University of Alberta

Cardioprotection by Drug-Induced Changes in Glucose and Glycogen Metabolism

by

Mohamed Abdalla Omar

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Pharmacology

Spring 2012
Edmonton, Alberta

Dedications

This thesis is dedicated to my wife, Aliaa Elkenany, and my parents, Dr. Abdalla Omar and Dr. Nabila Boraie.

Abstract

Myocardial energy substrate metabolism is subjected to significant changes during ischemia and reperfusion (I/R), which can greatly influence post-ischemic recovery of left-ventricular (LV) mechanical function. One of the main mechanisms that contribute to I/R injury is the accumulation of protons and resultant Ca^{2+} overload, which occurs mainly as a result of uncoupling of glycolysis and glucose oxidation. Pharmacological interventions that improve the coupling between glycolysis and glucose oxidation are of particular interest. In this thesis, we focus on the role of partitioning of glucose between different metabolic pathways on post-ischemic recovery of LV mechanical function. We examine different approaches to limit excessive glycolysis during reperfusion and identify various targets in the glycolytic pathway that can be modulated to protect against myocardial I/R injury.

Utilizing the isolated perfused working rat heart model, we demonstrate that pre-ischemic glycogen content is an important determinant factor controlling ischemia-induced changes in glucose metabolism. Our results challenge current dogma by demonstrating that ischemia-induced activation of 5'AMP-activated protein kinase (AMPK) results in accelerated glucose uptake only under conditions where pre-ischemic glycogen is depleted while in hearts with replenished pre-ischemic glycogen, ischemia-induced activation of glycogenolysis supplies sufficient substrate for glycolysis without the need to stimulate glucose

uptake. Furthermore, we show that inhibition of glycogen synthase kinase-3 (GSK-3) repartitions glucose-6-phosphate away from glycolysis as a result of stimulation of glycogen synthesis. This limits proton production from glucose metabolism and subsequently reduces intracellular Ca^{2+} overload, which limits LV mechanical dysfunction in early reperfusion. These results provide a novel mechanism to explain cardioprotection mediated by GSK-3 inhibition. In the final study in this thesis, we demonstrate that adenosine-induced cardioprotection is mediated by activation of Ser/Thr protein phosphatase activity that reduces the phosphorylation of AMPK, which subsequently inhibits phosphofructokinase-2 and slows glycolysis and proton production during reperfusion. Our results challenge the current paradigm by demonstrating the benefits of limiting excessive AMPK activation during reperfusion.

Overall, this thesis highlights the importance of glucose and glycogen metabolism in the pathogenesis of I/R injury and presents a number of approaches to manipulate the balance between glycolysis and glucose oxidation and thereby limit myocardial I/R injury.

Acknowledgments

First and foremost, I would like to thank Dr. Alexander Clanachan for allowing me to pursue my graduate studies in his laboratory. His excellent mentorship always gave me confidence that I can fulfill my goals. His door was always open for me to discuss new findings, chat about recent papers, exchange ideas, and for general advice.

I would also like to thank the members of my supervisory committee, Dr. Peter Light and Dr. Jason Dyck for their positive contributions, guidance, and encouragement during my studies.

Thanks to my friend and colleague Dr. Manoj Gandhi for teaching me how to master the isolated perfused heart model and for showing me the importance of being meticulous in doing my experiments.

Thanks to all the current and past members of the Clanachan laboratory. Thanks to Dr. Jay Jaswal for always sharing his experiences with me. Thanks to Dr. Lianguo Wang for helping me with the Ca^{2+} measurement studies. Thanks to Waleed Masoud for always encouraging me.

Thanks to the late Dr. Susan Dunn for her great advice and support at a time when I really needed help

Thanks to my wife, Aliaa Elkenany, for her love and support, for always believing in me, for her patience in listening to my complaints about experiments that do not work, and for her tolerance when I had to work late nights and weekends to finish my work. Thanks to my daughter, Jana Omar, and my son, Hussein Omar, for all the joy they bring to my heart and for giving me a reason to work harder so that I can spend more time with them.

Thanks to my father, Dr. Abdalla Omar, and my mother, Dr. Nabila Boraie, for always supporting me by all means, for always believing in me, and for raising me up to be the person I am today. Thanks to my sister, Noha Omar, for her encouragement and advice.

Thanks to all my friends that I met here in Edmonton as well as my friends back in Alexandria, Egypt for always supporting me and for shaping me into the person I am now.

Thanks for the funding agencies that provided financial support so that I can finish my studies: Canadian Institutes of Health Research, Killam Trusts,

Faculty of Graduate Studies and Research, and the Faculty of Medicine and Dentistry.

Thanks to everyone in the Department of Pharmacology as well as in the Cardiovascular Research Group, now renamed the Cardiovascular Research Centre. Thanks to all the administrative staff in the Department of Pharmacology for always answering my questions and solving my problems.

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^{18}FDG	^{18}F-deoxyglucose
5'NT	5'-nucleotidases
ACC	acetyl CoA carboxylase
ADO	adenosine
ADP	adenosine diphosphate
AF	aortic flow
AICAR	5-aminoimidazole-4-carboxamide-ribonucleoside
AMP	adenosine monophosphate
AMPK	5'-AMP activated protein kinase
AMPKK	5'-AMP activated protein kinase kinase
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
AR	aldose reductase
AS160	Akt substrate of 160 kDa
ATP	adenosine triphosphate
BMK	Big mitogen-activated protein kinase
CaMKKβ	Ca^{2+}/calmodulin-dependent kinase kinase β
CF	coronary flow
CO	cardiac output
CoA	Coenzyme A
CPT-I	carnitine palmitoyl transferase-I
CPT-II	carnitine palmitoyl transferase-II
Cr	creatine
CypD	cyclophillin-D
DHAP	dihydroxy acetone phosphate
ERK	extracellular signal-regulated kinases
JNK	c-jun NH2-terminal kinases
F-1,6-BP	fructose-1,6-bisphosphate
F-2,6-BP	fructose-2,6-bisphosphate
F-6-P	fructose-6-phosphate
FABP	fatty acid binding protein
FABPpm	plasma membrane fatty acid binding protein
FACS	fatty acyl CoA synthase
FATP	fatty acid transport protein
FBPase-1	fructose-1,6-bisphosphatase
FBPase-2	fructose-2,6-bisphosphatase
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
G-depleted	glycogen depleted

G-replete	glycogen replete
GAP	glyceradlehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GI	global ischemia
G_{in}	glycogen synthesis
GLUT	glucose transporter
G_{out}	glycogen degradation
GP	glycogen phosphorylase
GS	glycogen synthase
GSH	reduced glutathione
GSK-3	glycogen synthase kinase-3
GSSG	oxidized glutathione
I/R	ischemia/reperfusion
I_{Na}	sodium current
LC	lumped constant
LFI	low-flow ischemia
LV	left ventricular
LVW	left ventricular work
MAPK	mitogen-activated protein kinase
MCD	malonyl CoA decarboxylase
MPTP	mitochondrial permeability transition pore
NAD^+/NADH	nicotinamide adenine dinucleotide
NADP/NADPH	nicotinamide adenine dinucleotide phosphate
NBC	sodium bicarbonate cotransporter
NCX	sodium calcium exchanger
NEFA	non-esterified fatty acids
NHE	sodium hydrogen exchanger
NKA	sodium potassium ATPase
PCr	creatine phosphate
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PEG	polyethylene glycol
PET	positron emission tomography
PFK-1	phosphofructokinase-1
PFK-2	phosphofructokinase-2
P_i	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidyl-inositol-3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
PPP	pentose phosphate pathway
rmNCX	reverse-mode sodium calcium exchanger
ROS	reactive oxygen species

SDH	sorbitol dehydrogenase
TAG	triacylglycerols
TAK1	transforming growth factor-β-activated protein kinase-1
TCA	tricarboxylic acid cycle
UDP	uridine diphosphate
VDAC	voltage-dependent anion channel

1. Introduction

1.1 Myocardial ischemia/reperfusion injury

Cardiovascular disease is the leading cause of mortality and morbidity in adults in Canada accounting for 30% of all deaths in 2006 (1). Heart attacks (acute myocardial infarction) were responsible for 23% of these deaths while ischemic heart diseases in general caused 54% of them (1). On a global scale, the World Health Organization expects that by the year 2020 cardiovascular disease will surpass infectious diseases and become the leading cause of mortality worldwide (2). In 2001, ischemic heart diseases were estimated to be responsible for 11.8% of all deaths in low-income countries and 17.3% of all deaths in high-income countries (3). However, due to recent advances in both primary and secondary prevention of cardiovascular diseases, there has been a decline in the total number of deaths due to cardiovascular diseases in Canada (4). Thus, the number of Canadian living with the burden of cardiovascular disease has increased raising the economic burden on Canadian society (over 22.2 billion dollars per year in 2000) (5).

Myocardial ischemia occurs when coronary blood flow is compromised leading to a reduction of myocardial oxygen supply by the coronary vasculature, which becomes inadequate to meet the oxygen demand for the maintenance of normal heart function. Most of the cases of acute myocardial ischemia develop as a result of coronary atherothrombosis (6). To date, the most effective approach to

salvage the ischemic myocardium in patients with acute coronary occlusion is through rapid re-establishment of perfusion of the affected area. This involves either the use of drugs (thrombolytic agents), surgical (by-pass grafting), or non-surgical interventions (angioplasty or stents). Despite the effectiveness of reperfusion, its success is limited by the duration of the interval between the onset of ischemia and re-establishment of flow (7) as well as by the presence of other co-morbidities (8). In addition, post-ischemic contractile dysfunction is not caused only by the ischemic episode; establishment of reperfusion contributes to, and even extends this damage (9). This phenomenon, termed "myocardial reperfusion injury", limits the benefits of re-establishment of myocardial perfusion (10).

Current pharmacological approaches to treat ischemic heart disease focus mainly on improving hemodynamic parameters and reducing myocardial O_2 demand. Organic nitrates, calcium channel blockers, and angiotensin-converting enzyme inhibitors improve hemodynamics by reducing preload and systemic vascular resistance while β-adrenoceptor antagonists reduce myocardial O_2 demand because of their negative inotropic and chronotropic effects. However, these interventions do not directly protect the heart against ischemia/reperfusion (I/R) injury, which emphasizes the importance of defining new targets for cardioprotection. One novel and promising intervention is through manipulation of myocardial energy substrate metabolism to optimize myocardial energy

metabolism and limit the detrimental consequences of ischemia and reperfusion. The heart predominantly relies on fatty acids and glucose as energy substrates. The focus of this thesis is to provide a better understanding of the role of myocardial glucose and glycogen metabolism in I/R injury and the possible interventions that can manipulate this pathway for therapeutic benefit. The introduction will provide an overview of myocardial I/R injury followed by an overview of the major metabolic pathways in the heart with a focus on carbohydrate metabolism.

1.2 Mechanisms of myocardial ischemia/reperfusion injury

Most of the cases of acute myocardial ischemia develop as a result of thrombosis developing on a culprit coronary atherosclerotic plaque (6). Data from autopsy studies, clearly biased towards fatal outcomes, show that fatal coronary thrombosis usually develops as a result of a "through-and-through" disruption of the plaque's protective fibrous cap (11,12). Other possible mechanisms that can account for a minor proportion of fatal coronary thrombosis include superficial erosion, intraplaque haemorrhage, and the erosion of a calcified nodule (13).

Under resting conditions, the heart extracts 70-80% of the O_2 from each unit of blood delivered to it (14). Thus it is essential that the heart tightly couple oxygen supply and demand to maintain adequate tissue oxygenation. In the absence of coronary artery disease, coronary flow increases almost proportionately to increased myocardial oxygen consumption to prevent tissue ischemia (14). However under conditions of impaired coronary blood supply, myocardial ischemia develops resulting in an interruption of oxygen supply and significant reduction in the rates of oxidative phosphorylation. Thus, aerobic adenosine triphosphate (ATP) synthesis slows and eventually ceases and then ATP is produced mainly through anaerobic glycolysis, which is not sufficient to meet the energy demands of the heart. This results in depletion of ATP stores and accumulation of adenosine diphosphate (ADP), adenosine monophosphate (AMP) and other metabolite byproducts leading eventually to the rapid cessation of contractile function and dysregulation of ionic homeostasis (15).

Management of acute myocardial ischemia involves the re-establishment of coronary perfusion via thrombolytic treatment, percutaneous coronary intervention, or coronary artery bypass graft surgery. Reperfusion of previously ischemic tissue results in a wide range of pathological effects, collectively termed "reperfusion injury" (9). The concept of myocardial reperfusion injury was first suggested in 1960 by Jennings et al. (16) in their histological description of post-ischemic canine myocardium. Reperfusion injury can manifest as: a) reversible

impairment of mechanical function (myocardial stunning) (17), b) microvascular dysfunction (no-reflow phenomenon) (18), c) ventricular dysrhythmias (19), as well as d) irreversible cardiomyocyte death (lethal reperfusion injury) (10).

The relative proportion of injury occurring during ischemia versus that occurring during reperfusion is variable and depends on the duration of ischemia (20). Studies in animal models of acute myocardial infarction show that in case of early and successful reperfusion, reperfusion injury accounts for as much as 50% the final myocardial infarct size (9). This highlights the clinical importance of targeting reperfusion injury as it allows for pharmacological intervention during the critical reperfusion phase as a treatment strategy. A large number of cardioprotective strategies and drugs have been shown to be effective when initiated early at the onset of reperfusion. These include postconditioning (21), inhibition of glycogen synthase kinase-3 (GSK-3) (22,23), adenosine (ADO) and ADO receptor agonists (24-28), opioid receptor agonists (22,29,30), erythropoietin (31), mitochondrial permeability transition pore (MPTP) inhibitors (32), protein kinase C (PKC)-δ inhibitors (33), Na^+-H^+ exchanger (NHE) inhibitors (34) as well as other interventions.

I/R injury is a multifactorial pathology as there is no single mechanism that explains it. Contributing mechanisms involved include oxygen free radical generation (35), neutrophil infiltration (36), coronary endothelial and microvascular dysfunction (37), proteolysis of contractile proteins (38-40),

alterations in intracellular calcium homeostasis and myofilament sensitivity to calcium (20), opening of the MPTP (41) and altered myocardial metabolism (42). All these mechanism interact with each other and are interdependent to mediate I/R injury. Work in this thesis mainly involves the role of alterations in myocardial substrate metabolism and ionic dysregulation in the pathogenesis of I/R injury.

1.2.1 Ionic dysregulation and Ca^{2+}overload during ischemia and reperfusion

During ischemia, diminished oxidative phosphorylation and increased reliance on anaerobic metabolism results in limitation of ATP production and pronounced intracellular acidosis resulting in significant dysfunction of the ionic homeostasis in the cardiomyocyte. Intracellular H^+ ions generated during ischemia are removed from the cell by extrusion of weak acids such as lactic acid as well as by the action of NHE and Na^+-HCO_3^- cotransporter (NBC). This results in extracellular acidification, which eventually limits additional removal of H^+ from inside the cell (43). Concomitant assessment of intra- and extracellular pH during ischemia showed that pH decreases to final values of 5.9 and 5.5, respectively (44). In addition, inadequate supply of ATP during ischemia inhibits the activity of Na^+-K^+ ATPase (NKA), thereby limiting its capacity to extrude Na^+ against its large electrochemical gradient. Na^+ influx into the cardiomyocyte increases as well during ischemia, which, in combination with limited Na^+ efflux

via NKA, results in significant intracellular accumulation of Na^+ and depolarization of the resting membrane potential of the cardiomyocytes (20,45). Influx of Na^+ during ischemia occurs through multiple mechanisms. The increased reliance on anaerobic metabolism and the hydrolysis of glycolytically derived ATP results in a significant increase in H^+ content which stimulates its exchange with extracellular Na^+ via the activity of NHE (46) as well as its neutralization by increased activity of NBC (47). Another potential mechanism of increased Na^+ influx is through late or persistent Na^+ current (late I_{Na}) (48,49). Under resting conditions Na^+ entry via peak (transient) I_{Na} is minimal as a result of rapid inactivation of the voltage-gated sodium channels. However, ischemic conditions result in delayed Na^+ channel inactivation resulting in augmentation of late I_{Na}. The delayed inactivation of Na^+ channels during ischemia is mediated, in part, by the actions of 5'-AMP activated protein kinase (AMPK) (50), reactive oxygen species (ROS) (51-53), as well as ischemic metabolites (48). The increased accumulation of intracellular Na^+ results in reverse-mode (rm) activation of the Na^+-Ca^{2+} exchanger (NCX) so that 3 Na^+ ions are extruded in exchange with the influx of 1 Ca^{2+} ion resulting in intracellular Ca^{2+} overload (54).

During reperfusion, extracellular pH rapidly returns to normal values while the intracellular pH remains acidic initially. This creates a large pH gradient, which facilitates the extrusion of H^+ in exchange of Na^+ via NHE

resulting in increased Na^+ overload. This increased intracellular Na^+ can be extruded by either NKA or rmNCX resulting in sustained Ca^{2+} overload during the initial period of reperfusion (20,43).

Elevated intracellular levels of Ca^{2+} stimulate a number of detrimental pathways, including triggering of arrhythmias, activation of proteases and phospholipases, opening of MPTP, myocardial stunning, and induction of cardiomyocyte death (55). Different approaches that limit the extent of intracellular Ca^{2+} overload ameliorate I/R injury. These include pharmacological inhibition or genetic ablation of NHE (34,46,56) or NCX (54,57) as well as approaches that limit H^+ production from glucose metabolism (28,58,59).

In this thesis, we describe a number of approaches that reduce H^+ production and limit Ca^{2+} overload during reperfusion. In the study described in Chapter 4, we directly assess intracellular Ca^{2+} levels in the working rat heart during baseline, ischemia, and reperfusion, which enable us to concomitantly assess mechanical function, metabolism, as well as intracellular Ca^{2+} levels.

1.2.2 Role of MPTP

The increase in the permeability of the inner mitochondrial membrane associated with cell death, termed mitochondrial permeability transition and first discovered more than three decades ago (60), is mediated by the opening of the MPTP, a nonspecific channel that is thought to span both the inner and outer

mitochondrial membranes (61). The MPTP is nonselectively permeable to solutes up to 1.5 kDa (62). The opening of the MPTP is greatly enhanced by Ca^{2+} overload, adenine nucleotide depletion, elevated phosphate concentrations and oxidative stress, which are conditions known to accompany reperfusion following ischemic injury (63). Thus MPTP opening is suggested to be a major contributing event in the pathology of I/R injury. MPTP can undergo either transient or prolonged opening. Transient opening events of the MPTP are suggested to be involved in the physiological roles of the MPTP while the more prolonged openings are involved in pathological situations (64,65). A major consequence of MPTP opening is that the inner mitochondrial membrane no longer maintains a barrier for H^+, which causes equilibration of H^+ across the inner mitochondrial membrane and dissipation of the proton motive force. This resulting uncoupling of oxidative phosphorylation not only prevents mitochondrial generation of ATP, but also results in the reversal of activity of the H^+-translocating ATPase (63). This means that mitochondria with open MPTP will start to hydrolyze ATP produced by glycolysis and any remaining healthy mitochondria resulting in ATP depletion and bioenergetic failure of the cardiomyocyte (66,67). Another major consequence of the opening of the MPTP is the increased influx of water into the mitochondria causing swelling of the mitochondrial matrix. This can occur without rupture of the inner mitochondrial membrane because of the unfolding of the cristae but as the matrix expands further, this can put pressure on the outer membrane that eventually ruptures (66). This releases cytochrome *c* and other

pro-apoptotic mediators into the cytosol, which can direct the cell to apoptosis (68). However apoptosis predominates over necrosis only if the pore recloses and ATP levels are maintained to fuel the apoptotic pathway (67).

The structure of the MPTP was originally proposed to include the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane and cyclophillin-D (CypD) in the matrix (69). It was shown that VDAC, ANT, and CypD interact at membrane contact sites (70) and reconstitution of this complex in vesicles yields a Ca^{2+}-sensitive channel with similar properties to the MPTP (71). In addition, pharmacological inhibitors of ANT and CypD suppress MPTP opening and protect against I/R injury (72-75). However, recent genetic studies have casted a doubt on that proposed structure as it was shown in mitochondria and cells lacking all VDAC isoforms that VDAC is dispensable for MPT and is not a component of the MPTP (76). Results from mice lacking both isoforms of ANT also show that ANT is not a component of the MPTP and it only plays a regulatory role (77). This uncertainty in the exact structure of the MPTP limits the ability to fully understand its regulation and physiological and pathological roles.

Opening of the MPTP is influenced by a number of factors, most important of which are Ca^{2+}, inorganic phosphate (P_i), pH, oxidative stress, mitochondrial membrane potential, and adenine nucleotides (78). Matrix Ca^{2+} is

considered the main permissive factor that permits MPTP opening (79). The exact Ca^{2+} threshold mediating MPTP opening is difficult to define because it is dependent on various factors that vary together with the Ca^{2+} load (such as matrix P_i and pH) (78). As mentioned in the previous section, I/R injury is associated with significant intracellular Ca^{2+} overload. Reintroduction of oxygen during reperfusion and restoration of the mitochondrial membrane potential, together with elevated $Ca^{2+}{}_i$ levels results in significant Ca^{2+} uptake into the mitochondria through the mitochondrial Ca^{2+} uniporter, which can trigger activation of MPT (20). Matrix pH is also a major regulator of MPTP opening as it was shown that the optimum pH for MPTP opening in de-energized mitochondria is 7.3 (80). During ischemia, intracellular acidosis, oxygen deprivation and de-energization of the mitochondria strongly reduce the chances of MPTP opening (78). However, in respiring mitochondria (similar to the condition during reperfusion), exposure to acidic pH favors mitochondrial uptake of P_i, which is a strong activator of MPTP opening (81). This can explain the detrimental effects of post-ischemic intracellular acidosis.

Targeting MPTP to ameliorate reperfusion injury has been a subject of extensive research as it was shown in isolated cells and intact hearts that MPTP, which remains closed during ischemia, opens during reperfusion (64,75,82,83). Inhibition of CypD by cyclosporine-A or its analogues (sanglefehrin-A or Debio-025) protect against reperfusion injury and reduce infarct size in a number of

experimental models (32,74,75,83-86). In addition, transgenic mice deficient in CypD as well as mouse hearts depleted of CypD using siRNA are more resistant to I/R injury than wild-type mice (85,87,88). Several cardioprotective strategies were also found to inhibit the opening of the MPTP such as preconditioning (89), postconditioning (90), temperature preconditioning (91) as well as treatment with NHE inhibitors (92), urocortin (93) propofol (94), GSK-3 inhibitors (41,95), antioxidants (96,97), ADO receptor agonists (98,99), and opioids (100). An important study by Juhaszova *et al.* demonstrates that several cardioprotective interventions converge on a general mechanism that involves enhanced phosphorylation and inhibition of GSK-3 and subsequent limitation of MPTP induction (41). GSK-3 was suggested to disrupt the interaction between hexokinase and VDAC by phosphorylating VDAC. This dissociation between hexokinase and VDAC promotes MPTP opening (101). The recent discovery that VDAC is not a component or a regulator of MPTP casts doubt on the role of GSK-3 in MPTP modulation. In the study described in Chapter 4, we introduce a new mechanism by which GSK-3 inhibitors improve mitochondrial function during reperfusion and protect against I/R injury.

1.3 Myocardial energetics

The heart requires a constant supply of fuel and oxygen to maintain its intracellular ATP levels for uninterrupted pump function. It consumes more

energy than any other organ as it utilizes approximately 6 kg of ATP every day to circulate 10 tons of blood throughout the body (102). The heart has a relatively limited ATP content (5 μmol/g wet wt) and a rapid rate of ATP hydrolysis (0.5 μmol/g wet wt/sec) so there is complete turnover of the entire ATP pool in the heart every 10 sec (42). Thus the heart requires a continuous supply of ATP, which it acquires via converting chemical energy stored in fatty acids and glucose into the mechanical energy of the actin-myosin interaction (102). Approximately 60-70% of myocardial ATP hydrolysis is utilized to fuel myocardial contractility while the remaining 30-40% is utilized to fuel ion pumps to maintain ionic homeostasis in the cardiomyocyte (103). Under non-ischemic conditions, most of the ATP produced (95%) is derived from oxidative phosphorylation in the mitochondria while the remainder comes from glycolysis and GTP formation in the tri-carboxylic acid (TCA) cycle (42). Under normal conditions, 60-90% of the acetyl CoA that enters the TCA cycle is derived from β-oxidation of fatty acids and 10-40% comes from oxidation of pyruvate that is derived from glycolysis or lactate oxidation (104-108).

1.4 Glucose metabolism

Glucose is an important energy substrate that is utilized by almost all organisms via a common set of metabolic pathways. Our understanding of glucose metabolism dates back to the discovery of glycolysis by Louis Pasteur in

1860. The full glycolytic pathway was identified in 1937 by the work of Gustav Embden and Otto Fritz Meyerhof (109). Later, work done by Hans A. Krebs and William A. Johnson led to the discovery of the TCA cycle (110). More than 20 years later, Peter D. Mitchell hypothesized a chemiosmotic mechanism for ATP production (111) that resulted in the identification of the electron transport chain and oxidative phosphorylation and thus completing an understanding of the pathway of aerobic glucose metabolism.

Our current view of glucose metabolism is that glucose can be metabolized through several metabolic pathways that not only function as an energy supply but also affect a number of physiological and pathophysiological processes (Figure 1.1). The initial step of the metabolism of glucose is its transport followed by its conversion to glucose-6-phosphate (G-6-P), which is mostly converted by the glycolytic pathway to produce pyruvate that is utilized in the TCA cycle to provide energy. However, G-6-P can also be utilized in several other pathways such as glycogen synthesis, pentose phosphate pathway (PPP), or the aldose reductase (AR)/polyol pathway. Despite the glycolytic pathway being the main pathway of glucose utilization, these other pathways can play an important role, especially under pathophysiological conditions and can affect the partitioning of G-6-P among these pathways. Understanding the role of partitioning of G-6-P to its various metabolic pathways, especially between the

glycogen synthesis and the glycolytic pathways, in cardioprotection against I/R injury, is one of the main aims of this thesis.

1.4.1 Glucose uptake

Myocardial glucose uptake is a complex three-step process where glucose must first pass from the blood stream to the interstitial space followed by its transport to the intracellular space and finally its phosphorylation to form G-6-P (112). Little is known about the process of glucose transport through the walls of blood capillaries to the interstitial space; however, it is generally considered a process of passive diffusion through blood capillary endothelial cells down its concentration gradient (113). Because of its hydrophilic nature, glucose is not able to cross the lipid bilayer of the plasma membrane of cells other than capillary endothelial cells just by simple diffusion (113). Glucose entry to the intracellular space is facilitated by a family of glucose transporters (GLUTs) of which there are currently fourteen identified isoforms (114). In the cardiomyocyte, two members of the GLUT family are present, GLUT-1 and GLUT-4 (115,116). GLUTs are located both at sarcolemma as well as in intracellular compartments. The sarcolemmal pool is considered the functional pool for these transporters while the intracellular pool acts as storage depot (117). Glucose transport can be stimulated via increased translocation of GLUTs from their intracellular storage pools to the sarcolemma thus increasing the functional compartment (117). The translocation process is a vesicle-mediated process (118) that can be stimulated by

a number of stimuli such as insulin (119), contractility (120), and ischemia (121). GLUT-1 is generally considered to be available mainly in the sarcolemma to mediate basal glucose transport while GLUT-4 is translocated to the sarcolemma to increase glucose transport in response to stimulation (117). However, in the heart it was shown that GLUT-1 could also be translocated from the endosomal compartment to the sarcolemma in response to insulin and contraction (122). Cardiac GLUT-4 is stored intracellularly into two distinct compartments, a non-endosomal compartment and an endosomal compartment as part of the recycling endosomes (115). The non-endosomal compartment contains most of the intracellular pool of GLUT-4. Insulin induces translocation of GLUT-4 from the non-endosomal compartment to the recycling endosomes and from there to the sarcolemma while contraction mainly stimulates translocation of GLUT-4 from the endosomal compartment (117).

Insulin is one of the main activators of glucose transport. Insulin binds to its receptors on the surface of the cardiomyocyte resulting in activation of the tyrosine kinase moiety of the insulin receptor. This results in activation of insulin receptor substrate 1 and subsequent activation of the regulatory subunit p85 of phosphatidylinositol-3-kinase (PI3K), which catalyzes the formation of phosphatidyl-inositol-3,4,5-triphosphate (PIP_3). PIP_3 activates phosphoinositide-dependent kinase (PDK) which by turn activates Akt and the atypical protein kinase C (PKCζ). Both Akt and PKCζ where found to contribute to insulin-

induced GLUT-4 translocation (117). Activation of Akt results in phosphorylation and inhibition of the Akt substrate of 160 kDa (AS160), which is a negative regulator of GLUT-4 translocation (123). Thus, its inhibition downstream of insulin receptor activation relieves its inhibitory restraint on GLUT-4 translocation and results in increased glucose transport. GLUT-4 translocation can be stimulated as well by activation of AMPK, which occurs as a result of ischemia as well as increased contractility. This will be discussed in more detail in later sections.

Following transport into the cardiomyocyte, glucose gets rapidly phosphorylated by the action of hexokinase to form G-6-P. There are 2 isoforms of hexokinase in the myocardium, hexokinase-I and hexokinase-II. Hexokinase-I is predominant in the fetal heart while the insulin-sensitive hexokinase-II predominates in the adult heart (124). In general, the rate of glucose phosphorylation is higher than the rate of glucose transport maintaining a steep inward concentration gradient in favor of glucose entry (125). Thus, the rate of glucose transport by GLUTs is the rate-limiting step in the process of glucose uptake. However, under conditions when glucose transport is stimulated by insulin, hexokinase reaction may become limiting (126,127). Hexokinase is present intracellularly both in the cytosol as well as bound to the outer wall of mitochondria through its binding with VDAC (128,129). Binding of hexokinase to the mitochondria increases its activity, lowers its K_m for glucose, and

suppresses its inhibition by G-6-P (129,130). In addition, binding of hexokinase to mitochondria can modulate MPTP opening and mitochondrial function and plays an important role in preconditioning-induced cardioprotection (62,131).

Most of the G-6-P produced from the hexokinase reaction is shuttled through the glycolytic pathway with a smaller proportion directed towards storage as glycogen. A minor part of G-6-P is utilized by the PPP and the AR/polyol pathway.

1.4.2 Glycogen metabolism

Glycogen is the storage form for carbohydrates in virtually all organisms from yeast to primates. Most mammalian tissues, including the heart, store glucose in the form of glycogen. It occupies about 2% of the cell volume of adult cardiomyocytes and about 30% of the cell volume of neonatal and newborn cardiomyocytes (132). Glycogen is a polysaccharide of repeated glucose units linked by α1,4 glycosidic linkages with branch points introduced by α1,6 glycosidic linkages. Despite being considered as a passive storage repository, glycogen is a dynamic pool, which is in a constant state of turnover due to the concomitant activity of the processes of glycogen synthesis and degradation (Figure 1.2). Heart muscle, unlike skeletal muscle and liver, increases its glycogen content during fasting (133). This is due to the increased availability of fatty acids during fasting, which inhibits myocardial glycolysis to a greater extent

than its inhibition of glucose uptake thereby repartitioning G-6-P towards glycogen synthesis (124).

Glycogen synthesis

Glycogen synthesis follows a simple but strictly regulated process resulting in a complex structure (134). For G-6-P to enter in the glycogen synthesis pathway, it has to be converted first to glucose-1-phosphate (G-1-P) by the action of phosphoglucomutase. G-1-P then reacts with uridine triphosphate to form the active nucleotide uridine diphosphate (UDP)-glucose catalyzed by the enzyme UDP-glucose pyrophosphorylase. Glycogen synthase (GS) then utilizes UDP-glucose to add glucosyl residues by α1,4 linkages to an oligosaccharide primer, glycogenin. Glycogenin is a 37 kDa protein, which gets glucosylated on a specific tyrosine residue by UDP-glucose and acts as a primer on which GS adds further glucose residues (135). When the glycogen chain reaches at least 11 residues, another enzyme, the branching enzyme, creates branches in the glycogen molecule by transferring a chain of 7 molecules of the α1,4 chain to a neighboring chain to form an α1,6 linkage, thus establishing a branch (136).

GS catalyzes the rate-limiting step of the glycogen synthesis pathway. GS activity is tightly regulated through binding allosteric ligands, covalent phosphorylation, as well as enzyme translocation (134,137). This enzyme has been very well studied because it was one of the first examples of enzyme activity

control by covalent phosphorylation (138) and it is considered the first example of an insulin intracellular target (139,140). There are 2 isoforms of GS in mammals, a muscle and a liver isoform (141). All of the details described later in this section involve mainly the muscle isoform, which is the one present in the cardiomyocyte. GS is phosphorylated on nine Ser residues at both its NH_2- and COOH-termini by at least six protein kinases. GS phosphorylation results in its inhibition. These nine residues are located at the positions 7 (site 2a), 10 (site 2b), 640 (site 3a), 644 (site 3b), 648 (site 3c), 652 (site 4), 656 (site 5), 697 (site 1a), and 710 (site 1b) (139). For mammalian GS, two sites, site 3a and site 3b, appear to have the greatest influence on activity, resulting in suppression of the enzyme activity. Their phosphorylation is catalyzed by the action of GSK-3 through a hierarchal mechanism (142,143). Initial phosphorylation of site 5 by casein kinase II provides a recognition motif -S-X-X-X-S(P)- for GSK-3 which then sequentially phosphorylates sites 4, 3c, 3b, and 3a, respectively, leading to inhibition of GS. GSK-3 is phosphorylated and inhibited downstream of the insulin/PI3K/Akt pathway and is considered one of the main mechanisms by which insulin stimulates glycogen synthesis. The role of GSK-3 in modulating myocardial metabolism and I/R injury is the main aim of the study described in Chapter 4.

