University of Alberta

Short-term Calorie Restriction Improves Post-ischemic Recovery in the Spontaneously Hypertensive Rat

by

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DEDICATION

Thesis dedicated to my dearest husband Timothy S. Wozniak, parents Lidia and Stefan Lozyk, and late grandmother Katarzyna Kocan (30.11.1922 − 06.05.2012).

ABSTRACT

Ischemic heart disease (IHD) is associated with morbidity and mortality and is the leading cause of death in Western countries. The findings from this thesis has provided an important understanding how alterations in energy metabolism and the activation of Reperfusion Injury Salvage Kinase (RISK) pathway induced by short-term calorie restriction (CR) contribute to protecting the diseased heart from ischemia/reperfusion (I/R) injury. Our findings using the Spontaneously Hypertensive Rat (SHR) validate that short-term CR exhibits cardioprotection in the diseased rat model of hypertension and cardiac hypertrophy. Our data also suggest that improving glucose oxidation at the time of reperfusion as well as activating two members of the pro-survival anti-apoptotic RISK pathway, Akt and Erk1/2 MAPK, are possible mechanism by which short-term CR contributes to improving mechanical recovery of the heart during reperfusion following ischemia. Additionally, our data suggest that the effects of short-term CR in I/R injury may be mediated by an AMPK-independent mechanism as at the time of reperfusion following ischemia as the hearts from SHRs exhibited improved metabolic status in presence of significantly reduced AMPK activity. Based on all of our findings, we propose the short-term CR as a novel therapeutic approach that can be used to prevent I/R injury in the setting of cardiac surgery due to its effectiveness, practicality, inexpensiveness, and absence of side effects associated with cardioprotective pharmacological agents.

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LIST OF ABBREVIATIONS

µM…….....Micromolar
3-KAT……..…..3-Ketoacyl-CoA thiolase
ACC……...…….Acetyl-CoA carboxylase
AMPK……..…5’-adenosine monophosphate-activated protein kinase
AMPKK……...……AMPK kinase
ATP……..Adenine5’-triphosphate
bpm……...Beats per minute
Ca^{2+}……..……..……..Calcium ion
CABG……Coronary artery by-pass graft surgery
CaMKKβ……..……Ca^{2+}-calmodulin-dependent kinase kinase β
CAT……..……..Carnitine acetyl-transferase
CBS……..……..Cystathionine β-synthase
CF……...…….....Coronary flow
CHD……..…….Coronary heart disease
CIOMS…… ……...Council for International Organizations of Medical Sciences
CoA…….....Coenzyme-A
-COOH…….....Carboxyl group
CP……...…Cardiac power
CPT……....Carnitine palmitoyltransferase
CR……...…...…...Caloric restriction
DAG…….....Donkey anti-goat
DBP……..…Diastolic blood pressure
EF……...…….....Ejection fraction
ETS……..……Electron Transport System
F-2,6-bP……Fructose 1,6-bisphosphate
FA……..……..…Fatty acid
FABP……..Fatty acid-binding proteins
$FABP_{pm}$ …….....Plasma Membrane Fatty acid-binding protein
FACS……..Fatty acyl-CoA synthase
$FAD/FADH_2$…….…….....Flavin adenine dinucleotide
FFA…….……..….Free fatty acids
GAPDH……....Glyceraldehyde 3-phosphate dehydrogenase
GAR……..……..…….Goat anti-rabbit
GLUT…….....Glucose transporters
GPCR…….....G-protein coupled receptors
GPDH…….……...……..Glyceraldehyde 3-phosphate dehydrogenase
GSK-3β……..…Glycogen synthase kinase -3β
GTP……..……Guanosine 5’-triphosphate
H_2O……...……Water
HK…….....Hexokinase
HR…….....Heart rate

I/R……Ischemia/Reperfusion
IC……..……..Intracellular
IHD……Ischemic heart disease
IP……..…Intraperitoneal
IPC…….Ischemic preconditioning
LDH……...……Lactate dehydrogenase
LV…….Left ventricular
LVEDV……...Left ventricular end-diastolic volume
LVESV…….Left ventricular end-systolic volume
MAPK……Mitogen-associated protein kinase
MCD……...Malonyl-CoA decarboxylase
Mg^{2+}……..Magnesium ion
mmHg……...Millimeters of Mercury
mPTP…….....Mitochondrial permeability transition pore
mTOR……Mammalian target of rapamycin
mWatts.……..……...…….milliWatts
N_2…….....Nitrogen
$NADH/NAD^+$……Nicotinamide adenine dinucleotide
O_2……...……...….Oxygen
^oC……...Degree Celsius
PDC……..Pyruvate dehydrogenase complex
PDH……Pyruvate dehydrogenase
PDP……Pyruvate dehydrogenase phosphatase
PFK……...Phosphofructokinase
PGAL…….....Glyceraldehyde 3-phsophate
pH……Log[H^+]
PKA……..……...Protein kinase A
PM……..……..……...Plasma membrane
PSP……..Peak systolic pressure
RISK……Reperfusion Injury Salvage Kinase
SBP……..…….....Systolic blood pressure
SERCA……Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SHR……Spontaneously Hypertensive Rat
SR…….....Sarcoplasmic Reticulum
SV……..……..Stoke volume
TAG……..……...…..…Triacylglyceride
TAK-1.....Transforming growth factor-β-activated kinase-1
TCA……..Tricarboxylic acid
TCC…….....Tricarboxylate carrier
VLDL……...…Very low-density lipoprotein
VPR……...Volume-Pressure Recording

Chapter 1: INTRODUCTION

1.1 Cardiac Energy Metabolism - Introduction

A normal average adult heart beats 100,000 times and pumps about 2,000 gallons of blood per day [1]. In order to meet and sustain this substantial contractile function, in addition to its basal metabolism and to a lesser degree ionic homeostasis [2], the heart must continually produce and utilize enormous amount of energy in the form of adenosine 5'-triphosphate (ATP) [3, 4]. Consequently, although the myocardial ATP content is relatively low (5μmol/g wet wt), the ATP turnover rate is very high (~0.5 μmol · g wet wt^{-1} · s^{-1} or ~30 μmol· g wet wt^{-1}· s^{-1} at rest) with normally a complete turnover of the myocardial ATP pool approximately every ten seconds [4-9]; and resulting with the heart cycling from 3.5 to 5 kg of ATP per day to meet this considerable energy requirements [10, 11]. Due to this rapid consumption of energy, the pathways involved in the synthesis of ATP are closely controlled and under normal physiological conditions respond quickly to changes in the heart's energy demand [12, 13]. This close control of metabolic pathways and ability for rapid response allows for maintenance of equilibrium of metabolites in the cytoplasm and mitochondria over a large range of workloads [12].

Furthermore, under normal physiological conditions, cardiac metabolism is primarily aerobic and most of energy in the form of ATP is supplied *via* mitochondrial oxidative metabolism with small proportion derived from anaerobic catabolism − glycolysis [14, 15]. Specifically, in the normal heart almost all (>95%) generated ATP comes from mitochondrial oxidative phosphorylation, with the remainder derived from glycolysis and guanosine 5'-triphosphate (GTP) formed in the tricarboxylic acid (TCA) cycle [4, 14, 16, 17]. Mitochondrial oxidative phosphorylation is fuelled with energy derived from electrons transferred from carbon fuels by dehydration reactions to NAD^+ and FAD generating NADH and $FADH_2$ [16]. NADH and $FADH_2$ are electron carriers that are primarily produced through the fatty acid β-oxidation, the TCA cycle, and to a lesser extent from the glycolysis and pyruvate dehydrogenase reaction [16].

In the normal heart, the rate of oxidative phosphorylation is intricately linked to the rate of ATP hydrolysis, so that ATP level remains constant even with large increases in cardiac power [18-20] occurring during intense exercise or acute catecholamine stress [16]. To maintain this continuous and constant ATP level the normal heart is capable of utilizing various carbon substrates to generate ATP such as fatty acids, glucose, lactate, ketone bodies, and amino acids [2, 4, 5, 11, 21, 22]. Although, the total production of ATP is fairly constant, the source of ATP produced does change with available substrate [23]. The preferred substrate depends upon arterial concentrations, coronary blood flow, hormonal factors, nutrition status, oxygen supply, and workload [14, 16, 24, 25]. Under normal physiological conditions, the preferred substrates are fatty acids, glucose, and/or lactate − the production of energy is primarily derived from the catabolism of both fatty acids and pyruvate derived from either glycolysis or lactate [4].

Generally, a balance between these two pathways exists with fatty acid oxidation providing 60-70% of overall cardiac ATP supply [4, 8, 23, 26] and glucose and lactate providing the remainder one third of the heart's energy [4, 7, 8, 11, 17, 25, 27-30]. Of produced total ATP, approximately 60-70% fuels contractile function and the remaining 30-40% is primarily used for the activity of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and other ion ATP-dependent pumps [5, 31]. Indeed, although glycolysis contributes only small yields of ATP normally about 5% of total ATP produced by the aerobic heart [11], it is the glycolytic supply of ATP that is believed to be essential in maintenance of ionic stability and cell integrity [32]. Furthermore, even though glycolysis under normal physiological conditions is of small relevance, it is very important in the settings of myocardial ischemia, which results in decrease in oxygen supply to the heart and subsequent decrease in energy production [24]. This is because in the setting of the myocardial ischemia when oxygen is the limiting factor the utilization of the fatty acids as energy source is less oxygen-efficient [24]. Even though fatty acids produce more ATP than carbohydrates, they require approximately 10% more oxygen to produce an equivalent amount of ATP [4].

1.2 Cardiac Energy Metabolism – Overview of Pathways

Cellular respiration (Reviewed in [33, 34]) is a catabolic pathway that involves a controlled stepwise degradation of organic molecules from more reduced (higher stored potential energy) to more oxidized (lower stored potential energy) states and subsequent storage of extracted energy in the form of ATP. The two terms used frequently when discussing Redox reactions of cellular respiration are oxidation and reduction. The term reduction means loss of oxygen (O_2), or decrease in the number of bonds to oxygen, gain of hydrogen (H_2), or gain of electrons ($\bar{e}$) by an atom, ion, or molecule. Oxidation means the opposite. Furthermore, although cellular respiration is generally defined as the oxygen-requiring (aerobic) process in certain instances respiration can occur without presence of oxygen (anaerobic). The difference between the two types of respiration is the type of final electron acceptor. In the aerobic respiration, electrons are accepted by an exogenous electron acceptor such as O_2 producing water (H_2O) and carbon dioxide (CO_2).

A type of anaerobic respiration in eukaryotic cells is a non-oxidative/anaerobic glycolysis (Reviewed in [33, 34]). It is a process of energy derivation where an endogenous electrons acceptor is an organic molecule − pyruvate. Non-oxidative/anaerobic glycolysis is important under oxygen deprivation for the continuous production of ATP by glycolysis. In eukaryotic cells, this is accomplished by the regeneration of energy carrier the coenzyme NAD^+ (nicotinamide adenine dinucleotide) by reduction of pyruvate to lactate. The key energy carriers (Reviewed in [33]) involved in transporting energy between reactions during cellular metabolism are: ATP, coenzymes NAD^+, and FAD (flavin adenine dinucleotide). ATP is the cell's main short-term energy storage molecule. Direct synthesis of ATP is unfavourable, however the coupling of oxidation-reduction reactions in cellular respiration allows for the production of ATP. The main cellular processes in the oxidative catabolism resulting in energy production are glycolysis (splitting of glucose), β-oxidation of fatty acids (FAs), protein catabolism, oxidative decarboxylation by Pyruvate Dehydrogenase Complex (PDC), Tricarboxylic Acid cycle (TCA cycle),

Electron Transport System (ETS), and oxidative phosphorylation. All of these metabolic processes except protein catabolism are relevant to this thesis and will be discussed in details in the sections below.

Briefly, glycolysis (Reviewed in [33, 34]), a breakdown of simple sugar glucose, occurs in cytoplasm and does not require oxygen. During glycolysis 6-carbon glucose is partially oxidized and split in half to two 3-carbon pyruvate molecules. A small amount of ATP and NADH is produced in this anaerobic process. Prior to entering the TCA cycle in the mitochondrial matrix, pyruvate is oxidatively decarboxylated (oxidized to release CO_2 and produce NADH) and activated by the subsequent transfer of acetyl group to the coenzyme A (CoA) *via* action of the multi-complex of enzymes collectively referred to as Pyruvate Dehydrogenase Complex (PDC). β-oxidation (Review in [33]) is a repetitive four-step process by which fatty acid is converted into 2-carbon acetyl-CoA. At first, saturated fatty acid is activated in the cytoplasm in a process that requires two ATP. Once activated, fatty acid is transported into the mitochondrion and undergoes a series of β-oxidation cycles that convert it into 2-carbon acetyl-CoA fragments. With each round of β-oxidation cycle of a saturated fatty acid one molecule of acetyl-CoA, one NADH, and one $FADH_2$ are produced.

Glucose or fatty acid derived acetyl-CoA enters the TCA cycle (Reviewed in [33, 34]) localized to the mitochondrial matrix. During the TCA cycle, a 2-carbon acetyl group combines with a 4-carbon oxaloacetate to form a 6-carbon citrate. Citrate undergoing oxidative decarboxylations and isomerizations results in the regeneration of the original oxaloacetate ready for the next round of TCA cycle and the release of two molecules of CO_2 (exhaled by the organism). Oxidative decarboxylation was a reaction repeated earlier by the Pyruvate Dehydrogenase Complex, in which a molecule is oxidized to release CO_2 and produce NADH. In oxidative decarboxylation a molecule is reduced by one carbon, CO_2 is given off, and NADH is made from NAD^+. Upon completion of the one round of the TCA cycle, a modest quantity of ATP *via* GTP intermediate, a large quantity of NADH, and a small quantity of $FADH_2$ are generated. Although PDC and TCA cycle cannot occur without the presence of oxygen neither uses exogenous oxygen directly. Oxygen is necessary in the last stage of aerobic cellular

respiration − the electron transport and oxidative phosphorylation stage. *Via* function of Electron Transport Chain (Reviewed in [33]) located in the inner mitochondrial membrane NADH and $FADH_2$ are oxidized regenerating NAD^+ and FAD. Oxygen as the final electron acceptor is reduced to water and energy liberated by the electron transport chain is utilized to develop a proton gradient across the inner mitochondrial membrane, which subsequently is used in the synthesis of very large amounts of ATP. Aerobic cellular respiration is the most efficient catabolic pathway used by organisms to harvest the energy stored in nutrients such as carbohydrates, proteins, or fats. While anaerobic processes yield minimal amounts of ATP, aerobic cellular respiration yields very large amounts of ATP.

1.2.1 Glucose Metabolism

1.2.1.1 Glucose Uptake

The majority of glucose metabolized by the heart is obtained from the blood with its uptake facilitated by the transmembrane glucose gradient, and the concentration and the activity of glucose transporters (GLUT) located in the sarcolemma (GLUT-4 and GLUT-1) [35, 36]. In the absence of insulin glucose transport is generally rate limiting over any glucose phosphorylation processes within the cardiac myocyte [37]. This glucose transport is determined by the transmembrane glucose gradient, which in turn is determined by the interstitial and intracellular (IC) glucose concentrations [38]. The interstitial glucose level is a function of arterial glucose concentration and blood flow, both decreased during ischemia [38]. As the intracellular glucose level is normally low, glucose transporters facilitate glucose movement down its concentration gradient [36]. However, the membrane capacitance for glucose transport and the rate of glucose uptake [39, 40] is determined by the degree of translocation of glucose transporters to the sarcolemma.

The stimulation of myocardial glucose transport involves recruitment from intracellular storage sites to the sarcolemma of GLUT-1 and GLUT-4 [41]. In fact, GLUT-4 and GLUT-1 are the two isoforms of glucose transporters that have been identified in the sarcolemmal membrane and in the intracellular microsomal vesicles [39, 42-44]. GLUT-1 is responsible for maintaining basal glucose

uptake, whereas GLUT-4 translocates (and to a lesser extend GLUT-1) from intracellular storage site to the sarcolemmal membrane in response to increased workload/exercise, insulin stimulation, or ischemia [21, 35, 36, 45-52]. During time of energy demand, the translocation of GLUT-4 to the sarcolemma is also stimulated by action of 5'-adenosine monophosphate-activated protein kinase (AMPK) *via* a multistep pathway involving a phosphatidylinositol 3-kinase (PI_3K)-dependent mechanism [36, 48, 53]. This translocation of glucose transporters to the sarcolemmal membrane increases the membrane's capability for glucose transport [39, 40]. On the contrary, a decreased cardiac work and a presence of fatty acids as alternative energy substrates reduce cellular glucose uptake [45, 54].

1.2.1.2 Glycolysis

Glycolysis (Reviewed in [33, 34]) is the first stage in extraction of energy from carbohydrates. During glycolysis (Figure 1.1), 6-carbon glucose is partially oxidized and split in half to two 3-carbon pyruvate molecules. A small amount of ATP and NADH are produced in this anaerobic process. Specifically, this biochemical process converts glucose to pyruvate or lactate under aerobic or anaerobic conditions, respectively. Under aerobic conditions the series of ten enzymatically controlled reactions (three of which are irreversible) lead to the oxidative breakdown of glucose into two molecules of pyruvate (the ionized form of pyruvic acid), the reduction of NAD^+ to NADH, and the net production of two moles of ATP per one mole of exogenous glucose. The general strategy of glycolysis is to first phosphorylate the glucose molecule on both ends and then split it into two 3-carbon units, which then can enter the PDC and TCA cycle. This breakdown of the 6-carbon glucose into two molecules of the 3-carbon pyruvate occurs in ten glycolytic steps. Each step catalyzed by a different enzyme. First five steps constitute the investment phase where the energy is spent and the last five steps are the payoff phase where energy is gained.

The two phases of glycolysis can be broken into four major events – two events per phase. The two major events of the investment phase are: 1) the primary phosphorylation of glucose molecule, which utilizes two ATP; and 2) splitting of the 6-carbon glucose molecule into two 3-carbon intermediates. The

two primary events of the payoff phase are: 1) oxidation of 3-carbon molecule and formation of a high-energy phosphate bond *via* substrate-level phosphorylation to produce NADH and ATP; and 2) subsequent molecular rearrangement of the 3-carbon glucose intermediate to form a high-energy phosphate bond *via* substrate level phosphorylation to produce another ATP. At the end of glycolysis much of the initial energy of the glucose molecule is still stored in the pyruvate and depending on conditions pyruvate degradation can proceed in one of two directions. Under aerobic conditions, pyruvate is further oxidized during cell respiration in the mitochondria to generate large amount of ATP. Under anaerobic conditions in the process of non-oxidative/anaerobic glycolysis in eukaryotic cells, pyruvate is converted to lactate. This is because an oxidative step resulting in the production of NADH *via* conversion of glyceraldehyde 3-phsophate (PGAL) to 1,3-bisphosphoglycerate through the action of enzyme glyceraldehyde 3-phosphate dehydrogenase (GPDH) is rate-limiting.

To allow for glycolysis to continue in the absence of oxygen at a maximal rate, NAD^+ must be continually regenerated through the oxidation of NADH. In eukaryotes, this regeneration of NAD^+ under anaerobic conditions is accomplished by a reduction of pyruvate to lactate *via* action of enzyme lactate dehydrogenase (LDH) (Figure 1.1). Oxidative/aerobic and non-oxidative/anaerobic glycolysis refers to essentially the same process although their end products differ. Non-oxidative/anaerobic glycolysis refers to all of the reactions involved in glycolysis and the additional steps leading to the reduction of pyruvate to lactate. Non-oxidative glycolysis produces only two ATP per glucose molecule. The NAD^+ produced by reducing pyruvate anaerobically is available for reuse in the glycolytic pathway. However, there is a limit to the use of anaerobic glycolysis as an energy source − accumulation of lactate in the cells is toxic at high concentrations.

The rate of glycolysis, which can be affected by cardiac work [55] and oxygen supply/ischemia [16, 56], is controlled by multiple steps along the pathway [57]. Upon entering the cell, free glucose is rapidly phosphorylated by the enzymes hexokinase-1 (HKI) and /or hexokinase-2 (HKII) (the predominant form in the adult heart) [58-60] to form glucose-6-phosphate (G-6-P), which is the juncture

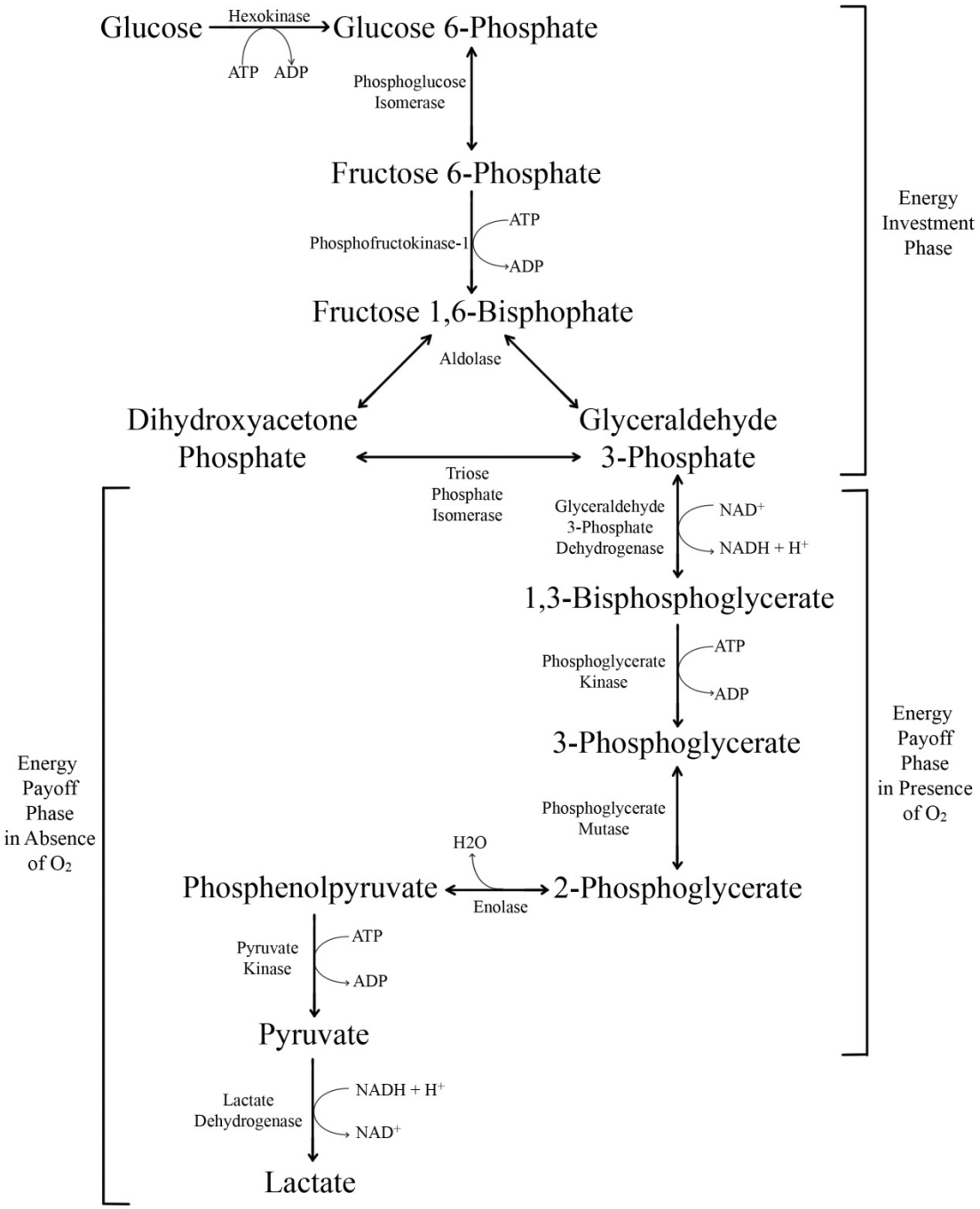
of the pathways of glucose uptake and glycogen breakdown. Accordingly, G-6-P can be used either for glycogen synthesis or it can proceed down the glycolytic pathway to form pyruvate. Glucose phosphorylation by hexokinase is irreversible and renders glucose impermeable to the cell membrane [21]. Insulin activates hexokinase in isolated rat hearts and causes its subsequent release from the outer mitochondrial membrane accelerating the uptake and phosphorylation of glucose, and thus the glycolytic flux [61-63]. Insulin also regulates hexokinase II by inducing mRNA transcription [64]. In addition, hexokinase activity is increased by phosphorylation of protein kinase A (PKA) [65] and inhibited by the accumulation of its product G-6-P [62, 63]. The hexokinase reaction product, G-6-P, is in flux equilibrium with fructose-6-phophate (F-6-P), which becomes the substrate for the primary irreversible reaction of the glycolytic pathway – the phosphorylation of F-6-P by an enzyme 6-phosphofructo-1-kinase (PFK-1).

