaptation to KDs increased the ability to oxidise lipid at higher intensities (Volek et al., 2016, Volek et al., 2015). However, the diet does not improve athletic performance (Burke, 2015). Indeed, the diet can impair performance as it depletes glycogen and decreases glycolytic capacity (Stellingwerff et al., 2006). Glycogen depletion in carbohydrate-deficient states may limit anaplerosis, blunting the ability to oxidise KBs. The elevated fatty acids may also inhibit glucose uptake(Randle et al., 1963b) however acyl-CoA activates pyruvate carboxylase, potentially stimulating anaplerosis (Scrutton and Utter, 1967).

KDs increase longevity and survival in mice (Roberts et al., 2018, Newman et al., 2017), associated with increased protein acetylation and decreased tissue-specific mammalian target of rapamycin complex 1 (mTORC1) signalling (Roberts et al., 2018). Hyperacetylation of mitochondrial protein lysine residues has been shown in both failing human and mouse hearts, where it may alter succinate dehydrogenase activity in the Krebs cycle, and alter NAD^+ homeostasis (Horton et al., 2016b). Cyclic KDs (KD alternated weekly with the control diet) can prevent obesity, reduce midlife mortality, and improve composite cardiac score, effects associated with suppression of insulin and insulin-like growth factor (IGF)-signalling, suppression of TOR activation and protein translation, and upregulation of fatty acid oxidation genes, including peroxisome proliferator-activated receptor-α (PPARα) (Newman et al., 2017), PPARα activation being required for full response to the ketogenic diet (Badman et al., 2007). However, KDs may cause hypercholesterolaemia and hypertriglyceridaemia (Kwiterovich et al., 2003, McPherson and McEneny, 2012b). The altered lipid profile and elevated blood fatty acids may increase atherogenic risk (Holloway et al., 2011). It has also been postulated that a maladaptive effect of KD can compromise endothelial vascular function and promote inflammation, as well as atherosclerotic lesions (Cai et al., 2017b), important in cardiovascular disease.

In a mouse model of HF, a KD for 4 weeks increased circulating βHB, enhancing KB delivery and utilisation (Wentz et al., 2010, Hasselbaink et al., 2003, Stanley et al., 2003c), but had no effect on left ventricular function by echocardiography (Aubert et al., 2016a). However, the authors comment that the myocardial metabolite profile was similar to that seen in HF mice on a standard chow diet, with elevated R- and S-C4OH-carnitine, consistent with increased uptake and oxidation of KBs (Aubert et al., 2016a).

The KD has not been well-studied in the management of diabetes, and specifically its effects on diabetic cardiac ketone metabolism and function are unknown, though induced ketosis may address cardiac dysfunction.

2. Exogenous ketosis and heart disease:

Ketosis can also be achieved by ingesting exogenous ketones or their precursors, which can result in high blood ketone concentrations (Table 1) and obviates the need for unpalatable diets, which are difficult to sustain. Since endogenous carbohydrate is not depleted, and glycogen stores are replete, endogenous metabolism is largely unaffected, with fatty acid levels low (or decreased, avoiding the deleterious effects of high fatty acid concentrations), and glucose and insulin profiles largely unchanged. However, there are general metabolic modifications due to the signalling properties of exogenous ketones from ketone drinks (Clarke et al., 2012c, Shivva et al., 2016b) or intravenous infusions (Balasse and Ooms, 1968b, Mikkelsen et al., 2015a), which inhibit lipolysis via GPR109A while directly elevating blood βHB concentration. This is a less "physiological" model than carbohydrate restriction, but holds beneficial potential by manipulating ketone availability in relative isolation from other substrates.

i. Medium chain triglycerides:

Medium chain triglyceride (MCTs) are a family of triacylglycerols containing medium chain fatty acids (MCFA), 6-12 carbon length aliphatic chains, including C6:0 (caproic), C8:0 (caprylic), C10:0 (capric), and C12:0 (lauric) fatty acids. Dietary sources include coconut oil and palm kernel oil. They are absorbed in the portal blood and are freely oxidised for hepatic ketogenesis (Traul et al., 2000, Marten et al., 2006). MCT diets (MCTD) can effectively increase blood ketones levels to 1 to 2 mM in humans (Ohnuma et al., 2016b, Yeh and Zee, 1976), but their use may be limited by adverse gastrointestinal effect such as cramps, diarrhoea and vomiting. Medium chain fatty acids do not require the carnitine

shuttle to enter mitochondria and, besides acting as hepatic ketogenic substrates, appear to undergo unregulated oxidation in the myocardium (Labarthe et al., 2008). Perfusion of hearts with medium chain fatty acid is associated with KB release (Vincent et al., 2000b), a function of high activity and reversibility of cardiac SCOT, hence their effect on ketosis is accompanied by a subtle change in FA availability for oxidative metabolism. MCTD has been successfully used for the treatment of refractory epilepsy (Liu, 2008) and an oral ketogenic supplement, AC-1202, containing glycerin and caprylic acid, was beneficial in Alzheimer's patients (Henderson et al., 2009). Beneficial effects have also been reported in the treatment of the metabolic syndrome (Nagao and Yanagita, 2010). MCTD have been advocated for the treatment of heart disease (see (Labarthe et al., 2008)), and have been found effective, concurrent with restored ketone body production, in the management of long chain FA oxidation defects (Duran et al., 1991, Brown-Harrison et al., 1996, Touma et al., 2001, Cox et al., 1998). Although persistent cardiac dysfunction was noted, despite improved clinical status (Touma et al., 2001), improvement of cardiomyopathy was only found by substituting even-carbon (C8) with odd carbon (C7) fatty acids (Roe et al., 2002b), raising the issue of anaplerosis in conjunction with KB oxidation. It is not clear if these benefits are from direct tissue MCFA oxidation or KB oxidation. MCTD may have a beneficial effect on cardiac hypertrophy and function in the spontaneously hypertensive rat, despite having no effect on blood pressure (Iemitsu et al., 2008, Hajri et al., 2001, Shimojo et al., 2004, Rupp et al., 1995). In PPARα-overexpressing mice, a long chain fatty acid-enriched diet exacerbated the cardiomyopathy and dysfunction, whilst a MCTD improved cardiac function (Finck et al., 2003). However, care must be taken interpreting studies of MCTD on cardiac function, as heart perfusion experiments have demonstrated beneficial effects of medium chain fatty acids in ameliorating cardiac hypertrophy and dysfunction (Allard et al., 2007, Labarthe et al., 2005), and ischaemia-reperfusion injury (Montessuit et

al., 2000, Madden et al., 1995) suggesting these effects are likely due to direct cardiac utilisation of the medium chain fatty acid itself rather than ketones derived from it.

ii. RS-1,3-Butanediol

Whilst not a medium chain triglyceride or fatty acid, RS-1,3-butanediol (RS-BD) is metabolised in the liver to produce two isoforms of βHB, D- and L-βHB or *R*- and *S*-βHB. Oral administration in dogs can achieve ketosis (0.8 mM) approaching KD (Puchowicz et al., 2000, Kies et al., 1973a). RS-BD administration attenuated salt-induced hypertension in Dahl salt-sensitive rats (Chakraborty et al., 2018, Place and Kanneganti, 2019), these rats having an attenuated ketosis response to fasting; the observed effect of RS-BD administration may have been due to restored βHB levels (about 1 mM) rather than supra-normal ketosis).

iii. Ketone salts:

As an alternative means to increase blood ketone concentration without dietary manipulation and widespread metabolic effects, synthetic ketones can be administered to achieve ketosis. However, the ketone anion (acetoacetate; β-hydroxybutyrate) must be formulated either as the acid, or as a salt with an appropriate cation (Na^+, K^+, Ca^{2+}). Ketone salts can be used intravenously (Mikkelsen et al., 2015a) or enterally to increase blood ketone levels (Balasse and Ooms, 1968b, Fery and Balasse, 1988, Keller et al., 1988, Mikkelsen et al., 2015a, Sherwin et al., 1975). Ketone salt preparations are typically racemic mixtures of two optical isoforms, D- and L-βHB, with only one of the stereoisomers (D-βHB) known to be biologically active; not detected in blood, the biological role of L-βHB is uncertain (Desrochers et al., 1992, Lincoln et al., 1987, Scofield et al., 1982, Webber and Edmond, 1977). One study showed that L-βHB is not readily oxidised by mammalian tissues (Desrochers et al., 1992, Webber and Edmond, 1977); others showed that L-βHB may be

converted to D-βHB and AcAc (Desrochers et al., 1992, Lincoln et al., 1987) or used for sterol biosynthesis (Webber and Edmond, 1977).