GS phosphorylation can also be regulated by active control of dephosphorylation by protein phosphatases (PPs). Most evidence suggests that

the PP involved in dephosphorylation of GS is of type 1, with a type 1 catalytic subunit in association with a regulatory subunit (G_M in striated muscles) (144). These regulatory subunits bind to PP1 and target it to the glycogen particle where all the glycogen-metabolizing enzymes are localized. Insulin was shown to phosphorylate and promote the binding of G_M to PP1 and its localization with glycogen to increase dephosphorylation of GS (145).

Allosteric modulators, most important of which is G-6-P, can also regulate GS. Binding of G-6-P to GS results in its allosteric activation by its unfolding leading to overriding the inhibitory effects of phosphorylation (134). In addition, G-6-P favors conformational changes in GS that facilitates its dephosphorylation (134). Modulation of the intracellular localization of GS in response to insulin, as well as to increased G-6-P levels, is the third mechanism by which GS activity can be regulated (141).

Glycogen degradation

Glycogen degradation or glycogenolysis is not the reverse of glycogen synthesis but is a separate pathway (136). Glycogen phosphorylase (GP) catalyzes the cleavage of α1,4 linkages to remove glucose molecules from the glycogen chain. This reaction is the rate-limiting step in the glycogen degradation pathway. When only 4 glucose units are available before a branch point, another enzyme, the debranching enzyme, catalyzes the transfer of 3 glucose residues to

an adjacent branch of the glycogen chain. The same enzyme then catalyzes the cleavage of the α1,6 bond to release the glucose molecule from the branch point to allow GP to resume its removal of glucose moieties from this chain (136). Glycogenolysis releases G-1-P from the glycogen molecule, which can be converted by the action of phosphoglucomutase to G-6-P to be utilized in glycolysis. Thus, glycogen degradation provides an already phosphorylated glucose moiety, whereas the metabolism of exogenous glucose requires the consumption of 1 mole of ATP per 1 mole of glucose to convert glucose to G-6-P. Therefore, more ATP per mole of glucose is produced from glycogenolysis than glycolysis of exogenous glucose. This is especially important in conditions of diminished energy supply such as during ischemia. In addition, glucosyl moieties released by glycogen degradation are preferentially oxidized rather than converted to lactate (146).

GP, like GS, is also regulated by allosteric ligand binding as well as by covalent phosphorylation. However, unlike GS, phosphorylation of GP results in its activation and it has only one phosphorylation site (Ser14) that is phosphorylated by a single kinase, phosphorylase kinase. Activation of phosphorylase kinase and subsequent activation of GP is mediated by increase in intracellular Ca^{2+} levels as well as by phosphorylation by cAMP-dependent protein kinase (PKA) (137). Both phosphorylase kinase and GP can also be regulated by the action of PPs. Similar to GS, the PP involved is most likely to be

composed of type 1 catalytic subunit in association with a glycogen-binding targeting subunit (G_M in muscles) (137). Binding of different metabolites can regulate GP allosterically. AMP is the main allosteric activator of G-6-P while ATP and G-6-P are allosteric inhibitors (147).

Dynamic relationship between glycogen synthesis and degradation

Glycogen is a dynamic pool where there is concomitant synthesis and degradation. Relative rates of these two processes determine whether there is a net glycogen accumulation or depletion. The balance between these two processes can be affected by several factors, including substrate availability and preference, myocardial energy status, insulin, as well as myocardial workload. In general, during periods of energy starvation (increased AMP and decreased ATP and G-6-P), glycogen degradation exceeds the rates of glycogen synthesis resulting in net glycogen loss. On the other hand, during periods of energy abundance (increased ATP and G-6-P and decreased AMP), glycogen synthesis predominates leading to net glycogen accumulation. It is worthwhile to note that under all conditions, both processes occur simultaneously as it was shown that glycogen synthesis still occurs at minimal rates during periods of severe ischemia (148). In the study described in Chapter 3, we assess the rates of both glycogen synthesis and degradation during low-flow ischemia (LFI) in heart with either depleted or replenished pre-ischemic glycogen stores.

1.4.3 Glycolysis

Glycolysis is the metabolic pathway by which glucose is converted into two molecules of pyruvate in the presence of oxygen or into two molecules of lactate in the absence of oxygen. Glycolysis occurs in the cytosol where all the enzymes involved in the glycolytic pathway are located (136). However, glycolytic enzymes are not distributed freely in the cytosol but rather they are found clustered near the sarcoplasmic reticulum and the sarcolemma (149-152) where the glycolytically produced ATP may be preferentially utilized to fuel sarcoplasmic reticulum Ca^{2+} uptake (152) and to maintain ionic homeostasis (153,154). The glycolytic pathway can be divided into three stages: a) the priming stage (reaction 1-3) involves the investment of two ATP molecules per 1 molecule of glucose to form fructose-1,6-bisphosphate (F-1,6-BP). b) The splitting stage (reactions 3-4) where F-1,6-BP is cleaved by aldolase to form the triose phosphates, glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP). These two triose phosphates can be interconverted by triose phosphate isomerase. c) The energy trapping stage (reactions 6-10) where 2 molecules of GAP are converted to pyruvate with the generation of 4 ATP molecules (155) (Figure 1.3). Thus, there is a net production of 2 molecules of ATP per 1 molecule of exogenous glucose metabolized via glycolysis. As mentioned in the previous section, glycolysis of glucose moieties released from

the glycogen pool releases 3 molecules of ATP per 1 molecule of glucose because it bypasses hexokinase reaction.

GAP dehydrogenase (GAPDH) catalyzes the oxidation of GAP to 1,3-bisphosphoglycerate with the production of NADH. In order to ensure flux through this reaction, NADH must be continually reoxidized to NAD^+. NADH is reoxidized under anaerobic conditions by the enzyme lactate dehydrogenase, which converts pyruvate to lactate. This ensures that glycolysis proceeds to produce ATP under anaerobic conditions. Under aerobic conditions, NADH is reoxidized by the mitochondrial electron transport chain (136).

Regulation of glycolysis

The glycolytic pathway can be regulated at multiple steps. It can be regulated by the control of the availability of G-6-P either at the level of glucose transport and hexokinase, as well by as glycogen synthesis and degradation. It is also regulated by direct effects on the glycolytic enzymes, most important of which are phoshofructokinase-1 (PFK-1), pyruvate kinase, and GAPDH (125).

PFK-1 catalyzes the first irreversible step in the glycolytic pathway and is considered the most important regulator of the glycolytic pathway (125). It utilizes ATP to phosphorylate fructose-6-phosphate (F-6-P) to produce F-1,6-BP. It is mainly regulated by allosteric control by a number of positive and negative effectors. It is activated by ADP, AMP, and P_i and inhibited by ATP, thus it acts

to increase flux through glycolysis during periods of energy depletion (42). ATP, despite being a substrate for PFK-1, allosterically inhibits it by inducing negative cooperativety of the second substrate, F-6-P (156). Citrate and protons are also negative modulators of PFK-1, which limit glycolysis during periods of increased intracellular acidosis and will link changes in mitochondrial oxidative metabolism to glycolysis (157,158). Despite the number of modulators for PFK-1, fructose-2,6-bisphosphate (F-2,6-BP) is considered the most important positive modulator as it was found that PFK-1, in the presence of physiological concentrations of other substrates and effectors, is almost completely inactive unless physiological (micromolar) concentrations of F-2,6-BP relieve its inhibition by ATP (159,160).

F-2,6-BP content is tightly regulated by the activity of the bifunctional enzyme phosphofructokinase-2 (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) which is responsible for its synthesis and degradation (Figure 1.4). The heart has 2 isoforms of this enzyme, which are generated from the same gene by alternative splicing (161). PFK-2 activity is controlled by phosphorylation, allosteric ligands, as well as by transcriptional control (162). A variety of stimuli can induce the phosphorylation and activation of PFK-2. Insulin was shown to increase PFK-2 phosphorylation at Ser466 and Ser483 via activating a yet unknown wortmannin-sensitive, insulin-stimulated protein kinase, 'WISK', which does not correspond to Akt or PDK1 (163). This results in a 2-fold increase in PFK-2 V_{max} with no change in K_m for F-6-P (164). Both PKC and PKA were also

shown to phosphorylate PFK-2; however, these effects do not translate into changes in heart PFK-2 activity (162). Another important regulator of PFK-2 phosphorylation is AMPK, which phosphorylates PFK-2 at Ser466 resulting in an increase in its V_{max} with no change in its K_m for F-6-P (165). The details on the role of AMPK in regulation of glucose metabolism will be discussed in later sections of this introduction.

The terminal step of the glycolytic pathway, which involves the conversion of phosphoenol pyruvate into pyruvate, catalyzed by pyruvate kinase, can also play a role in regulation of glycolysis. It is regulated by a feed-forward mechanism where F-1,6-BP, the product of PFK-1 reaction, stimulates pyruvate kinase (166). This ensures the progress of the glycolytic pathway and prevents accumulation of glycolytic metabolites. In addition, GAPDH can also play a regulatory role during periods of ischemia as well as a result of oxidative stress due to limitation of NAD^+ availability (167-169).

1.4.4 Pentose phosphate pathway

Another pathway for glucose metabolism is the PPP. It does not result in ATP production; however, it serves two main functions: a) Production of reducing equivalents in the form of NADPH, which is important for fatty acid synthesis and to convert oxidized (GSSG) to reduced glutathione (GSH) which is important to detoxify ROS and b) formation of ribose-5-phosphate, which is important in the

synthesis of nucleic acids and nucleotides (136) as well as in replenishing the adenine nucleotide pool following ischemia (170). The PPP consists of two phases: a) an irreversible oxidative phase that produces NADPH and ribulose-5-phosphate (precursor of ribose-5-phosphate) via reactions catalyzed by G-6-P dehydrogenase, lactonase, and 6-phosphogluconate dehydrogenase and b) a reversible non-oxidative phase that converts three molecule of ribulose-5-phosphate into two molecules of F-6-P and 1 molecule of GAP (171). PPP is mainly regulated at the G-6-P dehydrogenase and 6-phosphogluconate dehydrogenase reactions (171). Under normal conditions, PPP proceeds at relatively low rate in the heart as compared to other tissues due to the inhibition of G-6-P dehydrogenase by the abundant amounts of NADPH (170,172). Thus, it is unlikely that changes in flux through PPP will have a major effect on other glucose metabolic pathways. However, changes in G-6-P availability as well as changes in G-6-P dehydrogenase activity can affect flux through PPP and thereby affect the cardiomyocyte's antioxidant status. This becomes relevant under conditions of increased oxidative stress, such as during reperfusion, as GSSG activates G-6-P dehydrogenase and stimulates fluxes through PPP (173).

1.4.5 Aldose reductase/polyol pathway:

Excess intracellular glucose undergoes metabolism by AR and sorbitol dehydrogenase (SDH) to produce polyhydroxylated sorbitol and fructose (polyol) (174). This pathway is comprised of two steps involving two oxidoreductases,

AR and SDH. AR utilizes NADPH to reduce glucose to sorbitol, then SDH utilizes NAD^+ to oxidize sorbitol into fructose (171). This pathway is not of great importance under normal conditions as the K_m of AR, the rate-limiting enzyme, for glucose is relatively high (70 mM) (171). However, in uncontrolled diabetes, when blood glucose levels are higher than normal, this pathway becomes more important (171). In addition, it was shown that flux through AR is increased in the heart under ischemic conditions, even in the absence of diabetes (175,176). Excessive flux through AR pathway can affect a number of pathways including glycolysis, glucose oxidation, oxidative stress, PKC activation, and intracellular non-enzymatic glycation (177).

1.4.6 Glucose oxidation

Pyruvate produced by glycolysis is an intermediate at a branch point for several metabolic pathways (Figure 1.5). As discussed in previous sections, pyruvate in the cytosol can be reduced to lactate via lactate dehydrogenase to maintain NAD^+ supply for the GAPDH reaction. Pyruvate can also contribute to the replenishment of TCA cycle intermediates, also termed anaplerosis, through its transformation into oxaloacetate by pyruvate carboxylase or malic enzyme (178-180). However, under aerobic conditions, the majority of pyruvate, following entry into the mitochondria via a monocarboxylate transporter (181), is decarboxylated to form acetyl CoA that then enters the TCA cycle to undergo complete oxidation liberating CO_2 (124). This step is considered the key

irreversible step in carbohydrate oxidation that commits pyruvate to oxidation. It is catalyzed by pyruvate dehydrogenase complex (PDH), a multimeric enzyme complex present in the mitochondrial matrix (136). This pathway produces the majority of ATP generated from carbohydrate sources.

PDH is regulated by its substrates and products as well as by covalent phosphorylation. PDH kinase, which phosphorylates and inhibits PDH is activated by acetyl CoA and NADH and inhibited by pyruvate (182,183). On the other hand, PDH phosphatase, which dephosphorylates and activates PDH, is mainly stimulated by Ca^{2+} and Mg^{2+} (184,185). This later effect explains the observed increase in glucose oxidation as a response to increased myocardial function following β-adrenoceptor stimulation (185,186).

1.5 Fatty acid metabolism and utilization

Fatty acids are considered the most important fuel utilized by the heart. Under normal conditions, the adult heart obtains 50-70% of its ATP requirement from fatty acid catabolism (187). Plasma fatty acids are supplied to the heart as nonesterified free fatty acids (NEFA) bound to albumin or as fatty acids released from triacylglycerols (TAG) found in chylomicrons and very-low density lipoproteins (187,188). The heart can also utilize fatty acids from endogenous sources via breakdown of the myocardial store of TAG (189). The uptake of

NEFA by the heart depends mainly on their levels in plasma, which are dynamic and vary widely during different stages of development as well as during metabolic stress. Under normal conditions, plasma NEFA in adults ranges from 0.2–0.8 mM (42). These levels dramatically increase during periods of metabolic stress, such as uncontrolled diabetes as well as ischemia, to over 2 mM (190,191). It has long been thought the NEFA enters the cardiomyocyte by passive diffusion, a process that is governed by the physicochemical partitioning of NEFA between albumin and the plasma membrane (192). However, data from isolated cardiomyocytes showing that NEFA uptake process is saturable and sensitive to competitive inhibition suggest that protein-facilitated transport is involved (193-195). Four fatty acid transporters have been identified so far. These include the plasma membrane fatty acid binding protein (FABPpm) (196), fatty acid translocase/CD36 (197), as well as two members of the fatty acid transport protein (FATP) family, FATP-1 and 6 (198,199).

Once in the cytosol, NEFA bind to FABP and then are converted to long-chain acyl CoA esters by the action of fatty acyl CoA synthase (FACS). The long-chain acyl CoAs can then be either esterified to TAGs or phospholipids by the action of glycerolphosphate acyltransferase or converted to acyl carnitines by carnitine palmitoyl transferase I (CPT-I) for transport into the mitochondria and subsequent β-oxidation (42). In the healthy normal heart, it was shown that 70-

90% of NEFA taken up by cardiomyocytes are converted to acyl carnitines and oxidized while 10-30% are stored as TAG (200).

Mitochondrial β-oxidation occurs in the mitochondrial matrix. This necessitates that long-chain acyl CoAs be transported first to the mitochondrial matrix in order to get oxidized. The inner mitochondrial membrane is impermeable to long-chain acyl CoAs, thus they are transported from the cytosol to the mitochondrial matrix by a carnitine-dependent transport system (201). The first step involves the conversion of long-chain acyl CoAs into long-chain acyl carnitine by the action of CPT-I, located at the outer mitochondrial membrane. Second, acyl carnitines are transported across the inner mitochondrial membrane via carnitine acyl translocase. The last step involves the re-conversion of the acyl carnitines into acyl CoAs by the action of CPT-II located at the inner mitochondrial membrane (201). The rate-limiting step in this process is the reaction catalyzed by CPT-I where its activity is strongly inhibited by malonyl CoA binding at its cytosol side (201-203). Malonyl CoA is an important regulator of fatty acid oxidation, where an increase in its levels suppresses fatty acid mitochondrial uptake and oxidation (204,205) while a decrease in its levels promotes fatty acid oxidation (206-208). Myocardial malonyl CoA forms a dynamic pool with a half-life of 1.25 min that is regulated by both its synthesis and degradation (209,210). Malonyl CoA is synthesized by carboxylation of

acetyl CoA via the action of acetyl CoA carboxylase (ACC) and decarboxylated back to acetyl CoA by the action of malonyl CoA decarboxylase (MCD) (42).

Once in the mitochondrial matrix, long-chain acyl CoAs undergo catabolism via β-oxidation. β-oxidation is a multi-step process that involves the sequential liberation of two carbon units (acetyl CoA) from the acyl CoA chain. These reactions are catalyzed by acyl CoA dehydrogenase, 2-enoyl CoA hydratase, 3-hydroxacyl CoA dehydrogenase and 3-ketoacyl CoA thiolase (136). During the process of β-oxidation NADH and $FADH_2$ are generated together with the acetyl CoA that produces more NADH and $FADH_2$ via TCA cycle.

Randle's glucose/fatty acid cycle

The relation between rates of glucose and fatty acid oxidation is reciprocal. This was first demonstrated by the work done by Randle's group in 1963 and was termed Randle's "glucose/fatty acid cycle" (211). Elevated rates of fatty acid oxidation limit glucose oxidation via inhibition of PDH activity. This is mainly mediated by an increase in mitochondrial acetyl CoA/free CoA and $NADH/NAD^+$, which activates PDK leading to phosphorylation and inhibition of PDH (42). In addition, increased fatty acid oxidation also limits flux through glycolysis due to the inhibition of PFK-1 by citrate. However, inhibition of glycolysis is less than the inhibition of glucose oxidation (124). This results in a mismatch between the rates of glycolysis and glucose oxidation leading to

accumulation of lactate and H^+. On the other hand, stimulation of glucose oxidation can reduce rates of fatty acid oxidation (212). This mainly occurs as a result of CPT-I inhibition due to an increased production of malonyl CoA (204).

1.6 Myocardial substrate metabolism during ischemia and reperfusion

The limited O_2 supply occurring as a result of myocardial ischemia results in inhibition of oxidative phosphorylation and reduction in ATP production. As mentioned in previous sections, the heart has a relatively high rate of ATP turnover and limited ATP stores, thus the cessation of oxidative ATP production as a result of ischemia will compromise both contractile function as well as ionic homeostasis in the cardiomyocyte.

Glycolysis is the only metabolic pathway that is able to produce ATP in the absence of O_2, which increases its importance during periods of ischemia. Limited O_2 availability induces the heart to shift to anaerobic metabolism with the rapid stimulation of glucose uptake, glycogenolysis, and glycolysis (124). The extent of the reliance of the heart on glucose during ischemia is highly dependent on the degree of ischemia (124). Flux through the glycolytic pathway is stimulated during ischemia mainly through activation of PFK-1 as a result of increased AMP levels and decreased ATP and citrate levels (125,213). In

addition, ischemia induces an increase in the supply of G-6-P available for glycolysis via increased glucose uptake and glycogenolysis. Glycogen degradation is stimulated during ischemia due to activation of GP as a result of increased levels of AMP and cAMP as well as due to decreased ATP levels (147). Glycogen stores represent a more energetically favorable source of G-6-P in the ischemic heart as discussed previously in this chapter. In addition, ischemia was shown to induce the translocation of GLUT-4 and GLUT-1 from their intracellular compartment to the plasma membrane (121,214-216) resulting in increased glucose transport. This is suggested to be mediated by ischemia-induced activation of AMPK (217-220). On the other hand, other reports show that ischemia either reduces or has no effect on myocardial glucose uptake. Stanley *et al*. showed in open chest swine that acute myocardial ischemia increases glucose extraction but not glucose uptake (221). Similar results have been obtained in Langendorff-perfused (222) as well as working rat hearts (148,223,224). In addition, AMPK activation was shown to occur without concomitant increase in glucose uptake in skeletal muscle (225) as well as in aerobically perfused isolated working rat hearts (226). The study described in Chapter 3 examines this controversy through studying the interplay between glucose uptake and glycogen turnover and AMPK activation in the ischemic heart.

During mild to moderate ischemia, glycolysis can be maintained for longer periods as long as oxidized NAD^+ is available from the conversion of pyruvate to

lactate. The limited amount of ATP produced from the glycolytic process during ischemia is important in fueling ion pumps for the maintenance of ion homeostasis. Hydrolysis of glycolytically produced ATP produces two protons for each glucose molecule while oxidation of pyruvate via the TCA cycle utilizes one proton for each pyruvate molecule (two protons for each glucose molecule oxidized) (45). During ischemia the rates of glycolysis greatly exceed the rates of glucose oxidation resulting in a net accumulation of lactate and H^+, which can further contribute to the ionic imbalance and ischemic injury. During more severe ischemia, the limited washout of glycolytic byproducts inhibits flux through glycolysis mainly due to the effect of acidosis on PFK-1 and GAPDH (125).

After restoration of coronary flow during reperfusion, ATP production resumes rapidly to almost pre-ischemic rates. However, ATP production during reperfusion is not efficiently translated into mechanical work (227). This is mainly attributed to the fact that during the period of reperfusion, the recovery of ATP production is mainly due to the rapid recovery of mitochondrial fatty acid oxidation at the expense of glucose oxidation (228,229). Fatty acid oxidation, despite producing more ATP than glucose oxidation, utilizes more O_2 for a given rate of ATP synthesis than that utilized by carbohydrates (230). The ATP to oxygen ratio for glucose and lactate are 3.17 and 3.0, respectively, as compared to palmitate and oleate with values of 2.80 and 2.86, respectively (230).

This excessive use of fatty acids during reperfusion further worsens the mismatch in glucose metabolism resulting in an even more pronounced intracellular acidosis. In addition, the restoration of coronary flow washes out the accumulated H^+ resulting in rapid restoration of extracellular pH. This creates a greater transmembrane pH gradient across which H^+ are exchanged with Na^+ resulting in intracellular Na^+ overload which activates the rmNCX resulting in Ca^{2+} overload as described in previous sections in this chapter (20) (Figure 1.6).

Modulation of myocardial substrate metabolism to limit myocardial ischemia/reperfusion injury

As mentioned in previous sections in this chapter, one of the main mechanisms that contribute to I/R injury is the accumulation of H^+ and resultant imbalance in ionic homeostasis, which occurs mainly as a result of derangement in myocardial substrate metabolism. Pharmacological interventions that improve the coupling between glycolysis and glucose oxidation are of particular interest. This can be achieved either via stimulation of glucose oxidation or partial inhibition of glycolysis. Glucose oxidation can be stimulated by activating PDH either directly by dichloroacetate (228) or indirectly by reducing fatty acid oxidation such as by trimetazidine (231).

This thesis mainly focuses on glycolytic inhibition as a means to limit H^+ production and confer cardioprotection. It is established that various interventions

that reduce glycolysis are cardioprotective. These include preconditioning (58) as well as exogenous ADO (232). The study described in Chapter 5 investigates the underlying mechanism of ADO-mediated inhibition of glycolysis. In addition, glycolysis can be also inhibited indirectly by limiting the availability of G-6-P. The study described in Chapter 4 investigated the possible effect of stimulation of glycogen synthesis in repartitioning of G-6-P away from glycolysis and its role in cardioprotection.

1.7 Glycogen synthase kinase-3 and cardioprotection

GSK-3 is a multi-functional kinase involved in several signaling pathways regulating cell fate, protein synthesis, glycogen metabolism, mitosis, and apoptosis (233). It has 2 isoforms, α and β, with strong homology in their kinase domains (234). GSK-3 is constitutively active and is regulated by inhibitory phosphorylation by upstream kinases (e.g., Akt, some isoforms of PKC, PKA, P90RSK, and P70S6 kinase) on Ser 9 (β isoform) or Ser 21 (α isoform) (235). Insulin, through stimulation of the PI3K/Akt pathway, enhances GSK-3 phosphorylation causing its inhibition resulting in accumulation of active GS and increased glycogen synthesis (139).

GSK-3 is unique among other protein kinases in regard to its substrate specificity. Many of its substrates require to be phosphorylated initially by

another protein kinase at a Ser or Thr residue, termed "the priming phosphate", located 4 amino acids carboxy-terminal to the site of GSK-3 phosphorylation (142,236). In the case of GS, one of the main substrates of GSK-3, casein kinase II acts a the priming kinase which phosphorylates Ser 656 providing the recognition motif for GSK-3 to sequentially phosphorylate Ser 652, 648, 644, and 640 as described in previous sections in this chapter. This unique substrate-specificity of GSK-3 also plays a role in its regulation by upstream kinases. It was found that phosphorylated Ser 9 (β-isoform) or Ser 21 (α-isoform) on GSK-3 protein acts as a pseudo-substrate that block the site where the priming phosphate of the substrate binds preventing substrate binding and blocking access to the catalytic center (237).

In the heart, GSK-3 has been shown to play a number of important roles. It was shown that it actively inhibits hypertrophy and that events leading to its inhibition stimulate the development of cardiac hypertrophy (238-241). This effect was mediated via its action on protein synthesis through eIF2B, several transcription factors including GATA-4 and NFAT, as well as its effect on the cellular levels of β-catenin, c-Myc, and cyclin D1 (242). However, a recent report by Hirotani *et al.* showed a beneficial role of GSK-3 inhibition during cardiac hypertrophy and heart failure as it was shown that persistent GSK-3 inhibition results in compensatory hypertrophy rather than pathologic hypertrophy (243).

Recently, inhibition of GSK-3 has been implicated in cardioprotection following I/R. Tong *et al.* were the first to report that ischemic preconditioning-induced infarct size reduction was due to increased GSK-3β phosphorylation and its subsequent inhibition (244). Ischemic preconditioning, insulin administration, and other cardioprotective approaches are unable to protect cardiomyocytes from mice expressing a mutant GSK-3β, which cannot by phosphorylated and thus cannot be inhibited by upstream kinases (41). Furthermore, inhibition of GSK-3 was suggested as the underlying mechanism explaining cardioprotection induced by opioids (22), bradykinin (245), erythropoietin (246), ADO A_3 receptor agonists (98), isoflurane (247,248), and PKCδ inhibition (249). However, the downstream effects that mediate these beneficial effects of GSK-3 inhibition are still not fully understood. One proposed mechanism involves the possible role of GSK-3 inhibition on preventing the opening of the MPTP (41). However, the unknown structure of the MPTP as well as the lack of evidence of a direct interaction between GSK-3 and MPTP weakens this proposed mechanism. Interestingly, it is not known whether the metabolic effects of GSK-3 inhibition might mediate these effects. The study described in Chapter 4 examines the metabolic consequences of pharmacological inhibition of GSK-3 and how they are involved in cardioprotection. It also provides a novel mechanism by which GSK-3 can modulate MPTP opening.

1.8 Adenosine and cardioprotection

ADO is an endogenous nucleoside that acts as a major autocrine and paracrine regulator of cellular function. It generally exerts a range of beneficial actions on the heart and blood vessels. ADO has been termed a "retaliatory" metabolite as it is released from metabolically compromised cells and acts to optimize the balance between energy utilization and generation (250). The effects mediated by ADO in the cardiovascular system were first studied in 1929 by Drury and Szent-Györgyi where they found that various tissue extracts, rich in ADO, result in significant bradycardia, hypotension, and coronary vasodilation (251). This was further studied by Berne in 1963 who proposed the "adenosine hypothesis". He suggested that ADO provided the link between energy supply and demand in the heart through mediating coronary vasodilation during periods of energy deprivation (252).

ADO is generated endogenously mainly via hydrolysis of 5'-AMP (major) as well as via metabolism of s-adenosylhomocysteine (minor) (253,254). Dephosphorylation of AMP to form ADO is catalyzed by 5'-nucleotidases (5'NT) that are either located intracellularly (endo-5'NT) or membrane-bound extracellularly (ecto 5'NT) (255). ADO can be removed from the cell either by rephosphorylation to AMP by ADO kinase, deamination to inosine by ADO deaminase, or removal from the cell by the nucleoside transporters (255). During

ischemia, the imbalance between oxygen supply and demand results in increased breakdown of ATP and subsequently an increase in generation of ADO. ADO production can occur in the cardiomyocyte as well as endothelial and vascular smooth cells (256).

ADO exerts its effects on the cardiovascular system through binding to and activating its four known receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3 ADO receptors). All ADO receptor subtypes are G-protein coupled receptors linked to varying downstream signaling pathways, and are expressed in different cell types of the heart and blood vessels. It is well established that A_1 receptors are expressed in the adult ventricular cardiomyocyte. It is the only ADO receptor subtype identified in the ventricular cardiomyocyte by radioligand binding (257). There are also some reports that show that A_{2A} receptors are expressed in adult ventricular myocytes, but, presently, there is no evidence that either A_{2B} or A_3 receptors are expressed in the ventricular cardiomyocytes (258).

Activation of A_1 receptors in the heart results in a number of physiological effects that are mediated by activation of G_i and inhibition of adenylyl cyclase. These include reduced heart rate, slowed AV conduction, reduced atrial contractility, reduced activity of cardiac pacemaker cells, as well as inhibiting the stimulatory effects of catecholamines. On the other hand, activation of A_{2A} and A_{2B} receptors, located primarily in the vasculature, results in activation of G_s and

subsequent activation of adenylyl cyclase resulting in marked coronary and systemic vasodilation (259).

There have been a large number of studies describing the cardioprotective effects of ADO against I/R injury. ADO-induced cardioprotection is manifest as improvement of post-ischemic mechanical function (reduced myocardial stunning), reduced infarct size (reduced cell death), as well as reduced dysrhythmias. Initially, ADO was regarded to confer cardioprotection via matching energy supply and demand by enhancing coronary flow and reducing energy demand via its negative chronotropic and negative inotropic effects. However, it was found that ADO still exhibits cardioprotective properties in the absence of changes in myocardial perfusion (260-263) as well as in hearts subjected to global ischemia (28,264-266) and in hearts injured by toxic agents that do not limit coronary flow (267). Furthermore, early studies proposed that ADO could possibly act as a substrate for ATP regeneration (268). However, this theory is challenged by the fact that selective ADO receptor agonists are able to elicit cardioprotection and ADO receptor antagonists block ADO-mediated cardioprotection (258). It is now generally accepted that ADO-mediated cardioprotection is mainly mediated through its action on its G-protein coupled receptors. The role of A_1 receptors in mediating cardioprotection is the most widely studied as they are the most abundant ADO receptor subtype in the cardiomyocyte; however, more recent studies have implicated the other subtypes

in mediating cardioprotection either through direct effects on the cardiomyocyte or through effects on other cell types (258). Strong evidence indicates the involvement of adenosinergic receptors in mediation of intrinsic protection through preconditioning as well as postconditioning (269,270). Exogenous administration of ADO or ADO receptor agonists has been shown to reduce infarct size, improve post-ischemic recovery of function, and prolong time to ischemic contracture (250).

Downstream of ADO receptor activation, many signaling pathways have been shown to play a role in ADO-induced cardioprotection. These involve modulation of the activity of PKC, PI3K as well as mitogen-activated protein kinases (MAPKs) to alter mitochondrial targets such as the mitochondrial K_{ATP} channels and MPTP. ADO also exerts significant effects on myocardial energy substrate metabolism, and these play an important role in modulating the myocardial response to ischemia and reperfusion. In this thesis, one focus is on how the effects of ADO on myocardial glucose metabolism might be involved in cardioprotection.