PFK-1is a key enzyme in the glycolytic pathway [66] – it is a major regulatory point in glycolysis under aerobic conditions. It operates at the junction of pathways of glucose degradation (glycolysis) or storage (glycogenesis) and it is under direct and allosteric regulation. PFK-1 also mediates the final energy-consuming reaction in glycolysis with the phosphorylation of fructose-6-phosphate to fructose 1,6-bisphosphate (F-1,6-bP). This reaction is the first regulatory site that commits glucose to metabolic breakdown *via* glycolysis [67]. There are two isoforms of PFK denoted PKF-1 and PKF-2. PKF-1 phosphorylates fructose 6-phosphate to form fructose 1,6-bisphosphate, whereas PFK-2 forms fructose 2,6-bisphosphate from fructose 6-phsophate [68, 69]. PFK-1 activity is allosterically inhibited by increased levels of cytoplasmic ATP [70-72], citrate [73-76], and H^+ (especially important in severe ischemia) [67]. Alternatively, the activity is PFK-1 is stimulated by ADP, AMP, inorganic phosphate (P_i), calcium ions (Ca^{2+}), and F-2,6-bP [70-72] accelerating flux through glycolysis when the phosphorylation level is low. These regulators indicate adequate energy level and supply of acetyl-CoA to the TCA cycle, thus linking changes in in mitochondrial oxidative metabolism to glycolysis [16]. F-

2,6-bP is a potent stimulator of PFK-1 and is formed from fructose 6-phosphate by a bi-functional enzyme phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) [69, 70].

PFK-2 is allosterically inhibited by citrate, which by decreasing F2,6-bP levels is a second mechanism by which citrate can inhibit PFK-1 activity [37, 77]. In addition, AMPK can also phosphorylate PFK-2 explaining AMP-induced acceleration of glycolysis [78, 79]. The final major regulatory step in glycolysis is glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalysis of NAD^+ – dependent oxidation of glyceraldehyde 3-phophate to 1,3-bisphosphoglyceradehyde and NADH. This step is very important under conditions of oxygen deprivation such as in hypoxia or ischemia, because anaerobic conditions limit glycolysis. To ensure that the flux through GAPDH is not limited during anaerobic conditions, NADH must be continually regenerated (re-oxidized) *via* conversion of pyruvate to lactate by an action of lactate dehydrogenase enzyme [80] (Figure 1.1).

Figure 1.1: Schematic of glycolysis. Glycolysis is a biochemical process that converts glucose to pyruvate. Under aerobic conditions, the series of ten enzymatically controlled reactions (three of which are irreversible) lead to the oxidative breakdown of glucose into two molecules of pyruvate, the reduction of NAD^+ to NADH, and the net production of two moles of ATP per one mole of exogenous glucose. Under anaerobic conditions, in order to regenerate NAD^+ pyruvate is converted to lactate. Please refer to the text for details. Adapted from *Essential Biochemistry* 2nd Edition [33] and *Biochemistry* 7th edition [34].



1.2.1.3 Pyruvate Decarboxylation

Pyruvate, the end product of glycolysis produced in the cytoplasm, is transported into the mitochondrial matrix where as a part of aerobic respiration it undergoes pyruvate decarboxylation. Pyruvate decarboxylation (Reviewed in [33, 34]) is a biochemical reaction that *via* decarboxylation, acetylation, and dehydrogenation of pyruvate converts this 3-carbon molecule to 2-carbon acetaldehyde, acetyl-CoA in the process releasing CO_2 and reducing equivalent, NADH. Specifically, pyruvate is degraded to acetaldehyde, with the loss of CO_2, the acetaldehyde is then oxidized to acetic acid and attached to coenzyme A (CoA) to produce activated acetyl unit (acetyl-CoA), with NAD^+ being reduced to NADH in the process. Thus, pyruvate decarboxylation is also known as the pyruvate dehydrogenase reaction, because it also involves the oxidation of pyruvate. The acetyl-CoA enters the TCA cycle to undergo further oxidation.

Pyruvate decarboxylation reaction is known as the link reaction, because it links two important metabolic pathways – glycolysis and the TCA cycle. Pyruvate decarboxylation is the key irreversible step of glucose oxidation and is catalyzed by pyruvate dehydrogenase (PDH) [81], a complex of three closely linked enzymes located on the inside of the inner mitochondrial membrane in the mitochondrial matrix, which also translocates the acetyl-CoA into the matrix for entry into the TCA cycle [3]. The PDH complex is a very important regulatory enzymatic unit. It is competitively inhibited by its end-products acetyl-CoA and NADH, and also is subjected to a covalent inactivation *via* upstream Pyruvate Dehydrogenase Complex kinase (PDK) [81-86]. There are four isoforms of PDK(1-4) with PDK4 being the predominant form in the heart [87-89]. PDK is an ATP-dependent kinase that inactivates PDH *via* its phosphorylation. PDK is activated by the reaction end-products, acetyl-CoA and NADH, resulting in inactivation of pyruvate PDH [82, 86, 90]; while it is inhibited by pyruvate and by decreases in the acetyl-CoA and NADH levels [81, 82, 86, 90, 91]. Reactivation of PDH is achieved by the de-phosphorylation of PDH by a pyruvate dehydrogenase phosphatase (PDP) [81-86] – an enzyme that in turn is activated by high levels of Ca^{2+} and Mg^{2+} [92, 93]

1.2.2 Fatty Acid Metabolism

1.2.2.1 Fatty Acid Uptake, Activation, and Mitochondrial Transport

The rate of fatty acid uptake by the heart is primarily determined by the concentration of non-esterified fatty acids, as well as triacylglycerides (TAG) in the plasma [17, 26, 94-97]. Non-esterified fatty acids in healthy humans may vary from approximately 0.2 to 0.8 mM [4, 26, 94] and exceed 1.0 mM under metabolic stress such as ischemia [17, 98-100]. In the plasma, fatty acids are transported in the non-esterified form as free fatty acids (FFA) bound to albumin, but also as triglycerides contained in chylomicrons or bound to apolipoproteins in very low-density lipoprotein (VLDL) [17, 101-103]. Fatty acids in chylomicrons and in VLDL are released into the plasma from the triglyceride *via* hydrolysis by lipoprotein lipase – a protein bound to the outside of capillary endothelium [104-108]. FFAs, originating either from albumin or lipoprotein-TAG, enter the cardiomyocyte either by passive diffusion or facilitated diffusion mediated *via* protein mediated transport across the sarcolemma [109-112] involving either a fatty acid translocase (FAT)/CD36 or a plasma membrane (PM) fatty acid-binding protein ($FABP_{pm}$) [112-114].

Once in the sarcoplasm, free fatty acids are attached to one of several transport ligands collectively known as fatty acid-binding proteins (FABP) [115, 116]. FFAs bound to FABPs are activated (activation enhances FFA reactivity) by ATP-dependent esterification to long-chain fatty acyl-CoA by fatty acyl-CoA synthase (FACS) [117, 118]. This long-chain acyl-CoA can either be esterified to intracellular lipids intermediates such as TAG, diacylglycerol (DAG), or ceramides, or converted to long-chain fatty acyl-carnitine and taken up by the mitochondria for FA β-oxidation [17] [17]. Many studies have demonstrated that in the healthy normal heart 70-90% of fatty acids taken up by the heart are immediately oxidized [4, 17, 94]. Remaining 10-30% enter the intra-cardiac triglyceride pool [11, 17, 94, 119].

Fatty acid β-oxidation occurs primarily in the mitochondria and peroxisomes, with the mitochondria being the major site of FA oxidation [120, 121]. Since mitochondrial β-oxidation occurs in

the mitochondrial matrix and since the inner mitochondrial membrane is impermeable to the cytoplasmic long-chain acyl-CoA, the long-chain fatty acyl-CoA is transported from the cytoplasm into the matrix *via* a carnitine-dependent transport system [17, 122]. The key enzyme involved in this translocation is the carnitine palmitoyltransferase (CPT)-1 [121]. CPT-1 catalyzes the conversion of long-chain acyl-CoA to long-chain acyl-carnitine, which is subsequently shuttled into the mitochondria. Allosteric inhibition of CPT-1 by malonyl-CoA is a key mechanism by which CPT-1 activity is regulated [17, 122-130]. Following activation in the compartment between the inner and outer mitochondrial membranes (intramembranous space), long-chain acyl-CoA is converted to long-chain acyl-carnitine *via* action of CPT-1 [17, 117, 118, 123-130]. Carnitine acyl-translocase then transports this long-chain acyl-carnitine across the inner mitochondrial membrane in exchange for free carnitine. CPT-2, loosely associated with the inner site of the inner mitochondrial membrane, regenerates long-chain acyl-CoA for the next cycle of long-chain fatty acyl-CoA transport in the mitochondrial matrix with the release of carnitine back into the intramembranous space.

In the heart FA metabolism, the activity of CPT-1 is tightly regulated. CPT-1 controls the access of the long-chain fatty acids into the mitochondria comprising the rate limiting step in FA oxidation. Its activity is closely regulated *via* malonyl-CoA, a 3-carbon activated compound, formed by the action of acetyl-CoA carboxylase (ACC) on acetyl-CoA [131, 132]. Myocardial malonyl-CoA levels are dependent on the balance between its synthesis from acetyl-CoA *via* ACC [133-137] and its degradation *via* malonyl-CoA decarboxylase (MCD) [99, 112, 138-143]. Malonyl-CoA function is to inhibit activity of CPT-1. This results in reduction of the influx of long-chain acyl-CoA into the mitochondria and subsequent decrease in the FA β-oxidation [133-135, 144, 145]. A key determinant of the ACC activity in the heart is the activity of AMPK [146-148]. AMPK is an energy sensor that up-regulates FA β-oxidation during times of increased energy demand *via* phosphorylation and decrease of ACC activity [4, 147, 149, 150].

1.2.2.2 Fatty Acid β-Oxidation – Saturated Fatty Acids

The metabolism of long-chain fatty acyl-CoA of saturated fatty acids in the mitochondrial matrix occurs *via* β-oxidation pathway/spiral (Figure 1.2), which removes 2-carbon fragments as acyl-CoA from the carboxyl (-COOH) end of the chain by sequential action of acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (3-KAT) [151]. Each cycle of FA β-oxidation results in the shortening of the long-chain fatty acyl-CoA by two carbons and the production of reducing equivalents NADH and $FADH_2$. Peroxisomes are responsible for a partial oxidation of very long chain fatty acids, which are further metabolized in the mitochondria [151, 152]. Additionally, the β-oxidation of mono- or poly-unsaturated FAs (e.g. oleate) requires additional auxiliary enzymes that facilitate the formation of *trans*- double bond from *cis*- double bond necessary for further β-oxidation [151, 152]. All of the four enzymes of FA β-oxidation pathway exist in different isoforms that have different chain-length specificities [153]. They are also loosely organized into a multi-complex, in which the substrate upon entering and departing never leaves the complex moving onto the next enzyme in the FA β-oxidation spiral [153].

The enzymes of FA β-oxidation are under a high degree of transcriptional control – an increase in the expression of the four enzymes is associated with the up-regulation of β-oxidation [154]. Each of the enzymes is also sensitive to the feed-back inhibition by its products, $FADH_2$ and NADH. The key enzyme of the β-oxidation pathway is 3-ketoacyl-CoA thiolase. In the final reaction, 3-ketoacyl-CoA thiolase splits 3-ketoacyl-CoA to produce acetyl-CoA and a fatty acyl-CoA that is shortened by two carbons [151, 152, 155, 156]. This key regulatory enzyme is inhibited by the accumulation of acetyl-CoA during times of low metabolic demand [4]. Specifically, the decrease in mitochondrial acetyl-CoA/CoA ratio in response to an increased energy demand [157, 158] results in the release of suppression of 3-KAT activity and increase in the activity of L-3-hydroxyacyl-CoA dehydrogenase resulting in the acceleration of the FA β-oxidation pathway [159].

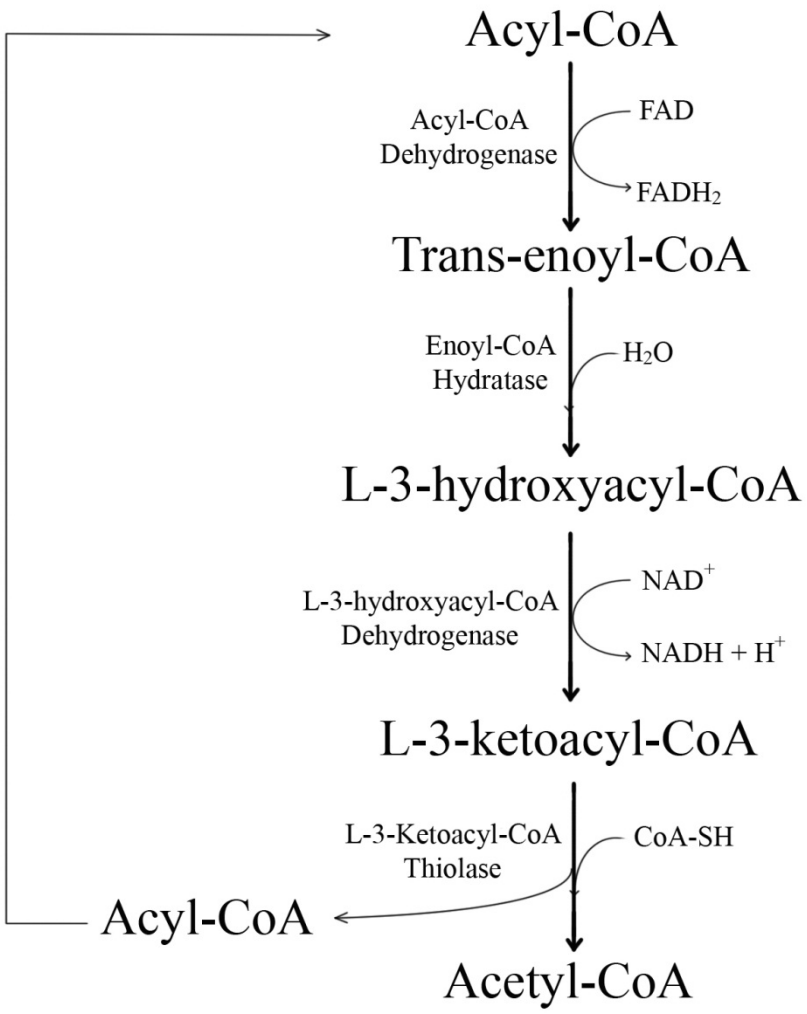
1.2.2.3 The Randle Cycle

How an increase in fatty acid supply to the myocardium decreases glycolysis and glucose oxidation was originally described by Randle's group [73, 83, 160]. Now this reciprocal relationship between fatty acids and glucose oxidative metabolism is generally referred to as the "Randle cycle" or is simply known as a glucose-fatty acid cycle. High rates of fatty acid oxidation inhibit PDH activity *via* an increase in mitochondrial acetyl-CoA/CoA and NADH/NAD^+ ratios, which activate PDK resulting in the phosphorylation and inhibition of PDH [161]. The increased supply of FA *β*-oxidation derived acetyl-CoA to the TCA cycle can also decrease glycolysis due to the inhibitory effects of citrate [a TCA cycle intermediate, which has gained access to the cytosol *via* the tricarboxylate carrier (TCC)] on PFK-1 [162]. Citrate from fatty acid *β*-oxidation derived acetyl-CoA can inhibit PFK-1, which in turn can lead to a lesser extent to an inhibition of hexokinase by G-6-P [163]. The inhibition of glucose oxidation is the predominant inhibitory effect of fatty acid *β*-oxidation on the pathways of glucose metabolism. Equally, inhibition of fatty acid oxidation increases uptake and oxidation of glucose and lactate − lowering acetyl-CoA and/or NADH levels in the mitochondrial matrix releases an inhibition of PDH [81, 164, 165]; and decreasing citrate levels releases an inhibition of PFK-1 [166].

Alternatively, increased contribution of glucose oxidation to the generation of acetyl-CoA and NADH can decrease fatty acid *β*-oxidation *via* feedback inhibition of 3-ketoacyl-CoA thiolase, acyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA dehydrogenase by acetyl-CoA [17, 21, 164, 165, 167-169]. Indeed, the mechanisms of the Randle cycle have been shown to involve interactions between three key enzymes in the heart: acetyl-CoA carboxylase (ACC), 5'- AMP-activated protein kinase (AMPK), and malonyl-CoA decarboxylase (MCD) [21]. An increase in glucose-derived acetyl-CoA results in the increase in the synthesis of the cytosolic malonyl-CoA, which inhibits fatty acid uptake and subsequent oxidation. Mitochondrial acetyl-CoA can be transferred to the cytosol by the formation of acetyl-carnitine *via* action of carnitine acetyl-transferase (CAT) [16, 170] and translocated into cytosol by carnitine acetyl-translocase [134, 170-172]. In the cytosol carnitine acetyl-transferase converts acetyl-

carnitine back to acetyl-CoA. Consequently, an increase in the intra-mitochondrial acetyl-CoA production due to increased glucose oxidation results in an increase in cytosolic acetyl-CoA levels, an increase in malonyl-CoA production by ACC, and subsequent inhibition of CPT-1 and fatty acid oxidation [21]. Thus, malonyl-CoA links the changes in acetyl-CoA supply to the rate of long-chain fatty acyl-CoA uptake by mitochondria. This mechanism explains how increases in glucose oxidation are able to down-regulate myocardial fatty acid oxidation. Furthermore, in times of energy demand, low AMP/ATP ratio results in the activation of AMPK and its down-regulation of ACC activity *via* phosphorylation of ACC at 265 [173] resulting in subsequent decrease in levels of malonyl-CoA, and thus increase of long-chain fatty acyl-CoA uptake and oxidation.

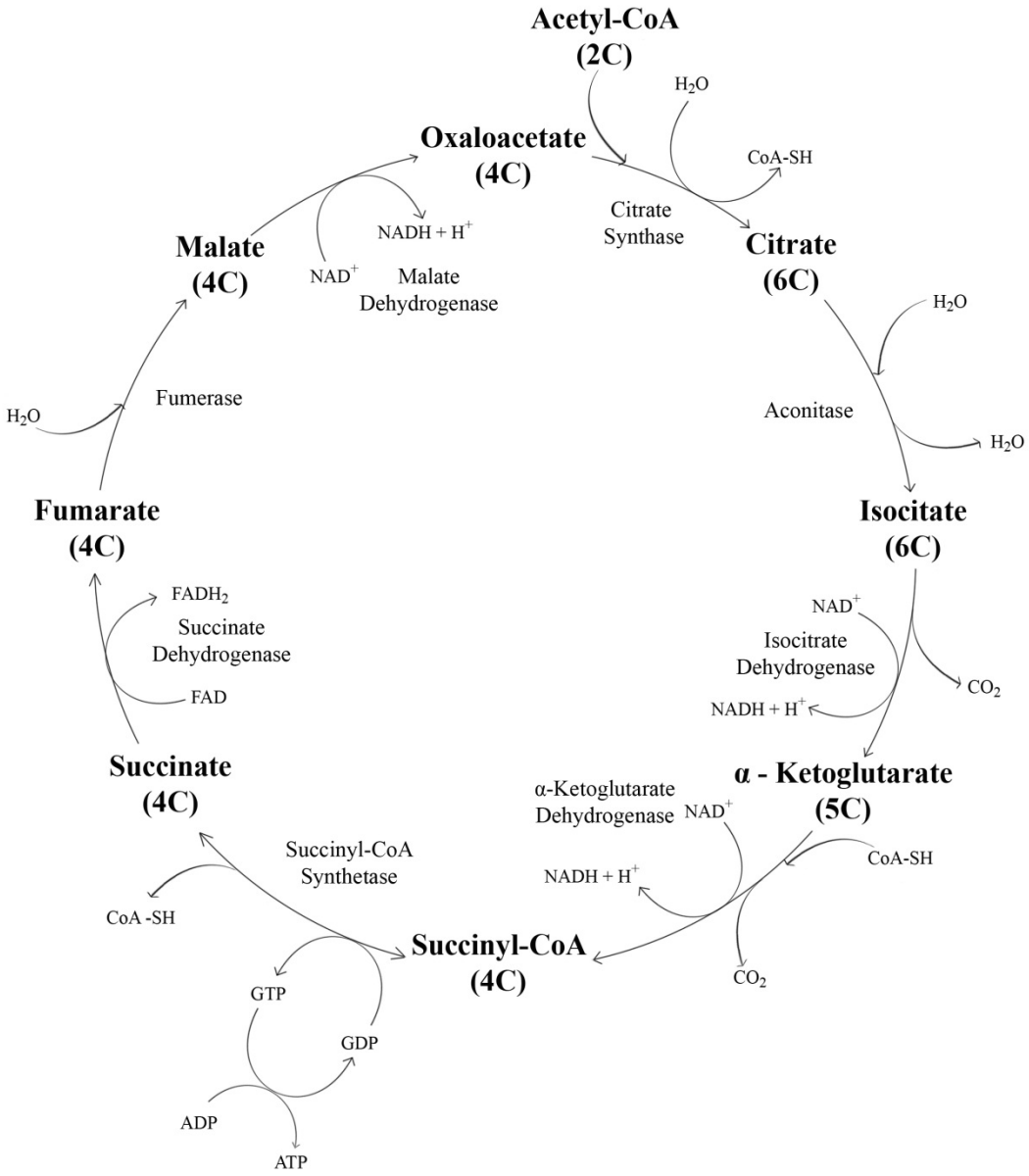
Figure 1.2. Schematic of fatty acid *β*-oxidation. The metabolism of long-chain fatty acyl-CoA of saturated fatty acids in the mitochondrial matrix occurs *via* the *β*-oxidation pathway/spiral, which removes 2-carbon fragments as acyl-CoA from the carboxyl (-COOH) end of the chain by sequential action of acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (3-KAT). Each cycle of FA *β*-oxidation results in the shortening of the long-chain fatty acyl-CoA by two carbons and the production of reducing equivalents, NADH and $FADH_2$. Please refer to the text for details. Adapted from *Essential Biochemistry* 2nd Edition [33] and *Biochemistry* 7th edition [34].



1.2.3 Tricarboxylic Acid Cycle

Glucose, lactate, or fatty acid derived acetyl-CoA to undergo further oxidation enter the tricarboxylic acid (TCA) cycle, also known as citric acid cycle or Kreb cycle (Figure 1.3), localized in the mitochondrial matrix. During the TCA cycle, this 2-carbon acetyl group combines with a 4-carbon oxaloacetate to form a 6-carbon citrate, which then undergoes oxidative decarboxylations and isomerizations to regenerate the original oxaloacetate [155]. Upon a completion of the one round of the TCA cycle one molecule of ATP *via* GTP intermediate, three molecules of NADH, and one molecule of $FADH_2$ are generated. The overall rate of the citric acid cycle is controlled by the flux though citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase. These fluxes are largely determined by the concentrations of substrates and end-products [174]. The availability of substrates, acetyl-CoA and oxaloacetate, limits the activity of citrate synthase, and thus the rate of citrate formation. The end-product, citrate as well ATP, can feedback and inhibit citrate synthase activity, while ATP inhibits isocitrate dehydrogenase activity [10, 155]. The end-product, NADH, inhibits activity of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase [175]. The activity of the TCA cycle increase with workload primarily due to low mitochondrial $NADH/NAD^+$ ratio, which acts to increase the activity of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase [10]. In addition, the flux though TCA cycle is dependent on the allosteric inhibition of enzymes. Inhibition of citrate synthase by ATP is relived allosterically by ADP [155]. In addition, citrate inhibits allosterically PFK-1and PFK-2 such that the rate of glycolysis is matched to the rate of TCA cycle. PFK-1 activity is greatly inhibited allosterically by increased levels of cytoplasmic ATP [70-72], citrate [37, 73-75], and H^+ (especially important in severe ischemia) [67]. PFK-2 is allosterically inhibited by citrate, which by decreasing F2,6-bP levels is the second mechanism by which citrate can inhibit PFK-1 activity [37, 77].

Figure 1.3. Schematic of Tricarboxylic Acid cycle. Glucose, lactate, or fatty acid derived acetyl-CoA to undergo further oxidation enters the tricarboxylic acid (TCA) cycle, also known as citric acid cycle or Kreb cycle, localized in the mitochondrial matrix. During the TCA cycle, this 2-carbon acetyl group combines with a 4-carbon oxaloacetate to form a 6-carbon citrate, which then undergoes oxidative decarboxylations and isomerizations to regenerate the original oxaloacetate. Upon a completion of the one round of the TCA cycle one molecule of ATP *via* GTP intermediate, three molecules of NADH, and one molecule of $FADH_2$ are generated. The overall rate of the citric acid cycle is controlled by the flux though citrate synthase, isocitrate dehydrogenase, *α*-ketoglutarate dehydrogenase, and malate dehydrogenase. Please refer to the text for details. Adapted from *Essential Biochemistry* 2nd Edition [33] and *Biochemistry* 7th edition [34].