Intake of large amounts of cations can be deleterious, causing acute metabolic alkalosis and increased oncotic pressure with fluid retention, which have health implications in the context of cardiac disease (Cahill, 2006b, Fery and Balasse, 1988, Muller et al., 1984). Long-term consumption of ketone salts and high levels of inorganic ions can cause hypertension (Dahl, 2005) with cardiovascular consequences. High salt intake is associated with adverse cardiovascular events, such as stroke (Strazzullo et al., 2009), whilst reduction in sodium intake can reduce blood pressure, stroke and fatal coronary heart disease in adults (Aburto et al., 2013). Ketone salts (such as a sodium/potassium β-hydroxybutyrate (βHB) mineral salt (BMS)) are not efficient in inducing hyperketonaemia in rodents (Kesl et al., 2016), and pharmacokinetics and pharmacodynamics of ketone salt supplementation in humans remains unclear.

Ketone salts provided a neuroprotective effect in two patients with hypoketotic hypoglycaemia (Plecko et al., 2002a) and exogenous ketone salt supplementation reduced anxiety-related behaviour in rats (Ari et al., 2016). In rat, ketone salt decreased atherogenic apolipoprotein B (apoB)-containing lipoproteins, low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) and increased anti-atherogenic HDL-cholesterol concentration (Caminhotto et al., 2017), suggesting an effect of ketones on reverse cholesterol transport. In athletes, oral ketone salt supplementation did not improve exercise performance, despite elevated blood ketone concentrations (Rodger et al., 2017), whilst ketone salt ingestion impaired exercise performance in healthy adult mice, despite increased fatty acid oxidation (O'Malley et al., 2017). A racemic salt mixture raised blood βHB levels to 0.76 mM in healthy male athletes but had no effect on cognition performance after exercise (Waldman et al., 2019). The acute cardiovascular effects of sodium

hydroxybutyrate intravenous infusion, to raise plasma βHB concentration from 0.4 to 3.3 mM, was accompanied by increased cardiac output (by 2 L/min) due to an increase in stroke volume and heart rate in patients with HF and reduced ejection fraction (Nielsen et al., 2019). Sodium hydroxybutyrate infusion increased left ventricular ejection fraction by 8% and MVO_2 without changing myocardial external energy efficiency (MEE; a similar cardiac output and MEE response was seen in matched controls) (Nielsen et al., 2019).

iv. Ketone esters:

An alternative synthetic strategy for exogenous ketone administration which avoids the need for a cation or acid load is to esterify the ketone body anion. Ketone ester compounds are made up of either βHB or AcAc, esterified to glycerol or (*R*)-1,3-butanediol. Free ketone (βHB; AcAc) is released into the circulation from the ingested ketone ester via the gastrointestinal tract. Ketone esters can elevate blood βHB higher than exogenous ketone salts. The first ketone ester, glycerol with acetoacetate, was used as an alternative energy source to glucose in rats (Birkhahn et al., 1977, Birkhahn et al., 1979). Brunengraber and colleagues showed that the oral ketone ester (R,S-1,3-butanediol acetoacetate) is a diffusible pH-neutral and sodium-free precursor of ketone bodies, which could rapidly increase blood ketone concentrations to 5 mM in pigs (Desrochers et al., 1992, Desrochers et al., 1995). Clarke and colleagues developed the first oral non-racemic human ketone monoester by trans-esterifying ethyl (*R*)-3-hydroxybutyrate with (*R*)-1,3-butanediol using lipase to produce the ketone monoester, (*R*)-3-hydroxybutyl-(*R*)-3-hydroxybutyrate, that can safely increase blood βHB concentrations up to 5 mM in healthy adults (Stubbs et al., 2017, Clarke et al., 2012a, Shivva et al., 2016b) and athletes (Cahill and Owen, 1968a, Cox et al., 2016b, Holdsworth et al., 2017a, Vandoorne et al., 2017). In gut, the ester bond is hydrolysed by esterases (Van Gelder et al., 2000), releasing (*R*)-1,3-butanediol and D-βHB into the portal circulation. The butanediol is metabolized by liver to form D -βHB and AcAc,