Adenosine and myocardial glucose metabolism

The effects of ADO on myocardial glucose metabolism are not completely understood and results from previous studies show conflicting results. A number of studies reported that ADO, as well as ADO receptor agonists, stimulates

myocardial glucose uptake (271-276) by a mechanism that either involves potentiation of insulin signaling (271-273,276,277) or mechanisms that are independent of insulin (274). ADO was also shown to stimulate myocardial glycolysis in isolated hearts perfused with glucose as the sole energy substrate (278,279). These effects of ADO were suggested to act to increase myocardial glucose utilization and ATP generation during ischemia and reperfusion thus ameliorating ischemic injury. However, as mentioned in previous sections of this introduction, the stimulation of myocardial glycolysis with no corresponding increase in glucose oxidation results in intracellular acidosis, which can exacerbate injury. On the other hand, several reports have shown that ADO, as well as activation of A_1 receptors, decreases the rate of myocardial glucose uptake and glycolysis (28,224,232,280-284). Gao *et al.* showed in isolated working guinea pig hearts that ADO receptor antagonism increases glucose uptake and glycolysis both during aerobic conditions as well as during mild hypoperfusion (280). Increased glycolytic rates were attributed to an increase in PFK-1 activity as measured by an increase in the ratio of F-1,6-BP to F-6-P. In the isolated working rat heart perfused with both glucose and palmitate as energy substrates, ADO inhibits glycolysis and proton production under aerobic conditions without affecting LV mechanical function (283). A similar inhibition of glycolysis and H^+ production induced by ADO during reperfusion following severe ischemia results in improved recovery of post-ischemic LV mechanical function (232,282). These effects of ADO are mimicked by A_1 selective agonists and are antagonized by A_1-

selective antagonists showing that activation of A_1 receptors plays a major role in these effects (28,224,284). Thus, ADO-mediated inhibition of glycolysis and subsequent inhibition of H^+ production seems to be causally related to improved post-ischemic recovery of mechanical function. In a different heart model, where hearts are acutely stressed by transient antecedent ischemia prior to an episode of more severe ischemia, ADO stimulates glycolysis and H^+ production and impairs recovery of LV work during reperfusion following severe ischemia (285). This highlights the importance of creating a better understanding of the mechanisms by which ADO modulates myocardial glycolysis. The study described in Chapter 5 investigates the mechanism by which ADO inhibits glycolysis, mainly looking at the role of stress-activated protein kinases and protein phosphatases in mediating these effects.

1.9 Stress-responsive protein kinases

1.9.1 5'-AMP activated protein kinase

AMPK was originally discovered in 1973 in the liver where it was found to act as a regulator of fatty acid and cholesterol biosynthesis (286,287), but it was not until 1995 that AMPK was first demonstrated to be expressed in heart (288). AMPK is named after its allosteric activator AMP (289). AMPK is now regarded as a cellular "energy sensor" as it is activated during periods of energetic stress to

increase catabolic ATP-producing processes, while inhibiting anabolic ATP-consuming pathways (290).

AMPK is a heterotrimeric Ser/Thr protein kinase comprised of a catalytic α subunit and regulatory β and γ subunits. Each subunit is encoded by multiple genes with a number of splice variants yielding at least 12 different heterotrimeric combinations (290). The N-terminus of the α subunit contains the catalytic domain and an important regulatory residue (Thr172) whose phosphorylation is required for AMPK activity (291) while its C-terminus is required for binding with the β and γ subunits (292). The β subunit has a central N-isoamylase domain that enables it to bind to glycogen (293,294). The significance of AMPK binding to glycogen and the physiological role of the β subunit is still not clear (290). The γ subunit contains 4 tandem repeats of a sequence called cystathionin-β-synthase domain which function in pairs to form what is called the Bateman domains which bind AMP or ATP (295-297).

The function of AMPK as an energy sensor relies on its sensitivity to changes in the ratio of AMP to ATP. AMP can bind to the Bateman domain on the γ regulatory subunit of AMPK to activate it by three independent mechanisms: a) allosteric activation of the phosphorylated enzyme; b) promotion of phosphorylation of Thr172 on the α catalytic domain by upstream kinases (AMPKK); c) hindering dephosphorylation of the same residue by PP2C (298). These multiple targets of AMPK regulation by AMP render AMPK

"ultrasensitive" to AMP where small changes in the concentration of free AMP may result in marked changes in AMPK activity (298). It is worthwhile to note that most of intracellular AMP is bound to proteins where free AMP content is orders of magnitude lower that total cellular AMP content. Thus changes in AMPK activity can occur as a result of small alteration in free AMP concentration in the absence of changes in total AMP content (299). More recently, two reports indicate that AMPK is not only sensitive to AMP and ATP but also to ADP (300,301). ADP, similar to AMP, hinders the dephosphorylation of Thr-172 but, unlike AMP, does not allosterically activate AMPK. Thus, both AMP/ATP ratios and ADP/ATP ratios contribute in AMPK regulation. AMPK may be regarded as direct adenylate charge-sensing protein kinase rather than specifically an AMP-activated protein kinase. This goes in line with Atkinson's "adenylate charge hypothesis" for regulating metabolic pathways which has been published more than 4 decades ago (302)

The identification of AMPKK has been a subject of extensive research. Several AMPKKs have been identified, including LKB1, Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ), and transforming growth factor-β-activated protein kinase-1 (TAK1) (303-311). However, it appears that there may be additional AMPKKs, which mediate ischemia-induced activation of AMPK in the heart (312). PPs also play an important role in regulation of AMPK activity. Both PP2C and PP2A are able to dephosphorylate Thr172 and reduce the activity

of AMPK (313). Binding of AMP to the γ-subunit of AMPK reduces the ability of PP2C to dephosphorylate AMPK (313). PP2A was also reported to dephosphorylate AMPK and a recent study showed that palmitate activates PP2A and inhibits AMPK in endothelial cells, an effect that can be blocked by the use of PP2A inhibitors (314).

Role of AMPK in regulation of myocardial energy substrate metabolism

One of the main roles of AMPK in the heart is to phosphorylate downstream targets involved in the control of myocardial glucose and fatty acid metabolism. Activation of AMPK stimulates GLUT-4 translocation in skeletal muscle during hypoxia (218) and pharmacological activation of AMPK in isolated rat ventricular papillary muscle increases GLUT-4 translocation as well as glucose uptake (219). In addition, activation of AMPK reduces endocytosis of GLUT-4, thereby increasing the sarcolemmal content of GLUT-4 (315). Russell *et al.* further supported the role of AMPK in mediating ischemia-induced increase in glucose uptake by studies using transgenic mice expressing a kinase dead form of AMPK, in which low-flow ischemia fails to enhance glucose uptake or glycolysis (220). On the other hand, some studies show a dissociation between AMPK activation and increased glucose uptake in both skeletal muscle and heart (225,226). The study described in Chapter 3 examines the role of myocardial glycogen content in the relationship among ischemia-induced activation of AMPK, GLUT-4 translocation and glucose uptake.

The role of AMPK in glycogen metabolism has gained great interest since the discovery of a mutation in the AMPK regulatory γ_3 subunit gene (PRKAG3) of purebred Hampshire pigs that causes significant glycogen accumulation in skeletal muscle (316). This was followed by the discovery of a number of mutations in the gene encoding the AMPK γ_2 subunit (PRKAG2) in the hearts of patients which result in a glycogen-storage cardiomyopathy characterized by ventricular preexcitation (Wolf-Parkinson-White syndrome) and progressive conduction system disease, which in some cases can be accompanied by marked cardiac hypertrophy (317-323). The best-characterized mutations in the PRKAG2 gene are the missense mutations that occur in the DNA encoding amino acids 488 and 302 of the γ_2 subunit (PRKAG2 N408I and PRKAG2 R302Q) (324). Hearts from transgenic mice over-expressing these mutations exhibit marked hypertrophy with excessive glycogen accumulation. However, hearts expressing R302Q mutations have decreased AMPK activity in contrast with hearts expressing N488I mutation, which have increased AMPK activity (325,326). This shows that the relationship between AMPK and glycogen metabolism is rather complex and can be affected by factors other than enzyme activity.

AMPK is also able to stimulate myocardial glycolysis via phosphorylation of PFK-2 at Ser466 resulting in its activation (165). This results in increased F-2,6-BP which acts as an allosteric activator of PFK-1 that catalyzes the rate-limiting step of the glycolytic pathway. The increased flux through glycolysis as

a result of AMPK activation is important during ischemia to stimulate anaerobic production of ATP (165). The study described in Chapter 5 investigates the possible role of AMPK in ADO-mediated inhibition of glycolysis.

AMPK also plays an important regulatory role in myocardial fatty acid metabolism. AMPK activation results in increased uptake of NEFA into the cardiomyocyte via increased abundance of the fatty acid transporters at the sarcolemma (327,328). AMPK also increases the recruitment of lipoprotein lipase to the coronary lumen thus increasing the availability of NEFA to the heart (329). In addition, AMPK is reported to phosphorylate and inhibit ACC resulting in reduced malonyl CoA levels, increased transport of fatty acids into the mitochondria, and increased fatty acid oxidation rates (208,330). Thus, AMPK plays a central role in mediating increased myocardial fatty acid utilization via increased cellular and mitochondrial uptake.

Role in ischemia/reperfusion injury

It is well established that AMPK is rapidly activated during myocardial ischemia (208,220,331). This activation is accompanied by significant changes in glucose and fatty acid metabolism, which are considered an adaptive response to reduce ATP utilization and increase ATP production during ischemia and reperfusion. Whether this adaptive response ameliorates or potentiates myocardial I/R injury remains controversial. A number of recent studies show

that pharmacological activation of AMPK during ischemia protects against ischemic injury (332,333) and transgenic models with an AMPK α_2 kinase-dead mutation exhibit increased injury following ischemia and reperfusion (220,334). In addition, treatments that activate AMPK, including metformin, adiponectin, 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR or acadesine) and caloric restriction, also ameliorate I/R injury (335-338). On the other hand, mouse hearts with a dominant-negative α_2-subunit of AMPK (a model with a milder degree of AMPK suppression) are not energetically compromised and exhibit improved post-ischemic recovery of LV function (339). Such conflicting results may have arisen due to differences in the extent of AMPK inhibition or potential differential consequences arising from inhibition during ischemia or during reperfusion (not controllable in transgenic models). This difficulty, and the lack of reliable pharmacological agents to alter AMPK activity, has limited examination of the role of AMPK during reperfusion, as distinct from ischemia. While the metabolic consequences of AMPK activation are potentially beneficial during ischemia when energy supply is limited, during reperfusion, AMPK-mediated effects may exacerbate the injury. Stimulation of glycolysis and fatty acid oxidation as a result of AMPK activation with subsequent limitation of glucose oxidation will result in increased intracellular acidosis and the activation of the coupled exchangers that promote Na^+ and Ca^{2+} accumulation. Thus, during ischemia, stimulation of glycolysis by AMPK activation may be beneficial, while during reperfusion activation of glycolysis may be detrimental. The study described in

Chapter 5 presents an experimental model in which AMPK is inhibited only during reperfusion with no effect on ischemia-induced AMPK activation. This will help to better understand the effects of modulating AMPK activity during the reperfusion period and how that can affect post-ischemic recovery of mechanical function.

1.9.2 p38 mitogen-activated protein kinase

p38 MAPK is a stress-activated protein kinase that belongs to the MAPK superfamily of protein kinases. The MAPK family is a highly conserved family of proteins from yeast to humans that plays an important role in a variety of biological processes ranging from gene expression, mitosis, movement, metabolism, and programmed cell death (340,341). It is comprised mainly of four subfamilies including extracellular signal-regulated kinases (ERK1/2), c-jun NH_2-terminal kinases (JNK1, -2, and -3), big MAPK (BMK), and p38 MAPK (341,342). ERK1/2 pathway is mainly stimulated by the action of growth factors (343) while JNK and p38 MAPK are stress-activated MAPK that are activated by physical, chemical, and physiological stressors. These include ultraviolet light, osmotic shock, oxidative stress, infection, cytokines, as well as ischemia and hypoxia (344).

All MAPKs are activated via a canonical cascade culminating into the dual phosphorylation of a Thr-X-Tyr motif in the regulatory loop of MAPK protein

leading to its activation (345,346). This is accomplished by the action of a three-level kinase cascade where a MAPK kinase kinase phosphorylates and activates a downstream MAPK kinase, which in turn phosphorylates and activates the MAPK. This is a universal pathway for all MAPKs, which allows for signal modulation and amplification and higher specificity of the signaling pathway in response to different stimuli (347). Recently, it was reported that p38 MAPK can be activated by alternative non-canonical pathways. This can be either by TAB-1 mediated autophosphorylation as well as via T-receptor-induced cell activation through ZAP70 (348,349).

Four different isoforms of p38 MAPK have been identified so far. These include the prototypical isoform p38α (often referred to as simply p38), p38β (350), p38γ (351), and p38δ (352). The α-isoform is the predominant isoform present in the heart; however, p38β is also expressed in the heart (342). p38 MAPK is highly and rapidly activated by myocardial ischemia and reperfusion (353,354). Some reports show that it is activated during ischemia and this activation is maintained during reperfusion (353,354) while others report that it is only activated during reperfusion due to increased oxidative stress (355). The role of I/R-induced activation of p38 MAPK in I/R injury remains controversial. Several reports show that reducing the extent of p38 MAPK activation during ischemia and reperfusion confers cardioprotection (356-361) while other reports support a protective role of p38 MAPK during ischemia and reperfusion (362-

364). The protective role of p38 MAPK is usually observed within the context of ischemic preconditioning where p38 MAPK is activated during the preconditioning cycles and its inhibition blocks the benefits of preconditioning. However, it is worth noting that preconditioning, despite activating p38 MAPK initially, results in a lower degree of p38 MAPK activation during sustained periods of ischemia following the preconditioning cycle as compared to non-preconditioned hearts (365-367). These results can be reconciled on the basis of the explanation that during preconditioning, the modest and transient activation of p38 MAPK activates a negative feedback mechanism resulting in its inhibition during sustained index ischemia (342). In addition, pharmacological inhibition of p38 MAPK as well as in p38 $\alpha^{+/-}$ mice, reduces p38 MAPK activity and results in cardioprotection (368,369).

Despite the great interest in the role of p38 MAPK in myocardial function and its role in I/R injury, little is known about its role in myocardial metabolism. In isolated perfused rat hearts, p38 MAPK mediates leptin-induced stimulation of fatty acid oxidation (370). p38 MAPK also participates in the increased glucose transport due to ischemia, uncoupling of oxidative phosphorylation, as well as chronic myocardial hibernation (371-373). These effects occur downstream of activation of AMPK, probably as a result of increased recruitment of p38 MAPK to TAB1 (372-375). Previous work from our group showed that p38 MAPK mediates ADO-induced stimulation of glycolysis and inhibition of glycogen

synthesis in hearts subjected to acute ischemic stress prior to a longer period of sustained ischemia (376,377). However, in that experimental model, p38 MAPK activation was observed to occur upstream of AMPK activation. The study described in Chapter 5 examines the role of p38 MAPK in ADO-mediated inhibition of glycolysis and cardioprotection in non-stressed hearts and the consequences of pharmacological inhibition of p38 MAPK on glycolysis and post-ischemic recovery of mechanical function.

1.10 Hypothesis and aims

1.10.1 Hypothesis

Myocardial energy substrate metabolism is subjected to significant changes as a result of myocardial ischemia and reperfusion. These changes can greatly influence outcomes following I/R injury. I hypothesize that the partitioning of glucose between the different pathways of glucose metabolism to limit excessive myocardial glycolysis during reperfusion should protect against myocardial I/R injury and enhance post-ischemic recovery of mechanical function.

1.10.2 Specific Aims

1. To examine the role of pre-ischemic glycogen content on ischemia-induced activation of AMPK and subsequent alterations in glucose metabolism (described in Chapter 3).

2. To determine the consequences of repartitioning G-6-P away from glycolysis by stimulation of glycogen synthesis through the use of pharmacological inhibitors of GSK-3 on calcium homeostasis, and recovery of post-ischemic LV mechanical function (described in Chapter 4).

3. To identify the mechanism underlying ADO-mediated inhibition of glycolysis and ADO-induced cardioprotection and to examine specifically the role of the stress-responsive protein kinases, AMPK and p38 MAPK, as well as PPs in mediating these effects (described in Chapter 5).

Figure 1.1 Schematic representation of the different metabolic pathways involved in glucose-6-phosphate metabolism.

Figure 1.1

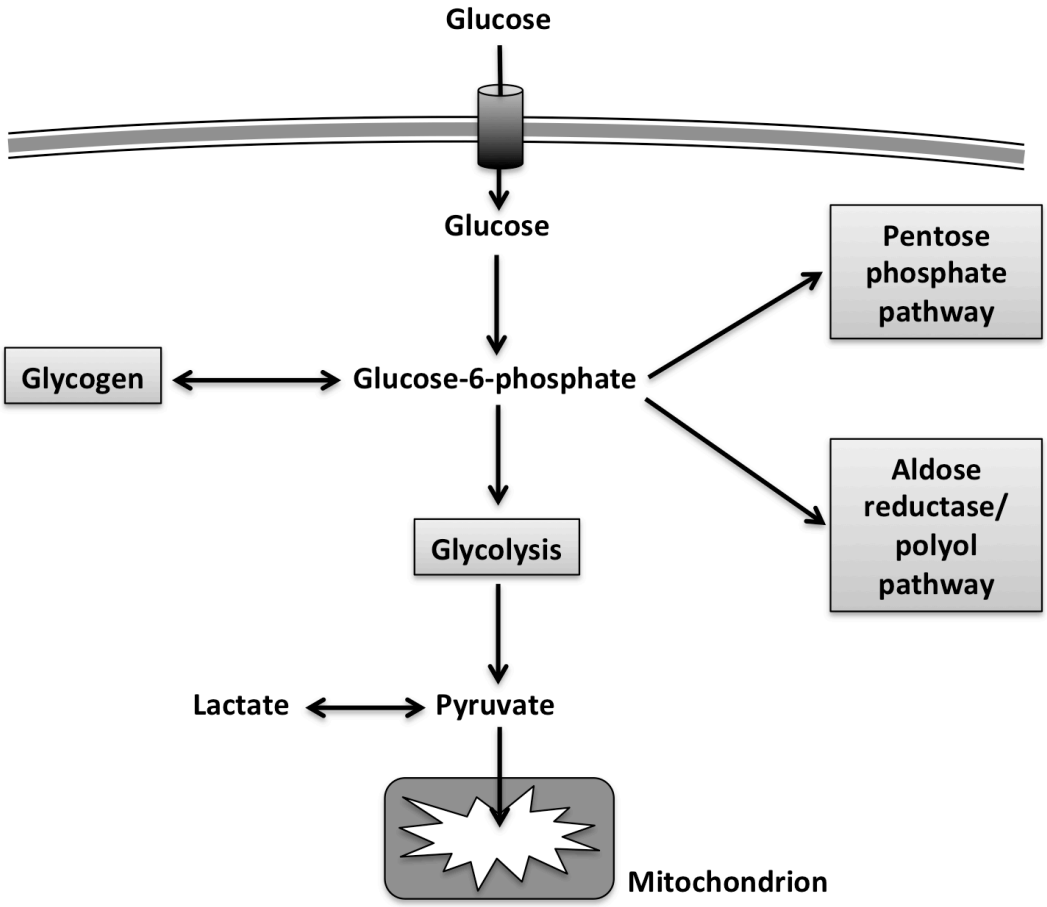

Glucose
Glucose
Pentose phosphate pathway
Glycogen
Glucose-6-phosphate
Aldose reductase/ polyol pathway
Glycolysis
Lactate
Pyruvate
Mitochondrion

Figure 1.2 Schematic representation of the pathways involved in glycogen turnover.

Figure 1.2

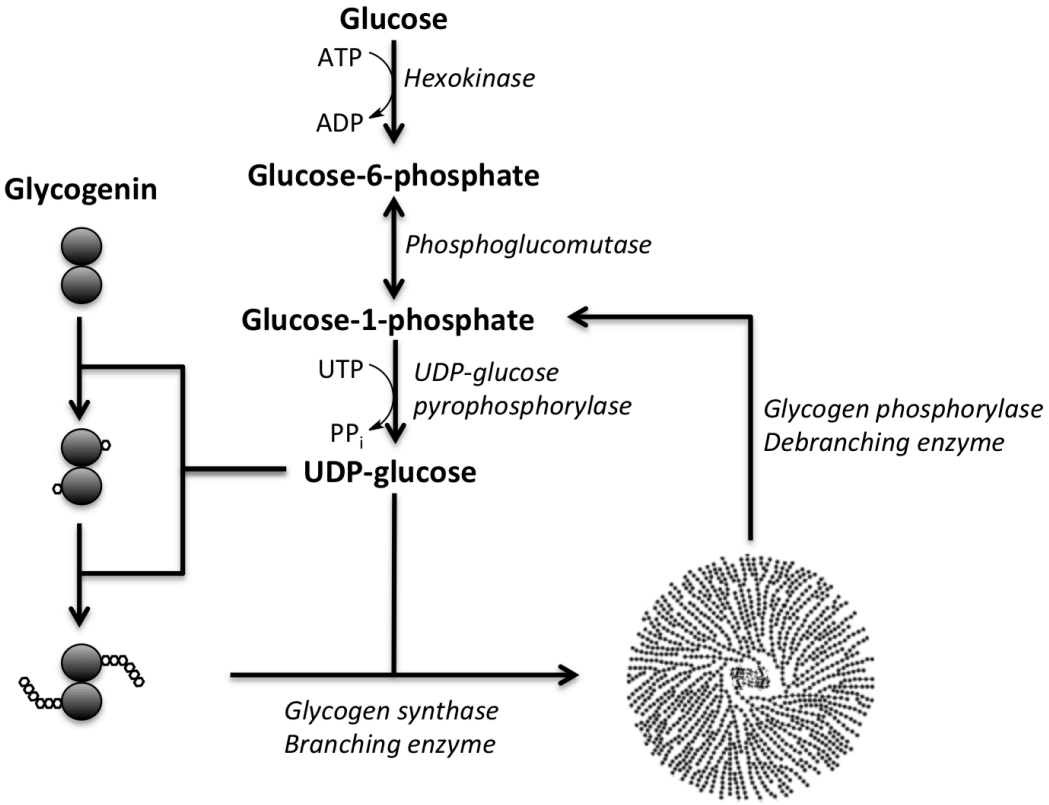

Glucose
ATP
Hexokinase
ADP
Glucose-6-phosphate
Glycogenin
Phosphoglucomutase
Glucose-1-phosphate
UTP
UDP-glucose
pyrophosphorylase
PPi
Glycogen phosphorylase
Debranching enzyme
UDP-glucose
Glycogen synthase
Branching enzyme

Figure 1.3 Schematic representation of the catabolism of glucose via glycolysis.

Figure 1.3

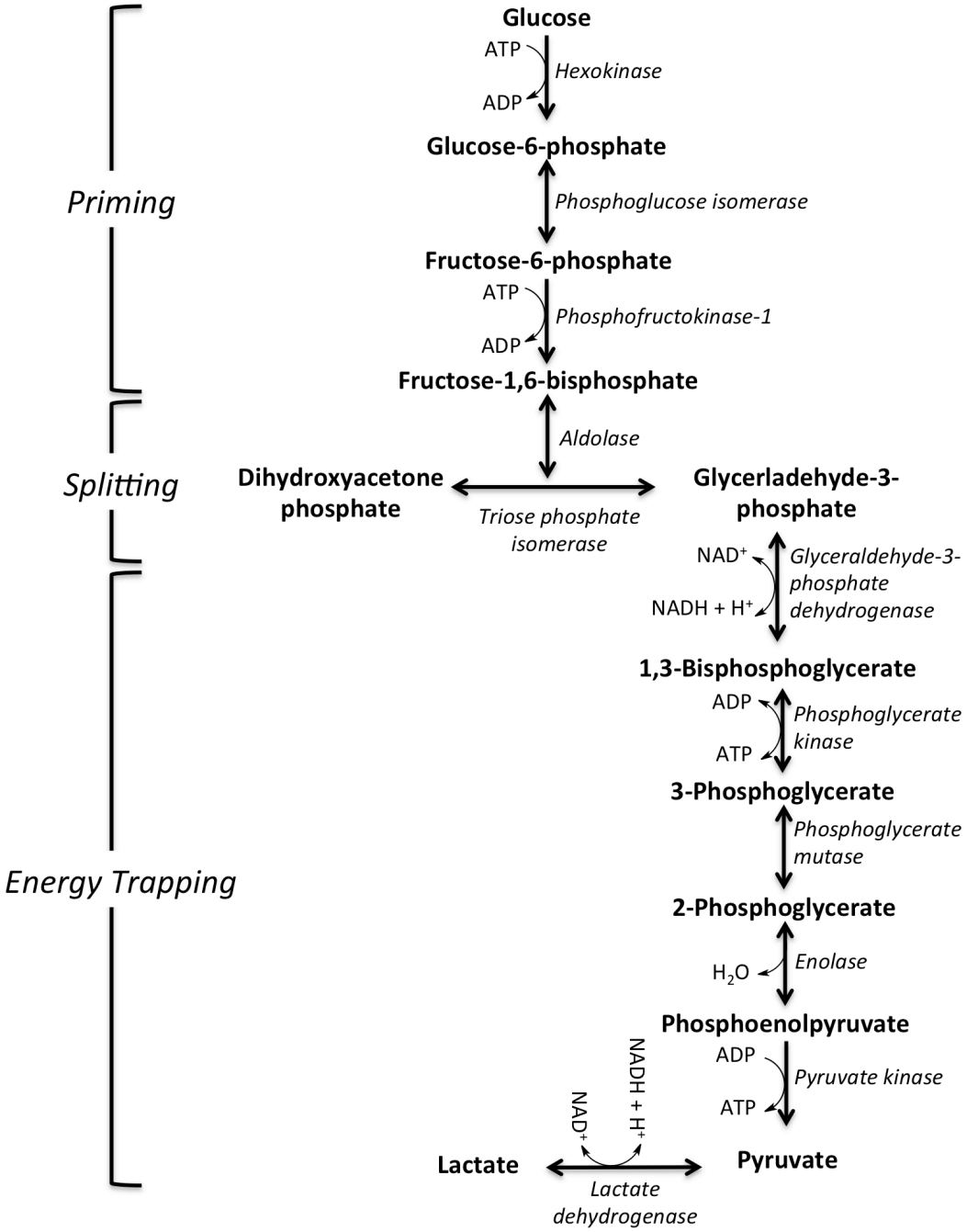

Glucose
ATP
ADP
Hexokinase
Glucose-6-phosphate
Phosphoglucose isomerase
Fructose-6-phosphate
ATP
ADP
Phosphofructokinase-1
Fructose-1,6-bisphosphate
Aldolase
Dihydroxyacetone phosphate
Triose phosphate isomerase
Glycerladehyde-3-phosphate
NAD+
NADH + H+
Glyceraldehyde-3-phosphate dehydrogenase
1,3-Bisphosphoglycerate
ADP
ATP
Phosphoglycerate kinase
3-Phosphoglycerate
Phosphoglycerate mutase
2-Phosphoglycerate
H2O
Enolase
Phosphoenolpyruvate
ADP
ATP
Pyruvate kinase
Lactate
NAD+
NADH + H+
Lactate dehydrogenase
Pyruvate
Priming
Splitting
Energy Trapping

Figure 1.4 Regulation of phosphofructokinase-1 (PFK-1).

Figure 1.4

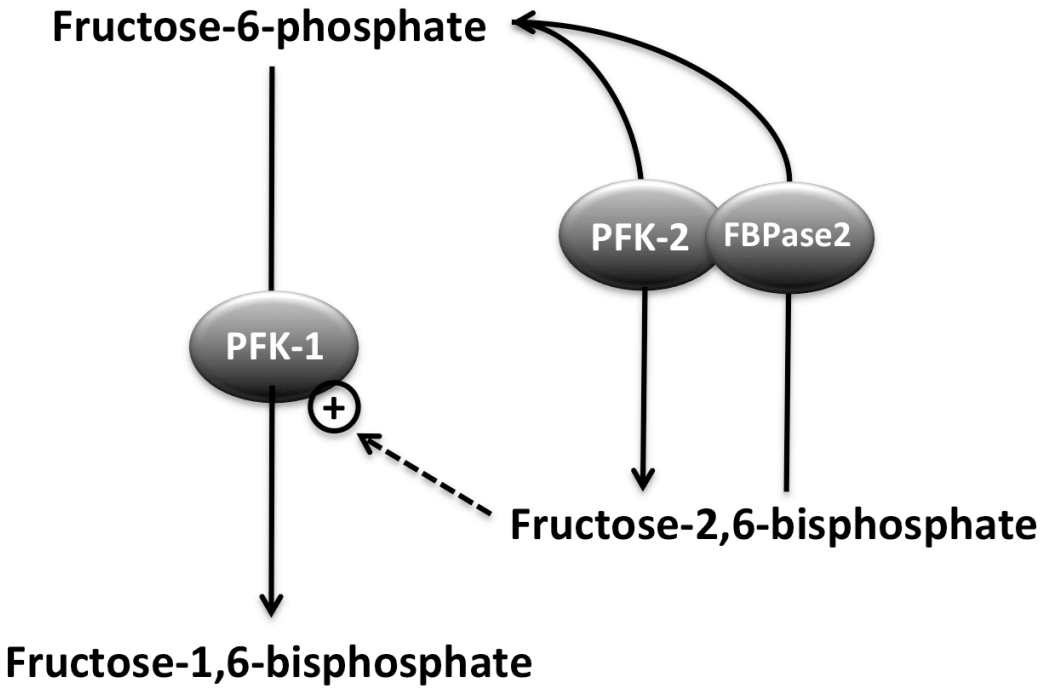

Fructose-6-phosphate
PFK-2
FBPase2
PFK-1
+
Fructose-2,6-bisphosphate
Fructose-1,6-bisphosphate

Figure 1.5 Metabolic fate of pyruvate.

Figure 1.5

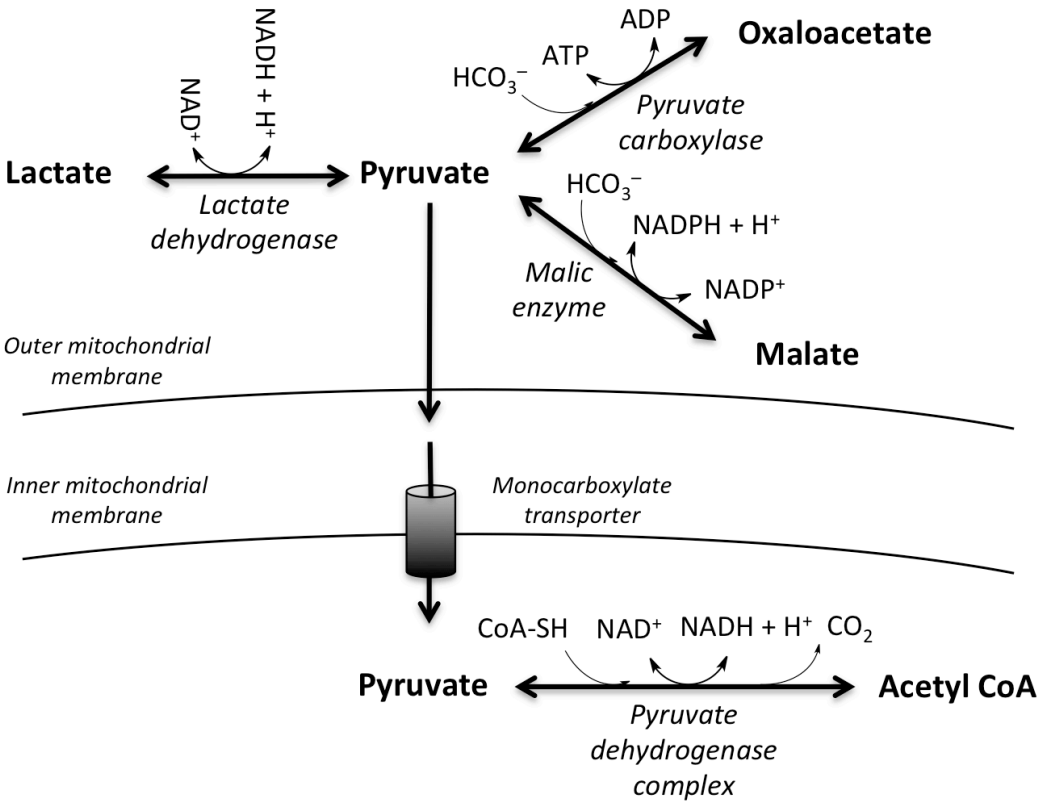
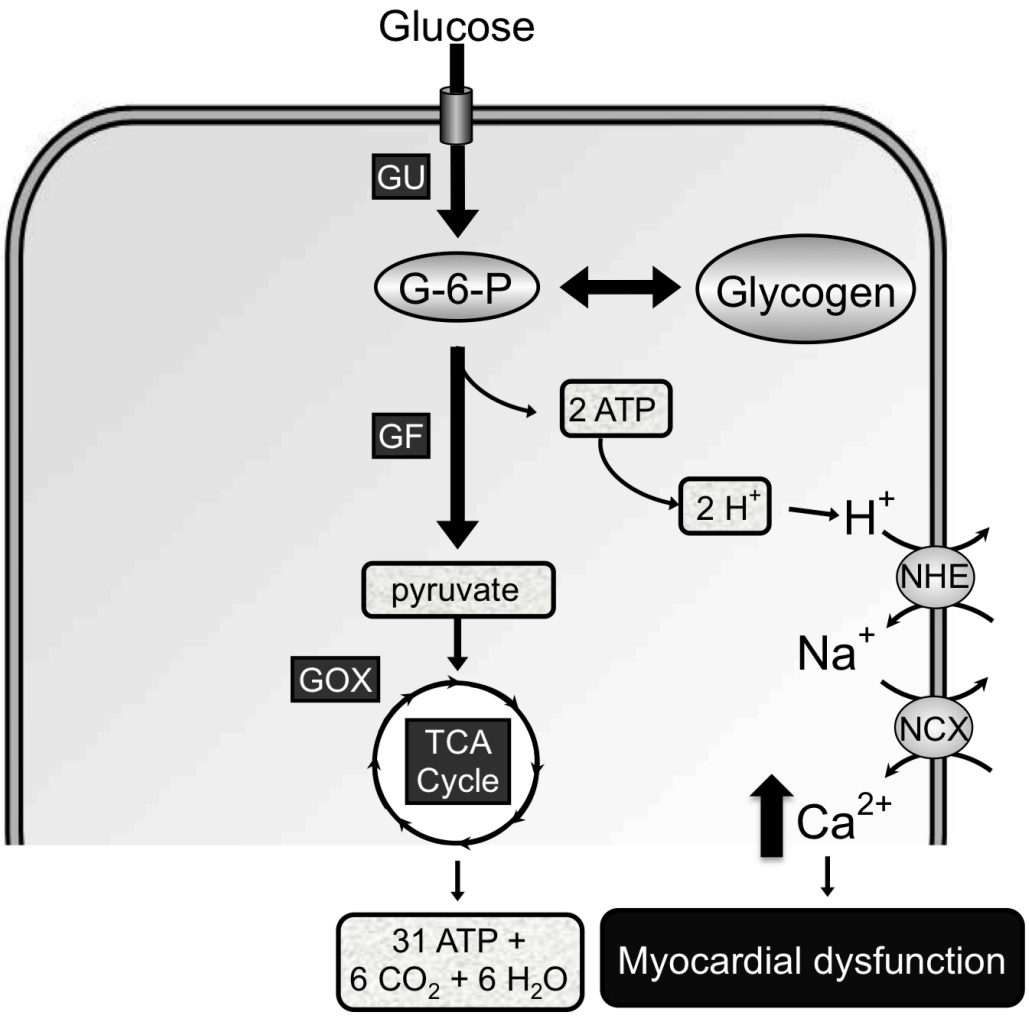

NADH + H+
NAD+
Lactate
Pyruvate
Lactate dehydrogenase
ADP
ATP
HCO3−
Oxaloacetate
Pyruvate carboxylase
HCO3−
NADPH + H+
Malic enzyme
NADP+
Malate
Outer mitochondrial membrane
Inner mitochondrial membrane
Monocarboxylate transporter
CoA-SH
NAD+
NADH + H+
CO2
Pyruvate
Acetyl CoA
Pyruvate dehydrogenase complex

Figure 1.6 Imbalance in the cardiomyocyte ionic homeostasis as a result of uncoupled glucose metabolism.