1.2.4 Electron Transport Chain and Oxidative Phosphorylation

In well-oxygenated tissue, reducing equivalents, NADH and $FADH_2$, generated throughout the cellular respiration (anaerobic and aerobic) are reconverted into $NADH^+$ and FAD in the last stage of cellular respiration − the electron transport and oxidative phosphorylation. Unlike substrate-level phosphorylation referring to a direct synthesis of ATP from inorganic phosphate coupled with the degradation of glucose, oxidative phosphorylation refers to coupling of oxidation with phosphorylation by redox reactions occurring during electron transport *via* reduced electron carriers NADH and $FADH_2$. Oxygen is a final electron acceptor and it is reduced to H_2O. The liberated energy is utilized to develop a proton gradient across the inner mitochondrial membrane, which subsequently is utilized in the synthesis of large quantities of ATP.

The respiratory chain [also termed Electron Transport Chain (ETC)] (Reviewed in [33, 34]) is a series of four large protein complexes embedded in the mitochondrial inner membrane and arranged in a specific sequence: complex I (NADH-ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (ubiquinone-cytochrome C oxidoreductase), and Complex IV (cytochrome C oxidase). The cytochrome complexes are electron-transferring proteins (containing iron heme groups) involved in the passage of the electrons. Their names specify their function. Reactions at the complexes I, III, and IV are associated with the ejection of protons from the mitochondrial matrix and transfer of electrons to oxygen to form H_2O. The oxidation of NADH and $FADH_2$ produces sufficient energy to pump ten and six protons, respectively, from the mitochondrial matrix into the intramembranous space. Since the inner mitochondrial membrane is impermeable to protons, this leads to accumulation of protons in the intramembranous space and development of the electrochemical gradient [176]. The movement of ejected protons down it electrochemical gradient into mitochondrial matrix from the intramembranous space *via* membrane proteins ionophores, called ATP synthetases, is utilized in the synthesis of the ATP.

1.3 AMP-Activated Protein Kinase and Energy Metabolism

1.3.1 AMPK Structure, Function, and Regulation

5' adenosine monophosphate-activated protein kinase, or simply AMPK, is a highly conserved heterotrimeric enzyme complex consisting of a catalytic α (63 kDa) subunit and regulatory subunits β (30 kDa) and γ (38-63 kDa) in a ratio $1\alpha{:}1\beta{:}1\gamma$ [177-179]. In mammals there are two kinds of α and β subunits and three kinds of γ subunits, which are encoded by distinct genes and are differentially expressed in various tissues [146, 177, 180-184]. Although the most common isoforms expressed in most tissues are the $\alpha1$, $\beta1$, and $\gamma1$, in addition to the $\alpha1$, $\beta1$, and $\gamma1$ heart primarily expresses $\alpha2$, $\beta2$, and $\gamma2$ isoforms [185-187]. The α-subunit of the AMPK enzyme complex contains the catalytic domain (including the regulatory Thr172 residue at the N-terminus, which is phosphorylated by upstream kinases) followed by an auto-inhibitory domain [188]. The β-subunit has glycogen binding domain [189, 190] and binds both α- and γ-subunits [191, 192]. The γ-subunit contains four conserved C-terminal CBS domains named after a similar motif in the enzyme cystathionine β-synthase. The CBS domains exist in tandem to form the basic functional unit called the Bateman domain [193] that binds AMP and regulate AMPK activity and phosphorylation [192, 194, 195]. Allosteric biding of AMP to the γ-subunit results in two to five fold increase in the AMPK activity [196]. Binding of AMP in the place of ATP unlocks γ-subunit resulting in the phosphorylation of the α-subunit Thr172 residue by an upstream kinase, subsequent release of the inhibition of the auto-inhibitory α-domain, and activation the kinase activity of AMPK [193, 194, 197-201].

AMPK is a key molecular regulator of energy metabolism [178]. In the heart AMPK importance increases under conditions of energy demand − during stresses such as ischemia, hypoxia, or excessive load. To ameliorate ATP deficiencies [202], AMPK inhibits energy-consuming pathways such as protein synthesis *via* direct inhibition of mTOR [203-206]; and up-regulates energy-generating pathways such as glucose up-take *via* the biogenesis and translocation of GLUT-4 to sarcolemma [53], activity of glycolytic pathway by phosphorylation and activation of PFK2 [39, 78, 207-216], and activity of β-oxidation of fatty

acids *via* inhibition of acetyl-CoA carboxylase [135, 142, 145, 202, 217-223]. AMPK is a serine/threonine kinase that responds to metabolic stresses that deplete cellular ATP, increase AMP, or increase the creatine/phosphocreatine (Cr/PCr) ratio *via* reversible decomposition of phosphocreatine [146, 148, 181, 224]. AMP/ATP ratio is a more sensitive indicator of cellular energy status than ADP/ATP ratio, because of the reciprocal relationship between AMP and ATP due to the action of adenlyate kinase enzyme (AMP + ATP ↔ 2ADP) [225, 226]. While AMPK activation usually is allosteric requiring changes in AMP/ATP or Cr/PCr proportions, cardiac AMPK activity can also be altered free of changes in nucleotide levels [202, 227]. In ischemia, the activation of unknown upstream AMPK kinase (AMPKK) significantly contributes to the activation of AMPK [227, 228]. Generally, AMPK can be phosphorylated by at least three different upstream AMPK kinases (AMPKKs): serine-threonine kinase 1 (LKB1), Ca^{2+}-calmodulin-dependent kinase kinase β (CaMKKβ), and transforming growth factor-β-activated kinase 1 (TAK1) [149, 229]. However, these identified AMPKKs are either expressed at very low levels in the cardiac tissue [146] or simply not activated by ischemia [227]. Finally, insulin inhibits myocardial AMPK activity [145, 212, 230, 231].

1.3.2 AMPK and Myocardial Ischemia

During mild or moderate ischemia, fatty acid and glucose oxidative metabolism is inhibited due to diminished oxygen supply to the heart [35]. At the same time, glucose transport and glycolysis are accelerated [35] allowing cells to have a continuous supply of ATP. Although under anaerobic conditions of ischemia the rate of glucose metabolism is up-regulated [232], the amount of ATP produced is down-regulated. Upon reperfusion when the heart is exposed to aerobic conditions, the ATP production increases and the rate of glycolysis decreases. This decrease in the glycolytic rate results from the indirect inhibition of PKF-2 *via* allosteric inhibition of the activity of PFK-1 (the third enzyme in the glycolysis pathway) by ATP. Thus, one of the central metabolic adaptations during ischemia in the heart is a change in substrate utilization to generate ATP primarily from glucose. AMPK plays a central role in this adaptation. Many studies have established that AMPK activation occurs during myocardial ischemia

[53, 78, 134, 202, 233]. Activation of AMPK presumably occurs to restore cardiac myocyte ATP levels primarily by turning on ATP generating pathways [146]. Thus, AMPK modulates glucose uptake [53] and glycolysis [78] in the ischemic heart. The glucose uptake in the heart involves facilitated diffusion across the sarcolemma *via* GLUT-1 and GLUT-4 [35, 234], and its rate is dictated by the number of the glucose transporters. Increased glucose transport is primarily attributable to the translocation of the GLUT-4 from intracellular storage site to the sarcolemma [35]. Many studies have demonstrated that AMPK is a critical mediator of glucose transport in the ischemic heart [53, 207, 235, 236] *via* insulin-independent mechanism (mechanism not mediated *via* PI_3K pathway responsible for insulin-stimulated uptake) [53].

AMPK also stimulates glycolysis *via* indirect activation of PFK-1, which is the rate-limiting enzyme in glycolysis and thus the primary regulatory step in the glycolytic pathway [78]. AMPK indirectly activates PFK-1 through phosphorylation of PFK-2, which converts fructose 6-phosphate to fructose 2,6-bisphosphate − an allosteric stimulator of PFK1 [78]. The second major metabolic consequence of AMPK activation during and flowing ischemia is the enhancement of fatty acid oxidation upon re-establishment of oxygen and substrate supply [145, 202, 233]. Persistent activation of AMPK during reperfusion promotes FA oxidation by phosphorylation and inhibition of ACC − the enzyme responsible for the synthesis of malonyl-CoA, which is a potent inhibitor of CPT-1 [140, 202]. CPT-1 is a rate-limiting mitochondrial enzyme responsible for internalization and rendering of FAs as substrates for oxidative phosphorylation [21]. Inactivation of ACC lowers malonyl-CoA levels relieving the inhibition of CPT-1 and subsequently resulting in enhanced FA oxidation. Activated AMPK has also other physiological effects that act to promote FA uptake and oxidation in the reperfused heart. AMPK activation increases protein expression and stimulates recruitment of FA transporter FAT/CD36 to the sarcolemma from the intracellular storage sites [237-239]. The availability of free FAs in the vicinity of cardiomyocytes is further enhanced by increased synthesis and recruitment of lipoprotein lipase to

capillary lumen [240] and by facilitating the uptake of FAs from triglyceride-containing lipoprotein particles by activation of lipoprotein lipase [240].

Since AMPK plays an important role in promoting glucose uptake and glycolysis [78, 241, 242], the activation of AMPK during ischemia may benefit the heart by increasing glucose utilization and subsequent anaerobic synthesis of ATP [146]. While glycolysis is a minor source of ATP in the normal heart, the small amounts of ATP that are generated by anaerobic glycolysis during ischemia are critical in maintaining cellular homeostasis, such as the activity of ATP-requiring ion pumps − SERCA, Na^+/K^+ ATPase, and sarcolemmal Ca^{2+}-ATPase [243]; and thus are beneficial to the heart during ischemia. AMPK also appears to be necessary in the mediation of adiponectin cardioprotective effects against ischemia-reperfusion injury [244]. The overall effect of activated AMPK is to increase ATP supply by accelerating the rates of FA oxidation and glycolysis in ischemia and reperfusion. Although FA oxidation plays an important role in the generation of ATP in the post-ischemic heart, excess FA oxidation can have detrimental effects on the recovery of the contractile function upon restoration of cardiac energy supply.

Ischemia-induced AMPK activation [53, 78, 134, 202, 233] and elevated levels of circulating FA during ischemia [98, 99, 245] promote FA oxidation in the early post-ischemic period, which subsequently significantly inhibit glucose oxidation *via* the Randle cycle [74]. According to Randle cycle, the fatty acid-derived acetyl-CoA is able to decrease the production of glucose-derived acetyl-CoA *via* inhibition of the pyruvate dehydrogenase complex [82]. This ultimately leads to an imbalance between glucose oxidation and glycolysis. Accelerated glycolysis results in increased lactate production and subsequent accumulation of lactate and protons [246, 247] perpetuating intracellular acidosis and promoting Ca^{2+}-overload during reperfusion [248]. Consequently, ischemia-induced activation of AMPK is detrimental to the ischemic heart primarily due to the stimulation of FA oxidation, the subsequent inhibition of glucose oxidation due to these high rates of FA oxidation, and finally high rates of FA oxidation contribution to decrease in the cardiac function and efficiency during the critical period of reperfusion.

1.4 Ischemia-Reperfusion Injury

Ischemia/reperfusion (I/R) injury is defined as damage to the heart when blood supply is restored to the ischemic areas (reperfusion) that results in cardiac dysfunction, metabolic inefficiency, and cellular damage of the myocardium including apoptosis and necrosis. I/R injury comprises of two phases: 1) ischemic injury, which is related to the damage associated with the lack of oxygen resulting from severe impairment of coronary blood supply [249, 250] (usually produced by thrombosis − formation of the blood clot in the blood vessel); and 2) reperfusion injury, which refers to oxidative cellular damage caused by the return of blood supply to the myocardial tissue following a period of prolong ischemia [251, 252]. However, the injury elicited by the reperfusion may be more severe than that caused by the ischemia due to further ionic and metabolic changes at the restoration of the oxygen supply to the heart. Furthermore, myocardial I/R injury can manifest itself in setting of acute myocardial infarction, heart block, and surgeries such as transplant surgery, heart valve replacement surgery, and coronary artery by-pass graft (CABG) surgery. Ischemia can occur during surgical procedures, because to work in the field void of blood the blood flow through the coronary arteries must be prevented at some point during the surgical operation. If blood flow is absent for a long period of time the ischemic tissue will die and for the heart to be salvaged the blood supply must be restored rapidly. Unfortunately, as mentioned above the reperfusion can act as a double-edge sword further exacerbating the damage that occurred during ischemia.

1.4.1 Cardiac Energy Metabolism During Ischemia-Reperfusion

Myocardial ischemia is an imbalance between the oxygen supply and demand in the heart due to an inadequate coronary blood flow. Impairment in the blood flow, usually due to thrombosis, results in an insufficient supply of oxygen to the cardiac tissue and restricted outflow of metabolites. The insufficient oxygen supply associated with inadequate blood flow results in the decreased mitochondrial oxidative metabolism (glucose and fatty acid oxidation) and subsequent significant reduction in the production of ATP [253]. Reduction in the oxidative ATP production leads to an increase in the rate of

anaerobic glycolysis in the attempt to compensate for this decrease in the oxidative ATP supply [4]. The increased glycolytic rate and continual low rates of glucose oxidation result in the uncoupling of anaerobic (increased glycolysis) from aerobic (decreased glucose oxidation) processes [4, 254]. Uncoupling of glycolysis from glucose oxidation leads to an accumulation of deleterious by-products such as protons (H^+) and lactate within the cardiac myocytes [255, 256]. Lactate accumulates in the state of oxygen deprivation due to the metabolism of pyruvate (generated from the glycolysis) to lactate instead to CO_2 and H_2O [255-258].

Furthermore, the continued production and intracellular accumulation of H^+ and lactate in the setting of ischemia results in a dramatic intracellular acidosis [255, 256] and decrease in both cardiac function and efficiency (cardiac work/oxygen consumed) [246, 259]. Cardiac function decreases due to the redirection of the myocardial contraction towards clearance of by-products of glycolysis [146]. Intracellular acidosis also decreases responsiveness of contractile proteins to Ca^{2+} resulting in severe reduction of the heart's contractile function during ischemia [260]. Finally, during severe/total ischemia due to the accumulation of H^+ flux through the glycolytic pathway, glycolysis is eventually inhibited at the level of PFK-1 and GAPDH [4, 68, 261] further aggravating disturbances in ionic homeostasis.

Ischemia also alters energy metabolism by disturbing the balance between fatty acid and glucose metabolism, which negatively influences cardiac function and efficiency [23]. Due to rapid ischemia-induced increase in the release of catecholamines [262] there is a rise in circulating plasma free fatty acids associated with increased adipose tissue lipolysis [99] during and after ischemia [17, 98, 173]. This raise in plasma free fatty acids increases delivery of fatty acids into cardiomyocytes [17]. The increased delivery of FAs to the myocardium in association with alterations in the intracellular control of FA oxidation keeps FAs as a major fuel during post-ischemic reperfusion [17]. Indeed, it has been shown that the FA oxidation can be dramatically accelerated during reperfusion with over 95% of acetyl CoA-derived ATP originating from fatty acids [173]. In addition, these high rates of fatty acid oxidation at reperfusion can dramatically inhibit glucose oxidation *via* the Randle cycle [263] – fatty acid-derived

acetyl-CoA decrease the production of glucose-derived acetyl-CoA *via* inhibition of PDH complex resulting in intracellular acidosis at reperfusion.

1.4.2 Impairment of Ionic Homeostasis in Ischemia-Reperfusion Injury

Although Ca^{2+} is vital in cellular function, an excessive amount of intracellular Ca^{2+} (intracellular Ca^{2+} overload) can have deleterious effects on myocardial function, structure, and metabolism. Ca^{2+} overload is a major cause of necrotic cellular damage and cardiac dysfunction in ischemic heart disease. During ischemia impairment of oxidative phosphorylation due to lack of oxygen leads to a rapid fall in intracellular ATP levels and subsequent stimulation of glycolysis though activation of phosphofructokinase (PFK). Accelerated glycolysis and decelerated glucose oxidation result in the accumulation of H^+ and lactate. Drop in intracellular pH causes inhibition of PFK and thus inhibition of glycolysis. Furthermore, during ischemia intracellular acidification resulting from the uncoupling of glycolysis and glucose oxidation stimulates Na^+/H^+ exchange *via* activation of reverse mode Na^+/H^+ antiporter [264-266]. The activation Na^+/H^+ antiporter to work in a reverse results in increase in the intracellular concentration of Na^+ (Na^+-overload) [264-266]. Additionally, a marked decrease in ATP production during ischemia compromises function of various ATP-dependent pumps involved in the regulation of intracellular ionic homeostasis. The Na^+ that enters *via* this route would normally be pumped out by the Na^+/K^+ ATPase, but greatly reduced level of ATP inhibits the efflux leading to a further progressive rise in intracellular Na^+. Hence, impaired activity of Na^+/K^+-ATPase, which is responsible for the extrusion of three Na^+ in exchange for two K^+ [267] and is crucial for regulating resting membrane potential [267], leads to a further increase in the intracellular Na^+ [268-270].

Early in ischemia activation of reverse mode Na^+/Ca^{2+} exchanger, which extrudes intracellular Na^+ in exchange for extracellular Ca^{2+}, leads to the development of intracellular Ca^{2+}-overload [270-272]. In addition, inhibition of the activity of the sarcolemmal and sarcoplasmic reticulum (SR) Ca^{2+}-ATPases following cardiac myocyte contraction due to a significant decrease of ATP production during ischemia also contribute to Ca^{2+}-overload [267]. Following cardiac myocyte contraction sarcolemmal and SR

Ca^{2+}-ATPases are responsible for the cytoplasmic Ca^{2+} extrusion and reuptake into SR, respectively [267]. Indeed, it has been demonstrated that Ca^{2+}-overload occurs as early as fifteen minutes of ischemia [270, 272] and results in severe contractile dysfunction [270, 272, 273]. Although reperfusion is essential in salvaging ischemic myocardium, it is also accompanied by a marked increase in the level of cytoplasmic Ca^{2+} [274, 275]. The normalization of post-ischemic extracellular pH promotes Na^{+}/H^{+} exchange [137, 276] further aggravating intracellular Na^{+}-overload, which also promotes reverse mode Na^{+}/Ca^{2+} exchange [137] triggering further Ca^{2+}-overload and Ca^{2+}-overload associated ischemia-reperfusion injury. The intracellular Ca^{2+}-overload is responsible for the necrotic cell damage *via* activation of different proteases and phospholipases leading to the disruption of cellular membranes integrity and ultrastructural injury. The re-oxygenation of cardiac tissue during reperfusion also further lowers energetic status of the cardiomyocytes. The re-oxygenation accompanied by mitochondrial Ca^{2+}-overload leads to the utilization of oxygen by the mitochondria in buffering of high level of Ca^{2+} instead of synthesis of ATP [269].

1.5 Cardiac Preconditioning

Myocardial preconditioning, association of the response of the heart to a stimulus, has been shown to protect the heart against I/R injury. They are many forms of cardiac conventional and non-conventional preconditioning. Conventional preconditioning includes ischemic preconditioning (IPC) (repeated short periods of ischemia prior severe ischemic period) [277-283], pharmacological preconditioning (a transient pre-treatment with certain pharmacological agents such as adenosine, bradykinin, opioid-peptides, angiotensin II, etc.) [284-290], physical-stress preconditioning (transient periods of rapid heart rate termed pacing or volume load termed stretching) [291], and heat-shock preconditioning (application of the temperature of 42^{o}C for fifteen minutes) [291, 292]. Non-conventional preconditioning includes short-lasting periods of physical workout (exercise) [293-295] or long/short-term calorie restriction (CR) [296-301]. All of these forms of cardiac preconditioning have demonstrated protection against ischemia/reperfusion injury in the heart [277-299, 301].

1.5.1 Ischemic Preconditioning

Ischemic preconditioning (IPC) is one of the strongest interventions that can protect heart from severe ischemia [277-283]. It involves an application of short-lived episodes of successive myocardial ischemia (IPC stimulus; less than fifteen minutes) and reperfusion prior to a long, irreversible infarct-producing period of ischemia (index ischemia; more than twenty minutes) that result in cell death [302]. The phenomenon was first described in dogs in 1986 by Murry and coworkers [277]. In this innovative study, a group of open-chest dogs exposed to four cyclic episodes of five minutes coronary occlusion followed by five minutes of reperfusion and then subjected to a prolong forty minutes ischemic insult demonstrated a reduction in cell death by 60-70% compared to control dogs [277]. Also, IPC was associated with delayed ATP depletion, reduced oxygen consumption, preservation of intracellular structures, and delayed or reduced cellular necrosis caused by a lack of ATP [277]. Unlike the case with previously reported interventions this phenomenon of cardiac preconditioning could be easily reproduced in every species tested including rats [303, 304], rabbits [284, 305], pigs [279, 306], as well in human

isolated myocytes [307] and muscle tissue [308]. There is also considerable clinical evidence that IPC occurs in humans *in vivo* [278, 280, 309].

IPC is a complex process that appears to have a bi-phasic pattern of protection. The first phase, termed "first", "early" or "classical" window of protection, protects the heart for one to two hours and then wanes; the "second", "delayed" or "late" window of protection, appears twenty four hours after the IPC protocol and last for three days in the rat heart [310, 311]. This distinction between the two phases of IPC results from the modification of existing myocardial proteins in "early" preconditioning and by synthesis of new cardioprotective proteins in the "late" preconditioning in the heart [312]. IPC is only effective at reducing infarct size induced by relatively short periods of index ischemia (thirty to forty five minutes in rodents and rabbits; sixty to ninety minutes in dogs and swine) [284, 305]. Furthermore, not all time combinations and durations of ischemia and reperfusion will trigger IPC and result in the myocardial protection. Preconditioning can be induced by a period of ischemia no less than three to five minutes followed by a minimal five minutes of reperfusion [277, 313].

1.5.2 Ischemic Preconditioning Signaling Mechanism

The major determinant of cardiomyocyte death following an episode of I/R injury is mitochondrial dysfunction arising from the formation of mitochondrial permeability transition pore (mPTP) [314-318]. mPTP is a non-selective pore permeable to any molecule less than 1.5 kDa and its opening is inhibited by low pH occurring during ischemia [315, 316, 319, 320]. However, the restoration of cellular pH coupled with a rapid elevation of mitochondrial Ca^{2+} levels due to the reconstitution of activity of respiratory chain leads to a rapid opening of the mPTP during reperfusion [315, 316, 319, 320]. Opening of mPTP results in uncoupling of the mitochondrial oxidative phosphorylation due to increased inner mitochondrial membrane permeability to H^+. The increased inner membrane permeability to H^+ leads to the dissipation of the H^+ gradient across the inner mitochondrial membrane and subsequent impairment of ATP synthesis. The impairment in the mitochondrial ATP production results in necrotic death [316, 320, 321]. In addition, mPTP opening may also induce mitochondrial swelling resulting in

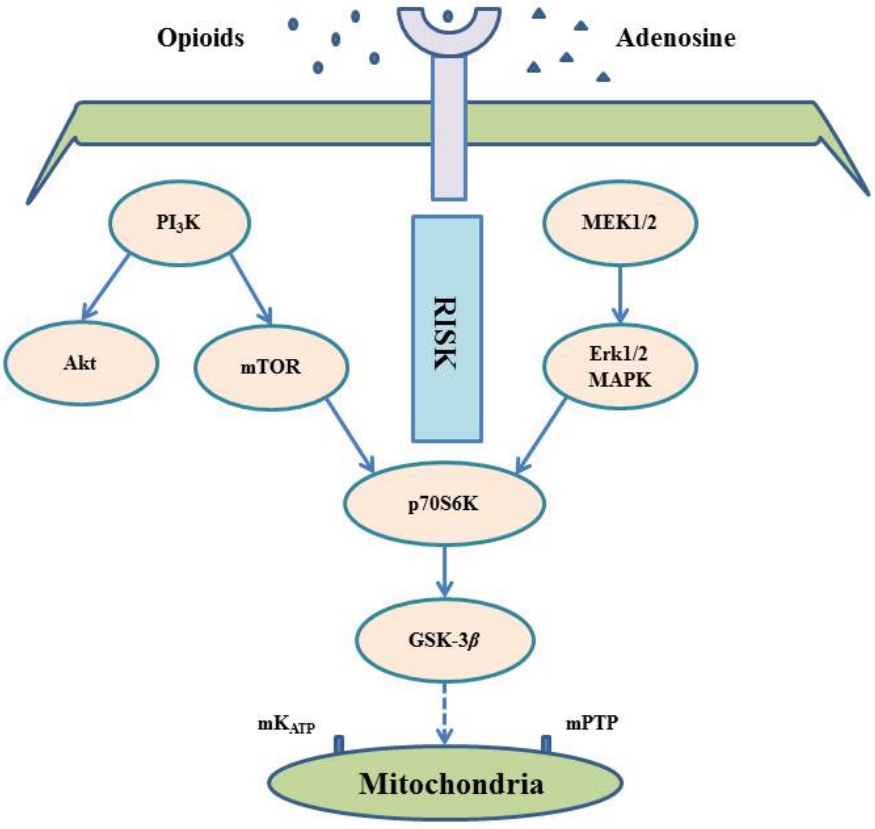
apoptotic cell death from the release of cytochrome C. Cytochrome C normally resides in the inner mitochondrial space bound to outer leaflet of the inner mitochondrial membrane and is required for electron transport by the cytochrome oxidase in Complex IV.