which are released into the systemic circulation. Increased plasma levels of βHB were attained within 20 minutes and the elimination half-life ranged from 0.8-3.1 h (Clarke et al., 2012c, Shivva et al., 2016b), suggesting that exogenous ketone monoester supplementation is a safe and simple method to elevate blood ketone levels (Soto-Mota et al., 2019).

Ketone esters modestly lower both blood glucose and fatty acid concentrations in fed and fasted subjects, with high insulin levels maintained in the fed state, suggesting that there was no synergistic effect of insulin and βHB in reducing blood glucose and fatty acid concentrations (Stubbs et al., 2017). Exogenous ketones may stimulate insulin secretion in animals by a direct action on pancreatic β cells (Madison et al., 1964, Miles et al., 1981a) and in isolated pancreatic islets (Biden and Taylor, 1983), causing mild hypoglycaemia. In humans, ketone ester drinks increased glucose uptake and plasma insulin in response to an intravenous glucose challenge (Holdsworth et al., 2017a). Hence ketone ester ingestion can maintain ketosis, allowing a normal meal pattern and providing an alternative dietary method to achieve ketosis.

Little data for the effects of exogenous ketone esters on cardiac muscle metabolism exist, but several studies have examined ketone ester metabolism in skeletal muscle. Ketone esters improve endurance performance and recovery from exercise by acting as oxidative substrates, but also potentially by inhibiting muscle glycolysis and increasing muscle glycogen (Holdsworth et al., 2017a, Cox et al., 2016b, Murray et al., 2016a, Vandoorne et al., 2017); ketosis also facilitated intramyocyte TAG oxidation, despite preserved glycogen stores. Ketone ester drinks may benefit athletes consuming normal mixed diets, as carbohydrate facilitates the ability of muscle to oxidise ketone bodies during exercise; the same may be true for cardiac muscle. Cox and colleagues showed that, in the glycogen replete state, ketone body oxidation from ketone ester drinks may account for ~10%-18% of the total oxygen consumption during exercise (Cox et al., 2016b), although the study did not

distinguish between skeletal and cardiac muscle oxidation. How these findings relate to human cardiac muscle performance is currently unknown.

Fatty acids are increased in advanced, severe dilated cardiomyopathy (DCM) and hypertrophy/HF associated with myocardial insulin resistance and impaired glucose uptake, with decreased PCr:ATP ratios and ATP levels (which predict mortality) (Conway et al., 1991, Nikolaidis et al., 2004, Neubauer et al., 1992). In human heart failure, plasma fatty acid levels correlated positively with mitochondrial uncoupling protein UCP2 and UCP3 (Murray et al., 2004b); UCPs can diminish cardiac efficiency by dissipating the mitochondrial intermembrane proton gradient and transporting fatty acid anions out of the mitochondrial matrix (Boehm et al., 2001b, Cole et al., 2011a, Fillmore et al., 2014, Murray et al., 2009b). However, the exogenous ketone ester lowers fatty acids, TAG and glucose (Myette-Cote et al., 2018, Stubbs et al., 2017), in agreement with studies in which ketosis by ketone infusion also decreased fatty acids levels under both fasting and fed conditions (Balasse and Ooms, 1968b, Miles et al., 1981a, Mikkelsen et al., 2015a). The anti-lipolytic effect of the exogenous ketone ester is even more pronounced with glucose ingestion; the decreased plasma fatty acids was associated with improved insulin sensitivity measured by oral glucose insulin sensitivity index in healthy young adults (Myette-Cote et al., 2018). Therefore, exogenous ketosis (from ketone esters or salts) can improve cardiac efficiency by decreasing lipolysis and plasma fatty acids, mediated through adipocyte GPR109A signaling, decreasing PPARα stimulation and hence lowering mitochondrial UCP levels (Tunaru et al., 2003a, Taggart et al., 2005a) (Figure 3). However, decreasing fatty acid levels using acipimox, which inhibits lipase through the GPR109A receptor, decreased cardiac work without changing oxidative metabolism in humans with idiopathic dilated cardiomyopathy, and hence decreased myocardial efficiency (Tuunanen et al., 2006).