Figure 1.6


Glucose
GU
G-6-P
Glycogen
GF
2 ATP
2 H+
H+
NHE
pyruvate
Na+
GOX
NCX
TCA Cycle
Ca2+
31 ATP +
6 CO2 + 6 H2O
Myocardial dysfunction

2. Materials and Methods

2.1 Materials

[U-^{14}C]glucose, D-[5-^{3}H]glucose and [9,10-^{3}H]palmitic acid were obtained from Perkin Elmer (Boston, Massachusetts). Bovine serum albumin (BSA fraction V, free fatty acid free) was purchased from Equitech-Bio, Inc. (Kerrville, Texas). Insulin (Novolin® ge Toronto) was obtained through the University of Alberta Hospital stores from Novo Nordisk (Mississauga, Ontario). Ecolite™ and Cytoscint™ Aqueous Counting Scintillation fluids were obtained from MP Biomedicals (Solon, Ohio). Hyamine hydroxide (1 M in methanol solution) was purchased from J.T. Baker (Phillipsburg, New Jersey). AG® 1-X4 anion exchange resin, chloride form, 4% crosslinkage, 200–400 dry mesh size was obtained from Bio-Rad Laboratories, Inc. (Hercules, California). Glucose assay kit was purchased from Sigma Diagnostics (St. Louis, Missouri). Protease inhibitor cocktail was obtained from Sigma-Aldrich, Inc. (St. Louis, Missouri). Phosphatase inhibitor cocktail was obtained from Calbiochem (Darmstadt, Germany). For HPLC analysis of adenine nucleotides, a Supelcosil™ LC-18-T Super Guard cartridge, 5 µm particle size, 2 x 4 mm and a Supelcosil™ LC-18-T, 5 µm particle size, 250 x 4.6 mm column were purchased from Supelco/Sigma-Aldrich (St. Louis, Missouri). Indo-1(AM) was purchased from TEFLabs, Inc. (Austin, Texas). Bio-Rad protein assay dye reagent concentrate, nitrocellulose membranes (Trans-Blot® Transfer Medium, 0.45 µm), Precision plus protein™

dual color molecular weight marker, and Mini-Protean III gel electrophoresis system were purchased from Bio-Rad Laboratories, Inc. (Hercules, California). Amersham ECL Plus Western blotting detection system was purchased from GE Healthcare (Buckinghamshire, UK). Fuji medical X-ray films (Super RX) were from FUJIFILM Europe Gmbh (Düsseldorf, Germany). Kodak BioMax MR films were from Carestream Health, Inc (Rochester, New York). Monoclonal and polyclonal antibodies for GLUT-4, Na^+-K^+ATPase α1, AMPK, phospho-AMPK (Thr172), p38 MAPK, phosph-p38 MAPK (Thr180 and Tyr182), LKB1, phospho-LKB1(Ser428) and peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from Cell Signaling Technology (Danvers, Massachusetts). Antibodies to phospho-PFK-2 (SER466) and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Aldolase (rabbit muscle), α-glycerophosphate dehydrogenase type I (rabbit muscle, glucose-6-phosphate dehydrogenase type VII (Bakers yeast), and phosphoglucose isomerase type III (Bakers yeast) were purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri). All other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri).

2.2 Methods

The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and complies with Canadian Council of Animal Care guidelines.

2.2.1 Isolated working rat heart

Hearts were cannulated for isolated working mode perfusion as described previously (378). Rats were deeply anesthetized using pentobarbital sodium (60 mg/kg, ip) and each heart was rapidly excised and placed in ice-cold Krebs-Henseleit solution followed immediately by aortic cannulation and perfusion in non-working Langendorff mode for 10 min. Hearts were then switched to working mode by clamping off the aortic inflow line and opening the left atrial and the aortic outflow lines, and perfused at 37 °C at constant workload (11.5 mmHg left atrial preload and 80 mmHg aortic afterload) and rate (paced at 300 beats/min). The perfusate (pH 7.4, recirculating, 100 ml) consisted of a modified Krebs-Henseleit solution containing (mM) 118.0 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 2.5 free Ca^{2+} along with insulin (100 mU/l) and both palmitate (1.2 mM, prebound to 3% BSA) and glucose (11 mM) as energy substrates. Gassing with carbogen (95% CO_2-5% O_2) in a thin-film oxygenator maintained perfusate pH and O_2 saturation. At the end of each perfusion protocol,

hearts were rapidly frozen using a Wollenberger clamp cooled to the temperature of liquid N_2. Frozen tissues were pulverized at the temperature of liquid N_2, and the resulting powders were stored at -80 ºC.

2.2.2 Left ventricular function measurement

During working mode perfusion, systolic and diastolic aortic pressures were measured using a Gould P21 pressure transducer attached to the aortic outflow line. Ultrasonic flow probes (Transonic T206), placed in the left atrial inflow line and the aortic outflow line, were used to measure cardiac output (mL/min) and aortic flow (mL/min), respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. Coronary vascular conductance (CVC, mL/min/mmHg/g dry wt) was calculated as the ratio of coronary flow and mean aortic pressure. Left ventricular (LV) work (Joules/min/g dry wt), calculated as the product of the cardiac output and LV developed pressure (systolic pressure – preload pressure) and normalized to the heart dry weight, was used as a continuous index of LV mechanical function.

2.2.3 Measurement of the rates of glycolysis, glucose and palmitate oxidation

Steady state rates of glycolysis and glucose oxidation were measured by perfusing hearts with [5-^{3}H/U-^{14}C] glucose while rates of palmitate oxidation

were measured by perfusing hearts with [9,10-^{3}H]palmitate as described previously (379). The radiolabelled substrates were included in the recirculating Krebs solution during the period of working mode perfusion. Two separate series of experiments were performed, one with [5-^{3}H/U-^{14}C]glucose and the other with [U-^{14}C]glucose and [9,10-^{3}H]palmitate. Rates of glycolysis were determined by the quantitative determination of $^{3}H_2O$ liberated from [5-^{3}H]glucose at the enolase step of glycolysis. $^{3}H_2O$ was separated from the perfusate by passing 100 μL of perfusate samples through AG® 1-X 4 anion exchange resin (200-400 mesh) columns as described previously (189). This was followed by washing the columns with 800 μL of water and the eluted water was collected in 5 mL scintillation vials and scintillation fluid was added (Ecolite, ICN) followed by counting the vials in a liquid scintillation counter. Rates of glucose oxidation were determined by the quantitative determination of $^{14}CO_2$ liberated from [^{14}C]glucose at the level of pyruvate dehydrogenase and in the TCA cycle. Both $^{14}CO_2$ gas released (captured in a hyamine hydroxide trap) as well as [^{14}C]bicarbonate retained in the perfusate (measured through reaction with 9N H_2SO_4) were accounted for (189). Rates of palmitate oxidation were measured by the quantitative determination of $^{3}H_2O$ liberated from [9,10-^{3}H]palmitate. Liberated $^{3}H_2O$ was separated from the perfusate by a vapor transfer method (380). This method consists of adding 500 μl of water into a 5 mL scintillation vial, then placing a lidless 1.5 ml micro-centrifuge tube containing 200 μL of the perfusate inside the scintillation vial and then the vial is capped. Scintillation

vials are then stored initially at 50 °C overnight and then at 4 °C for 24 hours. Following storage, the micro-centrifuge tube is removed, any water droplets on the wall of the micro-centrifuge tube was collected using a filter paper (1 cm X 1 cm) and dropped in the vial. Scintillation fluid is added and the vials are counted in a liquid scintillation counter. Perfusate samples were collected at 10 min intervals and rates (expressed as μmol substrate metabolized/min/g dry wt) were calculated for each time interval and were averaged for each phase of the perfusion protocol.

2.2.4 Calculation of the rate of proton production from glucose metabolism

The hydrolysis of ATP derived from glycolysis produces 2 protons for each glucose molecule while the oxidation of pyruvate via the TCA cycle utilizes 1 proton for each pyruvate molecule (2 protons for each glucose molecule oxidized) (45). Thus, if the rate of glycolysis exceeds the rate of glucose oxidation there will be net proton production, the rate of which was calculated as 2 X (glycolysis rate – glucose oxidation rate) as described previously (381). This method of calculation was validated previously as compared to pH_i measurement using ^{31}P NMR spectroscopy (381).

2.2.5 Measurement of glycogen content and turnover

Myocardial glycogen content (μmol glucosyl units/g dry wt) was determined as described previously. Glycogen was extracted from approximately 150 mg of ventricular tissue by boiling with 300 μL of 30% KOH for 1 hr in Corex® glass tubes. Samples were then cooled to room temperature followed by the addition of 200 μL of 1% Na_2SO_4 followed by 2 mL of absolute ethanol. Tubes were then covered and left overnight at -20 ºC. This was followed by centrifugation at 1000 g for 5 min and the supernatant was discarded. The remaining glycogen-rich pellet was then washed with 2 mL of 66% ethanol and allowed to dry. Glycogen in the pellet was converted to glucose by boiling with 2N H_2SO_4 for 3 hrs. The resultant solution was mixed with 0.5 mL 1M MOPS and neutralized to pH 6.8-7.0 using KOH. The neutralized solution was then centrifuged at 1000 g for 10 min and glucose content was measures in the supernatant using a Sigma® glucose analysis kit. In addition, the amount of radiolabelled glucose in glycogen extracts was also determined to evaluate the degree of incorporation of radiolabelled glucose into glycogen. The rate of glycogen synthesis was calculated from the increase of the content of radiolabelled glucose in the glycogen pool during the period of interest. The rate of glycogen degradation was calculated from the change in the content of the non-radiolabelled glucose in the glycogen pool during the period of interest.

2.2.6 Calculation of the rate of glucose uptake

Glucose uptake (μmol/min/g dry wt) was calculated as described previously (226) as the sum of the rates of glycolysis and glycogen synthesis. This method of assessment of glucose uptake accounts for each of the two main fates of glucose following its uptake into the heart.

2.2.7 Whole cell homogenate preparation

Frozen powdered heart tissue was homogenized (10% w/v) in buffer containing (mM) 20 Tris.HCl, 50 NaCl, 50 NaF, 5 sodium pyrophosphate, 250 sucrose, 1 DTT, protease inhibitor cocktail, and phosphatase inhibitor cocktail. This was followed by centrifugation of the tissue homogenate at 1000 g for 10 min at 4 ºC. The supernatant was aliquoted and stored at -80 ºC for further analysis. Protein levels in the supernatant were determined by Bio-Rad® protein assay.

2.2.8 Immunoblotting

Whole cell homogenates were diluted and boiled with SDS-protein sample buffer for 5 min. Equal amounts of protein for each sample were resolved using SDS polyacrylamide gel electrophoresis under denaturing conditions then transferred to nitrocellulose membranes. Membranes were then blocked for 1 hr at room temperature using 5% (w/v) milk dissolved in Tris-buffered saline

containing 0.1% (v/v) Tween-20 (TBST). This was followed by incubation at 4 ºC overnight with the primary antibody diluted in either 5% (w/v) milk or 5% (w/v) BSA in TBST. After extensive washing, membranes were incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000 dilution). After further washing, antibodies were visualized using the Amersham ECL Plus Western blotting detection system. X-ray films were scanned using a GS-800 calibrated densitometer (Bio-Rad) and analyzed using ImageJ software (382) (National Institute of Health, Bethesda, Maryland).

2.2.9 Statistical analyses

All data are represented as the mean ± SEM. Statistical analysis was performed using GraphPad Prism version 5.0d for Mac OS X (GraphPad Software, San Diego California USA). The specific statistical test used is described in each chapter. Values of $P<0.05$ were considered significant.

3. Ischemia-Induced Activation of AMPK Does Not Increase Glucose Uptake in Glycogen-replete Isolated Working Rat Hearts

HPLC analysis of nucleotides was performed by Ken Strynadka and Panakkezhum Thomas

Perfusion of glycogen-replete hearts was performed by Heather Fraser

A version of this Chapter has been published in the *American Journal of Physiology (Heart and Circulatory Physiology*). Omar MA, Fraser H, Clanachan AS. Ischemia-induced activation of AMPK does not increase glucose uptake in glycogen-replete isolated working rat hearts. 2008 Mar 1;294(3):H1266 -H1273.

3.1 Introduction

Under aerobic conditions, approximately 95% of the energy requirement of cardiac muscle is derived from the mitochondrial oxidation of fatty acids and carbohydrates while the remainder is provided by glycolysis. However, during ischemia, O_2 deprivation inhibits oxidative metabolism and glycolysis becomes a major source of myocardial ATP production (42). Thus, myocardial carbohydrate availability and metabolism are critical determinants of ischemic injury and post-ischemic left ventricular (LV) mechanical function.

Glucose transport, the first step of myocardial glucose utilization, involves the facilitated diffusion of glucose across the sarcolemmal membrane. The rate of glucose transport is determined by the trans-membrane concentration gradient of glucose as well as the abundance and affinity of glucose transporter proteins, GLUT1 and GLUT4 (383). Following transport, glucose is rapidly phosphorylated by hexokinase to glucose 6-phosphate that may be either utilized by glycolysis or stored as glycogen. Glycogen is an important store of endogenous glucose that undergoes simultaneous synthesis and degradation (148). Synthesis is accelerated by insulin and by increases in substrate availability, while degradation (glycogenolysis) is accelerated by ischemia or hypoxia (42). Thus, the rate of glucose uptake is influenced not only by glucose influx (transport), but also by the pathways of glucose metabolism that may be affected by endogenous

glycogen content, metabolic demand, availability of O_2 and other energy substrates, as well as by insulin concentration (384).

Several studies have investigated ischemia-induced alterations in glucose uptake, but these have yielded conflicting conclusions. More than three decades ago, Neely *et al.* showed that ischemia reduces glucose uptake and glycolysis (169,385). However, those results contrast with positron emission tomography (PET) studies that show an increase in the uptake of ^{18}F-deoxyglucose (^{18}FDG) in relation to coronary flow in patients with coronary artery disease (386,387). Similar observations *in vivo* (121) and *ex vivo* (216,388) were attributed to a translocation of GLUT4 to the cell surface, due to ischemia-induced activation of AMPK (220).

AMPK is a serine-threonine stress kinase and has been termed "the guardian of cardiac energy status" (389) as it is activated by energy deficient states to stimulate energy production while inhibiting energy consuming processes. AMPK stimulates GLUT4 translocation in skeletal muscle during hypoxia (218) and pharmacological activation of AMPK in isolated rat ventricular papillary muscle increases GLUT4 translocation as well as glucose uptake (219). The role of AMPK in mediating ischemia-induced increase in glucose uptake was further supported by studies using transgenic mice expressing a kinase dead form of AMPK, in which low-flow ischemia fails to enhance glucose uptake or

glycolysis (220). Indeed, it has become generally accepted that the activation of AMPK stimulates glucose uptake (324,390,391).

On the other hand, there is evidence that ischemia either reduces or has no effect on myocardial glucose uptake. Stanley *et al*. showed in open chest swine that acute myocardial ischemia increases glucose extraction but not glucose uptake (221). Similar results have been obtained in Langendorff-perfused (222) as well as working rat hearts (148,223,224). Indeed, AMPK activation fails to increase glucose uptake in skeletal muscle (225) as well as in aerobically perfused isolated working rat hearts (226). The lack of consistency regarding the effects of ischemia, as well as AMPK, on glucose uptake may be attributed to a number of factors, the most important of which concern the methods and conditions used to measure glucose uptake.

Given the importance of glucose uptake and utilization in cardiac function, this study was designed to compare the temporal changes in glucose uptake and utilization during ischemia in hearts with either normal or low levels of glycogen. Also, as the roles of AMPK in cellular metabolism and ischemia-reperfusion injury are the subject of intense investigation, we sought to determine the relationship between alterations in glucose uptake and utilization and the concomitant ischemia-induced activation of AMPK. Studies were performed in isolated working rat hearts that were perfused with Krebs-Henseleit solution containing insulin as well as glucose and fatty acids as energy substrates. LV

mechanical function, glucose uptake, glycogen turnover, glycolysis and AMPK activity were assessed during aerobic perfusion as well as during graded periods of severe low-flow ischemia (LFI).

3.2 Methods

3.2.1 Heart perfusions

Male Sprague-Dawley rat hearts were perfused in the working mode as described in Chapter 2. LFI was initiated by switching hearts to Langendorff mode and delivering oxygenated perfusate at a constant flow (0.5 mL/min) into the aorta via the aortic cannula (148,224). Two series of hearts with different pre-ischemic glycogen contents were studied. In the first series, glycogen replete (G-Replete), glycogen stores were replenished prior to the onset of LFI to near the normal levels reported for the rat heart *in vivo* (120-150 μmol/g dry wt) (392). These hearts were perfused initially for 10 min in Langendorff mode (unpaced) followed by 60 min of aerobic perfusion in the working mode. In the second series, glycogen depleted (G-Depleted), glycogen stores were depleted non-ischemically prior to the onset of LFI during an initial 35-min Langendorff perfusion with substrate-free solution (no glucose or palmitate) while pacing at 420 beats/min, as described previously (393). This was followed by 20 min of aerobic perfusion in working mode with modified Krebs-Henseleit solution

containing both glucose and palmitate to re-establish stable LV mechanical function. Hearts from both groups were then either rapidly frozen or were subjected to LFI (0.5 mL/min) for 10, 15, or 60 min and then frozen. Frozen tissues were pulverized at the temperature of liquid N_2 and the resulting powders were stored at -80°C.

3.2.2 Measurement of the total rate of glycolysis

Glycolysis rates were measured during aerobic baseline at 10 min intervals as described in Chapter 2 by the quantitative determination of ^3H_2O liberated from [5-^{3}H]glucose (at the enolase step of glycolysis). Rates (expressed as μmol glucose metabolized/min/g dry wt) were calculated for each time interval and were averaged for the period of aerobic perfusion. During LFI, glycolysis rates were calculated for the initial 10 min, 10-15 min, and 15-60 min periods. Total rates of glycolysis were calculated by the addition of the rate of liberation of ^3H_2O from labeled glucose (exogenous glucose as well as glucose liberated by glycogenolysis from labeled glycogen) and the rate of change of the unlabeled glycogen pool at the different time points.

3.2.3 Measurement of glycogen content and rates of glycogen turnover

Glycogen contents (μmol glucosyl units/g dry wt) in frozen tissues were determined as described in Chapter 2. In addition, the amount of radiolabelled glucose in glycogen extracts was also determined to evaluate the degree of incorporation of radiolabelled glucose into glycogen. The rates of glycogen synthesis (G_{in}) and degradation (G_{out}) were calculated as described previously (148). This method accounts for changes in both the unlabeled and labeled components of the glycogen pool during each phase of the perfusion protocol. This allows assessment of rates that are independent of the extent of labeling of the glycogen pool, such as might arise due to the "first-on, first-off" concept of glycogen turnover (148).

3.2.4 Glucose uptake and % extraction

Glucose uptake (μmol glucose/min/g dry wt) was calculated as described previously in Chapter 2 as the sum of the rates of glycolysis and the rate of incorporation of radiolabelled glucose into glycogen during the different phases of the perfusion protocol. This method of assessment of glucose uptake accounts for each of the two main fates of glucose (metabolism by the glycolytic pathway or incorporation into glycogen stores). Glucose extraction (%) was calculated from

the rate of glucose uptake as a percentage of glucose delivery (perfusate glucose concentration x coronary flow).

3.2.5 Measurement of ATP, AMP, creatine (Cr), and creatine phosphate (PCr)

Frozen heart tissue (100 mg) was homogenized and extracted with 6% perchloric acid. The tissue-perchloric acid mixture was centrifuged and the supernatant neutralized with 5M K_2CO_3 and then analyzed by high performance liquid chromatography for ATP, AMP, Cr, and PCr (394). Briefly, 100 µL of each sample was run through a SupelcosilTM LC-18-T guard cartridge and a SupelcosilTM LC-18-T column on a Beckman System Gold HPLC. Flow rate was set at 1.5 mL/min and analyte detection occurred at an absorbance of 260 nm on a Beckman System Gold model 168 diode array detector. The mobile phase consisted of buffer A (35 mM K_2HPO_4, 6 mM tetrabutyl-ammonium-hydrogen-sulfate, pH 6.0) and buffer B (a mixture of buffer A and acetonitrile in a ratio of 1:1 (v/v)). The gradient elution profile consisted of the following, 0 -10 min, 98 % A 2 % B; 10 - 20 min, 98 % A 2 % B to 45 % A 55 % B using Beckman's curve number 3; 20 - 25 min, 45 % A 55 % B. Peaks were integrated using the Beckman System Gold software.

3.2.6 Measurement of AMP-activated protein kinase activity

AMPK activity (nmol/min/mg protein) was determined in 6% polyethylene glycol (PEG) fractions extracted from 200 mg of frozen LV tissue. Activity of AMPK in the presence of 5'-AMP (200 μM) was measured in the 6% PEG fractions by following the incorporation of [γ^{32}P]-ATP into a Ser-79 phosphorylation site-specific synthetic SAMS [HMRSAMSGLHVKRR] peptide as described previously (331,395).

3.2.7 Plasma membrane GLUT4 content

Membrane fractions of frozen extracts of G-Replete and G-Depleted hearts were prepared as described previously (396). In order to validate that the procedure could detect changes in plasma membrane GLUT4 content, fractions were also prepared from additional working hearts perfused aerobically in the absence or presence of insulin (100 μU/mL). Powdered tissue was incubated for 30 min in a high-salt solution (2 M NaCl, 20 mM HEPES pH 7.4, and 5 mM NaN_3) at 4 °C. Thereafter, tissue was recovered by centrifugation for 5 min at 1000*g*, suspended in homogenization solution (20 mM HEPES pH 7.4, 2 mM EDTA, 1 mM $MgCl_2$, 250 mM sucrose) and centrifuged. The pellet was then homogenized (10% w/v) using a hand-held glass homogenizer (DUALL®, Kontes). Homogenates were sequentially centrifuged at 100g for 10 min and then 5000g for 10 min to prepare a plasma membrane fraction (397), confirmed by

enrichment of the plasma membrane marker Na^+-K^+ ATPase α1. Plasma membrane fractions were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and then immunoblotted using rabbit anti-Na^+-K^+ATPase α1 or rabbit anti-GLUT4 (1:1,000 dilution) (Cell Signaling Technology) as described in Chapter 2. After extensive washing, membranes were incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000 dilution) (Cell Signaling Technology). After further washing, antibodies were visualized using the Pharmacia enhanced chemiluminescence Western blotting and detection system (ECL Plus). Densitometric analyses of immunoblots (n = 4 per experimental group) were performed using ImageJ software. Densitometric values for GLUT4 protein were normalized to the corresponding value for Na^+-K^+ ATPase α1.

3.2.8 Statistical analyses

Results are expressed as means ± SEM of n observations. The significance of the differences for multiple comparisons was estimated by One-way analysis of variance (ANOVA). If significant, selected data sets were compared with Bonferroni's Multiple Comparison Test. Differences were considered statistically significant when $P<0.05$.

3.3 Results

3.3.1 LV mechanical function

LV work was similar in G-Replete and G-Depleted groups (2.81 ± 0.10 and 2.66 ± 0.13 Joules/min/g dry wt, respectively). Cardiac output, aortic flow, and coronary flow also remained stable throughout aerobic working mode perfusion in both groups (Table 3.1). During LFI, LV work was not detectable in either group.

3.3.2 Glycogen content of G-Replete and G-Depleted hearts

The stress associated with heart extraction markedly reduces glycogen content and, when perfused with Krebs-Henseleit solution containing glucose as the sole energy substrate, glycogen content remains low (148) relative to *in vivo* values (42,392). Thus, to generate G-Replete hearts, a 60-min period of aerobic perfusion was required to replenish glycogen content to 114±6 μmol/g dry wt (n=6). In G-Depleted hearts, the initial substrate-free Langendorff perfusion and the shorter period of aerobic working mode perfusion resulted in significantly lower myocardial glycogen content prior to LFI (71±6 μmol/g dry wt).

3.3.3 Effect of LFI on glycogen content

LFI rapidly reduced glycogen content during the initial 10 min of LFI in both G-Replete and G-Depleted groups reaching 66±9 μmol/g dry wt and 23±2 μmol/g dry wt, respectively. Glycogen content continued to decrease in the G-Replete group during more prolonged periods of LFI, while in G-Depleted hearts no further reductions were observed (Figure. 3.1A).

3.3.4 Effect of LFI on rates of glycolysis

Glycolysis during aerobic perfusion was lower in G-Depleted hearts by 44% as compared with the G-Replete group. In G-Replete hearts, total glycolysis was initially increased at 10 min LFI (unpaired t-test, P=0.08) and then returned to close to aerobic values during the remainder of LFI. In G-Depleted hearts, total glycolysis increased significantly during LFI to reach its peak at 15 min and it remained elevated during the remainder of LFI as compared with pre-ischemic values (Figure 3.1B).

3.3.5 Effect of LFI on glucose uptake and glucose extraction

In G-Replete hearts, glucose uptake was reduced by 59% during the first 10 min of LFI which was followed by gradual recovery to near aerobic values after 60 min of LFI. Glucose uptake was also reduced in G-Depleted hearts during the initial 10 min of LFI by 90% but this was followed by a significant

increase in glucose uptake at 15 min and a return to pre-ischemic values at the end of LFI (Figure 3.2A). Percent glucose extraction was very low during aerobic perfusion in G-Replete and G-Depleted hearts (0.64±0.09% and 0.58±0.07%, respectively). It increased gradually in G-Replete hearts during LFI to 21±5% after 60 min of LFI (Figure 3.2B). In G-Depleted hearts, it increased more rapidly to reach its maximum at 15 min (40±2%).

3.3.6 Effect of LFI on glycogen turnover

During baseline aerobic perfusion, G_{out} was similar in the two groups, while G_{in} was significantly higher in the G-Depleted group (Figure 3.3A). LFI for 10 min caused a marked increase in G_{out}. In G-Replete hearts, G_{out} recovered only partially during LFI but still remained higher than the pre-ischemic rate. In contrast, in G-Depleted hearts, the acceleration of G_{out} was not maintained (Figure 3.3A). LFI did not alter G_{in} in G-Replete hearts, but significantly reduced the higher pre-ischemic rate of G_{in} in G-Deplete hearts (Figure 3.3A). There was a significant inverse correlation between rates of glucose uptake and glycogen degradation (Figure 3.4). However, there were no significant correlations between glucose uptake and rates of glycolysis, glycogen synthesis, glycogen content, or AMPK activity.

3.3.7 Effect of LFI on AMPK activity

AMPK activity prior to LFI was similar to values reported previously for the aerobically perfused working rat heart (224,312,398). LFI elicited similar increases in AMPK activity in both G-Replete and G-Depleted groups. However, AMPK activity peaked at 15 min in G-Replete hearts and remained elevated throughout the rest of LFI, while in G-Depleted hearts, it peaked earlier (at 10 min) and then recovered to pre-ischemic values at the end of 60 min of LFI (Figure 3.3B).

3.3.8 Effect of LFI on high energy phosphates and their metabolites

Values for high-energy phosphates are presented in Table 3.2. The AMP/ATP and Cr/PCr ratios were similar in both G-Replete and G-Depleted groups during aerobic conditions. LFI resulted in a gradual increase in AMP/ATP ratio in both groups, which reached its maximum at 60 min of LFI (Figure 3.5A). However, LFI resulted in more rapid increases in Cr/PCr ratios in both G-Replete and G-Deplete hearts (Figure 3.5B).

3.3.9 Effect of LFI on sarcolemmal abundance of GLUT4

GLUT4 content in plasma membrane fractions was 3-fold higher when hearts were exposed to insulin (Figure 3.6A). LFI did not affect plasma

membrane GLUT4 content in G-Replete hearts, but caused a significant elevation in G-Depleted hearts (Figure 3.6B).

3.4 Discussion

This study investigated the effects of LFI on glucose uptake as well as glycogen turnover and glycolysis in working rat hearts perfused under conditions of normal (G-Replete) or low (G-Depleted) glycogen content. Relative to normal aerobic values, glucose uptake and GLUT4 translocation were not stimulated in G-Replete hearts either by short-term (10 min) or by longer-term (60 min) LFI. Indeed, glucose uptake was inhibited during short-term LFI and rates of glycolysis were maintained close to aerobic values by a marked acceleration of glycogenolysis. On the other hand, in G-Depleted hearts, glucose uptake decreased initially during LFI in association with accelerated glycogenolysis as well as inhibition of glycogen synthesis. After 15 min LFI, glucose uptake and GLUT4 translocation increased, effects that coincided with a decline in glycogenolysis as glycogen content became exhausted. As expected, LFI increased AMPK activity in both G-Replete and G-Depleted hearts, but this was not associated with acceleration of glucose uptake. These results suggest that LFI increases glucose uptake only when myocardial glycogen stores are partially depleted. Glycogen stores, if replenished to normal values prior to LFI, provide sufficient endogenous substrate to maintain glycolysis during prolonged LFI. Activation of AMPK by LFI is not sufficient to stimulate glucose uptake, but may alter glucose utilization by affecting glycogen turnover (inhibition of synthesis and acceleration of glycogenolysis).

The isolated working rat heart perfused with both glucose and palmitate provided an experimental system in which energy supply and demand, and hence rates of glucose uptake and utilization were close to physiological values. The presence of adequate sources of endogenous and exogenous energy substrates is fundamental to any study on energy substrate metabolism, but is of critical importance in studies on glucose uptake and utilization as glycogen provides a readily available source of glucose 6-phosphate for glycolysis. Heart extraction reduces glycogen and, as shown in this and other studies (58,148,226), aerobic perfusion for 45 to 60 min with Krebs-Henseleit solution containing glucose, insulin and palmitate is required to replenish glycogen content. Thus, in the G-Replete group, pre-ischemic glycogen content was close to normal values (120-150 μmol/g dry wt) (392).

Glycogen content is also an important consideration in studies designed to examine ischemia-induced signaling alterations. For example, there is an inverse correlation between glycogen content and AMPK activity in both skeletal (399,400) and cardiac muscle (377). Indeed, abundant stores of glycogen depress activation of AMPK by AICAR in skeletal muscles, as well as the associated acceleration of glucose uptake (399). Examination of the role of glycogen availability on the relationship between myocardial ischemia, glucose uptake and AMPK activation was achieved using an additional group of hearts (G-Deplete) in which glycogen levels were reduced to values reported for hearts that are either

perfused without insulin, perfused with glucose as the sole energy substrate, or where the pre-ischemic aerobic baseline period may be too short to permit adequate re-synthesis of glycogen. Importantly, in contrast to zero-flow ischemia models, the use of LFI in these experiments enabled the measurement of time-dependent changes in glucose uptake, glycogen turnover, and glucose utilization during the actual ischemic period in hearts with either normal or low levels of glycogen (148,224).