Ischemic preconditioning limits myocardial infarct size by preventing mPTP opening in reperfused hearts. However, at present the mechanism of mPTP inhibition in these settings is still unresolved. Several mechanisms of IPC have been proposed, which ultimate goal is inhibition of the mPTP pore at reperfusion. These mechanisms of IPC cardioprotection are divided into indirect and direct categories. Indirect mechanisms of mPTP inhibition are those in which mPTP inhibition arises as indirect consequence of the beneficial effect of IPC such as intracellular Ca^{2+} regulation [322-325], ATP preservation [311, 326-330], and oxidative stress and pH correction [326, 331, 332]. The other potential mechanisms of mPTP inhibition involves complex signalling transduction pathways, which underline IPC, and converge on the mitochondria resulting in mPTP inhibition through the phosphorylation and translocation of various protein kinases pathways, such as pro-survival anti-apoptotic Akt and Erk1/2 MAPK (acronym p44/42 MAPK) signalling pathways and inhibition of the pro-death GSK-3β (GSK-3β) pathway [333-338]. Indeed, in 2005 Hausenloy *et al*. [339] proposed that IPC protects the heart by inducing the activation of phosphatidylinositol-3 kinase (PI_3K)/Akt and the MEK1/2/Erk1/2 MAPK cascades at reperfusion. IPC at reperfusion leads to the activation of a group of protein kinases *via* activation of G-protein coupled receptors (GPCR) (opioid or adenosine receptors) or growth factor receptors, which are believed to initiate the signalling mechanism that confers a powerful cardioprotection and acts to promote cell survival (Reviewed in [340, 341]).

These pro-survival anti-apoptotic kinases constitute a Reperfusion Injury Salvage Kinase (RISK) pathway [318, 342], which involves at least two intermediate pathways. One pathway involves PI_3K acting on Akt and the mammalian target of rapamycin (mTOR), and the other involves mitogen-associated protein kinase (MAPK) - the extracellular signal-regulated kinase Erk 1/2 MAPK (acronym p42/44 MAPK) [339, 343-345]. Although the targets of the IPC are likely multiple, the key signalling

pathways ultimately converge on recovery of mitochondrial function following I/R injury and specifically on the prevention of the mPTP opening, which is believed to mediate mitochondrial injury. Thus, these two pathways converge on p70S6 kinase (p70S6K), which phosphorylates and inhibits GSK-3β involved in the pro-death pathway and whose inhibition acts to prevent mPTP opening [345-350]. As mentioned earlier, a mPTP is a non-specific pore of the inner mitochondrial membrane whose opening in the first few minutes of myocardial reperfusion mediates cell death by uncoupling oxidative phosphorylation and inducing mitochondrial swelling [314, 317]; and which opening is promoted by reperfusion and induces both apoptotic and necrotic cell death due to the release of mitochondrial proteins such as cytochrome C and loss of ATP-generating capacity. The link between the RISK pathway and the mPTP opening is still unresolved.

Figure 1.4. Schematic of RISK Signaling Pathway. IPC at reperfusion leads to activation of a group of protein kinases *via* activation of G-protein coupled receptors (GPCR) (opioid or adenosine receptors) or growth factor receptors that are believed to initiate the signalling mechanism that confers a powerful cardioprotection and act to promote cell survival. These pro-survival anti-apoptotic kinases constitute a Reperfusion Injury Salvage Kinase (RISK) pathway, which involves at least two intermediate pathways. One pathway involves phosphatidylinositol-3 kinase (PI_3K) acting on Akt and the mammalian target of rapamycin (mTOR); and the other involves mitogen-associated protein kinase (MAPK) – the extracellular signal-regulated kinase Erk1/2 MAPK (acronym p42/44 MAPK). Although the targets of the IPC are likely multiple, the key signalling pathways ultimately converge on recovery of mitochondrial function following I/R injury and specifically on the prevention of the mPTP opening, which is believed to mediate mitochondrial injury. Thus, these two pathways converge on p70S6 kinase (p70S6K),which phosphorylates and inhibits glycogen synthase kinase -3β (GSK-3β) involved in the pro-death pathway whose inhibition acts to prevent mPTP opening. Please refer to the text for details.



1.6 Caloric Restriction

Calorie restriction (CR) is a dietary regiment that aims on restricting calorie intake at the level 20-40% lower than a typical diet without malnutrition (maintaining an adequate amount of nutrients and vitamins) [351]. CR has been shown to provide numerous advantageous effects from increasing longevity to benefitting cardiovascular health. Pertaining to longevity, it increases the median and maximum life span in a variety of species, such as among yeast, fish, rodents (mice, rats), dogs, and primates [352-356]. A primate, Rhesus monkey, study conducted over a period of twenty years demonstrated a 30% increase in maximum life span with the CR-delayed onset of age-associated pathologies [352]. Generally, these animals have shown a reduced aging and significantly delayed onset of age related diseases such as cardiovascular disease, diabetes, cancer, and brain degeneration [355, 356]. Although significant progress has been made in the area of CR, the mechanism of action of CR is still not fully elucidated [351]. Of many proposed theories the current theory describe how CR diet benefits an organism by imposing a mild-intensity stressful condition, which elicits a defence survival response by altering metabolism and improving cellular defences that helps an organism to endure more severe stress [357]. The switch to a defence survival response may be controlled by longevity genes [358]. This is one of the key mechanisms that have been shown to promote the CR longevity in lower animals. In baker's yeast cells CR induces Sir2 or "silent information regulator 2", a member of the sirtuin family and NAD^+-dependent histone deacetylases [357, 359, 360], which are hypothesized to suppress DNA instability [13]. In mammals Sir2 is known as SIRT1 [361]. Sir2/SIRT1 gene may underline the effects of CR in mammals by protecting cells from dying under stress [362, 363] as it has been shown that the extension of life-span has been abolished in transgenic mice lacking SIRT1 [364].

Aging is associated with increased risk of cardiovascular diseases such as high blood pressure, cardiac hypertrophy, coronary artery disease, myocardial infarction, and heart failure. CR secondary to increase in longevity attenuates the risk factors for cardiovascular disease such as myocardial ischemic injury [296-298, 300, 365, 366] , left ventricular hypertrophy [367, 368], and diastolic dysfunction [367,

369]. Indeed, it was demonstrated that life-long CR restored cardioprotective effects of IPC in middle-aged (ten months) [300] and aged (twenty four months) rats [365]. This indicates that CR enhances defense mechanisms against ischemic stress [298]. In addition, to life-long CR short-term CR can also be advantageous for the heart and it has been demonstrated that short-term CR improves myocardial ischemic tolerance [298]. The cardioprotective effect of short-term CR is mediated by increased production of an adipokine hormone, adiponectin (peptide secreted by the adipose tissue) [370, 371] and the associated activation of AMPK [298]. CR significantly elevates serum levels of adiponectin in both young and old rats and increases myocardial levels of phosphorylated AMPK without affecting the myocardial AMP/ATP ratio [296, 298], which is a major regulator of AMPK activity [224, 372, 373]. Together, these findings suggest that the beneficial effects of short-term CR are mediated *via* adiponectin, which in turn activates the AMPK signalling pathway to produce the protective effects. Indeed, pharmacological inhibition of AMPK activity or genetic ablation of AMPK attenuates the advantageous cardioprotective effects of this hormone [298]. Unfortunately, at present the complete mechanism(s) by which adiponectin *via* actions of AMPK protects myocardium from ischemic injury has not been fully elucidated.

1.7 Rationale for the Thesis

Ischemic heart disease (IHD) is associated with high morbidity and mortality and is the leading cause of death in Western countries [374]. The most common cause of IHD is atherosclerosis of the coronary arteries, coronary embolus, or artery thrombosis [375, 376]. Coronary artery by-pass grafting (CABG) surgery is one of the most established procedures for the treatment of patients with severe IHD. While CABG is often successful in establishing flow to the myocardium, the surgery has been shown to cause tissue injury in 28.5 % of patients undergoing CABG [377]. The cross-clamping of the aorta during the procedure that causes a brief interruption of blood flow results in acute ischemia-reperfusion (I/R) injury, which can contribute to worse short-term and long-term clinical outcomes in this patient group [375, 376]. Consequently, to improve clinical outcomes in these high-risk cardiac patients, the development of new interventions for an optimal protection of the heart during cardiac surgery is required.

According to animal models and human studies [277-283], short periods of ischemia followed by reperfusion protects the myocardium against subsequent longer ischemic insults as evidenced by improved cardiac function, reduction in infarct size, and preservation of myocardial ATP [278]. Although research demonstrates that the human myocardium is responsive to Ischemic Preconditioning (IPC), due to its invasive nature it is unlikely that IPC will be an acceptable method of myocardial protection to the majority of patients undergoing surgery (Reviewed in [378]). In addition to mechanical IPC, an extensive use of pharmacological therapy has also failed due to its side-effects and drawbacks. Based on the above reasoning, there is a need for a novel preconditioning therapeutic approach that would be safe, simple, and cost-effect.

Indeed, it has been suggested that calorie restriction (CR) can induce cardioprotection by provoking a mild stress response resulting in enhanced cellular defence to a more severe myocardial ischemic insult [357, 379]. While many of these studies have utilized chronic CR to induce myocardial protection [300, 365-368], short-term CR could be of more benefit due to a much easier implementation

into clinical practice than life-long CR. Undeniably, it has been recently established that short-term CR is beneficial to healthy rodents by conferring protection against myocardial ischemia/reperfusion (I/R) injury [296, 298, 299, 380].

As a result of the aforementioned rationale, the aim of our study was to determine whether short-term CR could induce cardioprotection in diseased hearts. To address this, we subjected the Spontaneously Hypertensive Rat (SHR) model of cardiac hypertension and hypertrophy to I/R in order to test the effect of short-term CR in a rat model that would more closely represent patients at risk of myocardial infarction and/or ones that would undergo CABG. Indeed, the SHR provides a useful experimental model of human disease since it develops hypertension gradually, which contributes to severe cardiac hypertrophy [381-383]. In our study, we subjected ten-week old male SHRs who display signs of hypertension [299] to short-term CR. Our short-term CR protocol consisted of feeding SHRs 90% of their average caloric intake for three weeks followed by two weeks at 60% of the average caloric intake. The CR diet was formulated to ensure that CR-SHRs received the equivalent amount of vitamins, minerals, and salt equivalent to the *ad libitum* fed control-SHRs. To assess whether short-term CR improved post-ischemic functional recovery under the conditions of elevated levels of fatty acids normally observed during ischemia [98], control-SHR and CR-SHR hearts were subjected to *ex vivo* I/R using a Krebs-Henseleit solution containing 1.2 mM of palmitate.

Our data have demonstrate that hearts from CR-SHRs at reperfusion following ischemia exhibited significant improvement in cardiac function including heart rate (HR), peak systolic pressure (PSP), left ventricular developed pressure (LVDP), cardiac output (CO), coronary flow (CF), and cardiac power (CP). Collectively, the above data demonstrate that short-term CR is able to protect the heart against I/R injury in the diseased animal model of hypertension and cardiac hypertrophy. In addition, our findings suggest utilization of short-term CR as a novel therapeutic approach in prevention of the I/R injury in the setting of cardiac surgery such as CABG due to its effectiveness, practicality, inexpensiveness, and absence of side effects associated with cardioprotective pharmacological agents.

1.8 Hypothesis

Short-term Calorie-Restriction (CR) is a practical preconditioning approach that reduces ischemic injury in the healthy rodent heart. Based on this, we hypothesized that short-term CR will improve post-ischemic myocardial recovery in the Spontaneously Hypertensive Rat (SHR), which serves as our model of a diseased heart. We further hypothesize that the mechanism by which short-term CR improves post-ischemic myocardial recovery is by optimizing cardiac energy metabolism and by activating the pro-survival Reperfusion Injury Salvage Kinase (RISK) pathway.

Chapter 2: MATERIALS AND METHODS

2.1 Materials

As listed in Table 2.1, most of the primary antibodies utilized in this study were purchased from Cell Signaling Technology (Danvers, Massachusetts); except for the anti-α-actin and anti-α-tubulin primary antibodies as well as the goat anti-rabbit (GAR) and donkey anti-goat (DAG) secondary antibodies that were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Radiolabeled substrates for *Ex vivo* working heart perfusions were purchased from PerkinElmer Life Sciences, Inc. (Table 2.2). Other reagents and chemicals, and agencies they were purchased from are listed in Table 2.2. Reagents and chemicals that are not listed were purchased from Sigma-Aldrich (St. Louis, Missouri) and/or Invitrogen Canada Inc. (Burlington, Ontario).

Table 2.1 List of primary and secondary antibodies

Primary Antibodies:	
Anti-phospho-AMPKα (Thr172)	Cell Signaling Technology (Danvers, Massachusetts)
Anti-AMPKα	----------
Anti-phospho-Akt (Ser473)	----------
Anti-Akt	----------
Anti-phospho-p70 S6 kinase (Thr421/Ser424)	----------
Anti-phospho-p70 S6 kinase (Thr389)	----------
Anti-p70S6 kinase	----------
Anti-phospho-PDH-E1α (S293)	----------
Anti-PDH-E1α	----------
Anti-phospho-p44/42 MAPK (Erk1/2 MAPK) (Thr202/Tyr204)	----------
Anti-p44/42 MAPK (Erk1/2)	----------
Anti-α-tubulin	----------
Aanti-α-actin	----------
Secondary Antibodies:	Santa Cruz Biotechnology, Inc. (Santa Cruz, California)
Goat anti-rabbit (GAR)	----------
Donkey anti-goat (DAG)	----------

Table 2.2 List of reagents and radiochemicals

Reagents:	
Protease inhibitor cocktail No.1	Sigma- Aldrich (St. Louis, Missouri)
Phosphatase inhibitor cocktail	----------
DTT Dithiothreitol (1M)	Invitrogen Canada Inc. (Burlington, Ontario)
Bio-Rad Protein Assay	Bio-Rad Laboratories
Costar® EIA/RIA 96-well microtiter plate, no lid	Corning Incorporated
Trans-Blot® Transfer Medium (pure nitrocellulose)	BioRad (Richmond, California)
Western Lightning® Chemiluminescence Reagents Plus kit	Perkin Elmer Life Sciences (Woodbridge, Ontario)
KODAK/FUJI Medical X-ray films	Mandel Scientific (Guelph, Ontario)
Bovine serum albumin: (fraction V, FA free)	Equitech-Bio Inc. (Kerrville, Texas)
(fraction V)	Sigma Aldrich (St. Louis, Missouri)
Hyamine hydroxide (1 M in methanol solution)	NEN Research Products (Boston, Massachusetts)
Ecolite® Aqueous Counting Scintillation fluid	MP Biomedicals (Solon, Ohio)
Radiochemicals:	
[U-14C]glucose	Perkin Elmer Life Sciences (Woodbridge, Ontario)
D-[5-3H]glucose	----------
[9,10-3H-(*N*)]palmitic acid	----------

2.2 Methods

2.2.1 Animals

University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences (CIOMS). All Spontaneously Hypertensive Rats (SHRs) were obtained from Charles River Laboratories. The SHRs were housed in individual cages and maintained on a 12:12 hour dark-light cycle. Eight-week old SHRs had free access to food and water for a two-week acclimatization period. SHRs at age of ten week were randomly assigned into two groups - control (Control-SHR) and calorie restricted (CR-SHR) group. SHRs of the control group were fed once a day *ad libitum* an AIN-93G standard chow diet (Research Diets) for five weeks. SHRs of the calorie restricted group were subjected to a calorie restricted dietary regime that involved feeding once a day *ad libitum* of a calorie restricted AIN-93G standard chow diet (Research Diets) according to an established protocol for a period of five weeks [384, 385]. According to this calorie restriction protocol, for the first three weeks of the experiment CR-SHRs received 90% of the average calorie intake of the Control-SHRs followed by two weeks of the 60% of the average calorie intake of the control-SHRs. CR diets were enriched in vitamins, minerals, and salts to ensure that the calorie restricted animals were not malnourished and nutrient deficient relative to the baseline control animals.

2.2.2 Assessment of Cardiac Function and Blood Pressure

To assess cardiac function of the experimental animals, transthoracic echocardiography was performed on mildly isoflurane-anaesthetized SHRs utilizing a Vevo 770 High-Resolution Imaging System equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto). As previously described [386, 387], left ventricular (LV) ejection fraction (EF) was calculated as a measure of left ventricular systolic and diastolic cardiac functions. Stoke volume (SV), by definition the volume of blood ejected with each heartbeat, is a difference between the left ventricular (LV) end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV). Accordingly, the ejection fraction was obtained by

measuring the volumes of the heart's chambers during the cardiac cycle. The stroke volume, ejection fraction, and percentage ejection fraction were calculated utilizing following formulas:

Stroke Volume: $SV = LVEDV - LVESV$

Ejection Fraction is a quotient of SV and LVEDV: $EF = \frac{SV}{LVEDV} = \frac{LVEDV - LVESV}{LVEDV}$

Percentage Ejection Fraction: $\%EF = \frac{LVEDV - LVESV}{LVEDV} \cdot 100\%$

Utilizing the tail-cuff system, non-invasive blood pressure (BP) measurements were obtained according to manufacturer's instructions (IITC Life Science) of mildly isoflurane-anaesthetized SHRs. The tail-cuff system is a method that utilizes Volume-Pressure Recording (VPR). VPR indirectly measures the systolic (SBP) and diastolic (DBP) blood pressures in the experimental animals by directly determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff.

2.2.3 Isolated Working Rat Heart Perfusion

2.2.3.1 Heart Perfusion of Isolated Working Rat Hearts

Fifteen-weeks of age Control- and CR-SHRs were anaesthetized with 60 mg/kg intraperitoneal (IP) injection of sodium pentobarbital, hearts were excised, and immersed in Krebs-Henseleit bicarbonate solution consisting of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, and 2.5 mM $CaCl_2 \cdot 2H_2O$. These excised hearts once cannulated *via* aorta were perfused at a hydrostatic pressure of 60 mmHg with Krebs-Henseleit solution at 37^{o}C. As soon as the isolated hearts have stabilized, an excess remaining tissue and fat were trimmed and the hearts were allowed to equilibrate in the Langendorff perfusion mode (the Krebs-Henseleit solution is contained in the Langendorff apparatus and flows through Langendorff line to the heart). After equilibration in the Langendorff mode, perfusion was switched to the working mode by clamping off the inflow from Langendorff line and by opening the left atrial inflow lane. Rat hearts were perfused in the working ejecting mode with modified Krebs–Henseleit solution consisting of 1.2mM palmitate pre-bound to 3% fatty acid–free albumin, 5.5mM glucose, 2.5mM

Ca^{2+}, 100µU/mL insulin, and either ^{3}H-glucose (1mCi/mL) with ^{14}C-glucose (0.2mCi/mL) for glycolysis and glucose oxidation, or ^{14}C-palmitate (0.1mCi/mL) for palmitate oxidation as described previously [388, 389]. Spontaneously beating rat hearts at the rate of approximately 230 beats per min (bpm) were aerobically perfused at 37^{o}C for a period of a thirty minutes at a constant left atrial preload of 11.5 mmHg and a constant aortic afterload pressure of 80 mmHg. The modified Krebs–Henseleit solution perfusate was recirculated, and pH was re-adjusted to 7.4 *via* its oxygenation with a 95% O_2 and 5% CO_2 composition gas mixture in the oxygenator. Following a thirty minutes aerobic perfusion, the isolated hearts were subjected to a thirty minutes global, no-flow, ischemia, and subsequently were reperfused aerobically for two hours at 37^{o}C. At the completion of the perfusion, isolated rat hearts were immediately frozen in liquid nitrogen (N_2) and stored in the freezer at -80^{o}C.

2.2.3.2 Measurement of Mechanical Function in Isolated Working Rat Hearts

Herat rate [beats per minute (bpm)] and aortic pressure (mmHg) were measured with a pressure transducer (Gould P21, Harvard Apparatus) connected to the aortic outflow line. Cardiac output (mL/min) and aortic flow (mL/min) were measured with ultrasonic flow probes (Transonic T206) placed in the preload and afterload lines, respectively. Coronary flow (ml/min) was calculated as a difference between the cardiac output and aortic flow; or as the product of cardiac output and LV developed pressure, which is a difference between aortic systolic pressure and preload pressure. Cardiac power (mWatts) was calculated according to the equation: (cardiac output − peak systolic pressure − 2.22)/100. Data were collected utilizing an AcqKnowledge MP100 system (BIOPAC system Inc., USA).

2.2.3.3 Measurement of Glycolysis, Glucose and Palmitate Oxidation in Isolated Woking Rat Hearts

The perfusion of the hearts with radioactive [5-^{3}H/U-^{14}C]glucose and radioactive [9,10-^{3}H-(*N*)]palmitic acid allowed for the measurement of the glycolysis and glucose oxidation rates and the palmitate oxidation rates, respectively [388, 390]. Glycolysis and glucose oxidation rates were measured by collection of [5-^{3}H/U-^{14}C]glucose-derived $^{3}H_2O$ and $^{14}CO_2$, correspondingly; while the fatty

acid oxidation was measured by the collection of [9,10-^{3}H-(*N*)]palmitic acid-derived $^{3}H_2O$. Total myocardial production of $^{3}H_2O$ and $^{14}CO_2$ was determined at ten minutes intervals during thirty minutes of the aerobic perfusion and forty minutes of the reperfusion stages of the ischemia-reperfusion experiment. Glycolytic flux of [5-^{3}H]glucose was measured *via* separation of radioactive $^{3}H_2O$ from radioactive [5-^{3}H]glucose in the timed buffer samples utilizing a Dowex column − a separating column with an anion exchange resin. The separated out $^{3}H_2O$ was collected in the scintillation vials and counted utilizing the liquid scintillation counter. Glucose oxidation rates were determined by measuring the release of metabolically derived gaseous $^{14}CO_2$ trapped in the oxygenation chamber and as $^{14}CO_2$ dissolved in the perfusate buffer as $H^{14}CO_3^-$. The $^{14}CO_2$ vapour was trapped in hyamine hydroxide solution, while $H^{14}CO3^-$ ions were trapped on hyamine hydroxide saturated filter paper [388]. Specifically, gaseous $^{14}CO_2$ was collected utilizing 15 mL of 1mol/L of hyamine hydroxide solution [391]. The timed samples taken from the hyamine hydroxide were counted in a liquid scintillation counter. The $^{14}CO_2$ trapped in the timed sample buffers under mineral oil as $NaH^{14}CO_3$ was released by acidification with 9N H_2SO_4 of 1 mL of buffer sample in sealed 25 mL test-tubes. The data from the hyamine hydroxide samples and the buffer samples were used collectively to calculate glucose oxidation rates. Fatty acid oxidation rates were measured *via* collection of $^{3}H_2O$ derived from the metabolism of [9,10-^{3}H-(*N*)]palmitic acid [388]. Palmitate oxidation flux was measured through a separation of radioactive $^{3}H_2O$ from radioactive [9,10-^{3}H-(*N*)]palmitic acid, both present in the perfusate samples, utilizing the vapour transfer method [388, 392]. Vapour transfer method involves addition of 500 µl of water into a scintillation vial, followed by insertion of the lidless 1.5 ml microcentrifuge tube into the scintillation vial, and the addition of the 200 µl of the buffer sample into the microcentrifuge tube. To allow for the evaporation and subsequent condensation of the $^{3}H_2O$, capped scintillation vials were stored at 50^{o}C for 24 hours and then at 4^{o}C for another 24 hours. Following period of 48 hours and upon the removal of the microcentrifuge tubes, scintillation fluid was added to the scintillation vials containing condensed $^{3}H_2O$. The level of radioactivity of the condensed $^{3}H_2O$ was measured utilizing a liquid scintillation counter.

2.2.3.4 Calculation of Tricarboxylic Acid Cycle Acetyl-CoA Production

The rates of glucose oxidation and fatty acid oxidation were utilized in the calculation of the Tricarboxylic Acid Cycle (TCA) acetyl-CoA production. This calculation is based on the theoretical values that state that one mole of glucose produces two moles of acetyl-CoA *via* glycolysis and pyruvate dehydrogenase decarboxylation; and one mole of palmitate produces eight moles of acetyl-CoA *via* fatty acid β-oxidation.

2.2.3.5 Calculation of Metabolically Derived ATP Production

As previously described [3], glycolysis consumes 2 ATP and produces 4 ATP, with a net production of 2 ATP. Alternatively, glucose oxidation yields 32 ATP. Pyruvate dehydrogenation produces 1 molecule of $NADH_2$, which give rise to 2.5 ATP; and one turn of acetyl-CoA through the TCA cycle produces 9.75 molecules of $NADH_2$, which produces a total of 12.25 ATP per molecule of pyruvate. Consequently, full oxidation of glucose results in a net yield of 32 ATP per 1 molecule of glucose. In contrast, palmitate, a 16-carbon chain fatty acid, oxidation yields 105 ATP. Fatty acid oxidation initially consumes 2 high energy phosphates from ATP per molecule of FA in the process of fatty acid activation. In the FA oxidation of the palmitate, seven turns of the β-fatty acid oxidation spiral produces 7 molecules of $NADH_2$ relating to 17.5 molecules of ATP, and 7 molecules of FADH relating to 10.5 molecules of ATP. Palmitate derived 8 acetyl-CoA *via* TCA cycle will produce additional 78 molecules of ATP as 9.75 ATP is produced per acetyl-CoA per one turn of TCA cycle. This results in the overall energy yield of 105 ATP per 1 palmitate molecule.