Probably lowering FA levels using acipimox without providing an alternative oxidative substrate, such as βHB, would have "semi-starved" the heart.

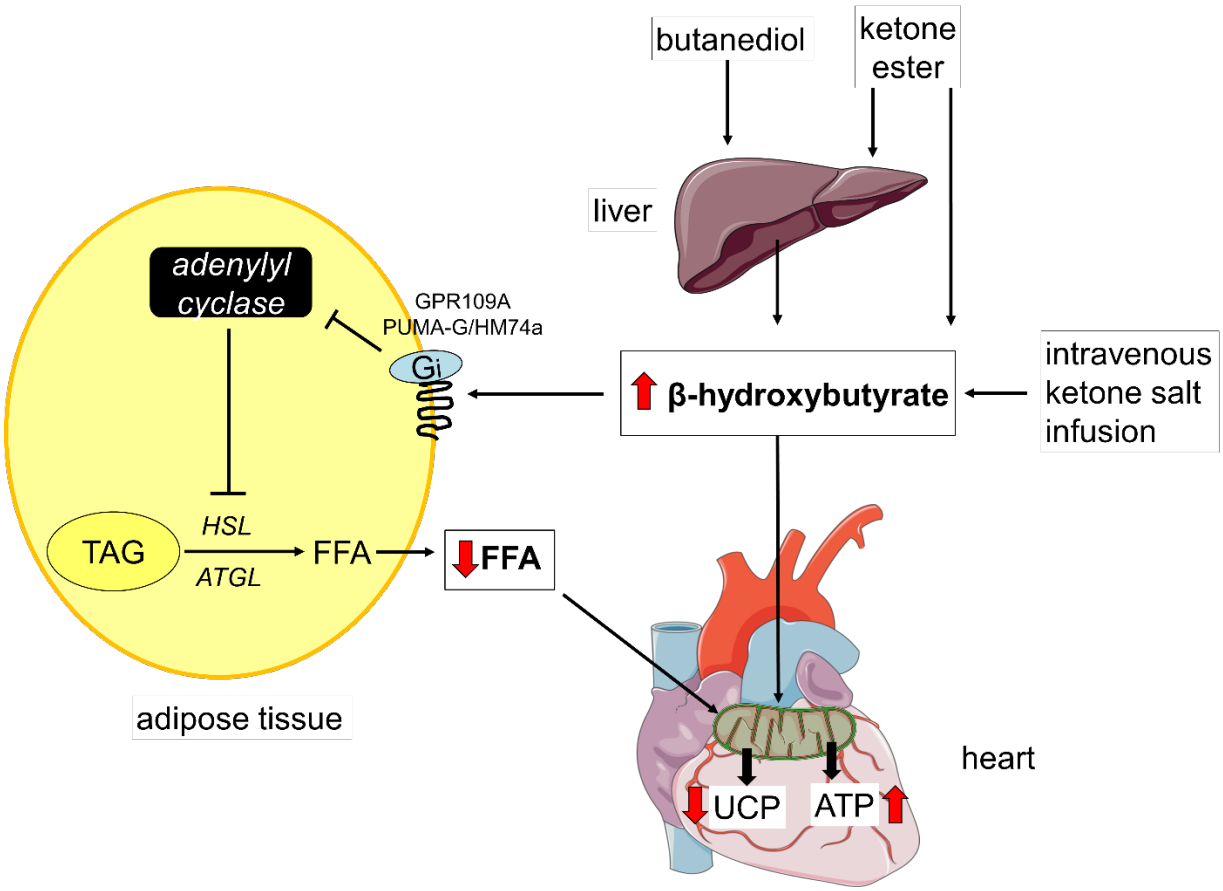


Figure 3. Putative mechanism for inhibition of adipocyte lipolysis by β-hydroxybutyrate (βHB) via the nicotinic acid receptor. *β-Hydroxybutyrate (βHB) from either ketone drinks or intravenous infusion activates nicotinic acid receptor (GPR109A) in adipose tissue, leading to inhibition of adipocyte adenylyl cyclase, results in a suppression of adipocyte lipases, such as hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). The suppression of the lipase reduces the amount of free fatty acids (FFA) as well as mitochondrial uncoupling protein (UCP). Oxidation of βHB increases the energy of (adenosine triphosphate) ATP hydrolysis (yielding more ATP per molecule of oxygen) by generating more NADH to be delivered to complex I within electron transport chain. TAG, triacylglycerol; ATP, adenosine triphosphate.*

Although direct evidence of a beneficial therapeutic effect of exogenous ketosis on cardiac function is lacking, evidence that increased KB oxidation may improve cardiac energetics in the (potentially energy-starved) failing heart is provided in a recent study of *ex-vivo* mouse hearts. Increasing βHB concentrations from 0.2 to 0.6 mM in pressure-overloaded, hypertrophied failing mouse hearts more than doubled ketone oxidation rates (without altering glycolysis, glucose oxidation or palmitate oxidation) and increased cardiac ATP production by 23%, with KB themselves providing some 18% of energy provision. Hence KBs appeared to restore the acetyl-CoA pool, Krebs cycle activity and ATP production rates. However, cardiac work and cardiac efficiency were unchanged. The authors postulated that the inhibitory effect of KBs on glucose or fatty acid oxidation may occur at a supra-physiological level (Ho et al., 2019). These results remain to be extended to human heart failure.

The ability of exogenous ketones to lower fatty acids and glucose implies that, perhaps paradoxically given their elevated endogenous levels and feared reputation, KBs may have a place in the management of diabetes, but their potential benefit in diabetic cardiomyopathy is unknown.

v. Pharmacologically-induced ketosis