Another important consideration in the present study is the approach used to assess glucose uptake. Glucose uptake is commonly measured using the non-metabolizable glucose analogues, 2-deoxyglucose or ^{18}FDG, but their utility has been questioned by the observation that the constant (termed Lumped Constant) used to account for the difference in their kinetic properties from glucose (124) depends upon the metabolic status of the heart as well as on the presence of insulin (401). Glucose uptake has also been assessed by the liberation of $^{3}H_2O$ from [2-^{3}H]glucose, but studies often neglect to account for incorporation of [2-^{3}H]glucose into glycogen, a process affected by numerous factors including insulin and ischemia. Glucose uptake may also be influenced by glycogen availability and turnover because glycogen synthesis is a route of glucose utilization and might enhance glucose uptake whereas glycogenolysis produces endogenous glucose phosphate that may inhibit glucose uptake. Glucose uptake in this study was measured as the sum of the rates of glycolysis and glycogen

synthesis and so accounts for both of the main fates of glucose following its uptake. Our data indicate that glycogen synthesis accounts for approximately 20% of glucose taken up during the initial aerobic phase in G-Replete hearts, but this increases dramatically in G-Depleted hearts to 66%. It should also be noted that while glucose uptake under these conditions is high (5 to 6 μmol/min/g dry wt), it is not maximal, as significant increases are demonstrable in response to removal of palmitate from the perfusate (402). Using these experimental approaches, glucose uptake is significantly inhibited during LFI in G-Replete hearts, a result that is in marked contrast with several reports that demonstrate ischemia-mediated increases in glucose uptake (121,216,220,388). Ischemia-induced changes in glucose uptake are clearly dependent on glycogen content, and an increase is only observed in G-Depleted hearts, and then only after glycogen stores are exhausted.

Inhibition of glucose uptake is not due to a lack of availability of exogenous glucose. While glucose extraction is significantly increased during LFI, glucose availability did not become rate limiting for uptake as extraction never exceeded 40%. Glucose uptake is likely inhibited because LFI elicits marked alterations in glycogen turnover. The rapid increase in glycogenolysis in G-Replete hearts provides more than 85% of glucose consumed through glycolysis during the initial 10 min of LFI and the elevation of glucose 6-phosphate availability suppresses hexokinase activity (134) and thereby slows the

rate of glucose uptake. Similar observations were reported for skeletal muscles during moderate exercise (299). Due to the finite supply of glycogen, glycogenolysis slows during more prolonged periods of LFI and consequently glucose uptake recovers to maintain substrate availability for glycolysis. In G-Depleted hearts, the greater inhibition of glucose uptake during the first 10 min of LFI is due to an increase in glycogenolysis as well as a marked inhibition of the accelerated rates of glycogen synthesis in this group. In the G-Depleted hearts, the lower pre-ischemic glycogen is almost exhausted after the initial 10 min of LFI and this leads to a stimulation of GLUT4 translocation and glucose uptake. Similarly, exercise-induced stimulation of glucose uptake in skeletal muscles is greater when glycogen content is low and unable to supply endogenous substrate for glycolysis (403,404).

The significant inverse correlation between the rate of glycogen degradation and the rate of glucose uptake throughout the different phases of perfusion suggests that these two processes interact to provide sufficient substrate for glycolysis. Also, during ischemia it is energetically more favorable for the heart to utilize endogenous, rather than exogenous, glucose. The degradation of glycogen provides a phosphorylated substrate for glycolysis, whereas the uptake of exogenous glucose requires the consumption of one mole of ATP per mole of glucose to convert glucose into G-6-P. Therefore, catabolism of each mole of glucose derived from glycogen yields one extra mole of ATP compared with the

catabolism of exogenous glucose. Thus, during ischemia when the heart is energetically compromised, glycogen degradation becomes the preferred source of glucose 6-phosphate for energy production by glycolysis as long as glycogen stores are sufficient. However, when glycogen stores are depleted, glucose uptake is increased to maintain the supply of G-6-P.

AMPK is a key kinase involved in the regulation of many aspects of cellular metabolism, including glucose metabolism (208,299,331,405). AMPK activation by AICAR in rat skeletal muscles is accompanied by an activation of glycogen phosphorylase that increases glycogenolysis (405) but no such relationship occurs in rat ventricular papillary muscle (219). However, our previous data have shown that the ADO-induced activation of AMPK in the stressed working heart does not affect glucose uptake, but is accompanied by an alteration in glycogen turnover manifest as an inhibition of glycogen synthesis (226). The current study extends those observations and indicates that ischemia-induced activation of AMPK is not sufficient to increase glucose uptake in G-Replete hearts, but is accompanied by alterations in glycogen turnover that comprise acceleration of glycogenolysis and inhibition of glycogen synthesis. While activation of myocardial AMPK has been shown to increase glycolysis via activation of phosphofructokinase-2 (165), glycolysis was only accelerated in G-Deplete hearts. The absence of such an effect in G-Replete hearts may be attributed to the higher pre-ischemic glycolysis rate and/or to a greater

accumulation of protons or glycolytic by-products. The failure of AMPK activation to increase glucose uptake in G-Replete hearts is not due to differences in the degree of AMPK activation between the G-Replete and G-depleted groups as peak AMPK activity is similar. Also, the rapid, ischemia-induced increase in the Cr / PCr ratio and the more gradual increase the AMP / ATP ratio, as has been reported previously (406), was similar in each group. Interestingly, the time-dependent increase in AMPK activity had a profile more related to the increase in Cr / PCr ratio, an early and sensitive indicator of energy deficiency, than to increase in the AMP / ATP ratio.

In addition to myocardial glycogen content, other factors may account for the marked difference between this study and others that support the view that AMPK activity regulates glucose uptake. Stimulation of glucose uptake by the AMPK activator, AICAR, is dependent upon both nutritional state (greater in fasted animals) and muscle fiber type (less in oxidative muscle) (407) suggesting that glucose uptake in G-Replete cardiac muscle may be less responsive to AMPK activation. However, other studies using isolated LV papillary muscle (219) have shown that AICAR-induced activation of AMPK increases [2-^{3}H]deoxyglucose uptake and that ischemia accelerates glucose uptake in wild-type mouse hearts, but not in hearts with a kinase dead mutation of AMPK (220). In both those studies (219,220), the effect of AMPK activation on glucose uptake was measured in the presence of either no or low (fasting) concentrations of insulin that may 1)

have limited glycogen re-synthesis during pre-ischemic conditions and/or 2) caused low aerobic (baseline) rates of glucose uptake. Our study indicates that LFI and subsequent AMPK activation can be accompanied by an increase in glucose uptake under conditions of glycogen depletion, which resemble the conditions under which many of the studies showing ischemia-induced increase in glucose uptake were performed. Lack of activation of glucose uptake in hearts with a kinase dead mutation of AMPK (220) may also be due to a lack of activation of glycolysis or inhibition of glycogen synthesis, effects that indirectly reduce the requirement for enhanced glucose uptake.

Conclusion

The present study provides clear evidence that in isolated working hearts ischemia-induced alterations in glucose uptake and utilization are dependent on pre-ischemic glycogen content. In G-Replete hearts, the marked and sustained activation of glycogenolysis by LFI supplies sufficient substrate for glycolysis and so glucose uptake is inhibited. The ability of LFI to markedly stimulate AMPK activity in both G-Replete and G-Depleted hearts, indicates that AMPK activation is not sufficient to accelerate glucose uptake. Instead, ischemia and AMPK may exert greater influence on the regulation of glycogen turnover and glycolysis, effects that are dependent on glycogen content.

Figure 3.1 Glycogen Content and Total Glycolysis.

Time-course of changes in glycogen content (μmol glucose/g dry wt) (A) and the rates of glycolysis (B) during LFI in glycogen-replete (left) and glycogen-depleted (right) hearts. Values are means ± SEM (n=6-8). **P*<0.05 compared with pre-ischemic value.

Figure 3.1

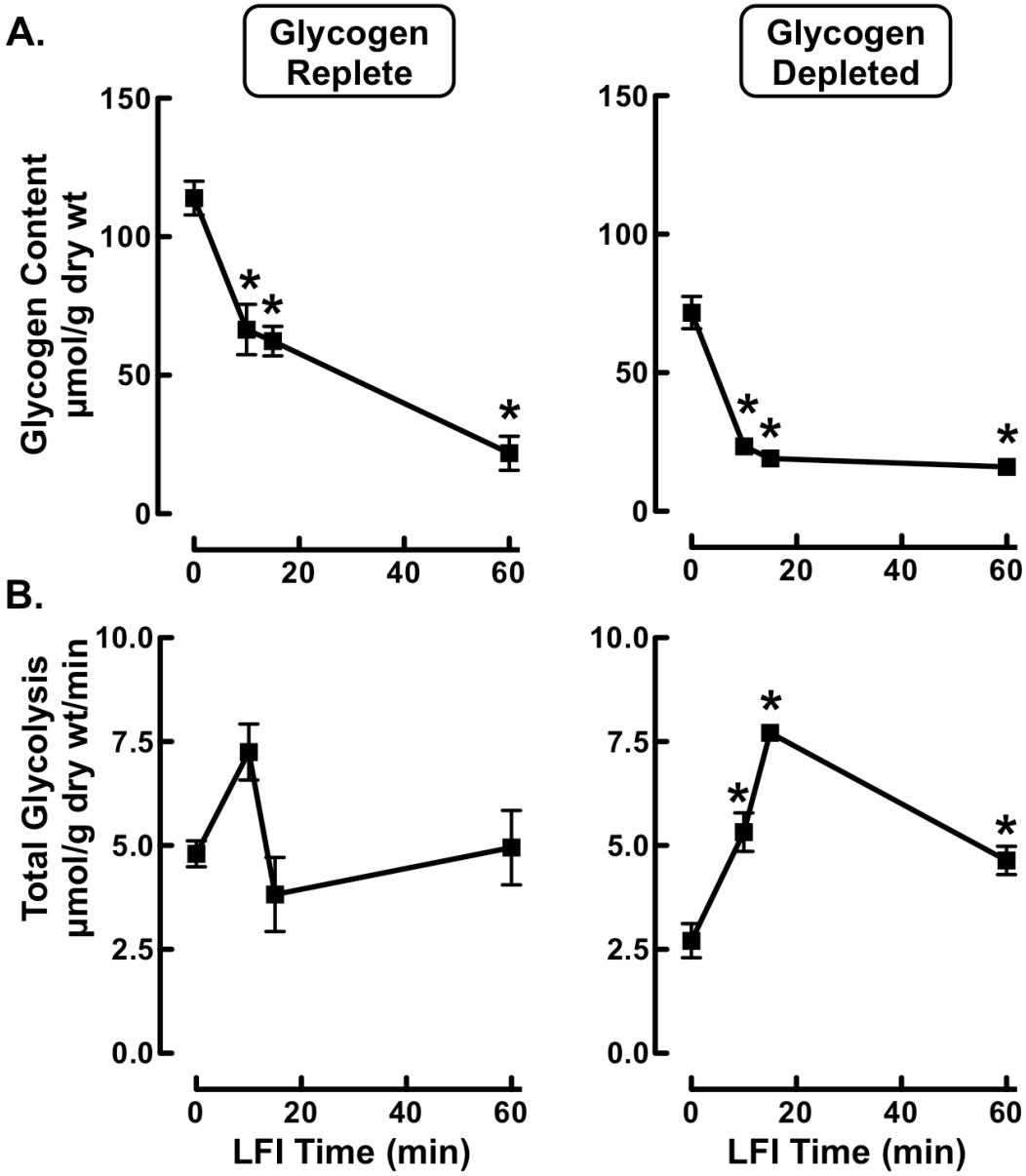

A.
Glycogen Replete
Glycogen Depleted
Glycogen Content
µmol/g dry wt
150
100
50
0
0
20
40
60
B.
Total Glycolysis
µmol/g dry wt/min
10.0
7.5
5.0
2.5
0.0
LFI Time (min)

Figure 3.2 Glucose Uptake and Extraction.

Time-course of changes in the rates of glucose uptake (μmol glucose/min/g dry wt) (A) and % glucose extraction (B) during LFI in glycogen-replete (left) and glycogen-depleted (right) hearts. Values are means ± SEM (n=6-8). **P*<0.05 compared with pre-ischemic value.

Figure 3.2

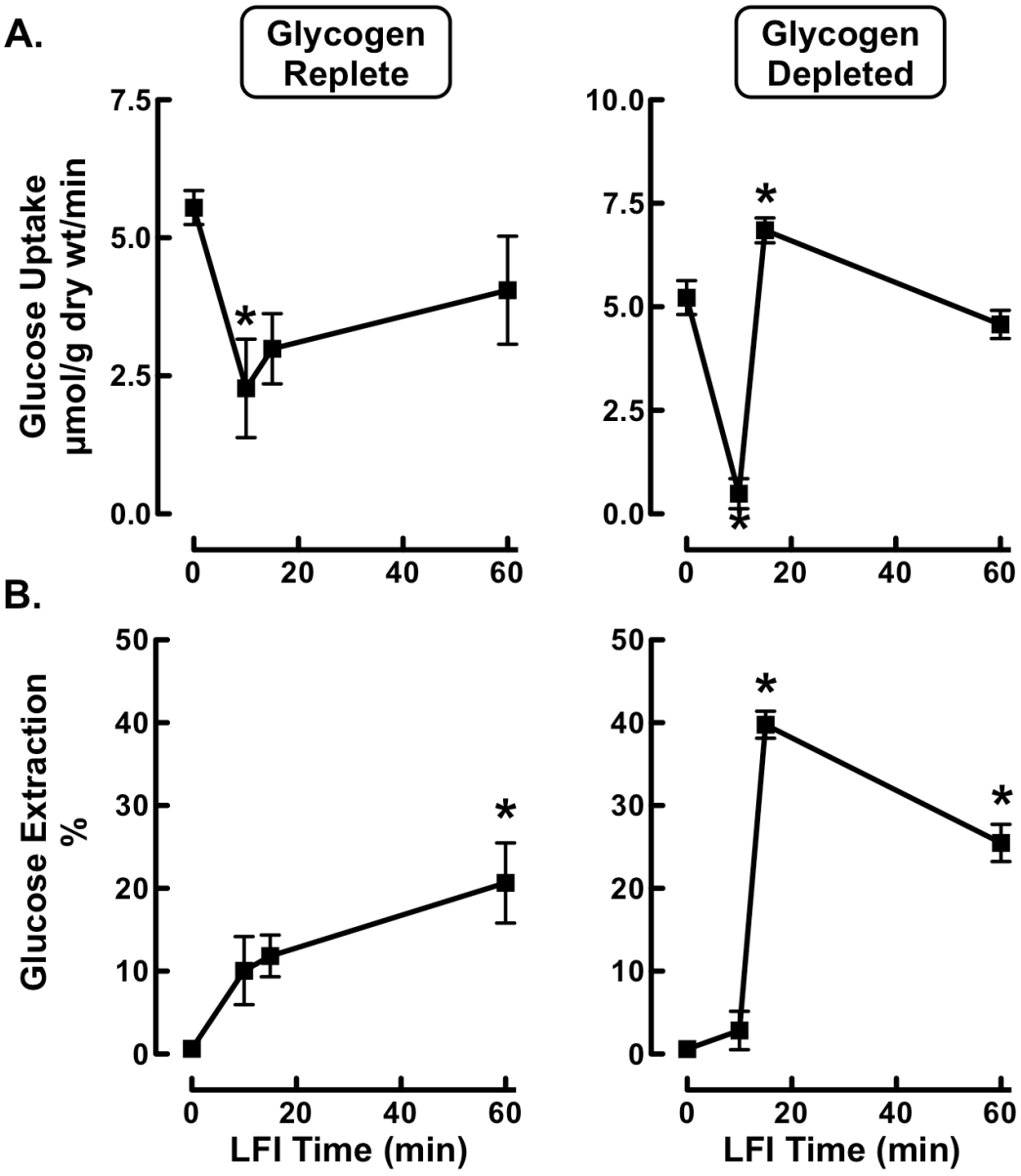

A.
Glycogen Replete
Glycogen Depleted
Glucose Uptake µmol/g dry wt/min
7.5
5.0
2.5
0.0
10.0
7.5
5.0
2.5
0.0
0
20
40
60
B.
Glucose Extraction %
50
40
30
20
10
0
LFI Time (min)
*

Figure 3.3 Glycogen Turnover and AMPK Activity.

Time-course of changes in glycogen synthesis (G_{in}, μmol glucose/min/g dry wt) and glycogen degradation (G_{out}, μmol glucose/min/g dry wt) (A) and AMPK activity (nmol/min/mg protein) (B) and during LFI in glycogen-replete (left) and glycogen-depleted (right) hearts. Values are means ± SEM (n=6-8). *P<0.05 compared with pre-ischemic value.

Figure 3.3

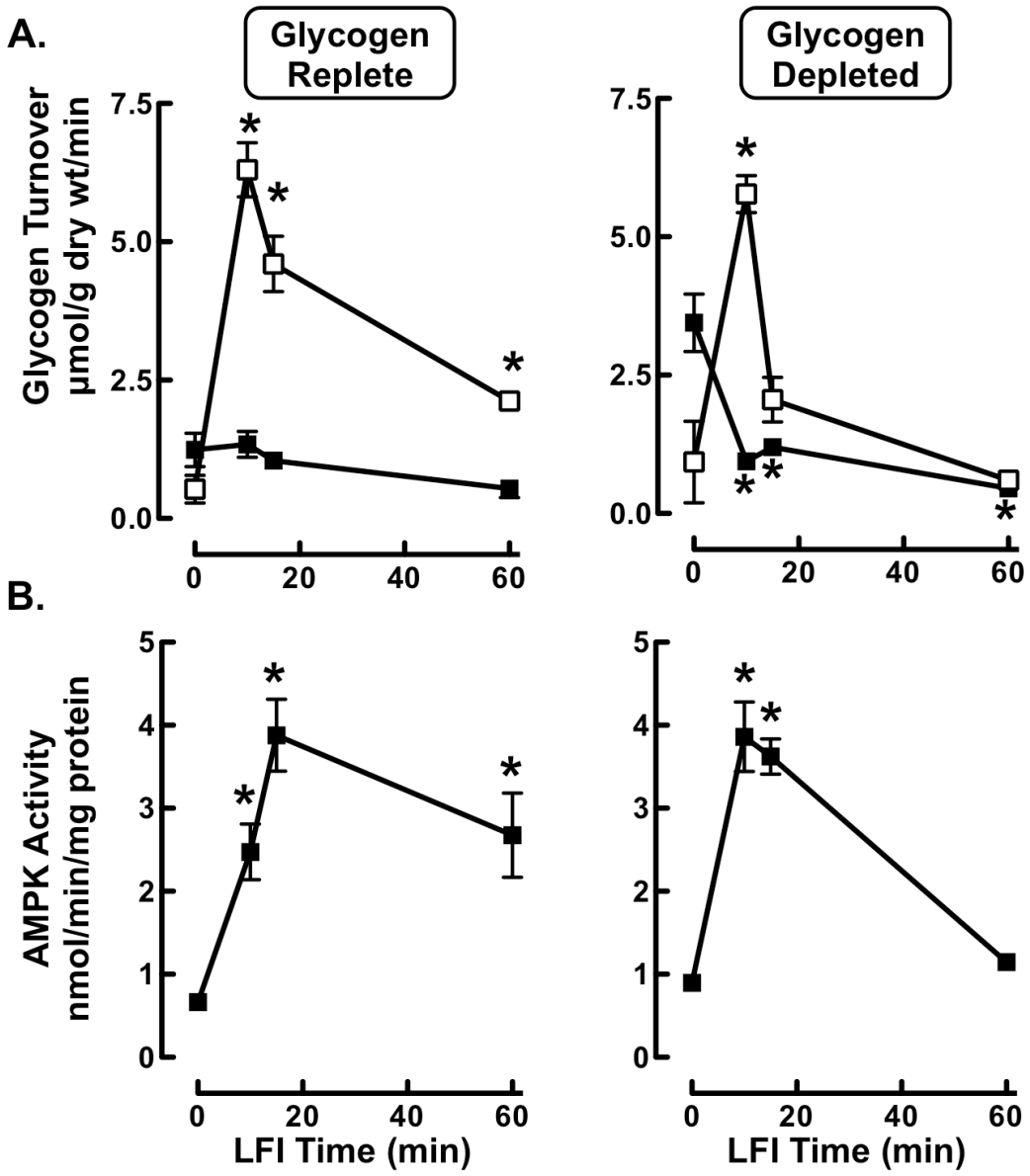

A.
Glycogen Replete
Glycogen Depleted
Glycogen Turnover
µmol/g dry wt/min
7.5
5.0
2.5
0.0
0
20
40
60
B.
AMPK Activity
nmol/min/mg protein
5
4
3
2
1
0
LFI Time (min)

Figure 3.4 Relationship between glucose uptake and glycogen degradation.

Correlation (by linear regression) of rates of glucose uptake (μmol glucose/min/g dry wt) and corresponding rates of glycogen degradation (G_{out}, μmol glucose/min/g dry wt) obtained in glycogen-replete and glycogen-depleted hearts subjected to graded durations of LFI ($r = -0.7$, $P < 0.0001$). Non-linear regression attempts did not yield significantly improved curve fits. The dotted lines represent the 95% confidence intervals.

Figure 3.4

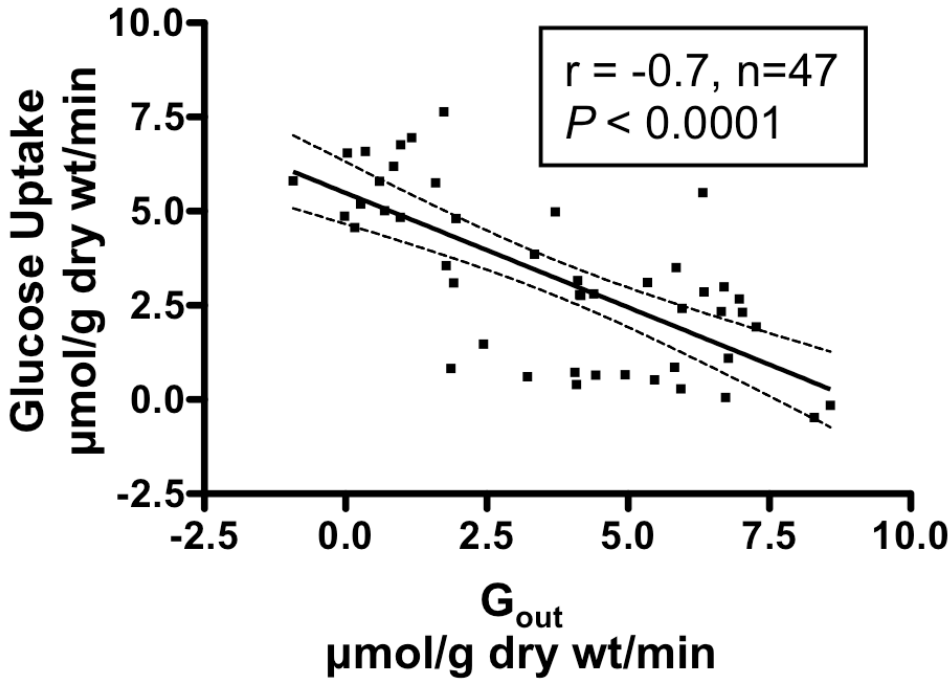

r = -0.7, n=47
P < 0.0001
Glucose Uptake
µmol/g dry wt/min
10.0
7.5
5.0
2.5
0.0
-2.5
-2.5
0.0
2.5
5.0
7.5
10.0
G out
µmol/g dry wt/min

Figure 3.5 AMP/ATP and Cr/PCr Ratios.

Time-course of changes in AMP/ATP ratio (A) and Cr/PCr ratio (B) during LFI in glycogen-replete (left) and glycogen-depleted (right) hearts. Values are means ± SEM (n=6-8). *P<0.05 compared with pre-ischemic value.

Figure 3.5

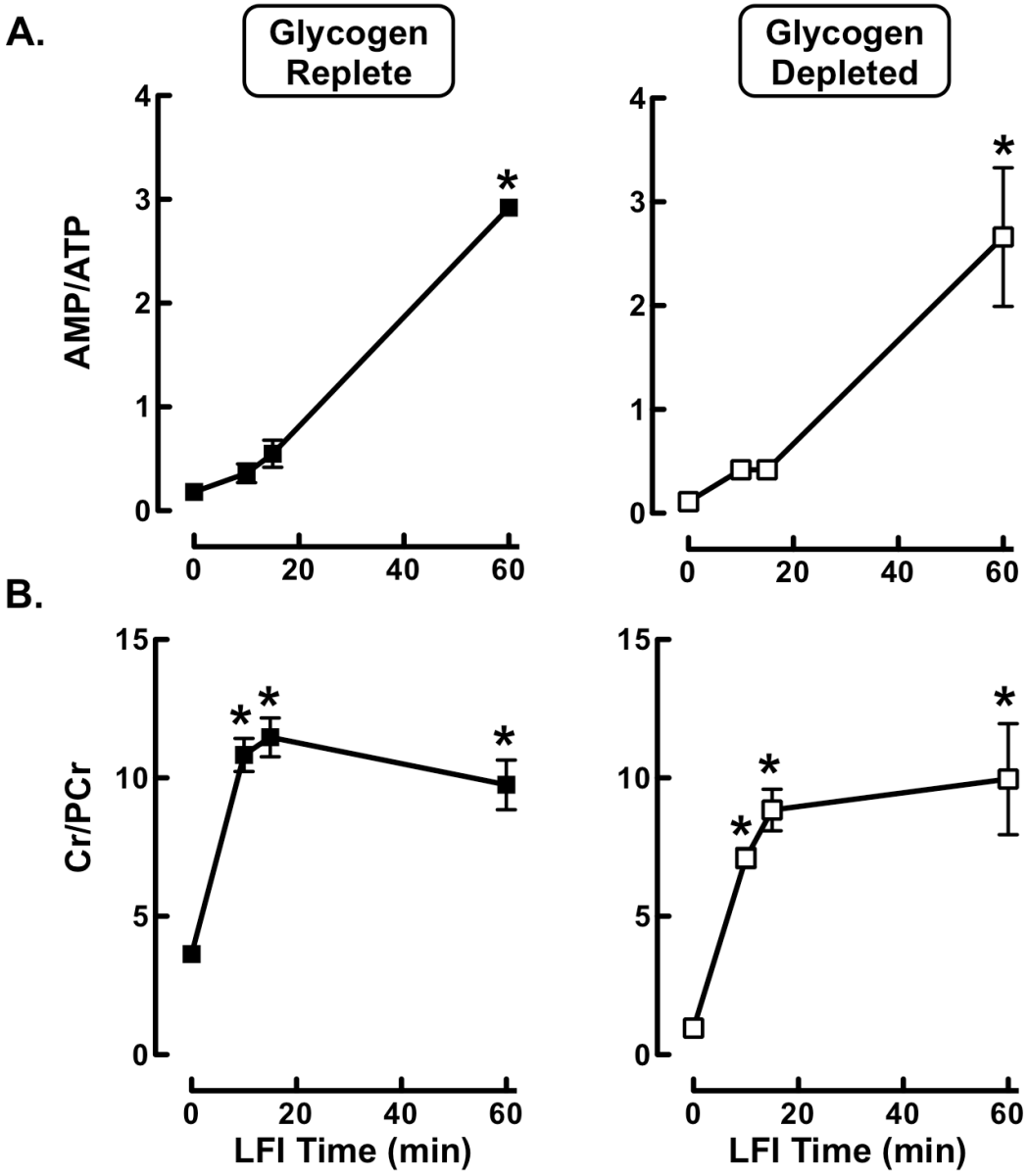

A.
Glycogen Replete
Glycogen Depleted
AMP/ATP
0
1
2
3
4
0
20
40
60
*
B.
Cr/PCr
0
5
10
15
LFI Time (min)

Figure 3.6 Plasma Membrane Abundance of GLUT4.

Immunoblots of Na^+-K^+ ATPase α1 and GLUT4 in plasma membrane fractions from hearts frozen after perfusion in either the absence or presence of insulin (left) or from G-Replete or G-Depleted hearts (right) frozen after aerobic baseline (A) or after 15 min low-flow ischemia (LFI). Histograms show results of immunoblot quantification for the effects of insulin (1) and LFI (2) on GLUT4 translocation where GLUT4 immunoreactivity is normalized to the plasma membrane marker, Na^+-K^+ ATPase α1. Values for (2) are means ± SEM (n=4). *$P<0.05$ compared with pre-ischemic value (unpaired t-test).

Figure 3.6

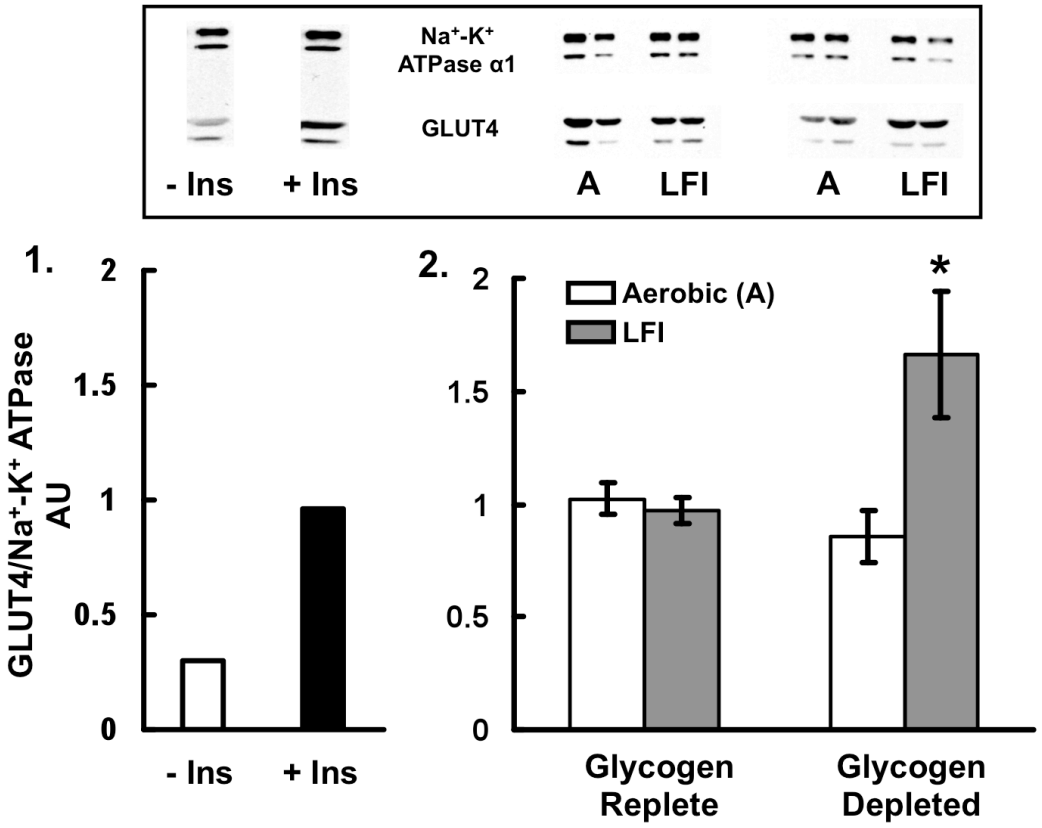

Na+-K+ ATPase α1
GLUT4
- Ins
+ Ins
A
LFI
A
LFI
1.
GLUT4/Na+-K+ ATPase AU
2
1.5
1
0.5
0
- Ins
+ Ins
2.
Aerobic (A)
LFI
*
Glycogen Replete
Glycogen Depleted

Table 3.1. Cardiac function for Glycogen-Replete and Glycogen-Depleted hearts during baseline perfusion

Cardiac Parameter	Glycogen-Replete Hearts	Glycogen-Depleted Hearts
CO (mL/min/ g dry wt)	205.1 ± 6.5	199.1 ± 7.9
AF (mL/min/ g dry wt)	130.0 ± 5.8	119.2 ± 7.0
CF (mL/min/ g dry wt)	75.0 ± 2.5	79.9 ± 4.4
LVW (Joules/min/g dry wt)	2.81 ± 0.10	2.66 ± 0.13

Values represent means ± SEM for cardiac output (CO), aortic flow (AF), coronary flow (CF), and LV work (LVW).

Table 3.2. Values for ATP, AMP, PCr, and Cr contents of LV tissue.

	Glycogen-Replete Hearts				Glycogen-Depleted Hearts			
	Aerobic	LFI			Aerobic	LFI		
		10 min	15 min	60 min		10 min	15 min	60 min
ATP	32.0±1.5	20.9±1.8*	19.1±1.7*	6.9±1.7*	18.0±0.9	8.6±0.3*	9.4±0.2*	2.8±0.3*
AMP	5.7±0.6	6.8±0.8	9.8±1.8	20.1±3.8*	2.0±0.1	3.5±0.2*	3.9±0.2*	6.2±0.6*
PCr	55.6±7.5	15.8±1.3*	15.1±0.7*	19.6±3.2*	52.6±3.0	7.8±0.2*	6.9±0.5*	8.7±1.9*
Cr	201±72	5.4±2.1	8±9.3	5.4±8.3	48.7±3.6	55.2±1.4	59.5±2.5*	64.2±2.0*

ATP, AMP, PCr, and Cr contents (μmol/g dry wt) were measured in extracts of both G-Replete and G-Depleted hearts frozen after baseline aerobic perfusion as well as after 10, 15 or 60 min of LFI. Values are means ± SEM (n=6-9). *P<0.05 compared with aerobic baseline value.