2.2.3.6 Calculation of the Index of ATP Utilization for Contractile Work

To calculate the rate of ATP production as an index of ATP utilization for contractile work, the cardiac power data set was converted from the units of mmHg x mL per min to Pascal x m^3 (equivalent to Joule) per minute. The converted value was divided by the empirical value of 10^{-19} Joule per ATP molecules [393] to calculate the rate of ATP production per minute.

2.2.3.7 Calculation of Metabolically Derived Proton Production

The calculations for proton (H^+) production were performed according to the following experimental evidence: hydrolysis of ATP from the glycolysis and lactate production results in the net production of $2H^+$ per molecule of glucose [17, 259]. In contrasts, if glycolysis is coupled to glucose oxidation the net production of H^+ of zero [394, 395]. Therefore, the overall rate of production derived from glucose utilization was calculated by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by 2.

2.2.4 Immunoblot Analysis

2.2.4.1 Tissue Homogenization

Post ischemia-reperfusion experiment, Control- and CR-SHR hearts were rinsed in ice cold 1X PBS (pH 7.4), snap fozen in liquid nitrogen (N_2), and stored at -80^{o}C. Cardiac tissue was powdered utilizing a motor and pestle chilled in liquid N_2. For homogenizations, 20 to 40 mg of the cardiac tissue was weighted out in a frozen in liquid N_2 2 mL clear eppendorff tubes. The tissue was lysed by mechanical homogenization twice on ice at medium speed for twenty second in 300 to 350 µl of ice cold AMPK lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 M sucrose, and 2 mM sodium orthovanadate supplemented with 1:1000 dilution of mammalian protease inhibitor cocktail (Sigma), 1:100 dilution of phosphatase inhibitor cocktail 1 (Sigma) to prevent tissue digestion by its own enzymes, and 1 mM dithiothreitol (DTT). Homogenized tissue samples were lysed for ten minutes on ice (to prevent protein denaturation), syringed four times each with 26-gauge syringe needle (to enhance cellular lysis), and then centrifuged at 800·g for ten minutes (to separate cellular debris from cellular homogenate) at 4^{o}C. Supernatant was collected into a fresh tube and pallet was discarded. 100 µL of the supernatant was allquoted into separate eppendorff tubes for determination of protein concentration *via* Bradford protein assay analysis. Eppendorff tubes containing supernatant were snap frozen in liquid N_2 and stored at -80^{o}C.

2.2.4.2 Bradford Protein Assay – Determination of Protein Concentration

The protein concentration of the supernatant was determined *via* a spectroscopic analytical procedure, the Bradford protein assay. This assay determines protein concentrations in solutions, which depends upon the change in absorbance in Coomassie Blue G-250 (Bradford reagent) upon biding of protein [396]. Bradford protein assay was performed utilizing a ready-to-use Bio-Rad Protein Assay Dye Reagent Concentrate stored at 4^{o}C. First, samples containing 100 µL of supernatant stored at -80^{o}C were defrosted on ice and diluted in 1 to 10 ratio of supernatant in APMK lysis buffer. Meanwhile the dye reagent concentrate was filtered utilizing Whatman filter paper, 1 to 5 dilution of the dye reagent

concentrate in double distilled water (ddH_2O) was prepared, and 1 ml of the diluted dye concentrated was aliquoted in triplicated into labelled 10 mL plastic tubes. For the generation of a standard curve enabling determination of the protein concentration (µg/µL) of the samples, a range of protein standards (0 µg to 10 µg) in triplicates were prepared with a commercially obtained 1mg/mL BSA solution (New England Biolabs). Subsequent to the preparation of the standard curve 2 to 4 µL of diluted sample supernatants were added in triplicate into each test-tube. Following three seconds vortexing, 250 µL of each standard and each supernatant both diluted in 1 mL of reagent solution were added to a 96-well microtiter plate. Following incubation for minimum of five minutes utilizing a spectrophotometer, the absorbance of the samples was measured at the wavelength of 595 nm (Bradford dye shifts from 465 nm to 595 nm when biding to protein occurs) [397]. Obtained spectroscopic absorbance values that give absorbance values close to those for the protein samples of interest were utilized in the calculation of volumes of ddH_2O needed to dilute the supernatant to load 20 µg of the protein for each sample, firstly by calculating the sample density and subsequently volumes of ddH_2O needed to obtain a constant loading volume for each sample equivalent to 20 µg of the protein.

2.2.4.3 Sample Preparation for Immunoblot Analysis

Stored at -80^oC supernatant collected from tissue homogenization was defrosted on ice. Once defrosted each sample (5-15 µL) was diluted in small volumes (30-40 µL) of ddH_2O as appropriate to enable loading of 20 µg of protein per sample in each well of 15-well sodium dodecyl sulphate (SDS) polyacrylamide gel. Following sample dilution in ddH_2O to generate sample volume associated with 20 µg of protein, one part of PARP reducing SDS loading buffer (6.25 mM Tris-HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% Bromophenol Blue, and 5% β-mercaptoethanol) was added to two parts of the diluted supernatant. After brief vortexing, the samples were boiled for five minutes at 95^oC, subsequently centrifuged at very low speeds to condense the vapours, and stored at -20^oC for future loading.

2.2.4.4 SDS-PAGE Gel Electrophoresis

20 µg of protein from boiled samples of cardiac tissue homogenates were subjected to SDS – polyacrylamide gel electrophoresis (also known as SDS-PAGE) at room temperature (RT) to separate proteins according to their size. Proteins were separated in gels containing 8% acrylamide using the Bio-Dad Mini-PROTEAN3 cell. The loading of 10 µL of the prestained molecular marker, the Bio-Rad Precision Plus Protein dual color standard, enabled estimation of the molecular weight and helped in the identification of the size of the protein of interest. Prior to loading of the standard and the samples, the SDS-PAGE apparatus was half filled with 1X protein reservoir buffer (also known as running buffer) prepared from 5X stock reservoir buffer consisting of 0.125 M Tris, 0.96 M Glycine, and 0.5% SDS (pH 8.3). Once samples of interest were loaded in an appropriate order, the apparatus was topped up with the 1X reservoir buffer. The gel was run at constant value of 60 V for approximately thirty minutes until the samples migrated though the stacking gel and reached the separating gel. Once the samples have entered the separating gel, the voltage has been increased to constant value of 130 V. SDS-PAGE gel electrophoresis was continued until the blue dye have reached bottom of the gel in a time frame of approximately one hour and forty minutes.

2.2.4.5 Protein Transfer to Nitrocellulose

Upon completion of SDS-PAGE gel electrophoresis, the glass plates enclosing the 8% gel were carefully separated, the stacking gel was trimmed off with the Bio-Rad plastic separator, and subsequently the separating gel was placed for fifteen minutes in the container containing cold wet transfer buffer consisting of 25 mM Tris, 0192 M glycine, and 20% methanol. A Bio-Rad nitrocellulose membrane was cut to an appropriate size and pre-equilibrated for at least fifteen minutes at RT in the wet transfer buffer on the shaker. To set up the protein transfer, the gel was sandwiched between sponges, Whatman filter paper, and nitrocellulose paper in the Trans-Blot holder from black side as bottom in the following order: sponge, Whatman filter paper, separating gel, nitrocellulose membrane, Whatman filter paper, and sponge. To allow for proper and complete protein transfer, a care was taken to ensure that there were no

air bubbles between a nitrocellulose membrane and the gel. The whole compilation was then inserted into the Tran-Blot module, which sat in the Bio-Rad Mini-PROTEAN3 cell apparatus with ice pack and cold wet transfer buffer. To drive over the negatively charged proteins to the positively charged nitrocellulose membrane, the transfer set up was such that the negative charge was on the side of the gel and the positive charge was on the side of the nitrocellulose membrane. Electrophoresis was used to drive the protein bands from the gel onto the nitrocellulose membrane by placing the whole apparatus in the 4^{o}C cold room and setting voltage at a constant value of 90 V for two hours and thirty minutes, with a change of the ice pack one hour and thirty minutes into the transfer. Upon completion of the transfer, apparatus was taken apart and the membrane protein transfer was visualized *via* staining of the nitrocellulose membrane with a Ponceau stain consisting of 0.1% weight to weight (W/W) of ponceau stain (Sigma P-3504) and 1% volume to volume (V/V) acetic acid in ddH_2O solution. Following the scanning of the ponceau stained membrane and the storage of the digital file for future reference, the membrane was washed three times for five minutes in 1X Tris-buffered saline (TBS) solution, and subsequently was dried on a piece of fresh Whatman filter paper. Once dried, membrane was labelled with a super fine black Sharpie pen. Gel was placed in Coomassie Blue Stain for twenty four hours, followed by incubation in the Coomassie Blue Destain for another twenty four hours. This allowed for the determination of the protein transfer efficiency.

2.2.4.6 Western Blotting Procedure

Membranes were blocked for nonspecific binding by incubation for one hour at RT or occasionally overnight in the cold room at 4^{o}C on the shaker in the 20 mL per membrane of Blocking buffer (TBSMT) consisting of 5% Carnation non-fat dry milk, 1X Tris-buffered saline (TBS), and 0.1% Tween 20. Following blocking, the membranes were rinsed with 1X TBST consisting of 1X Tris-buffered saline (TBS) and 0.1% Tween 20 (i.e., TBSMT minus the milk) to rinse off the milk. Membranes were immunoblotted (Western blotted) utilizing a variety of phospho-protein and protein specific primary antibodies by an incubation of the membranes overnight at 4^{o}C in the 50 mL Blue Max

Tubes on the rotator with primary antibodies at 1:500 or 1:1000 dilutions in 3 mL of the Primary Antibody Dilution buffer consisting of 5% fatty acid-free BSA, 1X TBS, and 0.1% Tween 20. Following twenty four hours incubation in the primary antibodies, membranes were vigorously washed at RT three times five minutes each in copious amounts of 1XTBST on the shaker. Following this extensive wash membranes were incubated for one hour at RT in the 20 mL of the secondary horseradish peroxidase (HRP)-conjugated antibodies diluted in TBSMT − with1:1000 dilution of the secondary antibody such as goat-anti-rabbit (GAR) or donkey-anti-dog (DAD) when appropriate. Following incubation in the secondary antibodies, the membranes were washed at RT three times five minutes each in 1X TBST.

To develop Western blots, antibodies following last wash were visualized *via* enhanced chemiluminescence (ECL) detection method utilizing the PerkinElmer Western Lightning chemiluminescence Western reagent plus system. In Western blotting, proteins have been immobilized on a membrane and labelled by complexing with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. In the chemiluminescence reaction, horseradish peroxidase catalyzes the oxidation of luminol to a light emitting reagent, which releases light during its decay. Since the oxidation of luminol is catalyzed by HRP, which is complexed with the membrane bound protein of interest, the amount and location of light that HRP catalyzes the emission of is directly correlated with the location and amount of the protein on the membrane. According to ECL protocol, equal volumes of Enhanced Luminol Reagent (brown bottle) and Oxidizing Reagent (white bottle) were mixed in the 50 mL Blue Max Tube. Immediately, 2 ml of the mixture were poured onto a saran wrap. Membrane was placed side down for one minute. The membrane was wicked away from the excess ECL on clean surface of the saran wrap and then wrapped in a fresh piece of the saran wrap and developed. Negatives were developed using KODAK X-ray film and film exposure times ranged from three seconds to fifteen minutes – the time was varied as needed for optimal detection.

2.2.4.7 Nitrocellulose Membrane Stripping and Re-probing

Term stripping describes removal of the primary and secondary antibodies from a Western blot membrane, and this method is used to investigate more than one protein on the same blot. After visualization, each membrane was transferred to a 50 mL Blue Max Tube containing Amersham Pharmacia stripping buffer consisting of 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCL (pH 6.7), and incubated in the hybridization oven at 55^{o}C for at least ninety minutes. Following a subsequent washing with 1X PBS three times ten minutes each time, membranes were ready for re-probing. Between immunoblotting, membranes were stored in TBS at 4^{o}C and -20^{o}C for short-term (up to three days) and long-term storage, respectively.

2.2.4.8 Western Blot Densitometry

Following the development of the Western blot membranes, negatives were scanned in the gray scale as tiff files. Then analysis of the band intensities was performed utilizing image analysis software, Image J. Obtained results were entered into Microsoft Excel spreadsheet for graphical and statistical analysis.

2.2.5 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). For the comparison of two groups at multiple time-points, a two way repeated measures analysis of variance (ANOVA) was used for the determination of statistical significance. For comparison of two groups and for comparison of three groups a two-tailed unpaired Student's *t*-test and analysis of variance (ANOVA) were utilized, respectively. A Bonferroni *post-hoc* analysis was employed to correct for multiple comparisons when appropriate. A value of $p < 0.05$ was considered significant.

Chapter 3: RESULTS

3.1 Improved Post-ischemic Functional Recovery in Hearts from Calorie Restricted SHRs

Since recent work by our laboratory [299, 301] as well as by others [298, 380] has shown that calorie restriction (CR) is beneficial in healthy rodents, we investigated whether short-term CR is also beneficial in diseased hypertrophied heart utilizing a commonly used model of cardiovascular disease, the Spontaneously Hypertensive Rat (SHR) [381, 384]. Male SHRs utilized in this study were between ten and fifteen weeks of age. During this period, SHR are reported to exhibit mild hypertension advancing rapidly to severe hypertension [398, 399] followed by the active phase of hypertrophic growth between sixteen to twenty weeks of age [384].

Control-SHRs were *ad libitum* fed for five weeks with control diet; while CR-SHRs were fed for three weeks at 90% of their average caloric intake (10% CR) followed by two weeks at 60% of the average caloric intake (40% CR) (Figure 3.1A). The CR diet was formulated to ensure that CR-SHR received equivalent amount of vitamins, minerals, and salt as the control-SHR. Before the initiation of the feeding protocol [298, 380], no differences in body weight were shown to exist between SHRs randomized into *at libitum-fed* (control SHR) or short-term (five weeks) CR group [299]. As previously reported by our laboratory, a five weeks of short-term calorie restriction significantly reduced weight gain in the CR group [299]. Additionally, it was reported that the tibia length compared to control-SHR weight was not altered indicating that control-SHR and CR-SHR were of similar size and CR has not impeded their growth [299].

To assess whether short-term CR improved post-ischemic functional recovery under the conditions of elevated levels of fatty acids normally observed during ischemia [98], control-SHR and CR-SHR hearts were subjected to *ex vivo* I/R using a Krebs-Henseleit solution containing 1.2 mM of palmitate. During the aerobic period prior to ischemia, percent cardiac functional parameters were similar between control-SHR and CR-SHR groups (Table 3.1). On the contrary, post-ischemic contractile

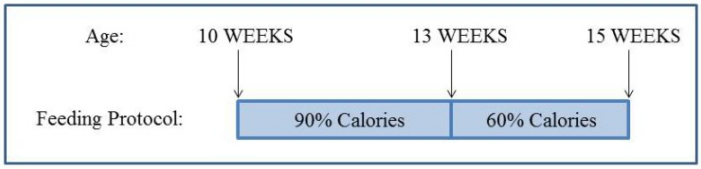
function was dramatically improved in hearts from CR-SHRs compared to control-SHRs (Table 3.1, Figure 3.1B, C, and D).

Table 3.1 Pre- and post-ischemia heart functional parameters in *ex vivo* perfused hearts from control- and CR-SHRs. Values are mean±SEM of hearts from control-SHR (n=14) and CR-SHR (n=15) rats perfused *ex vivo* following five weeks of diet. Control-SHRs were *ad libitum* fed for five weeks with control diet; while CR-SHR were fed for three weeks at 90% of their average caloric intake (10% CR) followed by two weeks at 60% of the average caloric intake (40% CR). Values shown represent averages for the thirty minutes (aerobic) and sixty minutes (reperfusion) perfusion period; *Significant difference ($P<0.05$) between Control-SHR and CR-SHR two-way ANOVA analysis of variance test accompanied by the Bonferroni *post hoc* multiple comparisons test; CR indicates calorie-restricted; SHR, Spontaneously Hypertensive Rat; HR, heart rate; bmp, beats per minute; PSP, peak systolic pressure; mmHg, millimeters of Mercury; LVDP, left ventricular developed pressure; CO, cardiac output; AO, aortic outflow; CF, coronary flow; CP, cardiac power; by definition CF = CO – AO.

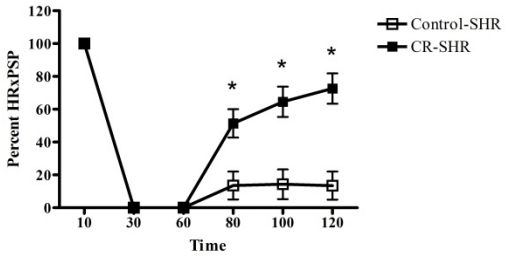
	Aerobic		Reperfusion	
	Control-SHR	CR-SHR	Control-SHR	CR-SHR
HR (bpm)	233.5 ± 6.6	221.4 ± 6.8	133.7 ± 17.2	207.2 ± 10.8 *
PSP (mmHg)	127.4 ± 3.3	133.2 ± 3.2	27.1 ± 10.6	93.6 ± 9.8 *
HRxPSP ($x10^{-3}$)	29.8 ± 1.2	29.5 ± 1.3	5.8 ± 3.1	20.3 ± 2.5 *
LVDP (mmHg)	68.6 ± 4.0	71.1 ± 3.4	12.1 ± 5.9	35.3 ± 4.6 *
CO (ml/min)	59.9 ± 2.6	58.3 ± 2.4	12.9 ± 4.9	30.0 ± 3.3 *
AO (ml/min)	34.6 ± 2.7	35.4 ± 2.0	4.9 ± 3.4	10.7 ± 1.9
CF (ml/min)	25.2 ± 0.5	22.9 ± 0.6	8.0 ± 1.7	19.3 ± 1.8 *
CP (ml/min*mmHg)	77.0 ± 4.9	77.7 ± 3.8	10.1 ± 6.4	32.3 ± 4.5 *

Figure 3.1 Myocardial functional parameters of *ex vivo* perfused hearts from control- and CR-SHRs during I/R. A) Five week feeding protocol for control-SHR and CR-SHR rats. Control-SHRs were *ad libitum* fed for five weeks with control diet; while CR-SHR were fed for three weeks at 90% of their average caloric intake (10% CR) followed by two weeks at 60% of the average caloric intake (40% CR); B) Percent Heart rate x peak systolic pressure (HR x PSP); C) Percent coronary flow; and D) Percent cardiac power hearts were subjected to *ex vivo* I/R using a Krebs-Henseleit solution containing 1.2 mM palmitate. Values are mean±SEM of hearts from control-SHR (n=14) and CR-SHR (n=15) rats perfused *ex vivo* following five weeks of diet; *P<0.05 represents significant difference between Control-SHR and CR-SHR. Two-way repeated measures ANOVA analysis of variance test accompanied by the Bonferroni *post hoc* multiple comparisons test was performed to detect differences in B, C, and D.

A)



B)



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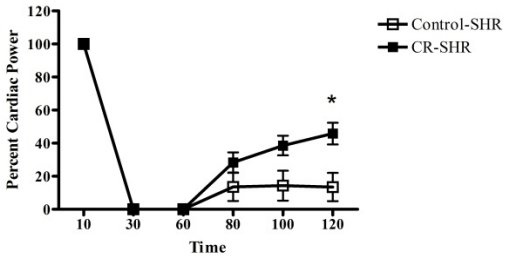

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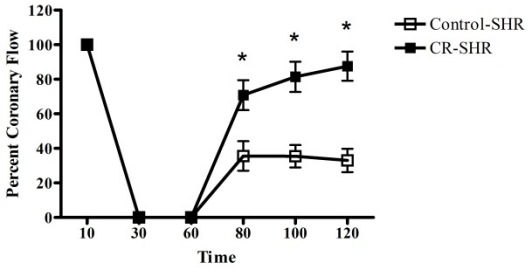
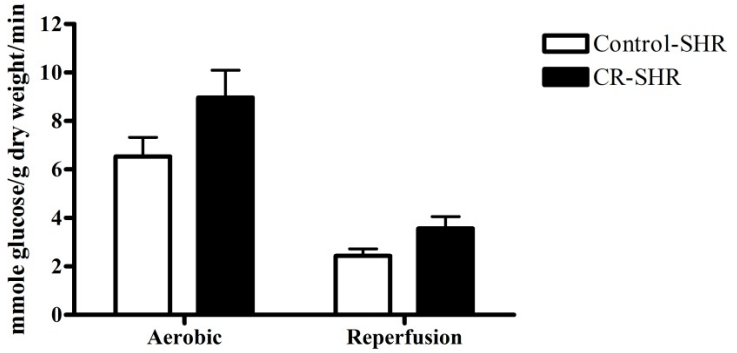


3.2 No Alteration of the Myocardial Metabolism at Baseline in Hearts from CR-SHRs

To determine if alterations in energy metabolism could contribute to improved functional recovery observed in hearts from CR-SHR, we measured glucose (glycolysis, glucose oxidation) and fatty acid (fatty acid oxidation) metabolism in these hearts. During the aerobic perfusion period prior to ischemia, there were no significant differences in the rates of glycolysis, glucose oxidation, and fatty acid oxidation between control-SHR and CR-SHR groups (Figure 3.2A, B, C).

Figure 3.2 Cardiac energy metabolism in *ex vivo* perfused hearts from control- and CR-SHRs during aerobic perfusion prior ischemia and aerobic reperfusion following ischemia. A) Glycolysis (n=7/control-SHR group; n=8/CR-SHR group); B) Glucose oxidation (n=7/control-SHR group; n=8/CR-SHR group); C) Palmitate oxidation (n=7/control-SHR group; n=7/CR-SHR group); Values are mean±SEM of hearts from control-SHR and CR-SHR rats perfused *ex vivo* following five weeks of diet; *P<0.05 represents significant difference between Control-SHR and CR-SHR. Two-tailed unpaired Student's *t*-test was performed to detect differences in A, B, and C.

A)



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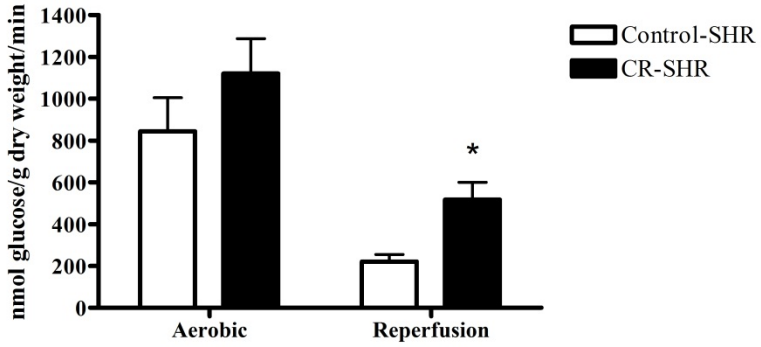

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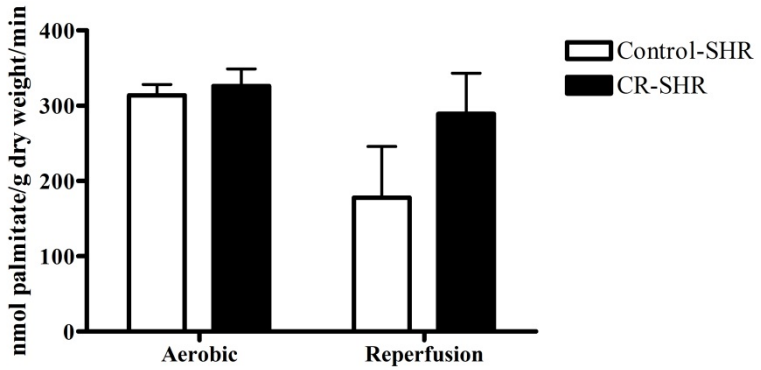


3.3 Association of Improved Post-ischemic Recovery in Hearts from CR-SHRs with Improved Myocardial Metabolism during Reperfusion

Since we did not observe any significant changes in the myocardial metabolism at baseline during aerobic phase prior to ischemia, we investigated whether alterations in myocardial energy metabolism at reperfusion following a period of ischemia may contribute to the improved functional recovery observed in hearts from CR-SHRs. Consistent with the metabolic profile of hearts prior to ischemia, glycolysis and palmitate oxidation were not altered between control-SHR and CR-SHR groups (Figure 3.2A and C). However, the glucose oxidation was significantly increased in hearts from CR-SHRs compared to control-SHR (Figure 3.2B).