The introduction of a novel class of oral hypoglycaemic drugs in the treatment of type 2 diabetes mellitus, the sodium-glucose cotransporter-2 (SGLT2) inhibitors such as empagliflozin, has unexpectedly provided new evidence of a role for ketone bodies in cardiac function. Treatment with SGLT2 inhibitors normalised blood glucose by inhibiting renal proximal tubular SGLT2-mediated glucose reuptake; SGLT2 is also expressed in pancreatic α-cells (Bonner et al., 2015) and SGLT2 inhibitors promote glucagon production (Prattichizzo et al., 2018, Taylor et al., 2015, Zinman et al., 2015). SGLT2 inhibition is associated with increased ketoacidosis, despite euglycaemia (Taylor et al., 2015), which has

been viewed anxiously by clinicians because the warning sign of hyperglycaemia is absent and diabetic ketoacidosis is considered to be a grave complication of uncontrolled diabetes. However, when the results of the EMPA-REG OUTCOME trial of empagliflozin treatment in type 2 diabetic patients with high cardiovascular risk were published (Zinman et al., 2015, Fitchett et al., 2016, Fitchett et al., 2018), there was a 35% relative risk reduction in hospitalisation for HF, a 38% reduction in cardiovascular death, and 75% reduction in death caused by worsening HF in patients taking the SGLT2 inhibitor. The benefits were observed at 3 months and continued for more than 3 years (Zinman et al., 2015, Fitchett et al., 2016, Fitchett et al., 2018). Short term empagliflozin treatment was associated with decreased left ventricular mass index and improved diastolic function (Verma et al., 2018). Empagliflozin appeared to rescue diabetic patients from cardiac decompensation. A small improvement in glycaemic control and visceral adiposity, together with mild natriuresis occurred, but insufficient to account for the observed benefits, so suggesting an alternative mechanism. SGLT2 inhibitors cause a mild, persistent hyperketonaemia (which can develop into ketoacidosis following metabolic stress); the mechanism for this is uncertain, but may relate to increased renal ketone body reabsorption secondary to the renal sodium effects caused by empagliflozin: the sodium-monocarboxylate cotransporter-1 (SLC5A8) mediates co-transport of sodium with acetoacetate and βHB (Taylor et al., 2015, Martin et al., 2006). Several authors have hypothesised that this changed metabolic profile increased myocardial KB utilisation at the expense of fatty acid oxidation; as ketones are more oxygen-efficient than fatty acids, the resulting increase in metabolic efficiency offered myocardial protection and may have improved cardiac function (Mudaliar et al., 2016a, Ferrannini et al., 2016a, Ferrannini et al., 2016b). SGLT2 inhibition with empagliflozin attenuated hypertrophy, decreased interstitial fibrosis, stimulated mitochondrial biogenesis, normalised uptake and oxidation of glucose and fatty acids and improved cardiac function in non-diabetic rats