4. Cardioprotection by GSK-3 inhibition: Role of Enhanced Glycogen Synthesis and Attenuation of Calcium Overload

Calcium measurements in perfused hearts were performed by Lianguo Wang.

A part of this chapter has been published in *Circulation* as an abstract. Omar MA, Wang L, Clanachan AS. Cardioprotection by Glycogen Synthase Kinase-3 Inhibition: Role of Reduced Proton Production From Glucose Metabolism and Attenuation of Calcium Overload. 2009 Nov 3;120(18, Suppl. 2):S812

A version of this chapter has been published in *Cardiovascular Research*. Omar MA, Wang L, Clanachan AS. Cardioprotection by GSK-3 inhibition: role of enhanced glycogen synthesis and attenuation of calcium overload. 2010 Jun 1;86(3):478-486.

4.1 Introduction

Glycogen synthase kinase-3 (GSK-3) is a multi-functional kinase that regulates signaling pathways affecting glycogen metabolism, protein synthesis, mitosis and apoptosis. It has 2 isoforms, α and β, that possess strong homology in their kinase domains (234). GSK-3 is constitutively active and is regulated by inhibitory phosphorylation by upstream kinases on Ser9 (β isoform) or Ser21 (α isoform) (235). In heart, GSK-3 has several important roles. It actively inhibits hypertrophy and its inhibition stimulates development of cardiac hypertrophy (238). Recently, inhibition of GSK-3 during ischemia and reperfusion (I/R) has been implicated as a cardioprotective mechanism. Tong *et al*. first reported that infarct size reduction by ischemic preconditioning is due to increased GSK-3 phosphorylation and its subsequent inhibition (244). Furthermore, inhibition of GSK-3 was suggested as a mechanism explaining cardioprotection induced by postconditioning (90), opioids (22), bradykinin (245), erythropoietin (246), ADO A_3 receptor activation (98), isoflurane (248), and PKCδ inhibition (249). However, mechanisms mediating these beneficial effects of GSK-3 inhibition are not fully understood. One proposed mechanism involves prevention of mitochondrial permeability transition pore (MPTP) opening (41) potentially due to effects on the voltage-dependent anion channel (VDAC) (101) or adenine nucleotide translocase (408). However, a direct interaction between GSK-3 and

the MPTP is still not established. In addition, recent evidence from mitochondria that are deficient in all isoforms of VDAC shows that VDAC is dispensable in MPTP opening (76). Other proposed mechanisms involve increased glucose utilization (409) and reduced mitochondrial ATP hydrolysis during ischemia (410), but these effects are unable to explain the protective effects of GSK-3 inhibition when added at the onset of reperfusion.

Interestingly, although the initial function (and naming) of GSK-3 was related to its effects on glycogen synthase (GS) activity, the contribution of alterations in glycogen or glucose metabolism by GSK-3 inhibition to cardioprotection has not been investigated. GSK-3 phosphorylates GS at Ser640 (site 3a) and Ser 644 (site 3b) via a hierarchal mechanism and thereby inhibits GS activity (143). In contrast, phosphorylation and inhibition of GSK-3, such as by insulin-mediated activation of the PI3K/Akt pathway, increases GS activity and accelerates glycogen synthesis (139). Thus, GSK-3 may influence the partitioning of G-6-P between the pathways of glycogen synthesis and glycolysis.

In this study we test the hypothesis that inhibition of GSK-3 will stimulate glycogen synthesis, repartition glucose partially away from glycolysis, improve the coupling between glycolysis and glucose oxidation and reduce the potential for intracellular acidosis. As acidosis initiates the intracellular accumulation of Na^+ and Ca^{2+} by enhanced activities of the Na^+-H^+ exchanger (NHE) and reverse-mode Na^+-Ca^{2+} exchanger (rmNCX), respectively (45), we

further investigated whether GSK-3 inhibition attenuates Ca^{2+}_i accumulation during ischemia and reperfusion.

4.2 Methods

4.2.1 Heart perfusions

Male Sprague-Dawley rats (350-400g) were used in this study. Rat hearts were cannulated for isolated working mode perfusion in a recirculating system under conditions of constant workload (11.5 mmHg preload, 80 mmHg afterload, rate 5 Hz) as described in detail in Chapter 2. Left ventricular (LV) work (Joules/min/g dry wt) was used as a continuous index of LV mechanical function.

4.2.2 Measurement of Ca^{2+}_i concentration in working rat hearts

Measurement of Ca^{2+}_i concentration was performed as previously described (411,412). Hearts were extracted as described in Chapter 2, then preloaded with the fluorescent Ca^{2+} indicator, indo-1AM (5 μM) during an initial working mode perfusion for 25 min. Any residual extracellular indo-1AM was washed from the hearts during a short period of Langendorff perfusion (5 min) before re-establishing working mode perfusion with fresh modified Krebs–Henseleit solution using a second perfusion circuit. Indo-1 fluorescence was measured from the epicardial surface of ~0.3 cm^2 area of the LV free wall using a

spectrofluorometer (Photon Technology International, London, Ontario, Canada) fitted with a bifurcated fiber optic cable containing both excitation (354 nm) and emission bundles. Signals were acquired at 500 Hz and the ratio of indo-1 fluorescence emitted at 405 nm and 485 nm was calculated to provide an index of diastolic $[Ca^{2+}]_i$ ($d[Ca^{2+}]_i$) and systolic $[Ca^{2+}]_i$ ($s[Ca^{2+}]_i$). Ca^{2+} transient amplitude was calculated as $s[Ca^{2+}]_i$ minus $d[Ca^{2+}]_i$.

4.2.3 Perfusion protocol

Ischemia-reperfusion protocol

After an initial 45 min of baseline aerobic perfusion, hearts were subjected to 17 min of global ischemia (GI) followed by 30 min of reperfusion. Hearts used for $Ca^{2+}{}_i$ concentration measurements were perfused for 15 min of baseline aerobic perfusion after the indo-1AM loading procedure. This was followed by 20 min of GI and 30 min of reperfusion. We had to use a longer duration of ischemia in the hearts used for $Ca^{2+}{}_i$ concentration measurements in order to obtain comparable degrees of injury as we observed that the Ca^{2+} chelating effect of indo-1AM could potentially reduce the extent of ischemic injury. The GSK-3 inhibitor, SB216763, (SB, 3 μM) or vehicle (DMSO, 0.25%) were added either 5 min prior to ischemia or at the onset of reperfusion. As hearts were perfused in a recirculating system, hearts were exposed to SB during ischemia and throughout reperfusion in the case of pre-ischemic administration or only throughout

reperfusion in the case of administration at the onset of reperfusion. This concentration of SB was shown previously to produce sufficient inhibition of GSK-3 and to induce cardioprotection in the isolated perfused rat heart (244,410).

Aerobic protocol

Two series of hearts with different pre-ischemic glycogen contents were studied. In the first series, glycogen replete (G-Replete), myocardial glycogen stores were replenished prior to administration of either SB (3 μM) or vehicle to near the normal level reported for the rat heart *in vivo* (120-150 μmol/g dry wt) (392). These hearts were perfused initially for 10 min in Langendorff mode (unpaced) followed by 45 min of aerobic perfusion in working mode with modified Krebs-Henseleit solution containing both glucose and palmitate. In the second series, glycogen depleted (G-Depleted), myocardial glycogen stores were depleted non-ischemically prior to administration of either SB or vehicle during an initial 35 min Langendorff perfusion with substrate-free modified Krebs-Henseleit solution (no glucose or palmitate) while pacing at 420 beats/min, as described previously (393). This was followed by 20 min of aerobic perfusion in the working mode with modified Krebs-Henseleit solution containing both glucose and palmitate. At the end of this baseline perfusion, glycogen levels were equivalent to those measured at the onset of reperfusion in the hearts subjected to GI (Figure 4.1).

At the end of each perfusion protocol, hearts were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid N_2. Additional hearts were frozen at the end of the baseline perfusion period as well as at the end of ischemia. Frozen tissues were pulverized at the temperature of liquid N_2 and the resulting powders were stored at -80°C for further biochemical analyses.

4.2.4 Measurement of glycolysis, glucose and palmitate oxidation and H^+ production

Steady state rates of glycolysis and glucose oxidation were measured by perfusing hearts with [5-^{3}H/U-^{14}C] glucose while rates of palmitate oxidation were measured by perfusing the hearts with [9,10-^{3}H]palmitate as described previously in Chapter 2. Rates of H^+ production were calculated as described in Chapter 2

4.2.5 Calculation of tricarboxylic acid cycle activity and ATP production

TCA cycle activity was calculated from the measured rates of glucose and palmitate oxidation on the basis that the oxidation of 1 mole of glucose produces 2 moles of acetyl CoA and 1 mole of palmitate produces 8 moles of acetyl CoA. ATP production was calculated from the measured rates of glycolysis, glucose and palmitate oxidation on the basis that 1 mole of glucose produces 2 moles of

ATP through glycolysis and 31 moles of ATP through glucose oxidation and 1 mole of palmitate produces 104 moles of ATP (413).

4.2.6 Measurement of glycogen content, glycogen turnover, and glucose uptake

Myocardial glycogen content (μmol glucosyl units/g dry wt) as well as rates of glycogen synthesis and degradation and glucose uptake were determined as described in detail in Chapter 2.

4.2.7 Statistical analyses

Results are expressed as means ± SEM of *n* observations. The significance of the differences for two group comparisons was estimated by Student's t-test. The significance of difference in time-course experiments was estimated by two-way ANOVA with repeated measures on time and a Newman-Keuls post-test. Differences were considered statistically significant when $P<0.05$.

4.3 Results

4.3.1 GSK-3 inhibition enhances recovery of LV mechanical function following ischemia

LV mechanical function was stable during the initial period of baseline perfusion with no differences among experimental groups (Figure 4.2A). SB216763 (3 μM) given 5 min before ischemia significantly enhanced recovery of LV mechanical function to 53.0±5.6% (n=11, $P<0.001$) compared with 21.1±5.3% (n=17) in vehicle-treated hearts (Figure 4.2B). This cardioprotective effect of SB is similar to effects observed in previous studies (244,410).

4.3.2 GSK-3 inhibition stimulates glycogen synthesis and reduces glycolysis and H^+ production during reperfusion

SB stimulated the rate of glycogen synthesis as measured by the degree of incorporation of radiolabelled glucose into glycogen by 118% ($P<0.01$) (Figure 4.3A). The rate of glycogen synthesis in individual hearts is directly correlated with its post-ischemic recovery of LV function ($r^2=0.51$, $P<0.005$, Figure 4.5A), providing evidence of a potential role of enhanced glycogen synthesis in cardioprotection.

Stimulation of glycogen synthesis by SB was associated with repartitioning of G-6-P away from glycolysis, causing a 62% inhibition ($P<0.01$) relative to vehicle-treated hearts (Figure 4.3B). Evidence for repartitioning of G-6-P between these pathways is strengthened by the significant inverse correlation between rates of glycogen synthesis and glycolysis ($r^2=0.59$, $P<0.001$) (Figure 4.5B).

Inhibition of GSK-3 also increased rates of glucose (Figure 4.3C) and palmitate oxidation (Figure 4.3D) during reperfusion by 65% and 125%, respectively. There was also a trend ($P=0.053$) towards lower rates of glucose uptake in SB-treated hearts (Figure 4.3E). The SB-mediated inhibition of glycolysis and acceleration of glucose oxidation during reperfusion reduced rates of H^+ production derived from glucose metabolism by 71% ($P<0.01$) (Figure 4.3F).

The increased rates of glucose and palmitate oxidation during reperfusion in SB-treated hearts resulted in a significant elevation in acetyl CoA and ATP production by 99% and 64%, respectively (Figures 4.3G and 4.3H). This increase in ATP production by SB resulted from a higher contribution of palmitate and glucose oxidation, while there was a lower contribution from glycolysis (Figure 4.3I).

4.3.3 Inhibition of GSK-3 attenuates Ca^{2+}_i overload during I/R

To explore further the mechanism by which inhibition of GSK-3 confers cardioprotection, we examined the effect of pre-ischemic administration of SB on Ca^{2+}_i levels (Figure 4.4). Both groups show equivalent $d[Ca^{2+}]_i$ (Figure 4.4D) as well as $s[Ca^{2+}]_i$ (Figure 4.4E) during aerobic baseline perfusion. After 5 min of GI, there was a significant increase in $d[Ca^{2+}]_i$ in both groups. In vehicle-treated hearts, $d[Ca^{2+}]_i$ continued to increase throughout the remaining period of ischemia. However, in SB-treated hearts there was a significant attenuation of diastolic Ca^{2+}_i accumulation after the initial 5 min of GI (by 43% at the end of GI, $P<0.01$) (Figure 4.4D). Ca^{2+}_i transients gradually ceased during GI, so values of $s[Ca^{2+}]_i$ were not detectable (Figure 4.4E). During reperfusion, both $d[Ca^{2+}]_i$ and $s[Ca^{2+}]_i$ recovered only partially in vehicle-treated hearts and remained significantly higher than aerobic values. Inhibition of GSK-3 resulted in a significant reduction of both diastolic and systolic Ca^{2+}_i overload (Figures 4.4D and 4.4E). This was associated with improved recovery of post-ischemic LV function to 71.8±5.2% of baseline ($P<0.001$) relative to 26.7±7.1% in vehicle-treated hearts. There were no significant differences in Ca^{2+}_i transient amplitude between groups (Figure 4.4F). Values for $d[Ca^{2+}]_i$ during reperfusion show a significant inverse correlation with the degree of recovery of LV function ($r^2=0.73$, $P<0.005$, Figure 4.5C).

4.3.4 GSK-3 inhibition results in similar reduction in H^+ production under non-ischemic conditions of glycogen depletion

In order to assess the exact role of the stimulation of glycogen synthesis induced by inhibition of GSK-3, it is important to delineate the cause and effect relationship between glycogen and glucose metabolism and improved LV function during reperfusion. For this purpose, we studied the effects of SB in aerobically perfused hearts (no ischemia) with normal (G-replete) or partially depleted glycogen stores (G-depleted) (Figure 4.2).

Glycogen-replete hearts

SB had no effect on LV work in G-replete hearts (2.41±0.12 *vs*. 2.44±0.25 Joules/min/g dry wt). SB elicited only a minor alteration in the rate of glycogen synthesis that was not significantly different from vehicle-treated hearts (Figure 4.6A). As a result, SB did not alter rates of glycolysis (Figure 4.6B), glucose oxidation (Figure 4.6C) or H^+ production (Figure 4.6D) in G-replete hearts.

Glycogen-depleted hearts

SB had no effect on LV work in G-depleted hearts (2.12±0.21 *vs*. 2.07±0.14 Joules/min/g dry wt). SB induced a significant increase in the rate of glycogen synthesis in G-depleted hearts by 40% ($P<0.05$) (Figure 4.6E) which was accompanied by reduced rates of glycolysis (by 31%, $P<0.005$) (Figure 4.6F)

and H^+ production (by 38%, $P<0.005$) (Figure 4.6G). SB did not affect glucose oxidation (Figure 4.6H).

These results confirm that inhibition of GSK-3, through its effects on glycogen synthesis, results in reduction of H^+ production independent of LV mechanical function and that these effects are dependent on the level of myocardial glycogen.

4.3.5 Inhibition of GSK-3 protects against reperfusion injury

Administration of SB (3 μM) only at the onset of reperfusion also improved the recovery of LV mechanical function to 66.9±7.3% (n=6, $P<0.0005$) compared with 21.1±5.3% (n=17) in vehicle-treated hearts (Figure 4.7A). This beneficial effect was also due to acceleration of glycogen synthesis (by 96%, $P<0.05$) (Figure 4.7B) and a subsequent inhibition of glycolysis (by 49%, $P<0.05$) (Figure 4.7C). Glucose oxidation was accelerated by SB (by 47%, $P<0.05$) (Figure 4.7D), H^+ production from glucose metabolism was reduced (by 58%, $P<0.05$) (Figure 4.7E), while glucose uptake was unaltered. Similar to our previous results, the reduction in H^+ production during reperfusion resulted in a significant attenuation of Ca^{2+}_i overload (Figure 4.7F).

4.4 Discussion

The first evidence for the role of GSK-3 in cardioprotection was obtained in studies showing that ischemic preconditioning results in phosphorylation and inhibition of GSK-3β and that pharmacological inhibition of GSK-3 mimics the cardioprotective effects of preconditioning (244). Now, extensive evidence supporting the role of GSK-3 inhibition in ischemic as well as various forms of drug-induced preconditioning and postconditioning is emerging (see reviews (414-418)) and GSK-3 is now attracting considerable research attention as it is considered to participate in a common final pathway of cardioprotection leading to inhibition of the opening of MPTP, and to improved cell survival (41). Our demonstration that SB, administered either before ischemia or at the onset of reperfusion, enhances recovery of post-ischemic mechanical function confirms that drug-inhibition of GSK-3 is cardioprotective (244,410,419). More importantly, this study provides new insights about mechanisms resulting from GSK-3 inhibition and indicates a role for altered glucose metabolism as an early and upstream event. Specifically, our data indicate that inhibition of GSK-3 increases glycogen synthesis during reperfusion, which partially repartitions G-6-P away from glycolysis. The reduced rate of glycolysis lessens intracellular acidosis during reperfusion and the potential for Na^+ accumulation (via NHE) that leads to the observed attenuation of Ca^{2+}_i overload (via rmNCX). The reduced Ca^{2+}_i overload is potentially an upstream event leading to enhanced mitochondrial

function during reperfusion and improved mitochondrial oxidative capacity. We also provide evidence that acceleration of glycogen synthesis is not a consequence of improved LV function, as similar metabolic alterations occur in glycogen-depleted aerobic hearts independent of changes in LV mechanical function.

In order to examine the relative rates of glycogen synthesis and glycolysis in the absence and presence of GSK-3 inhibition, studies were performed in isolated rat hearts that were perfused in working mode with both glucose and palmitate as energy substrates. These conditions ensure hearts are studied under conditions of physiological work-load (energy demand) as well as adequate energy supply. Moreover, aerobic perfusion conditions ensure the re-establishment of normal glycogen content (previously severely depleted during deep anesthesia and heart extraction), a key requirement for investigations of glucose and glycogen metabolism. Also, this experimental approach enables LV work to be measured simultaneously with rates of glucose, glycogen, and palmitate metabolism or with beat by beat analysis of $d[Ca^{2+}]_i$ and $s[Ca^{2+}]_i$.

Consistent with previous reports (46,412,420), ischemia results in significant $Ca^{2+}{}_i$ accumulation in untreated hearts that only partially recovers during reperfusion and this is associated with partial recovery of LV mechanical function. The significant attenuation of $Ca^{2+}{}_i$ overload by GSK-3 inhibition during reperfusion and the enhanced recovery of mechanical function are potentially direct results of inhibition of glycolysis, H^+ production and acidosis

(381) that lessens Na^+ accumulation and rmNCX activity. It is unlikely that GSK-3 inhibition exerts its action via changes in SR Ca^{2+} release, as no changes occur in the amplitude of Ca^{2+}_i transients.

GSK-3 inhibition also reduces Ca^{2+}_i overload during ischemia. This beneficial effect is independent of LV work, but as rates of glucose metabolism could not be measured during GI, the role of glucose metabolism in limiting Ca^{2+}_i overload during ischemia cannot be determined. While alteration of glycolysis may be involved, other mechanisms arising from GSK-3 inhibition during ischemia may contribute, such as improved ionic homeostasis due to reduced mitochondrial ATP consumption, an effect possibly due to interaction of GSK-3 with VDAC (410). Our demonstration that cardioprotection was similar in hearts treated with SB either before ischemia or at the onset of reperfusion suggests that the effect of GSK-3 inhibition on Ca^{2+}_i overload during ischemia is unlikely to play an important role in the enhanced recovery of mechanical function during early reperfusion.

A clear association between stimulation of glycogen synthesis, partial inhibition of glycolysis and attenuation of Ca^{2+}_i overload is demonstrable in hearts subjected to I/R. However, as LV mechanical function (energy demand) and energy substrate metabolism are interdependent, additional experiments were performed in aerobic hearts in order to determine if alteration of glucose partitioning might simply be a consequence, rather than a cause, of enhanced

recovery of LV function. The ability of SB to produce a similar re-partitioning of glucose metabolism in aerobic hearts that are partially depleted of glycogen to levels similar to the end of GI confirms that the alterations in metabolism are not a consequence of changes in LV function. Rather, it indicates that the enhanced recovery of LV function is due to the changes in metabolism.

Similar beneficial consequences of inhibition of H^+ production from glucose metabolism have been reported previously (28,58,59) and result mainly from reduced activation of the coupled exchangers, NHE and rmNCX, that reduce Na^+_i and Ca^{2+}_i accumulation, respectively, that lessens Ca^{2+}_i overload and LV mechanical dysfunction (45). Other approaches that limit Ca^{2+}_i overload also reduce I/R injury such as pharmacological inhibition or genetic ablation of NHE (34,46,56) or NCX (54,57). On the other hand, several studies have shown that prolongation of acidosis during reperfusion is cardioprotective (421-424). In order to assess the role of acidosis during reperfusion, it is important to distinguish between extracellular and intracellular acidosis. In those studies, reperfusion with acidic solution was utilized to prolong acidosis during reperfusion which reduces the trans-membrane proton gradient and so will inhibit the activity of the Na^+/H^+ exchanger, thereby limiting the exchange of intracellular H^+ with extracellular Na^+. This will eventually limit Ca^{2+}_i overload. Partial inhibition of glycolysis and H^+ production is an upstream event that

hinders activation of NHE and subsequently rmNCX, thereby reducing dysregulation of myocardial ionic homeostasis.

A well-described downstream consequence of GSK-3 inhibition is delayed opening of MPTP in response to ROS (41). The role of GSK-3 in limiting MPTP opening was proposed to arise by direct phosphorylation of VDAC and prevention of its binding to hexokinase (101), but more recent studies indicate that VDAC is not required for MPTP formation and does not possess a regulatory role in MPTP opening (76). Thus, the uncertain identity of the MPTP complex limits a clear interpretation of its interactions with GSK-3. However, direct interaction of GSK-3 with VDAC reduces adenine nucleotide transport across the outer mitochondrial membrane independent of PTP opening (410), thereby conserving ATP content by reducing mitochondrial ATP consumption. Such a preservation of ATP may facilitate ionic homeostasis and explain our observation that SB attenuates Ca^{2+}_i overload during ischemia. However, it cannot explain cardioprotection when SB is administered only at the onset of reperfusion, a period when ATP generation returns to close to pre-ischemic levels (227). Instead, we propose a cytosolic action of GSK-3 inhibition that may indirectly modulate MPTP opening, via reduced acidosis during reperfusion and attenuation of Ca^{2+}_i overload. Reintroduction of oxygen and restoration of the mitochondrial membrane potential during reperfusion, together with elevated Ca^{2+}_i levels, is expected to cause a significant Ca^{2+} uptake into the mitochondria

through the mitochondrial Ca^{2+} uniporter (20). As elevation of mitochondrial matrix Ca^{2+} levels is an essential factor for MPTP opening (79), the lower Ca^{2+}_i levels during reperfusion resulting from GSK-3 inhibition likely limits MPTP opening. Although the open probability of MPTP is reduced sharply in acidic pH in de-energized mitochondria (80,425), exposure of respiring mitochondria to an acidic environment, such as in early reperfusion, will favor mitochondrial inorganic phosphate uptake that facilitates MPTP opening (81). Thus, reduction in H^+ production during reperfusion may limit MPTP formation. Thus, we propose that GSK-3 inhibition and the re-partitioning of glucose metabolism is an early and upstream event that leads to less Ca^{2+}_i overload and enhanced recovery of LV function. Reduced acidosis and attenuation of Ca^{2+}_i overload may ultimately lead to inhibition of MPTP opening and improved cell viability. This may also explain the improved mitochondrial function, demonstrated by enhanced glucose and palmitate oxidation, during reperfusion in SB-treated hearts. The stimulated mitochondrial oxidation may also arise due to the improved recovery of LV function and higher energy demand in SB-treated hearts. Furthermore, a direct interaction is unlikely as GSK-3 inhibition has no effect on MPTP opening in isolated mitochondria (426).

Conclusion

Although GSK-3 was initially discovered and named for its role in regulating glycogen metabolism, this is the first study to link this important effect

on myocardial metabolism with cardioprotection. Our study highlights the ability of GSK-3 to regulate myocardial glycogen and glucose metabolism and demonstrates an additional mechanism linking GSK-3 inhibition with enhanced recovery of post-ischemic mechanical function. Inhibition of GSK-3 during reperfusion stimulates glycogen synthesis, which repartitions G-6-P away from the glycolytic pathway. The reduced rate of glycolysis lessens H^+ production from glucose metabolism and subsequently reduces Ca^{2+}_i overload. These effects limit LV mechanical dysfunction in early reperfusion and may contribute to improvements in mitochondrial function and cell viability.

Figure 4.1 Glycogen content in G-replete, G-depleted hearts, and in hearts subjected to GI

Glycogen content (μmol/g dry wt) in G-replete (n=7) and G-depleted (n=7) aerobic hearts, as well as in hearts subjected to 17 min of GI (n=5). Values are means ± SEM. *P<0.05 compared with G-replete hearts.

Figure 4.1

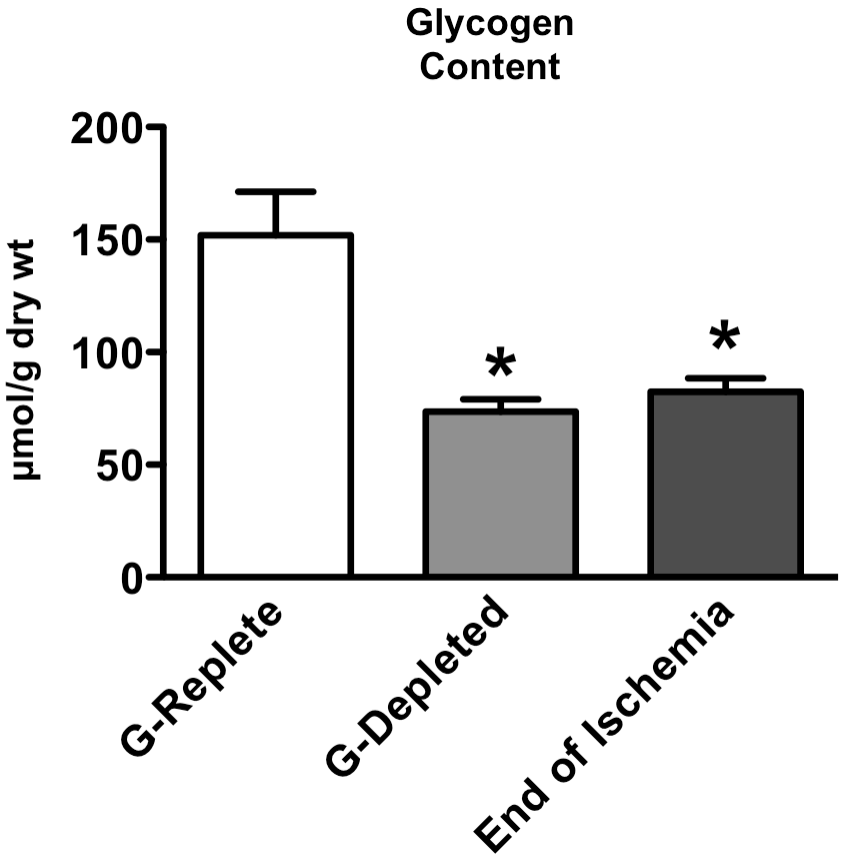

Glycogen
Content
200
150
100
50
0
µmol/g dry wt
*
*
G-Replete
G-Depleted
End of Ischemia

Figure 4.2 SB improves recovery of LV function during reperfusion

A, LV work (Joules/min/g dry wt) during I/R protocol in isolated working rat hearts treated with vehicle (DMSO, 0.25%, n=17, □) or SB (3 μM, n=11, ■) added 5 min prior to onset of global ischemia (GI) and present throughout reperfusion. B, Recovery of LV work expressed as % of baseline LV work. Values are means ± SEM. **P*<0.05 compared with vehicle-treated hearts as determined by repeated measures two-way ANOVA (A) or Student's t-test (B).

Figure 4.2

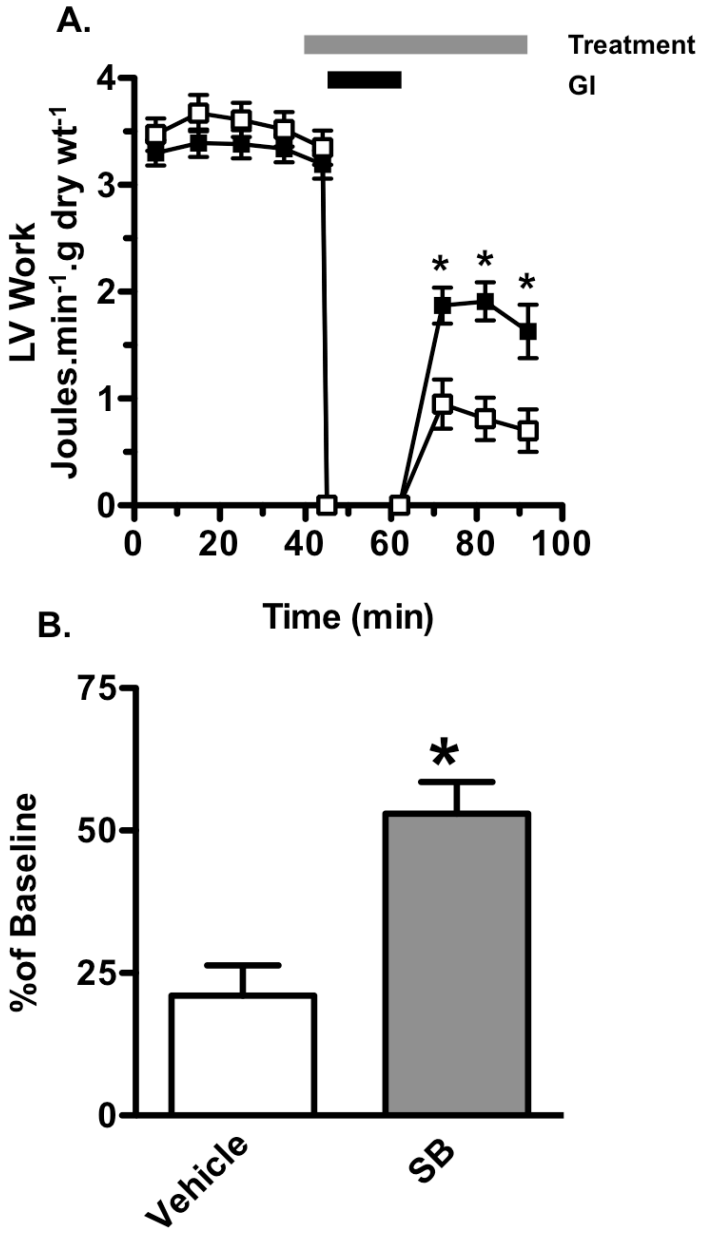

A.
Treatment
GI
LV Work
Joules.min-1.g dry wt-1
4
3
2
1
0
0
20
40
60
80
100
Time (min)
*
*
*
B.
%of Baseline
75
50
25
0
*
Vehicle
SB

Figure 4.3 SB-mediated alterations in energy substrate metabolism during reperfusion.

Values for metabolic rates (μmol/min/g dry wt) are shown for glycogen synthesis (n=10, 6) (A) glycolysis (GLY, n=10, 6) (B) glucose oxidation (GOX, n=17, 11) (C), palmitate oxidation (FOX, n=7, 5) (D), glucose uptake (n=10, 6) (E), H^+ production (n=10, 6) (F), acetyl CoA production (n=7, 5) (G), ATP production (H), and % ATP contribution (I). Values are means ± SEM. **P*<0.05 compared with vehicle-treated hearts as determined by Student's t-test.