3.4 Association of Improved Post-ischemic Recovery in Hearts from CR-SHRs with Improved Myocardial Energetics during Reperfusion

To investigate whether alterations in myocardial energy status at reperfusion following a period of ischemia may contribute to the improved functional recovery in hearts from CR-SHRs, we calculated metabolically derived ATP production rates of the *ex vivo* perfused hearts from control-CR and CR-SHRs during the aerobic reperfusion period following ischemia. In agreement with the metabolic profile of hearts prior to and following ischemia, glycolysis and palmitate oxidation contribution to ATP production were not altered between control-SHR and CR-SHR groups (Figure 3.3 A and D). However, the glucose oxidation considerably contributed to the metabolically derived ATP production rates in hearts from CR-SHRs compared to control-SHRs (Figure 3.3 B). The contribution of the glucose oxidation to the metabolically derived ATP production rates in hearts from CR-SHRs was so dramatic that we totalled the rates of metabolically derived ATP from glycolysis and glucose oxidation in Figure 3.3 C. In addition, calculated index of ATP utilization revealed that CR-SHR cardiac myocytes have significantly higher number of ATP molecules available for their contractile work compare to control-SHR cardiac myocytes (Figure 3.4).

Given the extensive research into cardiac efficiency and glucose utilization [131, 171, 172, 211, 261, 387, 394, 400-409], these data suggest that the larger contribution of the glucose metabolism to the

total metabolically derived ATP production rate in the CR-SHRs relative to control-SHRs results in the improvement of cardiac efficiency and ATP available for the contractile work. Indeed, although glucose oxidation generates less energy than oxidation of palmitate, it utilizes less oxygen per equivalent amount of energy generated compare to fatty acid oxidation making it a more efficient fuel. Furthermore, the overall rate of proton production between CR-SHRs and the control-SHRs derived from glucose utilization was statistically not significant suggesting no improvement in the proton coupling between glycolysis and glucose oxidation in C-SHRs (Figure 3.5).

To further validate the energy status of the hearts from CR-SHRs, we investigated the activity of pyruvate dehydrogenase (PDH) – a rate limiting step for oxidation of glucose by the TCA cycle. During ischemia, PDH is inhibited by rising NADH resulting in the inhibition of flux though this enzyme [82]. We have demonstrated that PDH activity did not change between CR-SHR and control hearts (Figure 3.6). By calculating TCA cycle acetyl-CoA production of *ex vivo* perfused hearts from control-SHRs and CR-SHRs during aerobic reperfusion following ischemia, we show that the energy level of the CR-SHR is equivalent to the energy level of the control-SHRs (Figure 3.7). To further demonstrate the improved metabolic status in the CR-SHR hearts, we investigated activation of master regulator of energy metabolism, AMP-activated protein kinase (AMPK) − an enzyme mainly regulated by the cellular AMP-to-ATP ratio [224, 372, 373]. Consistent with increased contribution of glucose oxidation to the metabolically derived ATP production in the hearts from CR-SHRs compare to control-SHRs (Figure 3.3 B), P-AMPK was significantly reduced in hearts from CR-SHRs compared to controls (Figure 3.8).

Figure 3.3 Calculated metabolically derived ATP production rates of *ex vivo* perfused hearts from control- and CR-SHRs during anaerobic reperfusion following ischemia. The rates of metabolically derived ATP from A) glycolysis (n=7/control-SHR group; n=7/CR-SHR group), B) glucose oxidation (n=7/control-SHR group; n=7/CR-SHR group), C) glucose and glucose oxidation combined, and D) palmitate oxidation (n=7/control-SHR group; n=7/CR-SHR group) were utilized in the calculation of the metabolically derived ATP production. This calculation is based on the theoretical values that state that 1 mole of glucose produces net 2 ATP *via* glycolysis and full oxidation of glucose results in a net yield of 32 ATP per 1 molecule of glucose. Palmitate oxidation results in the overall energy yield of 105 ATP per 1 palmitate molecule; Values are mean±SEM of hearts from control-SHR and CR-SHR rats perfused *ex vivo* following five weeks of diet; *P<0.05 represents significant difference between Control-SHR and CR-SHR. Two-tailed unpaired Student's *t*-test was performed to detect differences in A, B, C, D.

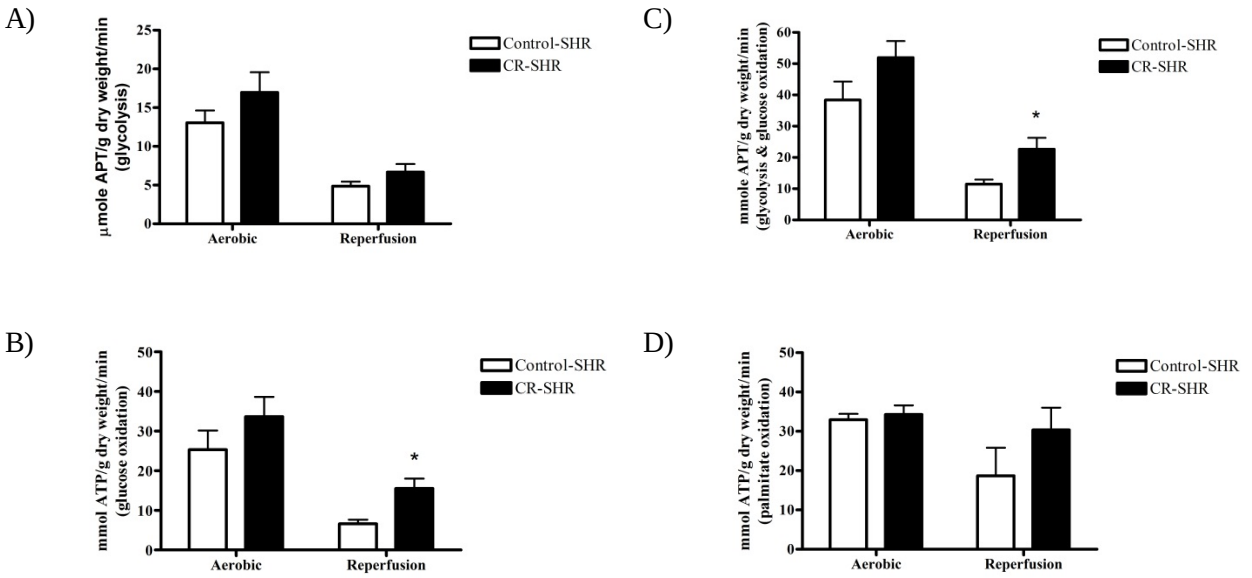


Figure 3.4 Calculated index of ATP utilization for contractile work of *ex vivo* perfused hearts from control- and CR-SHRs during anaerobic reperfusion following ischemia.
Calculated rate of ATP production utilizing cardiac power data set (n=14/control-SHR group; n=14/CR-SHR group) as an index of ATP utilization for contractile work. The cardiac power in mL x mmHg per minute was converted to Joule per minute and then divided by the empirical value of 10^{-19} Joule per ATP molecule [393] to calculate the rate of ATP production per minute; *P<0.05 represents significant difference between Control-SHR and CR-SHR. At perfusion P=0.0974 and at reperfusion P=0.0049. Two-tailed unpaired Student's *t*-test was performed to detect differences.

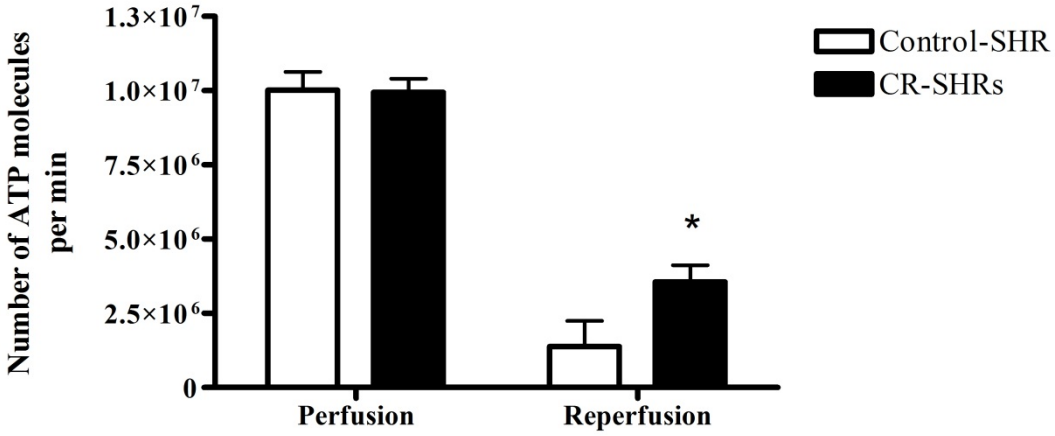


Figure 3.5 Calculated metabolically derived Proton production rates of *ex vivo* perfused hearts from control- and CR-SHRs during anaerobic reperfusion following ischemia. Calculated metabolically derived proton (H^+) production rates utilizing glycolysis (n=7/control-SHR group; n=7/CR-SHR group) and glucose oxidation (n=7/control-SHR group; n=7/CR-SHR group) data sets. The calculations for H^+ production were performed according to the following experimental evidence: hydrolysis of ATP from the glycolysis and lactate production results in the net production of $2H^+$ per molecule of glucose. In contrasts, if glycolysis is coupled to glucose oxidation the net production of H^+ is zero. Therefore, the overall rate of production derived from glucose utilization was calculated by subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by 2. Values are mean±SEM of hearts from control-SHR and CR-SHR rats perfused *ex vivo* following five weeks of diet; *P<0.05 represents significant difference between Control-SHR and CR-SHR. At perfusion P=1.5320 and at reperfusion P=0.1210. Two-tailed unpaired Student's *t*-test was performed to detect differences.

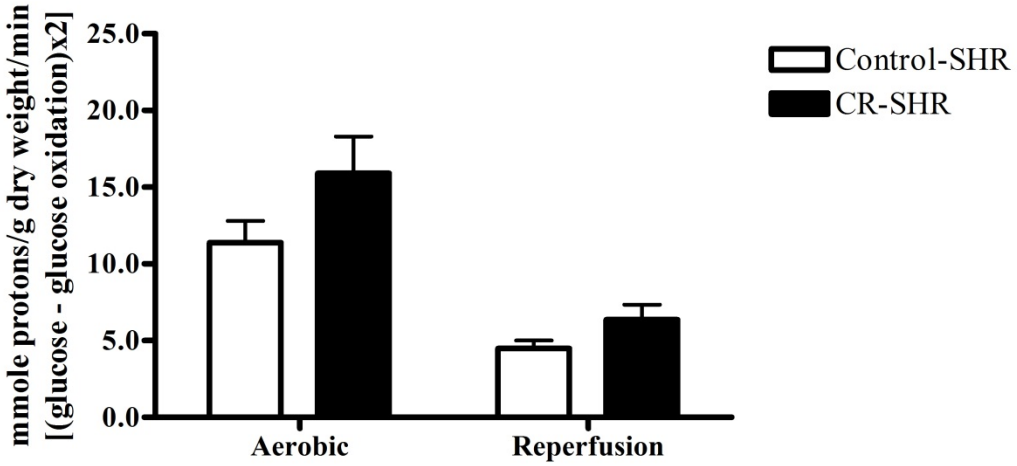
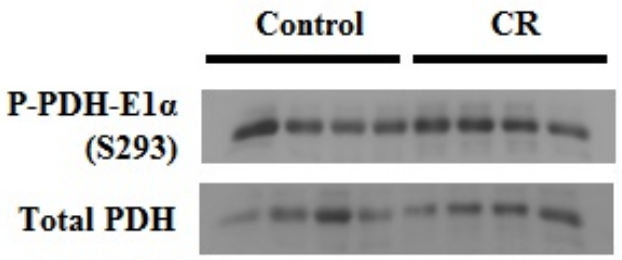


Figure 3.6 **Energetic status of *ex vivo* perfused hearts from control- and CR-SHRs during aerobic reperfusion following ischemia.** Immunoblot analysis performed on control-SHR (n=4/control-SHR group) and CR-SHR hearts (n=4/CR-SHR group) at the end of reperfusion with anti-phosphorylated PDH-E1α(S293) and anti- PDH-E1α; Values are mean±SEM of hearts from control-SHR and CR-SHR rats perfused *ex vivo* following five weeks of diet. *P=0.5981 represents no significant difference between Control-SHR and CR-SHR. Two-tailed unpaired Student's *t*-test was performed to detect differences.



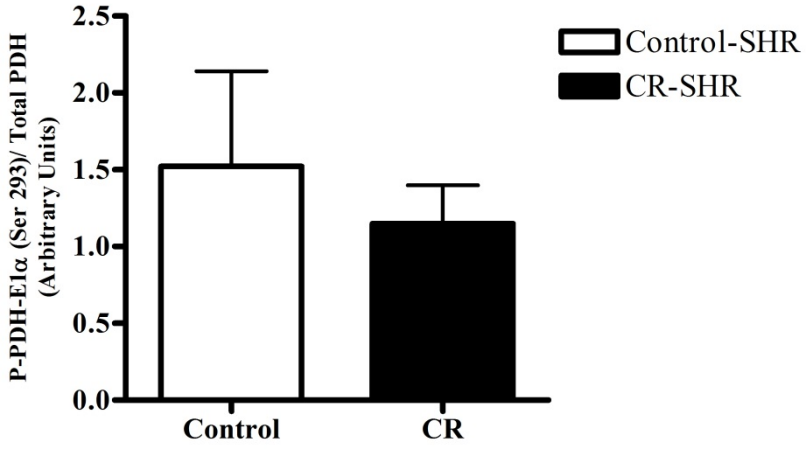


Figure 3.7 Calculated total TCA cycle acetyl-CoA production of *ex vivo* perfused hearts from control- and CR-SHRs during aerobic reperfusion following ischemia. The rates of glucose oxidation (n=7/control-SHR group; n=7/CR-SHR group) and palmitate oxidation (n=7/control-SHR group; n=7/CR-SHR group) were utilized in the calculation of the TCA acetyl-CoA production. This calculation is based on the theoretical values that state that 1 mole of glucose produces 2 moles of acetyl-CoA *via* glycolysis and pyruvate dehydrogenase decarboxylation; and 1 mole of palmitate produces 8 moles of acetyl-CoA *via* fatty acid *β*-oxidation; Values are mean±SEM of hearts from control-SHR and CR-SHR rats perfused *ex vivo* following five weeks of diet; *P<0.05 represents significant difference between Control-SHR and CR-SHR. At perfusion P=1.2650 and at reperfusion P=0.2299. Two-tailed unpaired Student's *t*-test was performed to detect differences.

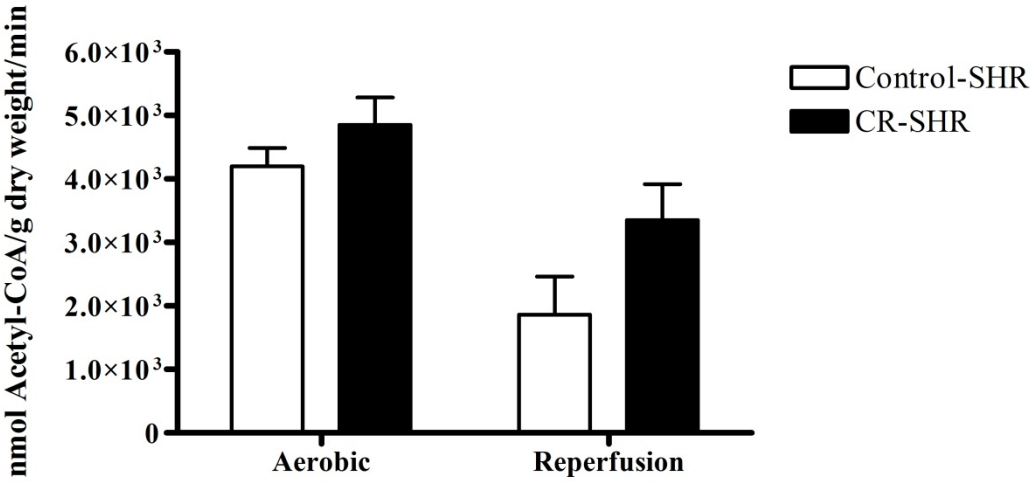
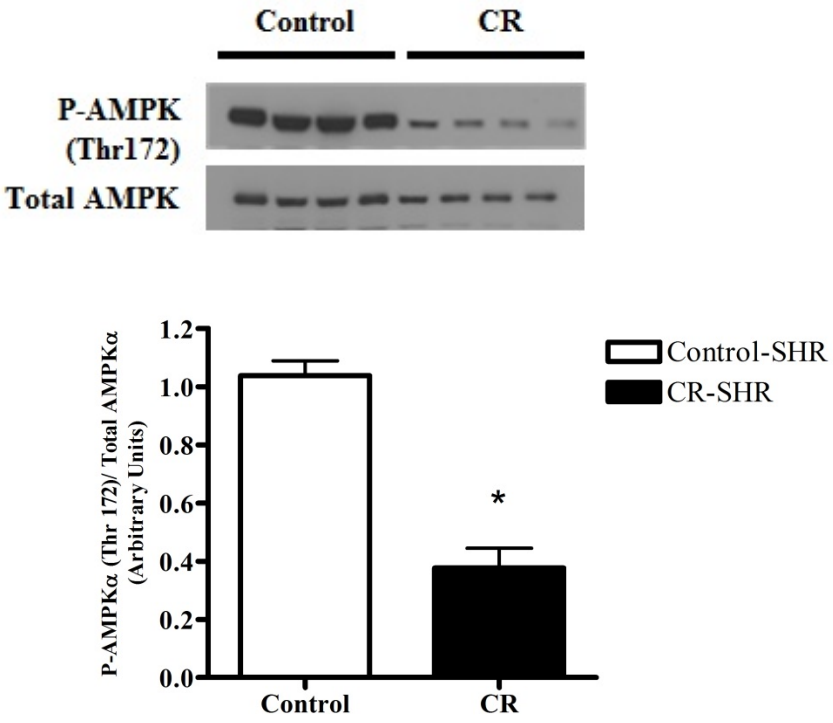


Figure 3.8 Energetic status of *ex vivo* perfused hearts from control- and CR-SHRs during aerobic reperfusion following ischemia. Immunoblot analysis performed on control-SHR and CR-SHR hearts (n=8/control-SHR group; n=8/CR-SHR group) at the end of reperfusion with anti-phosphorylated α-AMPK(Thr172) and anti-α-AMPK; Values are mean±SEM of hearts from control-SHR and CR-SHR rats perfused *ex vivo* following five weeks of diet; *P<0.0001 represents significant difference between Control-SHR and CR-SHR. Two-tailed unpaired Student's *t*-test was performed to detect differences.



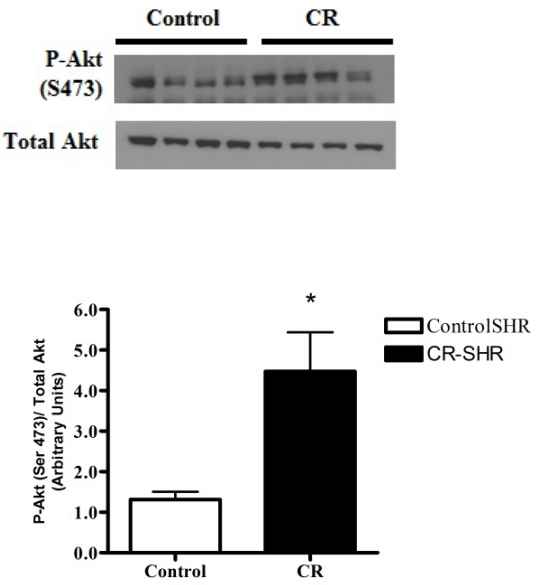
3.5 Elevated Myocardial RISK Signalling is Associated with Improved Post-ischemic Recovery in Hearts from CR-SHRs

Another mechanism that may contribute to the beneficial effects of short-term CR during reperfusion following ischemia is the activation of specific signalling pathways such as the Reperfusion Injury Salvage Kinase (RISK) signalling pathway that consists of kinases such as Akt and p44/42 MAPK (acronym Erk1/2 MAPK). Since activation of the Akt signalling pathway has been proposed to be essential in preconditioning-induced cardioprotection [339] and it was previously demonstrated that brief periods of CR increase phosphorylation of Akt in the heart [410], we investigated level of activation of Akt in the hearts from control-SHR and CR-SHR hearts during reperfusion following ischemia. Our data demonstrate that at reperfusion, the level of P-Akt was significantly increased in hearts from CR-SHRs compared to control-SHRs (Figure 3.9 A).

Additionally and consistent with the activation of the RISK pathway during reperfusion in classical preconditioning, we demonstrated a significant increase in the activity of p44/42 MAPK activity *via* its increase in the phosphorylation level at the end of reperfusion in the CR-SHR hearts compared to control hearts (Figure 3.9 B). Finally, as activation of p70S6K has been proposed as a point of convergence of Akt and p44/42 MAPK [411], we investigated the activity level of p70S6K by examining its phosphorylation at the phosphorylation sites Threonine 389 (T389) and Threonine T421/Serine 423 (T421/S423), respectively. We show that the phosphorylation status and thus activity of p70S6K at S389 and T421/S423, respectively, are significantly increased in the CR-SHR hearts at the end of reperfusion following ischemia compared to control hearts (Figure 3.10 A and B). Together, these data suggest that that the two members of the cardioprotective RISK signalling pathway (i.e. Akt and p44/42 MAPK) exert their effects on downstream kinases.

Figure 3.9 Calorie restriction increases activity of Akt and p44/42 MAPK of the pro-survival anti-apoptotic RISK pathway in CR-SHR heart during reperfusion flowing ischemia. Immunoblot analysis performed on heart homogenates from control-SHR (n=8/control-SHR group) and CR-SHR (n=8/CR-SHR group) (A, B). Levels of phosphorylated Akt and p44/42 MAPK (acronym Erk1/2 MAPK) were quantified by densitometry and normalized against total protein levels in hearts collected at end of reperfusion; *P=0.0062 and P=0.0710/0.0068 represent significant difference between Control-SHR and CR-SHR for Akt and p44/42 MAPK, respectively. Two-tailed unpaired Student's *t*-test was performed to detect differences in A and B.

A)



B)

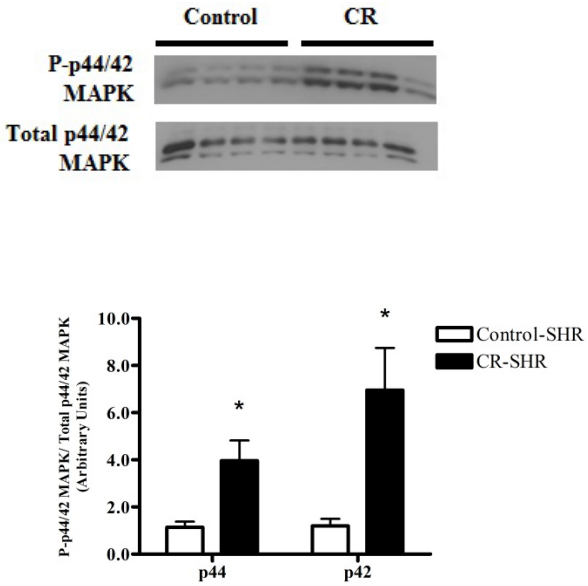
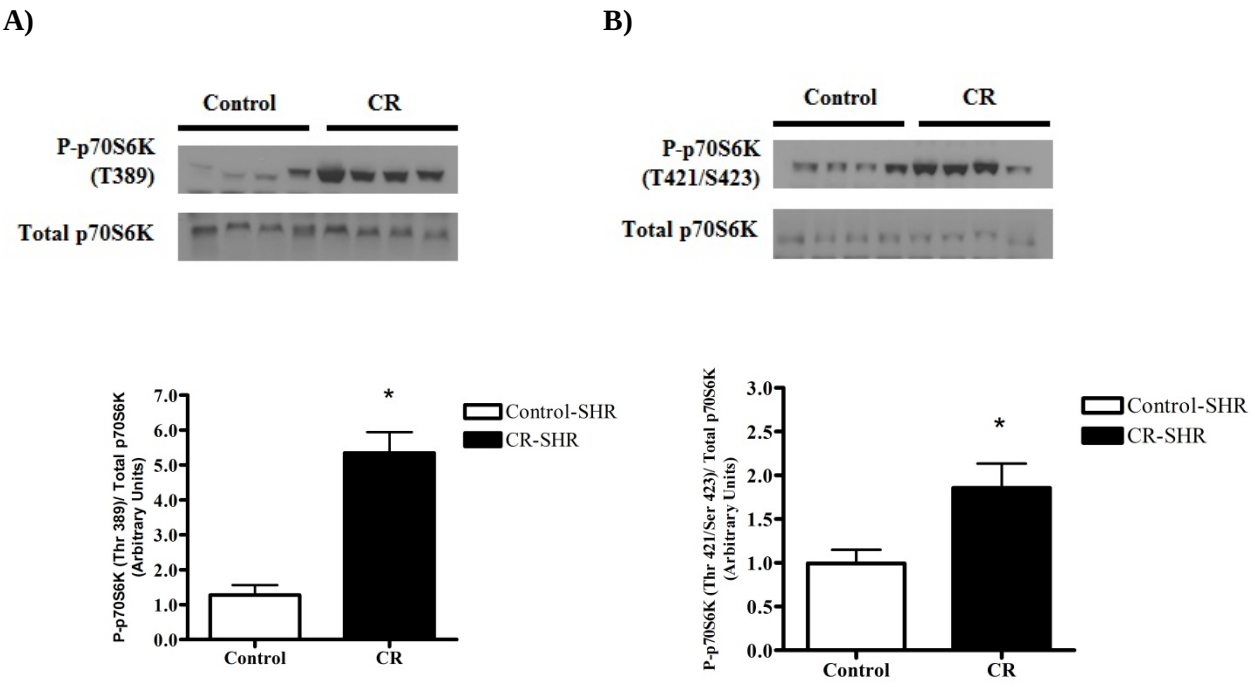


Figure 3.10 Calorie restriction increases activity of p70S6K(T389) and p70S6K(T421/S423) – a converge point of the pro-survival anti-apoptotic RISK pathway members, Akt and p44/42 MAPK, in CR-SHR heart during reperfusion following ischemia.
Immunoblot analysis performed on heart homogenates from control-SHR (n=8/control-SHR group) and CR-SHR (n=8/CR-SHR group) (A, B). Levels of phosphorylated p70S6K(T389) and p70S6K(T421/S423) were quantified by densitometry and normalized against total protein levels in hearts collected at end of reperfusion; *P<0.0001 and P=0.0165 represents significant difference between Control-SHR and CR-SHR for p70S6K (T389) and p70S6K (T421/S423), respectively. Two-tailed unpaired Student's *t*-test was performed to detect differences in A and B.