following myocardial infarction; this was associated with increased plasma ketone bodies, ketone transporters and BDH1 and SCOT enzymes, suggesting increased ketone body oxidation (Yurista et al., 2019). Empagliflozin decreased renal lipid accumulation in diabetic mice (Wang et al., 2017); a similar mechanism in the diabetic myocardium would potentially reduce the lipotoxicity, and hence cardiac dysfunction, in this condition.

Some authors have argued that the improvements are unlikely to be entirely metabolic and may be at least partly explained by improved haemodynamics (Abdul-Ghani et al., 2016). Empagliflozin has also been found to improve cardiac function and prevent cardiomyopathy in diabetic mice by inhibiting oxidative stress-mediated injury by the soluble guanylate cyclase (sGC)-cyclic guanosine monophosphate (cGMP)-cGMP-dependent protein kinase (PKG) pathway (Xue et al., 2019). Furthermore, investigation in animal models have failed to support the ketone body "superfuel" or "thrifty substrate" hypothesis in relation to the diabetic heart. Diabetic (db/db) mouse hearts were assessed for energy provision, and rates of glucose and ketone body oxidation were decreased, but fatty acid oxidation was increased, with overall decrease in cardiac ATP production (Verma et al., 2018); however, treatment with empagliflozin increased cardiac ATP production by about 30% (and prevented cardiac failure), but this was due to increased rates of glucose and fatty acid oxidation, with no change in ketone oxidation (Verma et al., 2018), arguing against a central role for myocardial ketone oxidation in diabetic cardiac function. Furthermore, fatty acids were found to protect diabetic rat heart against low-flow ischaemia, whilst ketone bodies did not (King et al.). However, the role of induced/exogenous ketosis in the context of cardiac function in diabetes remains unresolved, and it is possible that increasing cardiac ketone oxidation at the expense of fatty acid utilisation (though not necessarily at the expense of glucose availability) may be beneficial in diabetic heart disease and such a profile could be obtained by exogenous ketosis, for example by ketone ester administration.

Alternatively, the beneficial effects on cardiac function observed in SGLT2 inhibitor studies may be related to a signalling role of ketone bodies rather than an energetic substrate mechanism.

Conclusion:

From a recognition that ketone bodies are not merely metabolic waste products but have a vital role in energy provision, both as substrates and signals, it is now well established that KBs are important in cardiac energetics, in the diseased as well as the healthy heart. In addition, it is recently recognised that much heart disease has a metabolic aetiological basis, and altered myocardial KB metabolism is a characteristic of heart failure. The ability to manipulate ketosis, and hence KB provision, offers the possibility of managing heart disease, potentially through both substrate provision and signalling functions of ketones. The challenge remains to establish an acceptable regime, and to fully elucidate the pathways involved.

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Competing interest declaration:

AbAK is in receipt of a Malaysian Government (Ministry of Higher Education) Scholarship. The intellectual property, and patents covering the uses of ketone bodies and esters are owned by BTG Ltd, the University of Oxford, the NIH and TdeltaS Ltd. Should royalties ever accrue from these patents, KC as named inventor may receive a share of royalties as determined by the terms of the University of Oxford. KC is director of TdeltaS, a spin out company of the University of Oxford, aiming to develop and commercialise products based on the KE.

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