Figure 4.3

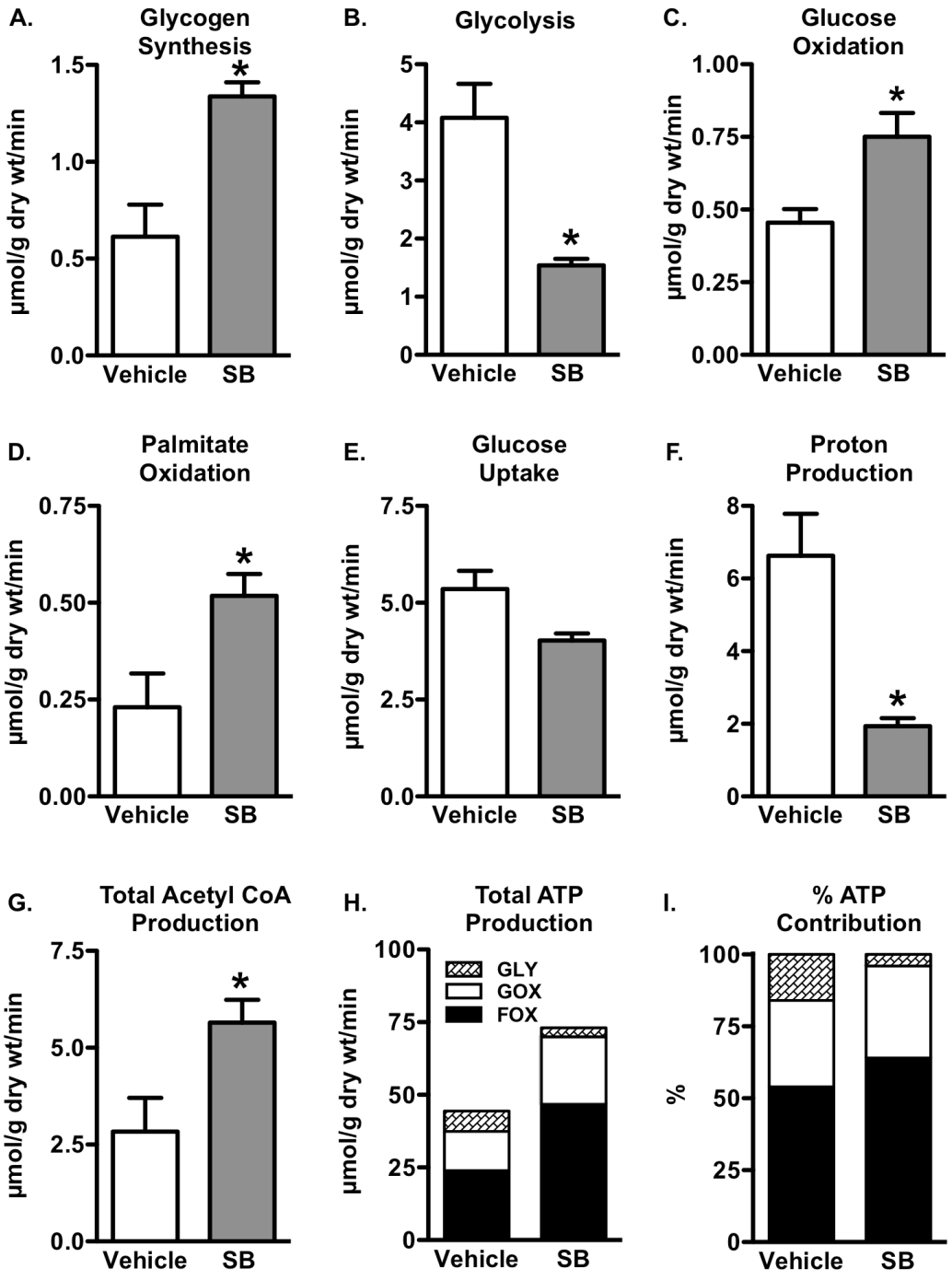

A.
Glycogen Synthesis
µmol/g dry wt/min
1.5
1.0
0.5
0.0
*
Vehicle
SB
B.
Glycolysis
µmol/g dry wt/min
5
4
3
2
1
0
*
Vehicle
SB
C.
Glucose Oxidation
µmol/g dry wt/min
1.00
0.75
0.50
0.25
0.00
*
Vehicle
SB
D.
Palmitate Oxidation
µmol/g dry wt/min
0.75
0.50
0.25
0.00
*
Vehicle
SB
E.
Glucose Uptake
µmol/g dry wt/min
7.5
5.0
2.5
0.0
Vehicle
SB
F.
Proton Production
µmol/g dry wt/min
8
6
4
2
0
*
Vehicle
SB
G.
Total Acetyl CoA Production
µmol/g dry wt/min
7.5
5.0
2.5
0.0
*
Vehicle
SB
H.
Total ATP Production
µmol/g dry wt/min
100
75
50
25
0
GLY
GOX
FOX
Vehicle
SB
I.
% ATP Contribution
%
100
75
50
25
0
Vehicle
SB

Figure 4.4 SB alters Ca^{2+}_i overload during ischemia and reperfusion.

Representative traces for Ca^{2+}_i oscillations at aerobic baseline (A), reperfusion in vehicle-treated hearts (B), and reperfusion in presence of SB (C). Dashed lines represent the average level of diastolic Ca^{2+}_i ($d[Ca^{2+}]_i$) (lower) and systolic Ca^{2+}_i ($s[Ca^{2+}]_i$) (upper) in aerobic hearts. Levels of $d[Ca^{2+}]_i$ (D) during I/R in hearts treated with vehicle (DMSO, 0.25%, n=5, □) or SB (3 μmol/L) added 5 min prior to the onset of ischemia and present throughout reperfusion (n=5, ■). Levels of $s[Ca^{2+}]_i$ (E) during I/R as well as Ca^{2+}_i transient amplitudes (F) are also presented. Values are means ± SEM. **P*<0.05 compared with vehicle-treated hearts as determined by repeated measures two-way ANOVA.

Figure 4.4

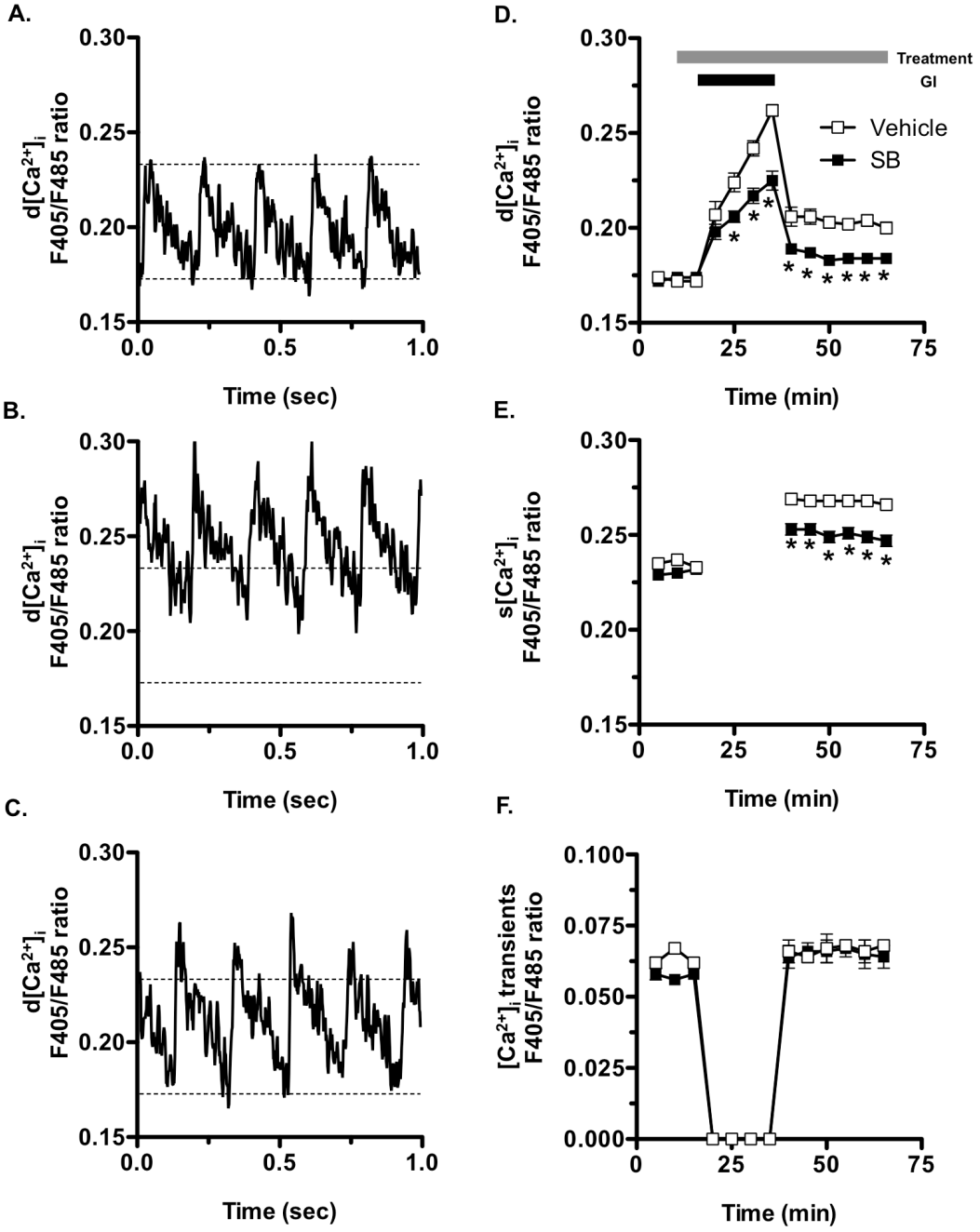

A.
B.
C.
D.
E.
F.
d[Ca2+]i F405/F485 ratio
s[Ca2+]i F405/F485 ratio
[Ca2+]i transients F405/F485 ratio
Time (sec)
Time (min)
Treatment
GI
Vehicle
SB
0.30
0.25
0.20
0.15
0.100
0.075
0.050
0.025
0.000
0.0
0.5
1.0
0
25
50
75

Figure 4.5 Correlations between rates of glycogen synthesis and post-ischemic recovery of LV work, rates of glycogen synthesis and rates of glycolysis, and post-ischemic recovery of LV work and levels of d[Ca^{2+}_i]

Correlations (by linear regression) between: A, rates of glycogen synthesis and % recovery of LV work during reperfusion (n=16, r^2=0.51, P<0.005), B, rates of glycogen synthesis and rates of glycolysis during reperfusion (n=16, r^2=0.59, P=0.0005), and C, levels of d[Ca^{2+}]$_i$ and % recovery of LV work during reperfusion (n=10, r^2=0.73, P<0.005).

Figure 4.5

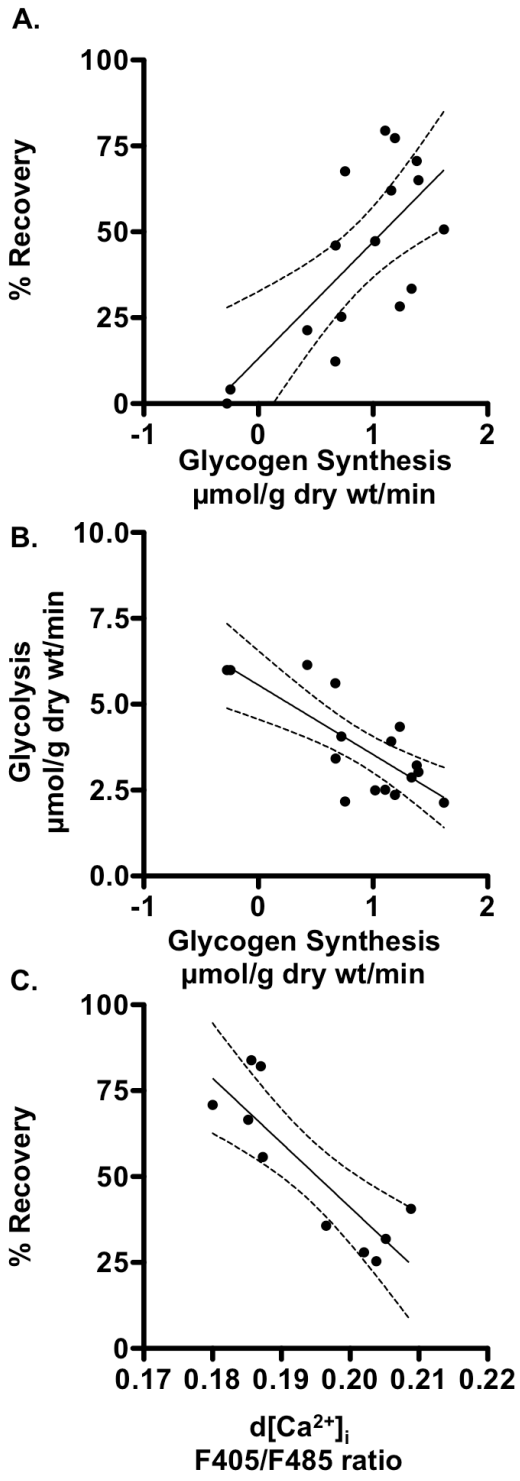

A.
100
75
50
25
0
% Recovery
-1
0
1
2
Glycogen Synthesis
µmol/g dry wt/min
B.
10.0
7.5
5.0
2.5
0.0
Glycolysis
µmol/g dry wt/min
-1
0
1
2
Glycogen Synthesis
µmol/g dry wt/min
C.
100
75
50
25
0
% Recovery
0.17 0.18 0.19 0.20 0.21 0.22
d[Ca2+]i
F405/F485 ratio

Figure 4.6: Effects of SB on glycogen and glucose metabolism in aerobic hearts.

Rates (μmol/min/g dry wt) of glycogen synthesis , glycolysis, glucose oxidation, and H^+ production from glucose metabolism are shown for G-replete (A,B,C, and D, respectively) as well as for G-depleted hearts (E,F,G, and H, respectively) (n=7 per group). Values are means ± SEM. **P*<0.05 compared with vehicle-treated hearts as determined by Student's t-test.

Figure 4.6

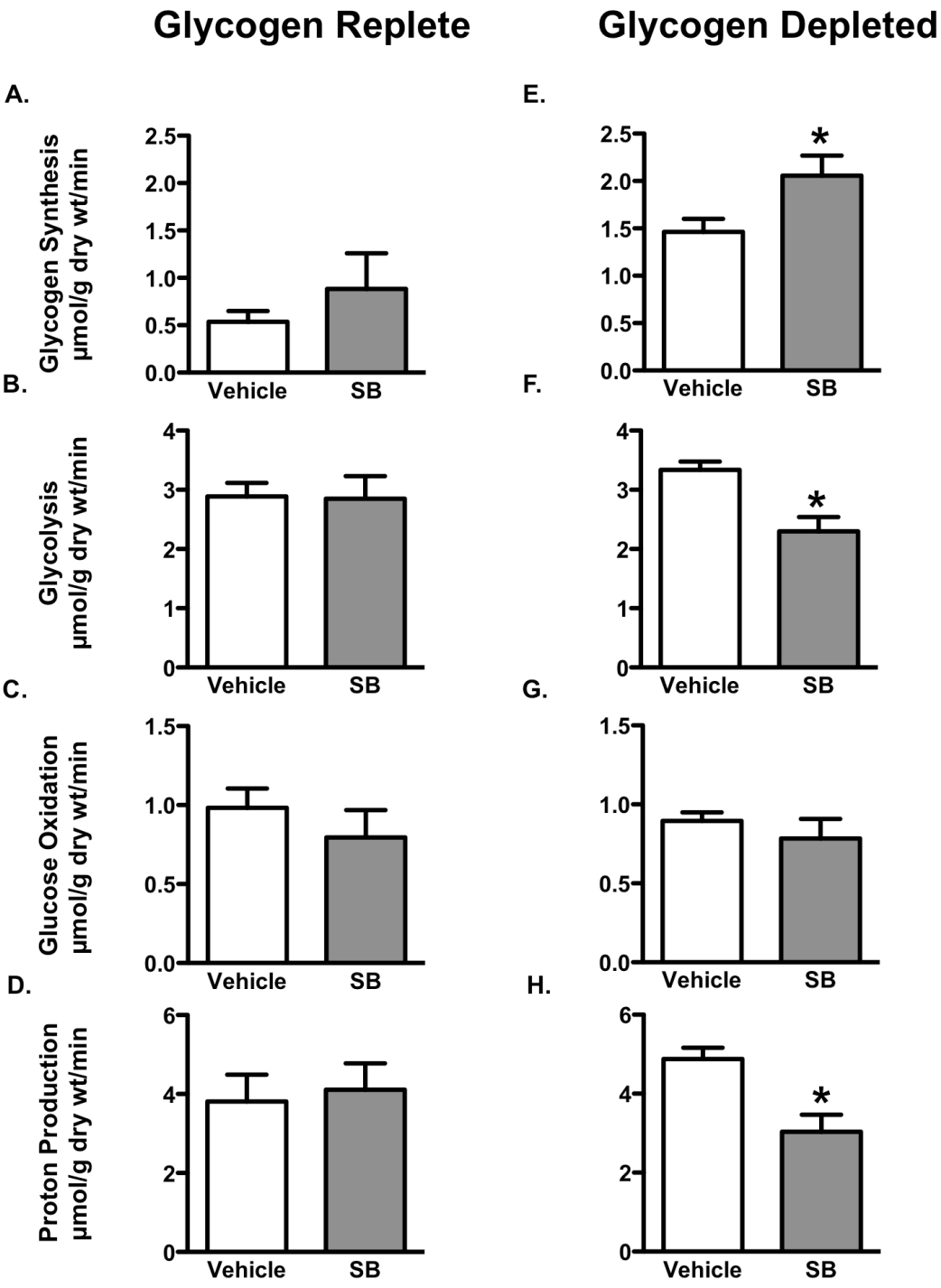
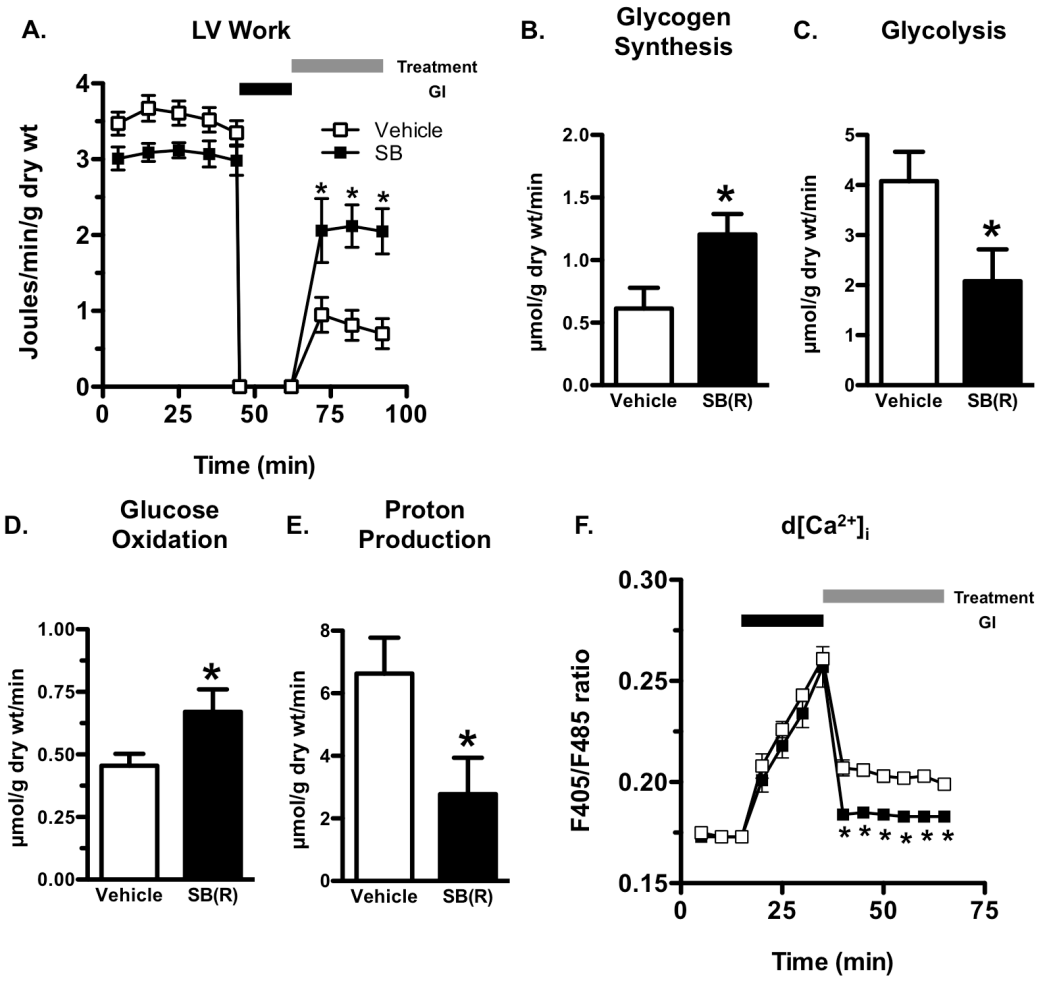

Glycogen Replete
Glycogen Depleted
A.
E.
Glycogen Synthesis µmol/g dry wt/min
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
SB
*
B.
F.
Glycolysis µmol/g dry wt/min
4
3
2
1
0
C.
G.
Glucose Oxidation µmol/g dry wt/min
1.5
1.0
0.5
0.0
D.
H.
Proton Production µmol/g dry wt/min
6
4
2
0

Figure 4.7: Effects of SB when administered at the onset of reperfusion.

A, LV work during I/R protocol in isolated working rat hearts treated with vehicle (DMSO, 0.25%, n=17, □) or SB (3 μM, n=6, ■) added at the onset of reperfusion and present throughout the remaining perfusion protocol. Values for metabolic rates (μmol/min/g dry wt) are shown for glycogen synthesis (B), glycolysis (C), glucose oxidation (D), and H^+ production (E). F, levels of $d[Ca^{2+}]_i$ during I/R protocol. Values are means ± SEM. **P*<0.05 compared with vehicle-treated hearts as determined by repeated measures two-way ANOVA (A,F) or Student's t-test (B,C,D,E).

Figure 4.7


A.
LV Work
Treatment
GI
Vehicle
SB
Joules/min/g dry wt
Time (min)
B.
Glycogen Synthesis
µmol/g dry wt/min
Vehicle
SB(R)
C.
Glycolysis
µmol/g dry wt/min
Vehicle
SB(R)
D.
Glucose Oxidation
µmol/g dry wt/min
Vehicle
SB(R)
E.
Proton Production
µmol/g dry wt/min
Vehicle
SB(R)
F.
d[Ca2+]i
Treatment
GI
F405/F485 ratio
Time (min)

5. Cardioprotection by Adenosine: Protein Phosphatase Activation Inhibits 5'-AMP-Activated Protein Kinase and p38 Mitogen-Activated Protein Kinase and Lessens Glycolysis and Proton Production during Reperfusion

5.1 Introduction

Adenosine (ADO) is a major autocrine and paracrine regulator of tissue function, especially under conditions of diminished energy supply. During myocardial ischemia, reduced aerobic metabolism, accelerated catabolism of ATP, and limited washout of metabolites results in ADO accumulation that limits cardiomyocyte injury (250). While endogenous ADO participates in the cardioprotective benefits of preconditioning (269) and postconditioning (270), exogenous ADO or selective ADO receptor agonists, when present throughout ischemia and/or reperfusion, improve recovery of left ventricular (LV) post-ischemic mechanical function and reduce infarct size (250).

A number of mechanisms have been identified to explain the range of cardioprotective actions of ADO, and a key factor for the improved recovery of LV mechanical function during post-ischemic reperfusion is inhibition of intracellular Ca^{2+} overload (427). In untreated hearts, Ca^{2+} overload arises during ischemia-reperfusion (I/R) due to stimulation of sodium-calcium exchanger (NCX) activity by increases in intracellular Na^{+}, mediated, in part, by stimulation of sodium-hydrogen exchanger (NHE) activity in response to intracellular proton accumulation (20). An important source of intracellular protons that drives these coupled exchangers is the hydrolysis of ATP derived from high rates of glycolysis that exceed rates of glucose oxidation (termed uncoupled glucose metabolism)

(45). Thus, drug-induced inhibition of glycolysis or acceleration of glucose oxidation lessens proton production and thereby reduces Ca^{2+} overload (59,381). Indeed, ADO, as well as A_1-selective ADO receptor agonists, inhibit glycolysis in reperfused hearts, and the associated inhibition of proton production lessens activation of the coupled exchangers, reduces Ca^{2+} overload and so enhances recovery of LV mechanical function (28,232,427). Thus, elucidation of the mechanism(s) of glycolytic inhibition by ADO may help identify additional drug targets to limit proton production and enhance post-ischemic LV function.

Glycolysis is a well-characterized pathway for the metabolism of glucose to pyruvate, and a key rate-limiting step is the conversion of fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-BP) catalyzed by phosphofructokinase-1 (PFK-1). 5'-AMP activated protein kinase (AMPK), a stress-activated protein kinase, stimulates myocardial glycolysis via activation of that rate-limiting step through its action on phosphofructokinase-2 (PFK-2) (165). As we have shown previously that ADO, as well as ADO A_1 receptor agonists, inhibit AMPK (224,226), we determined whether ADO-mediated inhibition of glycolysis is due to reduced AMPK-mediated PFK-2 phosphorylation. p38 mitogen activated protein kinase (MAPK) is another stress-responsive protein kinase that is activated by ischemia (428) and has similar actions as AMPK on myocardial glucose metabolism (429) and fatty acid oxidation (370). Interactions between p38 MAPK and AMPK, as well as between p38 MAPK and ADO, have

been identified (372,375,377), but the role of p38 MAPK in the regulation of myocardial glycolysis has not been determined.

The activities of AMPK and p38 MAPK are regulated by the extent of phosphorylation, and regulation by their respective upstream kinases has been well characterized (430,431). However, dephosphorylation by Ser/Thr protein phosphatases (PP) (PP2A and PP2C) also plays an important role (313,314,432). As activation of ADO A_1 receptors in cardiomyocytes activates PP2A (433) and cantharidin, a PP2A and PP1 inhibitor, limits the anti-adrenergic effects of ADO A_1 receptor agonists (434), we hypothesized that AMPK and/or p38 MAPK activity may be inhibited by ADO-induced stimulation of PP activity.

In this study, we utilized isolated perfused working rat hearts to determine the role of the stress-activated kinases, AMPK and p38 MAPK, in ADO-mediated inhibition of glycolysis and cardioprotection. We tested the hypothesis that ADO-induced inhibition of glycolysis and cardioprotection are the result of PP-mediated reduction of AMPK and p38 MAPK activation during reperfusion, an effect that limits activation of the glycolytic pathway by PFK-2.

5.2 Methods

5.2.1 Heart Perfusions

Male Sprague-Dawley rat hearts were cannulated for isolated working mode perfusion as described in Chapter 2. Systolic and diastolic aortic pressures (mmHg), cardiac output (ml/min), and aortic flow (ml/min) were measured as described in Chapter 2. Coronary flow (ml/min/g dry wt), LV work (Joules/min/g dry wt), and CVC (ml/min/mmHg/g dry wt) were calculated as described in Chapter 2

5.2.2 Perfusion protocols

Hearts were perfused for an initial aerobic baseline period of 45 min followed by 17 min of no-flow global ischemia (GI) and 30 min of reperfusion. This duration of GI was sufficient to produce marked LV dysfunction that is partially reversed during post-ischemic reperfusion. Hearts were either untreated (Control) or were exposed to ADO (500 μM, added 5 min prior to the onset of GI and present throughout reperfusion) or a selective p38 MAPK inhibitor (SB202190, 10 μM, added at the onset of reperfusion). Inhibition of Ser/Thr protein phosphatases was achieved by adding cantharidin (5 μM) to heart perfusate 10 min prior to ADO. In order to examine the effects of ADO in the absence of I/R-mediated changes in LV work, additional groups of hearts were

perfused aerobically for 80 min, either in the absence or presence of ADO (500 μM, present between 45 and 80 min).

At the end of each perfusion protocol, hearts were rapidly frozen using a Wollenberger clamp cooled to the temperature of liquid N_2. In order to examine hearts immediately before or after GI, additional groups were perfused and harvested at the end of aerobic baseline perfusion or following GI. Frozen tissues were pulverized at the temperature of liquid N_2, and the resulting powders were stored at -80 ºC.

5.2.3 Measurements of rates of glucose metabolism

Rates of glycolysis, glucose oxidation, H^+ production, and glucose uptake (μmol/min/g dry wt) were measured as described in Chapter 2. Samples of perfusate were collected every 10 min during the perfusion protocol and rates were calculated for each time interval and then averaged for each phase of the perfusion protocol.

5.2.4 Measurements of glycogen content and glycogen turnover

Myocardial glycogen content (μmol glucosyl units/g dry wt) was determined as described in Chapter 2. The net rate of glycogen synthesis (μmol/min/g dry wt) was determined as described previously from the rate of incorporation of radiolabelled glucose into myocardial glycogen. The net rate of

glycogen degradation was measured from the change in the unlabeled myocardial glycogen pool.

5.2.5 Measurement of glycolytic metabolites

G-6-P, F-6-P, and F-1,6-BP contents in heart tissue (nmol/g dry wt) were determined by a coupled enzymatic assay as described previously (435). Briefly, heart tissue samples (100 mg) were deproteinized with perchloric acid and supernatants were adjusted to pH 3.5 using K_2CO_3 (5 M). Supernatant concentrations of G-6-P were measured by oxidation to gluconolactone-6-phosphate by G-6-P dehydrogenase with the production of NADPH (measured by increase in absorbance at 339 nm). F-6-P was measured by a similar approach after it was initially converted to G-6-P by phosphoglucose isomerase. F-1,6-BP was measured by initially converting it to dihydroxyacetone phosphate (DAP) and glyceraldehyde-3-phosphate (GAP) by addition of aldolase. The resultant GAP was converted to DAP by triose phosphate isomerase. Glycerol-3-phosphate dehydrogenase catalyzed the reduction of DAP into glycerol-3-phosphate with the consumption of NADH (measured as the decrease in absorbance at 339 nm

5.2.6 Gel electrophoresis and immunoblot analysis

Frozen powdered heart tissue was homogenized and subjected to immunoblot analysis using a range of primary antibodies raised against phosphorylated and total AMPK, p38 MAPK, and LKB-1 (Cell Signaling

Technology, Danvers, Mass), as well as phosphorylated PFK-2 and actin (1:1,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, California). Densitometric analyses of immunoblots were performed using ImageJ software (National Institute of Health, Bethesda, Maryland). Densitometric values for phosphorylated protein were normalized to total protein (normalized to actin in case of P-PFK-2). Values are presented relative to values in control hearts.

5.2.7 Statistical analyses

Results are expressed as means ± SEM of *n* independent observations. The significance of the differences for two group comparisons was estimated by Student's *t*-test. The significance of the differences between three or more groups was estimated by one-way ANOVA and Bonferroni post-test. The significance of differences in time-course experiments was estimated by two-way ANOVA with repeated measures on time and Bonferroni post-test. Differences were considered statistically significant when $P<0.05$. Statistical analysis was performed using GraphPad Prism.

5.3 Results

5.3.1 ADO enhances recovery of post-ischemic LV work

LV work was stable during the initial 45 min of baseline aerobic perfusion with no differences among experimental groups. During reperfusion of control hearts, LV work recovered partially to 29.4±6.7% (n=15) of pre-ischemic values (Figure 5.1a). In addition, coronary flow during reperfusion was depressed to 71.7±9.3% of pre-ischemic values while there was no change in CVC. Relative to control hearts, ADO significantly improved recovery of post-ischemic LV mechanical function to 56.4±6.3% (n=12, $P<0.01$) of pre-ischemic values (Figure 5.1A). Also, coronary flow was restored to near aerobic values in ADO-treated hearts and was significantly higher ($P<0.05$) than in control hearts (Figure 5.1B). CVC was not different between control and ADO-treated groups (Figure 5.1C).

5.3.2 ADO reduces rates of glycolysis and proton production during reperfusion

Rates of glycolysis, glucose oxidation, or proton production were similar in all experimental groups during baseline aerobic perfusion (data not shown). As reported previously for fatty acid perfused working rat hearts (28,232,283), ADO reduced rates of glycolysis (by 43.5%, $P<0.001$, Figure 5.2A), proton production (by 50.1%, $P<0.001$, Figure 5.2C), and glucose uptake (by 35.4%, $P<0.001$,

Figure 5.2D) during reperfusion relative to control hearts. ADO did not alter rates of glucose oxidation (Figure 5.2B).

5.3.3 ADO preserves myocardial glycogen content following reperfusion

To determine whether ADO-induced inhibition of glycolysis was due to a re-partitioning of G-6-P away from glycolysis and towards glycogen synthesis, myocardial glycogen content was measured at the end of reperfusion. In ADO-treated hearts, glycogen content was higher relative to control hearts (by 57.8%, $P<0.005$, Table 5.1). As ADO had no effect on the rate of glycogen synthesis, preservation of glycogen was due to a 94.1% reduction in the rate of glycogen degradation ($P<0.01$) (Figures 5.2E and F).

5.3.4 ADO inhibits glycolysis at the step catalyzed by PFK-1

To identify the target enzyme by which ADO inhibits glycolysis, the myocardial content of three metabolites in the glycolytic pathway, G-6-P, F-6-P, and F-2,6-BP were measured in reperfused hearts (Table 5.2). ADO significantly increased myocardial content of G-6-P (by 28.6%, $P=0.005$) and there was a trend towards an increase of F-6-P (by 26.5%, $P=0.098$). On the other hand, F-2,6-BP content was significantly lower in ADO-treated hearts (by 38.5%, $P<0.005$). The ratio of F-6-P to G-6-P, a useful surrogate measure of phosphoglucose isomerase

activity, was not affected by ADO while the ratio of F-1,6-BP to F-6-P, a measure of PFK-1 activity, was reduced by ADO (by 46.9%, $P<0.005$).