Chapter 4: DISCUSSION

4.1 Calorie Restriction as a Practical Method of Protecting the Heart in Cardiac Surgery

Ischemic heart disease (IHD) is associated with morbidity and mortality and is increasing globally [374]. At present, it is the leading cause of death in Western countries [374]. The most common cause of IHD is coronary heart disease (CHD) such as atherosclerosis of coronary artery, coronary embolus, or artery thrombosis [375, 376]. CHD accounts for more than 50% of cardiovascular events and according to the World Health Organization is the leading cause of death world-wide nova days. The coronary artery by-pass grafting (CABG) surgery is one of the most established procedures for the treatment of patients with severe CHD and remains the procedure of choice for coronary artery revascularization in larger number of these patients. One of the important causes of myocardial injury during cardiac surgery resulting from cross-clamping of the aorta associated with brief interruption of blood flow is acute ischemia-reperfusion (I/R) injury [412]. While reperfusion is necessary for tissue survival, it can also cause tissue damage contributing to the worse prognosis of post-operative recovery.

Furthermore, the profile of patients undergoing CABG surgery is changing due to factors such as aging population and increasing incident of age-related diseases. The increasing number of elderly patients with CHD is suffering from comorbidities such as hypertensions, hypertrophy, diabetes mellitus, etc. Furthermore, patients with poorer ventricular function and more diffusely spaced diseased vessels are coming forward for surgery [378]. Consequently, with increasingly higher-risk patients being operated upon and surgeons taking on more complex operations, CABG surgeries are increasingly associated with worse short-term and long-term clinical outcome and even with significant morbidity and mortality in this patient group [375, 376]. Accordingly, to improve clinical outcomes in these high-risk cardiac patients and allowing for extra time to perform technically complicated procedures, development of new interventions for an optimal protection of the heart during the cardiac surgery is required.

Indeed, “conditioning” of the heart to improve its resistance to an episode of acute myocardial ischemia/ reperfusion (I/R) injury by harnessing its endogenous cardioprotective capabilities using brief ischemia, pharmacological agents, or dietary modifications can be applied to the clinical setting of CABG. This would result in prolonging the window during which revascularization therapies can be effectively instituted by delaying myocardial necrosis, reduce myocardial injury, and preserve LV systolic function [249]. Short burst of ischemia followed by reperfusion according to animal models and human studies, protect the myocardium against subsequent longer ischemic insult (index ischemia) as evidenced by better cardiac function, reduction in infarct size, preservation of myocardial ATP [278], and reduction in the release of cardiac troponin T (a marker of apoptosis and necrosis as it is released from cardiac myocytes following myocardial damage and loss of plasma membrane integrity) [413]. Although research demonstrates that human myocardium is responsive to ischemic preconditioning, this does not imply that the clinical benefits will inevitably precipitate. While IPC potential clinical application is restricted to scenarios in which the index ischemic episode can be reliably predicted, such as for patients undergoing CABG surgery, and while repeated clamping of the aorta to render the heart ischemic may indeed result in protective preconditioning, it is unlikely that IPC possibly will be an acceptable method of myocardial protection to the majority of cardiac surgeons (Reviewed in [378]).

Many surgeons would be very hesitant to practice repeated aortic clamping especially in elderly patients with atherosclerothic disease in their aorta (Reviewed [285]). The repeated cross-clamping of the aorta could carry the possibility of the stroke by inducing distal embolization of atherosclerotic plaque debris. Additionally, surgeons are reluctant to apply an invasive preconditioning protocol that might prolong the duration of surgery (Reviewed in [285]). Finally, there is a lack of clarity on the frequency and number of preconditioning cycles required to elicit cardioprotection in humans [285]. Consequently, despite the clinical studies reporting beneficial effects with IPC in cardiac surgery, the routine application of IPC in cardiac surgery has not emerged. The problem of IPC could be avoided by a use of safer pharmacological agents that by targeting the molecular mechanisms of IPC without actually inducing

ischemia are capable of recapitulating protection elicited by IPC, thereby avoiding the use of an invasive myocardial preconditioning protocol.

Accordingly, from the therapeutic perspective, preconditioning with pharmacological agents would have been of a greater clinical interest. However, no pharmacological therapy is without interactions, side-effects, and drawbacks. Just like with any drug there are additional challenges with the pharmacological agents that induce cardioprotection from I/R injury. These include unwanted side-effects associated with the lack of drug specificity and selectivity, and the development of tolerance toward the cardioprotective agents following its prolonged use. Thus, while above conventional forms of IPC (ischemic and pharmacological preconditioning) are associated with drawbacks, other non-conventional methods [calorie restriction (CR) and exercise] have also shown to have beneficial effects in protecting the heart from I/R injury [414]. One such non-pharmacological and non-conventional approach that appears to improve ischemic tolerance in rodents is a brief reduction in calorie intake, a short-term calorie restriction [296-298]. Short-term CR is a preconditioning approach that would be much easier to maintain in the modern society than the long-term CR, and which can exert a significant cardioprotective effect with relatively short period of time – a two week regiment [296].

Additionally, while CR preconditioning shares common features with conventional forms of IPC, it also displays distinctive characteristics. Evidence supports that IPC interventions are affected by age, sex, disease, and interactions with pharmacological agents. It is plausible that some of these drawbacks of IPC could be ameliorated by implementation of CR as a cardioprotective regiment and perhaps even may confer additional benefits. Short-term CR, unlike IPC, is able to effectively restore failed cardioprotective response and preconditioning in aged myocardium [365, 415, 416]. Additionally, an experimental study exploring the impact of CR on the pathogenesis of decompensated pressure-overload hypertrophy in the rat model has demonstrated that CR reduced hypertension and cardiac hypertrophy, while improving diastolic filling parameters and cardiac index (relates heart performance to the individual

size by comparing cardiac output to body surface) indicating that CR intervention, unlike IPC, may protect diseased heart.

Based on the above reasoning and due to aging population and prevalence of the ischemic heart disease in this age group, and for the vast majority of the studies employing juvenile of adult animals devoid of underlying cardiovascular disease, and due to numerous drawbacks of IPC, we investigated the role of CR in protecting the diseased heart from I/R injury. Consequently, in this study we have investigated the role of energy sensing kinase –AMPK, myocardial energy metabolism, and kinases involved in the Reperfusion Injury Salvage Kinase (RISK) signal transduction pathway in the diseased hypertrophied heart utilizing animal model of hypertension and hypertrophy – the spontaneously hypertensive rat (SHR).

4.2 Short-term CR Improves Post-ischemic Functional Recovery in CR-SHRs

It has been suggested that CR is cardioprotective by provoking a mild stress response resulting in enhanced cellular defence [357, 379]. Indeed, it has been recently established outside [296, 298, 380] and in our laboratory [299, 301] that calorie-restriction is beneficial in healthy rodents by conferring protection against myocardial ischemia/reperfusion (I/R) injury. All of the above mentioned studies [296, 298, 301, 380] except for one from our laboratory, which utilized male Spontaneously Hypertensive Rats (SHRs) [299], concentrated on healthy rodents. Therefore, the aim of our study was to determine whether short-term CR could induce cardioprotection in the setting of high-risk/diseased heart. As a result, our study has utilized a diseased model of cardiac hypertension and hypertrophy, SHR, to mimic more real situations of diseased heart. Actually, SHR is the most commonly used model of cardiovascular disease often with the Wistar Kyoto rat (WKY) as the normotensive control [381]. SHRs are descendants of an outbred Wistar male with spontaneous hypertension from a colony in Kyoto, Japan, mating with a female with an elevated blood pressure, and then inner mating of the offspring with selection for spontaneous hypertension defined as a systolic blood pressure of over 150 mmHg persisting for more than one month [382]. The SHR provides a useful experimental model of pressure overload that develops hypertension gradually and then hypertrophy demonstrating many similarities to humans [381-383].

Furthermore, short-term CR is much easier to include into clinical practice than life-long CR. Consequently, we have studied cardioprotective effects of non-conventional form of cardiac preconditioning – the short-term CR. Short-term CR protocol consisted of feeding of control-SHRs *ad libitum* fed for five weeks with control diet −feeding CR-SHRs for three weeks at 90% of their average caloric intake (10% CR) followed by two weeks at 60% of the average caloric intake (40% CR) (Figure 3.1A). The CR diet was formulated to ensure that CR-SHRs received the equivalent amount of vitamins, minerals, and salt compare to the control-SHR. Present study has utilized the same SHRs as our previous study focusing on CR prevention of hypertension and cardiac hypertrophy in the SHR [299]. Thus, as previously reported by our laboratory, a five weeks of short-term calorie restriction significantly reduced

weight gain in the calorie-restricted group without impediment of growth [299]. To assess whether short-term CR improved post-ischemic functional recovery under the conditions of elevated levels of fatty acids normally observed during ischemia [98], control-SHR and CR-SHR hearts were subjected to *ex vivo* I/R using a Krebs-Henseleit solution containing 1.2 mM of palmitate.

We have observed that during the aerobic period prior to ischemia, percent cardiac functional parameters were similar between control-SHR and CR-SHR groups (Table 3.1, Figure 3.1B, C, and D). On the contrary, post-ischemic contractile function was dramatically improved in hearts from CR-SHRs compared to control-SHRs (Table 3.1, Figure 3.1B, C, and D). Hearts from CR-SHRs at reperfusion following ischemia demonstrated significant improvement in heart rate (HR), peak systolic pressure (PSP), left ventricular developed pressure (LVDP), cardiac output (CO), coronary flow (CF), and cardiac power (CP) (Table 3.1, Figure 3.1B, C, and D). The intriguing observation was that beneficial effects of short-term CR is maintained even in the presence of high concentrations of fatty acids that would normally be observed in the plasma of patients following ischemia [98]. The beneficial effects of short-term CR in the presence of high concentrations of fatty acids were also reported previously by our laboratory in healthy animals, the C58B16 mice [301] and SHRs [299]. Our latter study has reported that short-term CR could reduce hypertension and prevent cardiac hypertrophy inherent to the non-obese SHR. They have demonstrated that after five weeks of *ad libitum* feeding, SHRs subjected to short-term CR had lower systolic blood pressure (BP) and reduced left ventricular wall thickness [299].

Furthermore, it was also previously demonstrated that the moderate CR improved diastolic dysfunction in the Dahl-SS rat [367] – a salt animal model of hypertension and hypertrophy. The Dahl salt-sensitive rat which develops gradual hypertension-associated diastolic dysfunction [referring to the decline in performance of ventricle(s) during the time phase of diastole] was used to assess the impact of CR upon decompensated pressure-overload hypertrophy [367]. Compared to high-salt fed group, the high-salt CR group showed reduced blood pressure, lower cardiac weight, improved diastolic chamber function, and cardiac index, which relates cardiac output to body surface area, thus relating heart

performance to the size of the animal [367]. Although this salt model demonstrates that CR improves cardiac function, because it relies on the impairment of kidney capacity and salt loading to rapidly induce hypertension and hypertrophy it is not a very realistic model for many human hypertensive patients [381]. Furthermore, it was demonstrated IPC can induce myocardial protection in hypertrophied myocardium in the rat whose hypertrophy was induced by hypertension *via* administration of saline drinking fluids and subcutaneous deoxycortisosterone acetate for four weeks [417]. Most of the patient cases of hypertension and hypertrophy are not caused by hypersecretion of aldosterone, lack of second kidney, or a diet copious in salt intake [381]. In addition these models progress very rapidly to severe hypertension and hypertrophy − a process much more gradual in humans [381]. SHR utilized in our study is a realistic model of human hypertrophy. In addition, it represents stable disease, produces predictable and controllable symptoms, and allows for measurements of relevant functional, metabolic, and cell signalling parameters [381].

Briefly, hearts from CR-SHRs at reperfusion following ischemia demonstrated significant improvement in cardiac function including heart rate (HR), peak systolic pressure (PSP), left ventricular developed pressure (LVDP), cardiac output (CO), coronary flow (CF), and cardiac power (CP) even in the presence of high concentrations of fatty acids that would normally be observed in the plasma of patients following ischemia such as during cardiac surgery [98]. In adult patients undergoing cardiac surgery, plasma fatty acid levels are significantly elevated during cardiopulmonary bypass (CPB) surgery and at the time of reperfusion following of the release of the aortic cross clamp [98, 418]. This could brand a non-conventional precondition by short-term CR a feasible cardioprotective method utilized to protect against I/R injury during cardiac surgery.

4.3 Short-term CR Improves Post-ischemic Cardiac Metabolism and Cardiac Energy Status in CR-SHRs

Ischemia alters energy metabolism by disturbing the balance between fatty acid and glucose metabolism, which also negatively influences cardiac function and efficiency [23]. Indeed, it has been demonstrated that in ischemic cardiomyopathy there is a development of left ventricular (LV) dysfunction as a result of alterations in substrate metabolism [419]. Due to the rapid ischemia-induced increase in catecholamine release [262], there is a rise in circulating plasma free fatty acids associated with increased adipose tissue lipolysis [99] during and after ischemia [17, 98, 173]. This raise in plasma free fatty acids increases the delivery of fatty acids into cardiomyocytes [17]. The increased delivery of FAs to the myocardium in association with the alterations in the intracellular control of FA oxidation keeps FAs as a major fuel during post-ischemic reperfusion [17]. Indeed, it has been shown that the FA oxidation can be dramatically accelerated during reperfusion with over 95% of acetyl CoA-derived ATP originating from fatty acids [173]. Additionally, these high rates of fatty acid oxidation at reperfusion can dramatically inhibit glucose oxidation *via* the Randle cycle [263] – fatty acid-derived acetyl-CoA decrease the production of glucose-derived acetyl-CoA *via* inhibition of PDH complex resulting in intracellular acidosis at reperfusion. Furthermore, the component of ischemia/reperfusion (I/R) injury and subsequent ability to recover depends on the type of substrate being metabolized by the heart during and following ischemia [390, 420-423]. It has been demonstrated that improving the coupling of glucose metabolism by stimulating glucose oxidation accelerates the recovery of intracellular pH and improves both mechanical function and cardiac efficiency [423].

We have demonstrated that the short-term CR does not alter the myocardial metabolism at baseline in the hearts from CR-SHRs by measuring glucose (glycolysis, glucose oxidation) and fatty acid (fatty acid oxidation) in these hearts (Figure 3.2A, B, C). During the aerobic perfusion period prior to ischemia, there were no changes in rates of glycolysis, glucose oxidation, and fatty acid oxidation between control-SHR and CR-SHR groups (Figure 3.2A, B, C). This is contrary to what was observed in our previous study utilizing healthy mice [301]. They have reported that during the aerobic period prior

to ischemia, there was no changes in rates of glycolysis and palmitate oxidation between the control and CR-mice [301] – a finding consistent with our observation in diseased animal model. However, they have reported that glucose oxidation was increased nearly twofold in hearts from CR-mice compared to control-mice [301]. This is inconsistent with our finding where we have observed no difference in glucose oxidation at baseline during aerobic perfusion prior ischemia in the hearts from CR-SHRs subjected to short-term CR. Our findings pertaining to SHR on the contrary to prior findings pertaining to healthy mice demonstrate that prior ischemia the energy metabolism is not improved in the CR-SHR hearts relative to control-SHRs.

The reason for this discrepancy is not clear, but it could be species related or could be associated with the pathophysiology and pathobiology of the diseased hypertrophied heart. As cardiac myocyte size increase due to hypertrophy, the reduced myocardial perfusion results in a mild ischemia [424]. The adaptive response to this reduced oxygen availability in this chronic reduction in myocardial perfusion is a significant alteration in cardiac energy metabolism [424, 425] – a reduction in oxidative metabolism such as glucose and fatty aid oxidation and increase in glycolysis [395, 426-428] resulting in increased oxygen efficiency. Thus, although in the early stages of pressure or volume overload induced hypertrophy cardiac metabolism is dominated by fatty acid oxidation [395] due to decreased perfusion associated with increasing cardiac myocyte size, there is a switch from adult to fetal metabolism due to downregulation of expression of enzymes involved in fatty acid oxidation and upregulation of the expression of enzymes involved in glucose uptake and glycolysis [424, 429]. Perhaps this switch to fetal metabolism in the hypertrophied heart resulting in the up-regulation of the expression of enzymes involved in glucose uptake and glycolysis already improves the energetic status of the CR-SHR hearts making increase in glucose oxidation prior ischemia a redundant process.

Since we did not observe any significant changes in the myocardial metabolism at baseline during aerobic phase prior ischemia, we investigated whether alterations in myocardial energy metabolism at reperfusion following a period of ischemia may contribute to the improved functional recovery observed

in hearts from CR-SHRs. Consistent to the metabolic profile of hearts prior to ischemia, glycolysis and palmitate oxidation were not altered between control-SHR and CR-SHR groups (Figure 3.2A and C). However, the glucose oxidation was significantly increased in hearts from CR-SHRs compared to control-SHR (Figure 3.2B). Indeed this finding is consistent with our previous study that demonstrated improved cardiac metabolism due to enhanced glucose oxidation [301].

Furthermore, by calculating metabolically derived ATP production rates of *ex vivo* perfused hearts during a period of aerobic reperfusion following ischemia, we have demonstrated that alterations in myocardial energy status at reperfusion may contribute to improved functional recovery in hearts from CR-SHRs. We have shown that consistent with the metabolic profile of hearts prior and following ischemia, glycolysis and palmiate oxidation contribution to ATP production were not altered between control-SHR and CR-SHR groups (Figure 3.3 A and D). However, the glucose oxidation considerably contributed to the metabolically derived ATP production rates in hearts from CR-SHRs compared to control-SHRs (Figure 3.3 B). A larger contribution of the glucose metabolism to the total metabolically derived ATP production rate in the CR-SHRs relative to control-SHRs results in the improvement of cardiac efficiency (defined as cardiac work/oxygen consumed) [430].

The amount of ATP produced per molecule of oxygen differ depending on the type of energy substrate utilized (Reviewed in [80]). Although, oxidation of palmitate generates over three times as much energy, it utilizes more oxygen per equivalent amount of energy generated compared to glucose oxidation making palmitate a less efficient substrate for ATP synthesis (Review in [80]). Hence, an increased dependence on oxidation of palmitate relative to glucose as an oxidative fuel during reperfusion following ischemia decreases cardiac efficiency (Review in [80]). However, we have demonstrated that short-term CR in the CR-SHR improves function leading to improved cardiac efficiency by improving ATP production *via* stimulation of glucose oxidation. Indeed, as mentioned above, it has been demonstrated that improving the coupling of glucose metabolism by stimulating glucose oxidation improves both mechanical function and cardiac efficiency [430]. These findings are consistent with our

previous observations demonstrating that improved post-ischemic recovery in hearts from healthy CR-mice is associated with improved myocardial energetics during reperfusion [301].

To additionally demonstrate the improved metabolic status in the CR-SHR hearts, we have investigated activity of 5'-adenosine monophosphate-activated protein kinase (AMPK). AMPK is a key molecular regulator of energy metabolism [178] that mainly responds to metabolic stress that deplete cellular ATP, increase AMP, or increase the creatine/phosphocreatine (Cr/Pcr) ratio [56, 148, 181, 224] such as ischemia. To ameliorate ATP deficiencies [202], AMPK inhibits energy-consuming pathways such as protein synthesis [203-206] and activates energy-generating pathways such glucose up-take [53], up-regulates glycolysis [39, 78, 207-216] and β-oxidation of fatty acids [135, 142, 145, 202, 217-223, 233]. A controversy exists whether the activation of AMPK during energy demand is damaging or protective to the ischemic heart [146, 202, 207, 224, 431]. A number of studies demonstrate that the activation of AMPK attenuate myocardial I/R injury by improving myocardial ischemic tolerance, for instance, by maintain energy reserves [298] and prevention of post-ischemic cardiac dysfunction and apoptosis [202, 207, 224, 431]. However, AMPK-dependent acceleration of fatty acid oxidation during reperfusion following ischemia may be damaging to the heart in the setting of I/R [202]. As previously mentioned, although oxidation of fatty acids generates considerably more energy than the oxidation of carbohydrates, it utilizes more oxygen per equivalent amount of energy generated making fatty acids a less efficient substrate for ATP synthesis (Review in [80].

Hence, an increased dependence on oxidation of fatty acids relative to glucose as an oxidative fuel during reperfusion following ischemia decreases cardiac efficiency (Review in [80]. We have demonstrated that consistent with increased contribution of glucose oxidation to the metabolically derived ATP production in the hearts from CR-SHRs compare to control-SHRs (Figure 3.3 B) leading to the elevated ATP levels, AMPK activity was significantly reduced in hearts from CR-SHRs compared to controls (Figure 3.6). Reduction of activity of AMPK during reperfusion following ischemia in association to short-term CR suggests improved energy status in hearts from CR-SHRs compare to

controls, and thus improved cardiac efficiency associated with improved glucose oxidation brought by short-term CR. Indeed findings in this study are consistent with previous observations reported by our laboratory indicating that short-term CR improves cardiac function and metabolism in healthy mice independent of AMPK activation [301]. Interestingly, we have demonstrated that PDH is not inhibited in CR-SHR hearts relative to control-SHR hearts (Figure 3.4)

4.4 Short-term CR Improves Post-ischemic Recovery in CR-SHR Hearts *via* Activation of the Pro-survival Anti-apoptotic RISK Pathway

Signalling through phosphatidylinositol-3 kinase (PI_3K) has been demonstrated to confer protection against ischemia/reperfusion (I/R) injury [432, 433], through its activation of the serine-threonine kinase, Akt [434]. The Erk1/2 MAPK (acronym p42/44 MAPK) is a member of the mitogen-activated protein kinases (MAPKs) (a family of serine-threonine kinases regulating cell survival, proliferation, and differentiation), and when activated in the setting of I/R can mediate cellular protection [435, 436]. These pro-survival anti-apoptotic kinases constitute a Reperfusion Injury Salvage Kinase (RISK) pathway [318, 342]. Indeed, it has been demonstrated that the IPC results in phosphorylation of both Akt and Erk1/2 MAPK during the first few minutes of reperfusion [437]. Furthermore, PI_3K/Akt and MEK1/2/Erk1/2 MAPK signalling kinase cascades that are activated in response to I/R injury initiate myocardial protection through their anti-apoptotic actions *via* involvement in a numerous cellular processes through phosphorylation of diverse array of substrates. One of such substrates that these two pathways converge is p70S6 kinase (p70S6K) [411]. Activated p70S6K subsequently phosphorylates and inhibits glycogen synthase kinase -3β (GSK-3β kinase) involved in the pro-death cascade whose inhibition acts to prevent mitochondrial permeability transition pore (mPTP) opening [345-350]. Consequently the RISK pathway links closure of the mPTP to cardioprotection [346].

Many agents that initiate cardioprotection when given at the time of reperfusion following ischemia appear to activate either one or both of these pro-survival kinase cascades comprising the RISK pathway implicated in IPC [437]. In addition, it was reported that SHRs, a model of hypertrophied myocardium, exhibits increasing vulnerability to necrotic damage [438, 439]. Based on this, we have investigated whether short-term calorie restriction (CR) exerts its cardioprotection *via* induction of these two cascade components of the RISK pathway at reperfusion following ischemia in the model of cardiac hypertension and hypertrophy, the SHR. We have demonstrated that at reperfusion the activity if Akt was significantly increased in hearts from CR-SHRs compared to control-SHR (Figure 3.7A). We also demonstrated that the activity of another member of RISK pathways, p44/42 MAPK (acronym Erk1/2

MAPK)) significantly increase at the end of reperfusion in CR-SHR hearts (Figure 3.7B). Indeed, we have demonstrated similar findings in our previous study of short-term CR utilizing healthy mice [301]. Furthermore, our study has demonstrated that non-conventional cardiac pre-conditioning, the short-term CR, can exhibit cardioprotection like IPC *via* activation of the components of the RISK pathway in the rat species [303, 304].

4.5 Proposed Mechanism of Short-term CR Cardioprotection in CR-SHRs

Changes in myocardium metabolism, calcium handling, and inflammatory response accompanying the development of cardiac hypertrophy may account for the observed increases sensitivity of the hypertrophied myocardium to ischemia/reperfusion (I/R) injury [440]. Accordingly, with cardiac hypertrophy playing a critical role, it has been demonstrated that the hearts from SHR rats seems to be more sensitive to I/R injury as compared to aged-matched Wistar rats [441]. In our study we have demonstrated that pre-conditioning induced by short-term CR is preserved in the hypertrophied myocardium. This is in accordance with a number of previous studies utilizing classical IPC protocol and various experimental methods of induction of cardiac hypertrophy in animal models, which have also demonstrated pre-conditioning response is preserved in the hypertrophied heart. The cardioprotective effects of IPC was demonstrated in rats with abdominal/thoracic aortic banding-induced cardiac hypertrophy [442-444], in salt animal model of hypertrophy [417], in salt-sensitive rats [445], and in SHRs [446, 447]. Furthermore, we have demonstrated that short-term CR improves post-ischemic functional recovery and cardiac metabolism and cardiac energy status in CR-SHRs. Consistent with our findings, hearts exposed to IPC have a better metabolic metabolism and ionic homeostasis during ischemia [448]. In addition, it has been demonstrated that IPC protects by reducing ATP consumption during myocardial ischemia [311, 326, 327] and preserving mitochondrial energy production during myocardial ischemia and at reperfusion [327-330]. However, improvement of the metabolic status of the cardiomyocytes is not thought to be the main mechanism by which IPC is cardioprotective, but rather the signalling pathways that are activated by IPC that distinguish IPC hearts from the not pre-conditioned hearts [448].

Previous experimental studies have identified the IP_3K/Akt and MEK1/2/Erk1/2 MAPK components of the RISK pathway to be a major transduction pathway in mediating cardioprotection due to IPC at the time of myocardial reperfusion following ischemia [449]. It is recruited by ischemic preconditioning [450, 451] and can be stimulated by a variety of cardioprotective pharmacological agents

[333, 437]. The actual mechanism through which the activation of PI_3K/Akt and MEK1/2/Erk1/2 MAPK (acronym p44/42 MAPK) components of the Reperfusion Injury Salvage Kinase (RISK) pathway mediating IPC induced cardioprotection have not been yet fully elucidated. However, it has been demonstrated that an important downstream target of both Akt and Erk1/2 MAPK is GSK-3β [334-337, 346]. Collectively above studies have established that phosphorylation and subsequent inhibition of constitutively active GSK-3β activity at the time of reperfusion leads to the inhibition of the mPTP opening resulting in IPC induces cardioprotection. The mechanism through which GSK-3β inhibition actually mediates mPTP inhibition is however unclear. We have also established that the short-term CR improves post-ischemic recovery in CR-SHR hearts *via* activation of the pro-survival anti-apoptotic RISK pathway. The question is how the activation of the member cascades of the RISK pathway, the IP_3K/Akt and MEK1/2/Erk1/2 MAPK, is cardioprotective in the hypertrophied rat heart. Due to large similarities in cardioprotection mechanisms between IPC and short-term CR and particularly findings that in the adult rat cardiac myocytes the pharmacological inhibition of PI_3K or Akt abrogates the inhibitory effect on mPTP mediated by insulin [346], we propose that the preconditioning associated with short-term CR may involve Akt and Erk1/2 MAPK indirect inhibition of the mitochondrial permeability transition pore(mPTP) *via* phosphorylation and inhibition of constitutively active GSK-3β.

Another question is how the activation of the member cascades of the RISK pathway, the IP_3K/Akt and MEK1/2/Erk1/2 MAPK, improve cardiac metabolism and energetic status in the hypertrophied rat heart? It has been proposed that the regulation of glycolysis in setting of IPC may involve translocation of hexokinase II (HKII), an enzyme responsible for catalyzing the first step of glycolysis, to mitochondria explaining many of the phenomena described for IPC [452]. Association of HK with mitochondria is kinetically advantageous as it increases affinity of HK for substrate ATP, decreases its sensitivity to inhibition by its product (glucose-6-phosphate), and brings glycolysis under the control of oxidative phosphorylation [453]. In addition to up-regulation of GLUT 4 transporters at the cardiomyocyte sarcolemma, this may explain often observed increase in glucose oxidation in the hearts at

the time of reperfusion [454]. Resulting coupling of anaerobic glycolysis with oxidative phosphorylation (aerobic glucose oxidation) may lead to a decrease in production of glycolytic protons and subsequent attenuation of intracellular acidosis associated with ischemia [394].

Furthermore, it has been experimentally demonstrated that IPC and its mimetics (agents that mimic IPC) such as resveratrol significantly enhance phosphorylation of Akt upon reperfusion [339, 344, 455], induce mitochondrial translocation of HK [452], and inhibit opening of mPTPs upon reperfusion [348]. In addition, it has been demonstrated that Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of translocated to mitochondria HKII [456]. Due to similarities between the conventional IPC and non-conventional short-term CR mechanisms of cardioprotection, we propose that short-term CR may improves cardiac metabolic and energetic status in the hypertrophied heart *via* Akt mediated translocation of HKII to mitochondria and subsequent improvement in coupling of glycolysis with oxidative phosphorylation. Although this notion has to be investigated further. Collectively, above findings suggests that Akt contributes to cardioprotection *via* inhibition of mPTP through GSK-3β mediate mechanism and improved cardiac metabolism *via* Akt-associational translocation of HKII to mitochondria and subsequent improvement in coupling of glycolysis with oxidative phosphorylation.

Chapter 5: JUSTIFICATION OF METHODOLOGY AND EXPERIMENTAL LIMITATIONS

5.1 Spontaneously Hypertensive Rat

Rats as animal models have many advantages. They are economical and many techniques have been established to measure functional, metabolic, and biochemical parameters. The normal life span in rat is relatively short − two and a half to three years and one and a half to two and a half years in normotensive and SHRs, respectively, allowing easy monitoring of the disease progression [457]. Moreover, human hypertension is usually a slowly-developing disorder of middle to old age, which predisposes to cardiovascular diseases in the aged population [458] and the incidence of hypertension significantly varies by gender and race [459]. Hypertension is the most common cause of development of cardiac hypertrophy, as heart responds to pressure overload by hypertrophy in the form of myocardial wall thickening and a reduced ventricular cavity. As hypertrophy in humans is usually associated with chronic hypertension, the SHR is a realistic model of human hypertrophy. Another advantage of the SHR is that it follows the same progression of hypertension as human hypertension with pre-hypertensive, developing, and sustained hypertensive phases with each phase lasting several weeks [460].

The disadvantage is that in our study we have implemented short-term CR protocol when SHRs were ten to fifteen weeks old representative of young SHRs. Unfortunately, cardiovascular disease is uncommon in young humans but significantly increases with age [461]. For that reason in the future study we would like to utilize much older rats reaching mid-adulthood, fifty two to sixty four weeks [381], as these animals would be more representative of the ageing human population inflicted with age-related disease(s). Moreover, the SHR model is also suitable for gender study. Gender differences in have been observed in human [462, 463], as well in some animal studies [464-468] such as rat [465, 466]. Gender differences in cardioprotection against ischemia/reperfusion (I/R) injury have been described in adult rat hearts [465]. An experimental study utilizing a global ischemia model in a perfused rat heart reported improved smaller infarcts and improved post-ischemic recovery in female hearts compare to male heart. We utilized male SHRs for our study, as male SHRs are commonly used as a model of

established human hypertension [381] and we have not considered gender-differences. However, due to the described above sex difference observed in humans and rat animal models, we may consider to investigate the effects of short-term CR in the female SHRs that may lead to improved post-ischemic recovery in female hearts compare to male hearts.

5.2 Isolated Working Heart Perfusions

The isolated working small mammalian heart is a widely used experimental model. It is practical, relatively simple and quick, and of fairly low cost. Also despite these practical and economic advantages it is a very powerful tool in the study of cardiac energy metabolism and its relation to cardiac function, and ischemia and reperfusion induced injury. In addition, the experimental data is highly reproducible and measurements can be made in the absence of the neurohormonal influence of other organs on the heart. Although, this feature of the isolated working heart model may also act as a disadvantage, as the collected data may not reflect the realistic situation *in vivo*. However, this deficit can be compensated for by including exogenous peripheral factors such as catecholamine or other neurotransmitters in the perfusate buffer. Furthermore, cardiac energy metabolism can be measured under relevant physiological and pathologically applicable preload and afterload conditions in the presence of varying concentrations of energy substrates, such as glucose and fatty acids [381]. Also, isolated heart perfusion model is a powerful tool for assessing many aspects of ischemia and reperfusion induced injury. In ischemia/reperfusion (I/R) studies, the preparation allows for the induction of various levels of global or regional ischemia and various degrees of oxygen deprivation can be also studied.

It has been recognized that as an *ex vivo* preparation, the isolated heart is constantly deteriorating preparation, nevertheless it is capable of study for several hours. The limitations with our isolated working heart perfusion model was that the isolated SHR hearts had a higher rates of deterioration and thus did not allowed us to collect data for longer periods at the time of reperfusion following ischemia. Another limitation of our isolated working heart perfusion model was the use of cystalloid buffer in lieu of blood to perfuse the hearts. While this buffer is prepared to closely mimic ionic constitution and pH of

the blood, unfortunately it lacks other vital components of the blood such as haemoglobin, white blood cells (WBCs), neurotransmitters, and hormones. This is of importance as the large part of the myocardial I/R injury comprises of the inflammatory response [469, 470]; and lack of these WBCs may not realistically portray the injury elicited by ischemia and reperfusion. As this component of the I/R injury being missing the degree of injury may be underestimated. Finally, advantage of the isolated hearts is that following perfusions the hearts can be further utilized for investigation of the transduction signalling pathways employing SDS-page gel electrophoresis and Western blotting.

5.3 *Ex vivo* Measure of Energy Metabolism

The use of inexpensive radiolabeled ^{3}H and ^{14}C substrates in *ex vivo* perfused hearts permits for direct steady-state assessment of fluxes though various metabolic pathways *via* measurement of metabolic products such as H_2O or CO_2 production rate. *Ex vivo* measurement of energy metabolism, because it focuses on the measure of the products of the specific pathways directly allows for an easy quantification of these metabolic parameters [471, 472]. One limitation of using *ex vivo* hearts to measure energy metabolism is that the substrates used in the perfusate do not accurately reflect situation *in vivo* [381]. Used substrates in the perfusate such as glucose and more representative fatty acids such as palmitate or oleate is not reflective of the complete substrate composition of blood at given time, as the blood may contain other carbon substrates that are not accounted for in the prefusate, as well the different substrate ratio in the blood stream during the course of the day may change.

Another limitation of *ex vivo* measure of energy metabolism is that use of certain ^{14}C-radioisotopes may not accurately reflect metabolism though specific metabolic pathway [472]. For instance, $^{14}CO_2$ production from ^{14}C glucose not only originate from oxidative phosphorylation of glucose, but it also originate *via* a pass though the pentose phosphate shunt – advantageously not vary active in the heart [472]. An incomplete metabolism of the ^{14}C-labeleld carbon substrate also poses a limitation resulting in erroneous values [472]. Finally, disadvantage of *ex vivo* measurements of energy

metabolism is the lack of effects on energy metabolism exerted by neurotransmitters and hormones due to their absence in the perfusate buffer [472].

5.4 Limitations of the Study

Our findings using the SHR validate that short-term CR exhibits cardioprotection in the diseased rat model of hypertension and cardiac hypertrophy. Thus, based on our data utilizing *ex vivo* perfused hearts of short-term CR- and control-SHR we hypothesized a use of short-term CR as a novel therapeutic approach that can be used to prevent ischemia/reperfusion (I/R) injury in the setting of cardiac surgery such as coronary artery by-pass grafting (CABG) surgery. However, the intent of this study was to first demonstrate that short-term CR could prevent I/R injury and thus we did not use a model that represents CABG surgery but instead represented acute myocardial infarction (MI) in high-risk cardiac patients.

We suggested the use of short-term CR in the setting of CABG surgery due to its predictability –the time of cardiac surgery is known and can be prepared for by placing the patient on short-term CR. Conversely, it is impossible to predict when the high-risk cardiac patient will undergo acute MI. As such, while our findings may be more applicable to protective strategies to lessen ischemic damage during CABG surgery, our model does not test this. One limitation of our model for that precludes us from extrapolating our findings to CABG surgery is the difference in the cardiac metabolic status of a model of CABG surgery and the *ex vivo* perfused SHR hearts. For example, the metabolism of our *ex vivo* perfused isolated working SHR heart may not be representative of the depressed metabolism exhibited by human heart during CABG surgery rendering our data less applicable for the justification of the use of the short-term CR in the protection against reperfusion injury during the CABG surgery. In the *ex vivo* isolated working heart perfusions there is no alteration in the cardiac metabolism due to change in the normal contractile function of the heart. During *ex vivo* heart perfusion the isolated hearts are fibrillating during the period of ischemia and actively pumping warm crystalloid solution at 37^{o}C during reperfusion [388, 472]. Quite the opposite, during CABG surgery heart's metabolism is lowered to allow the surgeon

to work in a motionless and bloodless field. The heart is arrested by bringing on asystole *via* infusion of usually cold cardioplegic solution into the coronary circulation [473].

Additionally, during CABG surgery, cardiac metabolism is further lowered on by-pass machine/ heart-lung machine by rendering heart ischemic [474]. The by-pass machine, which takes over the function of the heart (circulation of the blood) and the lungs (exchange of oxygen and carbon dioxide) isolates the heart from the rest of the blood circulation through required cross-clamping of ascending aorta and pulmonary artery [474]. Thus, the heart on by-pass machine is ischemic as it is not receiving blood and thus oxygen for metabolism [474]. In addition to lowering cardiac metabolism during CABG surgery *via* cardioplegia and unavoidable and unfavourable ischemia, the cardiac metabolism is further lowered through deliberate introduction of cardiac hypothermia. Immersion of the heart in the crushed ice lowers the heart's temperature further depressing its metabolism and sparing intracellular energy stores [475]. The relative difference in the contractile function and thus cardiac metabolic status during *ex vivo* isolated working SHR heart perfusion and during CABG surgery may question the appropriateness of our data to justify the proposed novel approach of use of the short-term CR in the protection against myocardial reperfusion injury during the CABG surgery.

Recruitment of RISK pathway at the time of reperfusion proves to be a target for the cardioprotection against reperfusion injury, and we have demonstrated that just like in ischemic preconditioning (IPC) or remote ischemic preconditioning RISK pathway is a target of short-term CR in healthy [301] and diseased (present study) rodents. However, volatile anaesthetics extensively used in cardiac surgery are also cardioprotective against myocardial reperfusion injury [475, 476]. Like IPC or short-term CR preconditioning, volatile anaesthetic preconditioning exhibits similar mechanism of cardioprotection against myocardial reperfusion injury *via* stimulation of signalling pathways that converge on protection of the mitochondria by controlling influx of calcium resulting in necrosis and apoptosis [476]. Since ultimately both short-term CR preconditioning and volatile anaesthetic preconditioning protect the myocardium from the reperfusion injury their function may be redundant and

the implementation of the short-term CR may not carry on additional protection. Indeed, a recent study demonstrated that remote IPC applied in conjunction with volatile anaesthetic displayed no additional benefit to cardiac patients undergoing CABG surgery [477]. Specifically, a remote preconditioning consisting of five minutes cycles of 300 mmHg cuff inflation/deflation of the leg before aortic cross-clamping during inhalation of isoflurane did not provide further protection to the myocardium [477]. Accordingly, since volatile anaesthetic preconditioning appears to be sufficient in cardioprotection against myocardial reperfusion injury, just like remote ischemic preconditioning short-term CR may not contribute additional protection to myocardium at reperfusion during CABG surgery.

The major limitation to the clinical use of the short-term CR is that the intervention must be performed prior to the onset of the clinical symptoms of acute MI. This restricts short-term CR use to the predictable settings of ischemia such as cardiac surgery [478]. Due to acute MI unpredictability only way to implement CR to improve post- acute MI recovery would be to subject at risk patients to a long-term CR – a very unrealistic task. However, long-term use of CR mimetics would be a more suitable approach. One of such agent that mimics short-term CR is resveratrol (3,4',5-trihydroxystilbene) [479]. This natural compound has not only been shown to protect the heart from ischemia/reperfusion injury [480] but it also has been demonstrated to mimic short-term CR in neonatal rat cardiac myocytes [481] making it a likely CR mimetic substitute in lieu of short-term CR regiment to improve clinical outcomes of acute myocardial infarction. The further benefits of supplementation with resveratrol prior to acute MI or even generally are: delivery of additional antioxidant cardiac and whole body protection and the lack of the side effects frequently associated with the pharmacological agents. Most of the studies pertaining to resveratrol toxicity demonstrated lack of the adverse effects unless unrealistically high doses of resveratrol were administered [482]. No toxicity was observed in rats fed 300 mg of resveratrol per kg per day for four weeks − a dose comparable to 21 g of resveratrol consumed by an average 70 kg adult male [482]. When unrealistically high doses of resveratrol equivalent to 3000 mg of resveratrol per kilogram body weight per day were consumed renal toxicity was observed in the experimental rat population [482]. Overall, to date no adverse effects were observed in humans upon administration of

high doses of resveratrol [483, 484]. Collectively, above findings establish resveratrol as an excellent short-term CR mimetic suitable for use in the medical setting to improve the clinical outcomes of acute MI if the implementation of the short-term CR proves to be difficult.

Chapter 6: FUTURE DIRECTIONS

The phosphatidylinositol-3 kinase (PI_3K)/Akt and MEK1,2/Erk1,2 MAPK (acronym p44/42 MAPK) signalling cascades of the Reperfusion Injury Salvage Kinase (RISK) pathway participate in numerous cardioprotective cellular processes at the time of reperfusion in IPC *via* phosphorylation of a diverse array of substrates including GSK-3β, apoptotic proteins (BAD, BAX, BIM, p53), GLUT-4 vesicles involved in glucose metabolism, transcription factors (IKK-α), p70S6K, eNOS, and PKC [411]. Since our study examining the effects of short-term CR on the improvement of cardiac recovery at reperfusion post-ischemia in CR-SHRs culminated on investigation of activity of one of the anti-apoptotic substrates of IP_3K/Akt and MEK1/2/Erk1/2 MAPK cascades, p70S6K, our future direction would be to investigate activity of other possible pro-apoptotic substrates of these signalling cascades, such as BAD, BAX, BIM, and p53. Indeed, it has been previously demonstrated that activation of these signalling cascades results in the phosphorylation of BAD [485] leading to the prevention of apoptosis. Furthermore, it was demonstrated that the activation of either the PI_3K/Akt or the MEK1/2/Erk1/2 MAPK signalling cascade prevents apoptosis *via* inhibition of the translocation of BAX to mitochondria [486, 487], inhibition of expression of protein BIM [488, 489], or prevention of degradation of p53 [490]. As our future goal, we would like to investigate if these pro-apoptotic substrates of the PI_3K/Akt and MEK1/2/Erk1/2 MAPK signalling cascades play a role in the cardioprotection elicited by short-term CR at the time of the reperfusion in the rat model of cardiac hypertension and hypertrophy, SHR.

Another conversion point of multiple cardioprotective pathways in IPC including the pro-survival anti-apoptotic PI_3K/Akt and MEK1/2/Erk1/2 MAPK signalling cascades during the time of reperfusion following ischemia is GSK-3β. This constitutively active Ser/Ther kinase has a major role in the cardiovascular system in glucose metabolism (enzyme involved in conversion of glucose to glycogen) [491], cardiac hypertrophy [492], and also plays important roles in cardiomyocytes apoptosis and necrosis. GSK-3β activity promotes mitochondrial permeability transition pore (mPTP) opening, and thus promote cellular death in response to reactive oxygen species (ROS) and/or Ca^{2+} overload [337,

493]. Inactivation of GSK-3β by phosphorylation at Ser9 is a common mechanism of protection of cardiac myocytes against necrosis in many cardioprotective interventions [334, 335, 494, 495]. Indeed, a number of model-dependent studies have reported the importance of P-GSK-3β in protection from lethal ischemia/reperfusion (I/R) injury upon reperfusion [335, 336, 493, 494, 496-499]. Moreover, since most of the earlier studies on mPTP regulation *via* GSK-3β inhibition utilize healthy animals, their findings may not be applicable to pathological cases. Ischemic heart disease develops as a consequence of number of etiological risk factors and always co-exists with other disease states, for instance, such as systemic arterial hypertension and related left ventricular hypertrophy [500]. For instance, it has been demonstrated that the activity of GSK-3β is up-regulated increasing vulnerability to cell death in the hypertrophied myocardium [501]; and in these circumstances perhaps a larger stimulus would be needed to protect the heart against I/R injury.

The observations from animal models with underlying pathological condition suggest that an agent proven to be protective in healthy young animals is not necessarily effective in aging patients with underlying age-related diseases. In aging diseased population, there is always a possibility that the cardioprotective signalling pathways may become dysfunctional in the setting of the disease rendering cardioprotective agents ineffective or requiring administration of higher doses of the agent. Additionally, it has been reported that the ischemic pre-conditioning (IPC) is impaired in the aged hypertensive and hypertrophied rats involving effects of both hypertension and/or hypertrophy and age [502, 503]. Accordingly, we would like to investigate the effects of short-term CR on the GSK-3β activity, and thus its indirect effects on the mitochondrial permeability transition pore (mPTP) opening in the diseased model of cardiac hypertension and hypertrophy, SHR. Furthermore, since it has been suggested that IPC improves cardiac metabolism *via* Akt associational translocation of HKII to mitochondria [452] and subsequent improvement in coupling of glycolysis with oxidative phosphorylation, we would like to investigate whether activation of RISK pathway at time of reperfusion observed in the setting of short-term CR results in the translocation of HKII to mitochondria.

In our study we have implemented short-term calorie restriction (CR) protocol when SHRs were ten to fifteen weeks old representative of young SHRs. Unfortunately, cardiovascular disease is uncommon in young humans but significantly increases with age [461]. For that reason in the future study we would like to utilize much older rats reaching mid adulthood, fifty two to sixty four weeks [381], as these animals would be more representative of the ageing human population inflicted with age-related disease(s). Moreover, it has been reported that in many animal models of hypertrophy, females have improved survival and/or improved contractile function [464, 504, 505]. Gender differences in cardioprotection have been described in some animal models. Compared to males, premenopausal females have reduced ischemia/reperfusion (I/R) injury [465, 466]. Comparably, the animal gender-based differences are comparable to the human's gender-based differences. According to epidemiological studies, females have reduced risk for cardiovascular disease [462, 463]. Compared to age-matched males, pre-menopausal women have a lower incidence of left ventricular hypertrophy, cardiac remodelling following myocardial infarction, and coronary artery disease [506]. Also, a number of studies that have examined sex differences in morbidity and mortality outcomes following coronary revascularization, such as coronary bypass graft surgery, [507-512] have reported significantly higher mortality in females than males [512]. Our study investigating cardioprotection imposed *via* short-term CR have utilized male SHRs and taking into consideration above findings we would like to investigate the effects of short-term CR on the female SHRs. This culminating study would allow us further establish any gender-based differences in cardioprotection associated with short-term CR.

CONCLUSION

Overall, this thesis has provided important understanding how alterations in energy metabolism and the activity of Reperfusion Injury Salvage Kinase (RISK) pathway contribute to the cardioprotective effects of non-conventional pre-conditioning, the short-term calorie restriction (CR) against ischemia/reperfusion (I/R) injury in the diseased animal model of hypertension and cardiac hypertrophy, the Spontaneously Hypertensive Rat (SHR). Findings presented in thesis validates that short-term CR indeed exhibits cardioprotective effects in the diseased animal model of hypertension and cardiac hypertrophy. Moreover, obtain experimental data demonstrate that alteration in glucose metabolism including improved glucose oxidation, and the activation of the two members of the pro-survival anti-apoptotic RISK pathway elicited by this non-conventional methodology may contribute to mechanical recovery of the already diseased heart and result in cardioprotection at the time of reperfusion following ischemia. This thesis further establishes that the effects of short-term CR in I/R injury may be mediated by an AMPK-independent mechanism by demonstrating the at the time of reperfusion flowing ischemia SHR isolated working hearts exhibited improved metabolic status in presence of significantly reduced AMPK activity. In addition, this investigation recommends utilization of short-term CR as a novel therapeutic approach in prevention of the ischemia/reperfusion (I/R) injury in the setting of cardiac surgery due to its effectiveness, practicality, inexpensiveness, and absence of side effects associated with cardioprotective pharmacological agents.

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