5.3.5 ADO reduces the phosphorylation status of AMPK, p38 MAPK, and PFK-2

To investigate whether ADO altered AMPK activity, immunoblotting was used to measure tissue content of phospho-AMPK (P-AMPK) that was phosphorylated at its activation site (Thr172) and expressed as ratio to total AMPK (Figure 5.3). ADO markedly reduced levels of P-AMPK relative to control hearts by 82.1% ($P<0.005$), but had no effect on the phosphorylation of LKB-1, an upstream activator of AMPK. ADO also inhibited p38 MAPK phosphorylation by 85.7% ($P<0.01$) as assessed by levels of p38 MAPK phosphorylated at sites Thr180 and Tyr182 in its activation loop (430).

It is established that activation of myocardial AMPK 1) increases phosphorylation of PFK-2 at Ser466 resulting in its activation (165), 2) accelerates the synthesis of F-2,6-BP, a key allosteric regulator of PFK-1 activity, and 3) stimulates rates of glycolysis (160). To determine if ADO-induced inhibition of AMPK might lessen rates of glycolysis by inhibiting PFK-2, we used immunoblotting to compare PFK-2 phosphorylation (Ser466) in control and ADO-treated hearts. In accordance with a role of ADO-induced inhibition of

AMPK, PFK-2 phosphorylation (normalized to actin levels) was significantly lower in ADO-treated hearts (by 40.3%, $P<0.01$) (Figure 5.3).

5.3.6 ADO-mediated inhibition of AMPK and p38 MAPK occur during reperfusion

To determine whether the inhibitory action of ADO on AMPK and p38 MAPK phosphorylation observed at the end of reperfusion was due to an effect that occurred during ischemia or during reperfusion, AMPK and p38 MAPK phosphorylation were measured in additional groups of control and ADO-treated hearts that were frozen immediately before (Baseline) or after GI, and compared with values obtained following reperfusion (Figure 5.4). As expected, GI caused a significant activation of both AMPK and p38 MAPK in untreated hearts relative to aerobic baseline values. P-p38 MAPK remained elevated during reperfusion while P-AMPK partially returned to baseline values by the end of reperfusion. ADO, when administered 5 min prior to the onset of GI, did not significantly alter either P-AMPK or P-p38 MAPK at the end of ischemia. It was only in reperfused hearts that the ADO-mediated inhibition of AMPK or p38 MAPK was evident (Figure 5.4).

5.3.7 ADO inhibits glycolysis and AMPK phosphorylation in aerobic hearts

In order to investigate the effects of ADO on myocardial glucose metabolism independent of changes in LV work, the effects of ADO were examined in aerobically perfused non-ischemic hearts. ADO inhibited glycolysis (by 30.2%, $P<0.05$, Figure 5.5A), proton production (by 58.9%, $P<0.05$, Figure 5.5C), glucose uptake (by 27.5%, $P<0.05$, Figure 5.5D) and had no effect on rates of glucose oxidation (Figure 5.5B). ADO had no effect on either glycogen synthesis or glycogen degradation in aerobic hearts (Figures 5.5E and F). As in reperfused hearts, ADO significantly reduced phosphorylation of AMPK (by 82.4%, $P<0.05$) and PFK-2 (by 20%, $P<0.05$). On the other hand, p38 MAPK phosphorylation was not inhibited by ADO in aerobically perfused hearts (Figures 5.5G and H). ADO also increased tissue levels of G-6-P (by 24.2%, $P<0.005$) and F-6-P (by 36.8%, $P<0.05$), but did not alter levels of F-1,6-BP. There was a trend towards a lower ratio between F-1,6-BP and F-6-P ($P=0.09$) (Table 5.2).

5.3.8 ADO inhibits AMPK via Ser/Thr PP activation

To determine whether ADO-induced inhibition of AMPK phosphorylation was mediated by inhibition of the up-stream AMPK-kinase, LKB-1 (431,436), we assessed the level of phosphorylation of LKB-1 at its Ser428 site as this is well correlated with LKB-1 activity and AMPK phosphorylation (437-439). However,

relative to control hearts, ADO had no effect on LKB-1 phosphorylation (expressed as a ratio of total LKB-1) (Figure 5.3). As an alternate mechanism to explain ADO-induced inhibition of AMPK phosphorylation, the role of Ser/Thr PP was investigated by examining the effects of cantharidin, a PP-1 and PP-2A inhibitor, in control and ADO-treated hearts. Cantharidin had no effects *per se* on LV work during baseline aerobic perfusion or on the recovery of LV work in control hearts (Figure 5.6A). However, the administration of cantharidin (5 μM) 10 min prior to ADO, prevented the ADO-mediated improvement in the recovery of post-ischemic LV work (Figure 5.6A). Cantharidin also antagonized the ADO-mediated inhibition of glycolysis and proton production (Figures 5.6B and D) and had no effect on glucose oxidation (Figure 5.6C). In addition, cantharidin prevented the ADO-mediated preservation of glycogen content (Table 5.1) and partially reversed the effect of ADO on the F-1,6-BP to F-6-P ratio (Table 5.2). Moreover, cantharidin completely reversed the inhibitory effects of ADO on the phosphorylation of AMPK and p38 MAPK (Figures 5.6E and F).

5.3.9 Inhibition of p38 MAPK mimics the beneficial effects of ADO independent of AMPK

To determine the role of p38 MAPK in cardioprotection and inhibition of glycolysis by ADO, the effects of the selective inhibitor of p38 MAPK, SB202190 (10μM) were examined. SB202190, when added at the onset of reperfusion, significantly improved the recovery of post-ischemic LV work

(Figure 5.7A). SB202190 also reduced rates of glycolysis and proton production to an extent comparable with ADO (Figures 5.7B and D). As expected, SB202190 markedly reduced the phosphorylation of p38 MAPK; however, unlike ADO, SB202190 did not reduce AMPK phosphorylation (Figures 5.7E and F). Interestingly, the profile of glycolytic metabolites in the presence of SB202190 was distinct from that caused by ADO. SB202190 did not increase levels of G-6-P, but caused a trend towards lower levels of F-6-P (by 20.6%, P=0.067), and a significant increase in F-1,6-BP (by 42.2%, P<0.05). Accordingly, SB202190 decreased the ratio between F-6-P and G-6-P by 20.6% (P<0.001) and increased the ratio between F-1,6-BP and F-6-P by 85.4% (P<0.05). This shows that SB202190-induced inhibition of glycolysis does not involve PFK-1 but rather involves an alternative site in the glycolytic pathway distal to PFK-1.

5.4 Discussion

ADO is a well-known cardioprotective agent but mechanisms underlying its beneficial action have not been clarified. As inhibition of myocardial glycolysis during reperfusion is an important contributor to ADO-induced cardioprotection (28,232,283), this study investigated whether glycolysis is altered by ADO-induced changes in AMPK or p38 MAPK, two stress-activated protein kinases implicated in I/R injury. Our data show that ADO slows glycolysis during reperfusion directly by inhibition of PFK-1, likely as a consequence of marked inhibition of AMPK and subsequent inhibition of PFK-2. Although activity of p38 MAPK is also inhibited by ADO, and direct pharmacological inhibition of p38 MAPK inhibits glycolysis and is cardioprotective, ADO and p38 MAPK inhibition slow glycolysis by distinct mechanisms. As ADO-mediated inhibition of AMPK, p38 MAPK and glycolysis were completely reversed by cantharidin, it appears that inhibition of AMPK and p38 MAPK is due to ADO-induced activation of Ser/Thr PPs.

In order to examine carefully the effects of ADO on myocardial glucose and glycogen metabolism, studies were performed on intact rat hearts that were isolated and perfused in working mode with both glucose (plus insulin) and palmitate as energy substrates. This experimental model allows the study of changes in myocardial mechanical and metabolic function under conditions of

physiological work-load (energy demand) and adequate energy supply. In addition, the initial aerobic baseline perfusion ensures the re-establishment of normal glycogen content, which is severely depleted as a result of deep anesthesia and hypoxia/ischemia associated with heart extraction. This is an important consideration as we have shown previously that glycogen content can greatly influence AMPK-mediated changes in glucose metabolism (440). Also, our experimental design included comparison of the effects of ADO in reperfused hearts with hearts subjected to aerobic perfusion. This approach enables us to distinguish whether signaling and/or metabolic changes observed in reperfused hearts are a cause or a consequence of changes in LV mechanical function.

Consistent with previous observations (232,282,427), ADO significantly improves recovery of post-ischemic LV mechanical function, an effect mediated directly on the myocardium as CVC is not altered. While cardioprotection is associated with lower rates of glycolysis and proton production during reperfusion, the ability of ADO to inhibit glycolysis and proton production in aerobically perfused hearts provides evidence that inhibition of glycolysis is a cause, and not a consequence, of improved post-ischemic LV function. The role of glycolysis in ADO-induced cardioprotection is controversial and earlier reports have shown that ADO stimulates myocardial glycolysis (278,279), an effect that may be related to the use of glucose as the sole energy substrate, absence of insulin and/or inadequate restoration of glycogen to physiological levels.

Various interventions that limit proton production arising from glucose metabolism are cardioprotective, e.g., inhibition of glycolysis by ADO (232) or by preconditioning (58) as well as indirect inhibition of glycolysis through repartitioning G-6-P towards glycogen synthesis (23) or inhibition of glycogen degradation (441). Another approach to limit proton production arising from glucose metabolism is through stimulation of glucose oxidation by activation of the rate-limiting enzyme, pyruvate dehydrogenase complex, either directly by dichloroacetate (228) or indirectly by reducing fatty acid oxidation by trimetazidine (231).

A number of potential mechanisms may explain how ADO slows glycolysis, including inhibition of one or more of the glycolytic enzymes, or limitation of glycolytic substrate (G-6-P) availability due to inhibition of glucose uptake, impaired glycogenolysis, or stimulation of glycogen synthesis. We have shown previously that inhibition of glycogen synthase kinase-3 stimulates glycogen synthesis and repartitions G-6-P away from glycolysis thereby limiting proton production and Ca^{2+} overload (23). The demonstration in this study that ADO inhibits glucose uptake and glycogen degradation during reperfusion but has no effect on glycogen synthesis, together with increases in G-6-P content provides clear evidence that ADO inhibits glycolysis directly and not through modulation of glucose uptake or glycogen turnover. In support, ADO inhibits glycolysis in aerobically perfused hearts, with no effect on either glycogen synthesis or

degradation. The reduced ratio of F-1,6-BP to F-6-P in ADO-treated hearts shows that ADO inhibits glycolysis at the step catalyzed by PFK-1, the initial irreversible reaction in the glycolytic pathway (442).

PFK-1 is key regulatory enzyme in the glycolytic pathway that is under allosteric control by fructose-2,6-bisphosphate (F-2,6-BP), levels of which are influenced by its synthesis catalyzed by PFK-2 as well as its hydrolysis catalyzed by fructose-2,6-bisphosphatase (FBPase-2). Both the PFK-2 and FBPase-2 reactions are catalyzed on the same polypeptide of a homodimeric protein (162). As an increase in V_{max} without a change in its K_m for F-6-P occurs following phosphorylation of PFK-2 at Ser466 by AMPK accelerates glycolysis (165), we determined whether inhibition of PFK-2 phosphorylation due to inhibition of AMPK might explain ADO-mediated inhibition of glycolysis.

Activation of AMPK by ischemia elicits a number of alterations in myocardial glucose and fatty acid metabolism, in addition to acceleration of glycolysis. It stimulates glucose uptake in glycogen-depleted hearts by increasing translocation of glucose transporter-4 (GLUT4) to the cell surface (121,219,440). AMPK also stimulates fatty acid oxidation by phosphorylation and inhibition of acetyl CoA carboxylase (208). Although the roles of AMPK in cardiac metabolism and I/R injury are extensively characterized, it remains controversial whether its inhibition is beneficial or detrimental. In recent studies, augmentation of AMPK during ischemia protects against myocardial ischemic injury (332,333)

while transgenic mouse hearts with an AMPK α_2 kinase-dead mutation are more prone to I/R injury (220). On the other hand, mouse hearts with a dominant-negative α_2-subunit of AMPK (a model with a milder degree of AMPK suppression) are not energetically compromised and exhibit improved post-ischemic recovery of LV function (339). Such conflicting results may have arisen due to differences in the extent of AMPK inhibition or potential differential consequences arising from inhibition during ischemia or during reperfusion (not controllable in transgenic models). This difficulty, and the lack of reliable pharmacological agents to alter AMPK activity, has limited examination of the role of AMPK during reperfusion, as distinct from ischemia. While the metabolic consequences of AMPK activation are potentially beneficial during ischemia when energy supply is limited, ATP generation rapidly resumes to normal levels during reperfusion (227). Instead, poor recovery of LV mechanical function is due to inefficiency in ATP utilization due to the diversion of ATP to correct ionic imbalances arising from intracellular acidosis and the activation of the coupled exchangers that promote Na^+ and Ca^{2+} accumulation. Thus, during ischemia, stimulation of glycolysis by AMPK activation may be beneficial, while during reperfusion activation of glycolysis may be detrimental. Our results showing that ADO does not affect AMPK activation during ischemia while a marked inhibition of AMPK occurs during reperfusion support this concept and agrees with previous work showing that activation of ADO receptors only during reperfusion is cardioprotective (28,443).

Despite the extensive evidence regarding the marked cardioprotective effects of ADO in animal models, such effectiveness has been difficult to demonstrate clinically. In a previous study comparing the cardioprotective properties of ADO in healthy with acutely stressed hearts (285), we discovered a loss of protection in stressed hearts that was due to an ADO-induced activation of AMPK and p38 MAPK (226,377), and that inhibition of p38 MAPK restores the cardioprotective effects of ADO (376). Thus, we sought to examine the role of p38 MAPK in ADO-mediated cardioprotection. While it has no effect on basal p38 MAPK phosphorylation in aerobic hearts or on elevated levels in post-ischemic hearts, ADO causes a marked inhibition of p38 MAPK phosphorylation during reperfusion. This results in inhibition of glycolysis, an effect mimicked by a pharmacological inhibitor of p38 MAPK (SB202190) when present only during reperfusion. Interestingly, the p38 MAPK inhibitor inhibits glycolysis by a mechanism distinct from that of ADO; AMPK was not inhibited and examination of tissue contents of glycolytic intermediates in SB202190-treated hearts shows differences from ADO-treated hearts, particularly in F-1,6-BP content (decreased by ADO but increased by SB202190). It appears that the inhibitory action of SB202190 is distal to PFK-1 and deserves further examination as it may represent a new target for glycolytic inhibition and cardioprotection. Moreover, inhibition of glycolysis at two distinct sites probably explains the beneficial interaction observed for an ADO-SB202190 combination in hearts stressed by acute ischemia (376).

ADO-induced inhibition of glycolysis is mimicked by selective A_1 receptor agonists and inhibited by selective A_1 receptor antagonists (28), but the signaling mechanisms underlying glycolytic inhibition by ADO A_1 receptors has not been addressed. As N^6-cyclohexyladenosine, a selective A_1 ADO receptor agonist, also inhibits AMPK in reperfused hearts (224), we sought to examine the effect of ADO on mechanisms that control AMPK phosphorylation. LKB-1, the predominant AMPK kinase in the heart (431,436), is not affected by ADO, whereas inhibition of Ser/Thr PPs by cantharidin blocks ADO-mediated inhibition of AMPK and the subsequent inhibition of glycolysis. This supports a role for Ser/Thr PP activation by ADO and agrees with previous studies showing that A_1 ADO receptor stimulation activates PP2A via increased carboxymethylation and targeting to the particulate fraction of cardiomyocytes (433). In addition, in endothelial cells, activation of PP2A inhibits AMPK activation (314). Thus, the inhibition of ADO effects by cantharidin suggests PP2A activation as the mechanism of ADO-mediated inhibition of AMPK. The concentration of cantharidin used in this study (5 μM) is relatively non-selective for PP1 and PP2A (IC_{50} values for PP1 and PP2A of 10^{-6} and 10^{-7} M, respectively) (444). Thus, additional studies will be required to address the role of each Ser/Thr PP in ADO-mediated effects.

The role of PPs in the pathogenesis of myocardial I/R injury remains controversial. Despite the extensive investigation of the role of kinase activation

in I/R injury, relatively little is known about the respective role of PPs. Pharmacological inhibitors of PP1 and PP2A were found to protect isolated rabbit cardiomyocytes against ischemic injury (445) while in isolated perfused rat hearts, they blocked the cardioprotective effects of preconditioning and impaired post-ischemic functional recovery when given prior to the onset of ischemia (444). Our results provide evidence that ADO-mediated cardioprotection is possibly mediated via activation of Ser/Thr PPs indicating a role for PPs in mediating cardioprotection.

Conclusion

In conclusion, our results provide new insights into the mechanism of ADO-induced cardioprotection. ADO activates Ser/Thr PP activity that reduces the phosphorylation of AMPK, which subsequently inhibits PFK-2 and slows glycolysis and proton production during reperfusion. These effects of ADO are not evident during ischemia, and so potential beneficial effects of AMPK-mediated metabolic changes during ischemia are not impaired. This study provides further evidence in favor of the benefit of limiting AMPK activation, glycolysis and proton production during reperfusion.

Figure 5.1: ADO improves recovery of LV work during reperfusion.

(A) LV work, (B) coronary flow, and (C) coronary vascular conductance (CVC) during I/R protocol in isolated working rat hearts that were either untreated (Control, n=14, □) or treated with ADO (500 μmol/L, n=12, ■) added 5 min prior to the onset of global ischemia (GI) and present throughout reperfusion. Values are means ± SEM. **P*<0.05 compared with control hearts as determined by repeated measures two-way ANOVA and Bonferroni post-tests.

Figure 5.1

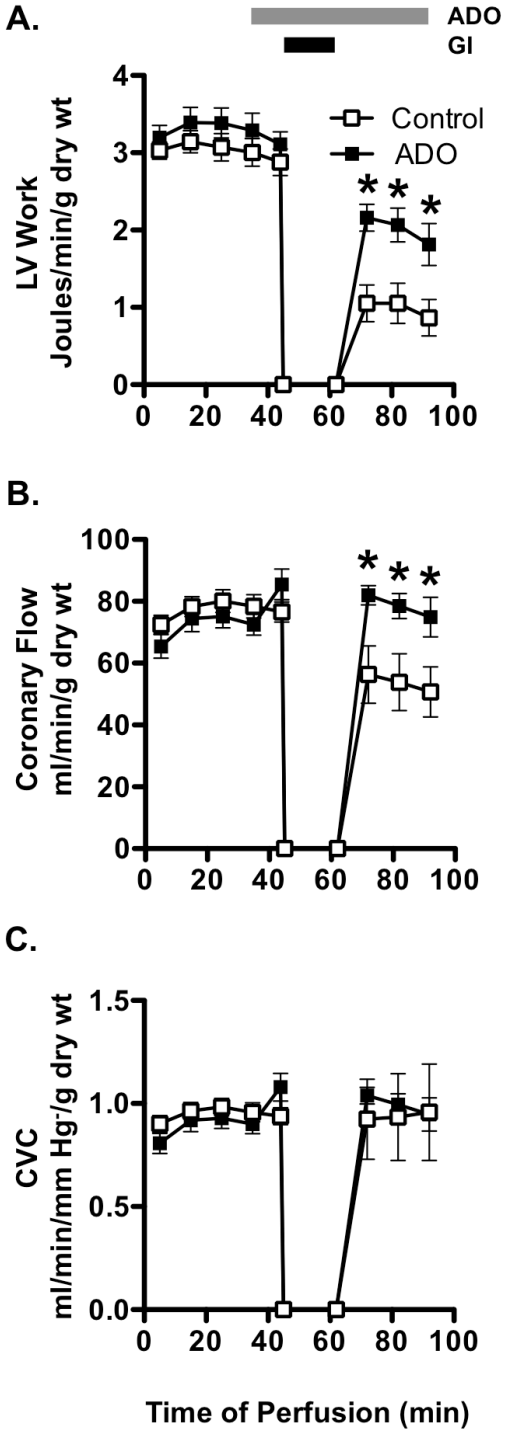

A.
ADO
GI
LV Work
Joules/min/g dry wt
Control
ADO
B.
Coronary Flow
ml/min/g dry wt
C.
CVC
ml/min/mm Hg-/g dry wt
Time of Perfusion (min)

Figure 5.2: ADO-mediated alteration in glucose metabolism during reperfusion.

Values for metabolic rates (μmol/min/g dry wt) are shown for control and ADO-treated hearts, respectively, for (A) glycolysis (n=14, 12), (B) glucose oxidation (n=14, 12), (C) proton production (n=14,12), (D) glucose uptake (n=12, 11), (E) glycogen synthesis (n=12, 11), and (F) glycogen degradation (n=12, 11). Values are means ± SEM. **P*<0.05 compared with control hearts as determined by Student's *t*-test.

Figure 5.2

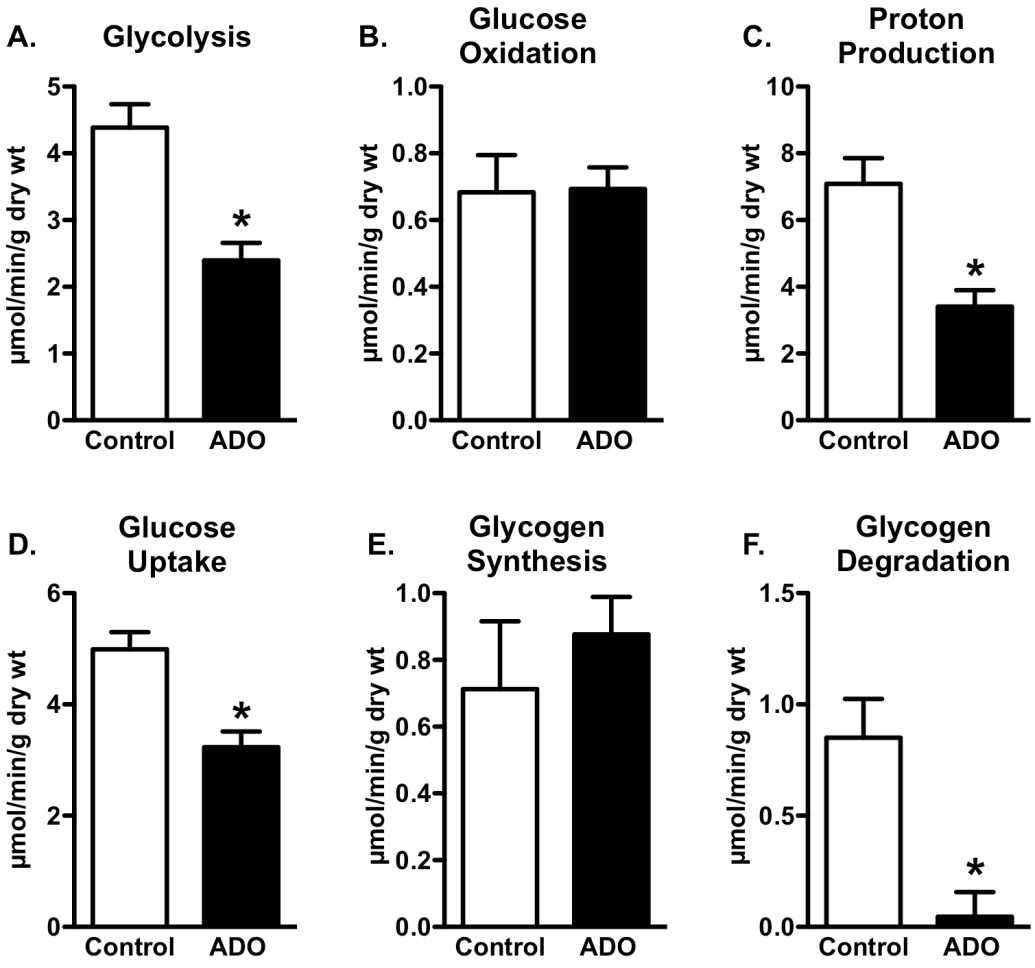

A.
Glycolysis
µmol/min/g dry wt
5
4
3
2
1
0
*
Control
ADO
B.
Glucose Oxidation
µmol/min/g dry wt
1.0
0.8
0.6
0.4
0.2
0.0
Control
ADO
C.
Proton Production
µmol/min/g dry wt
10
8
6
4
2
0
*
Control
ADO
D.
Glucose Uptake
µmol/min/g dry wt
6
4
2
0
*
Control
ADO
E.
Glycogen Synthesis
µmol/min/g dry wt
1.0
0.8
0.6
0.4
0.2
0.0
Control
ADO
F.
Glycogen Degradation
µmol/min/g dry wt
1.5
1.0
0.5
0.0
*
Control
ADO

Figure 5.3 Phosphorylation status of AMPK, p38 MAPK, LKB-1, and PFK-2 at the end of reperfusion

(A) Representative immunoblots of relative amounts of phosphorylated and total protein content of AMPK, p38 MAPK, and LKB-1 as well as phosphorylated PFK-2 and actin in tissue homogenates from hearts frozen at the end of reperfusion. (B) Quantification of a series of immunoblots measuring the ratio of phosphorylated to total protein for P-AMPK/AMPK, P-p38 MAPK/p38 MAPK, P-LKB-1/LKB-1, and P-PFK-2/actin. Results (means ± SEM) are expressed relative to control values for each protein. *$P<0.05$ compared with control hearts as determined by Student's t-test.

Figure 5.3

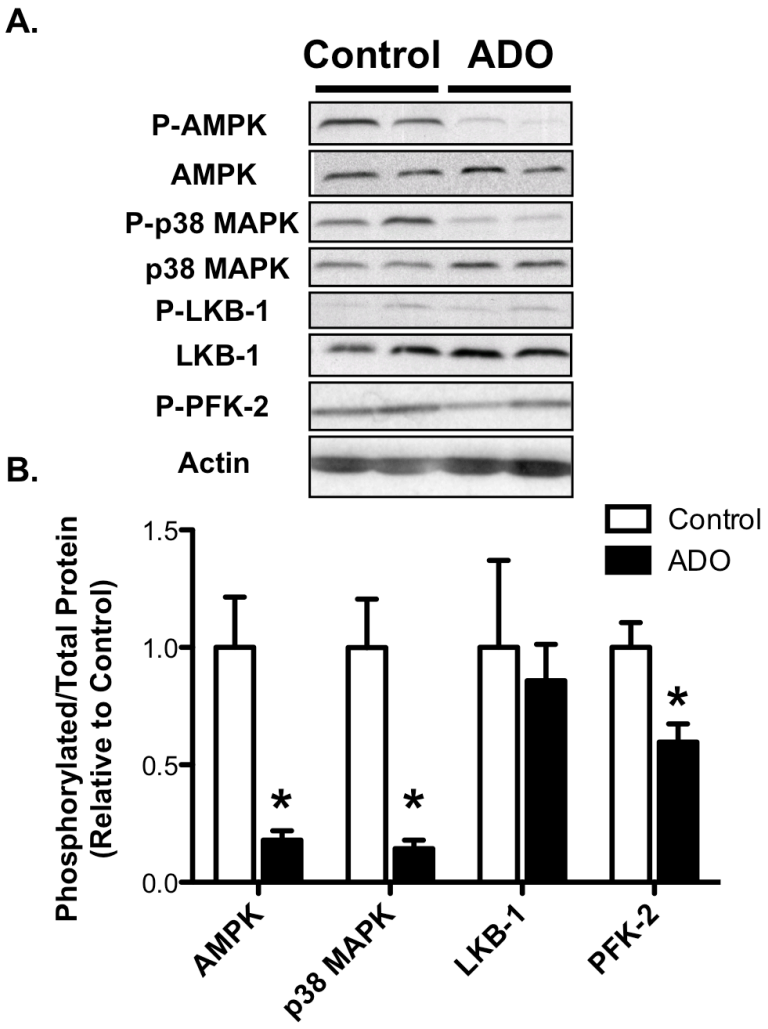

A.
Control
ADO
P-AMPK
AMPK
P-p38 MAPK
p38 MAPK
P-LKB-1
LKB-1
P-PFK-2
Actin
B.
Phosphorylated/Total Protein
(Relative to Control)
1.5
1.0
0.5
0.0
Control
ADO
*
*
*
AMPK
p38 MAPK
LKB-1
PFK-2

Figure 5.4 Changes in the phosphorylation state of AMPK and p38 MAPK at the end of ischemia and at the end of reperfusion

Representative immunoblots of relative amounts of phosphorylated and total protein content of (A) AMPK and (B) p38 MAPK in tissue homogenates in control hearts as well as ADO-treated hearts that were frozen at the end of baseline perfusion (Baseline), at the end of ischemia (C+I and ADO+I) or at the end of reperfusion (C+IR and ADO+IR). Quantification of a series of immunoblots measuring the ratio of phosphorylated to total protein for (C) P-AMPK/AMPK and (D) P-p38 MAPK/p38 MAPK. Results (means ± SEM) are expressed relative to baseline values for each protein. **P*<0.05 compared with baseline, #*P*<0.05 compared with control hearts at the end of reperfusion as determined by one-way ANOVA and Bonferroni post-tests.

Figure 5.4

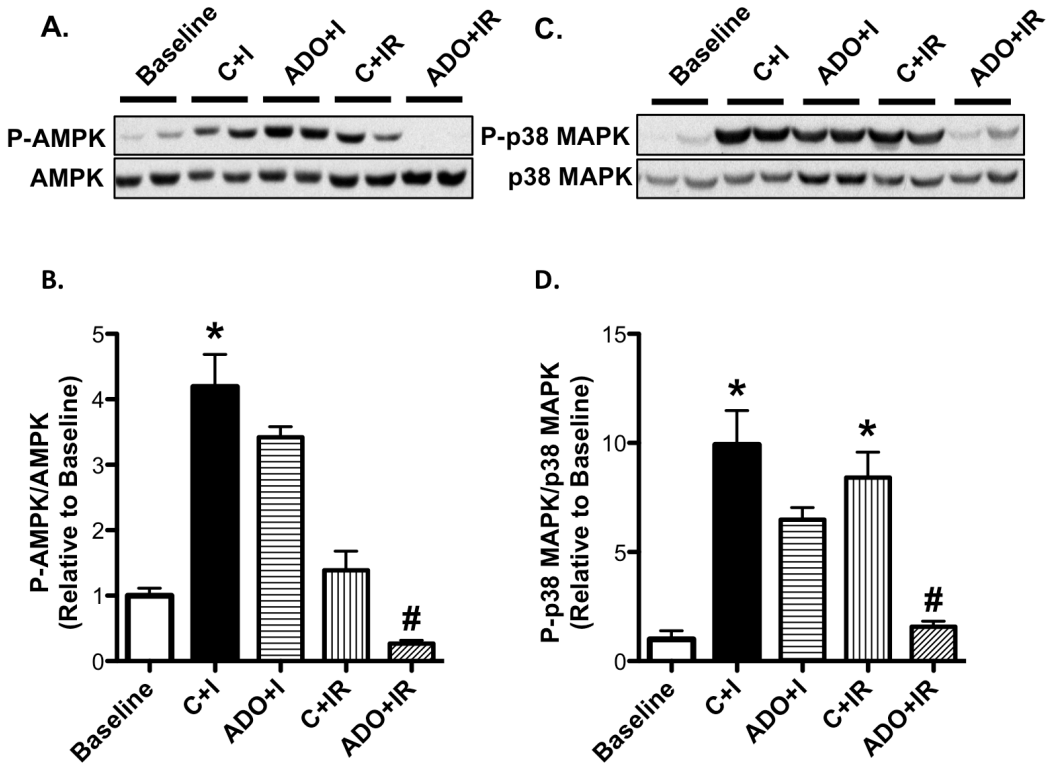

A.
Baseline
C+I
ADO+I
C+IR
ADO+IR
P-AMPK
AMPK
C.
P-p38 MAPK
p38 MAPK
B.
P-AMPK/AMPK
(Relative to Baseline)
D.
P-p38 MAPK/p38 MAPK
(Relative to Baseline)

Figure 5.5: ADO-mediated alteration in glucose metabolism during aerobic perfusion.

Values for metabolic rates (μmol/min/g dry wt) are shown for control and ADO-treated hearts, respectively, for (A) glycolysis (n=9, 8), (B) glucose oxidation (n=9, 8), (C) proton production (n=9, 8), (D) glucose uptake (n=9, 8), (E) glycogen synthesis (n=9, 8), and (F) glycogen degradation (n=9, 8). Values are means ± SEM. (G) Representative immunoblots of relative amounts of phosphorylated and total protein content of AMPK and p38 MAPK as well as phosphorylated PFK-2 and actin in tissue homogenates from hearts frozen at the end of aerobic perfusion. (H) Quantification of a series of immunoblots measuring the ratio of phosphorylated to total protein for P-AMPK/AMPK, P-p38 MAPK/p38 MAPK, and P-PFK-2/actin. Results (means ± SEM) are expressed relative to control values for each protein. *$P<0.05$ compared with control hearts as determined by Student's *t*-test.

Figure 5.5

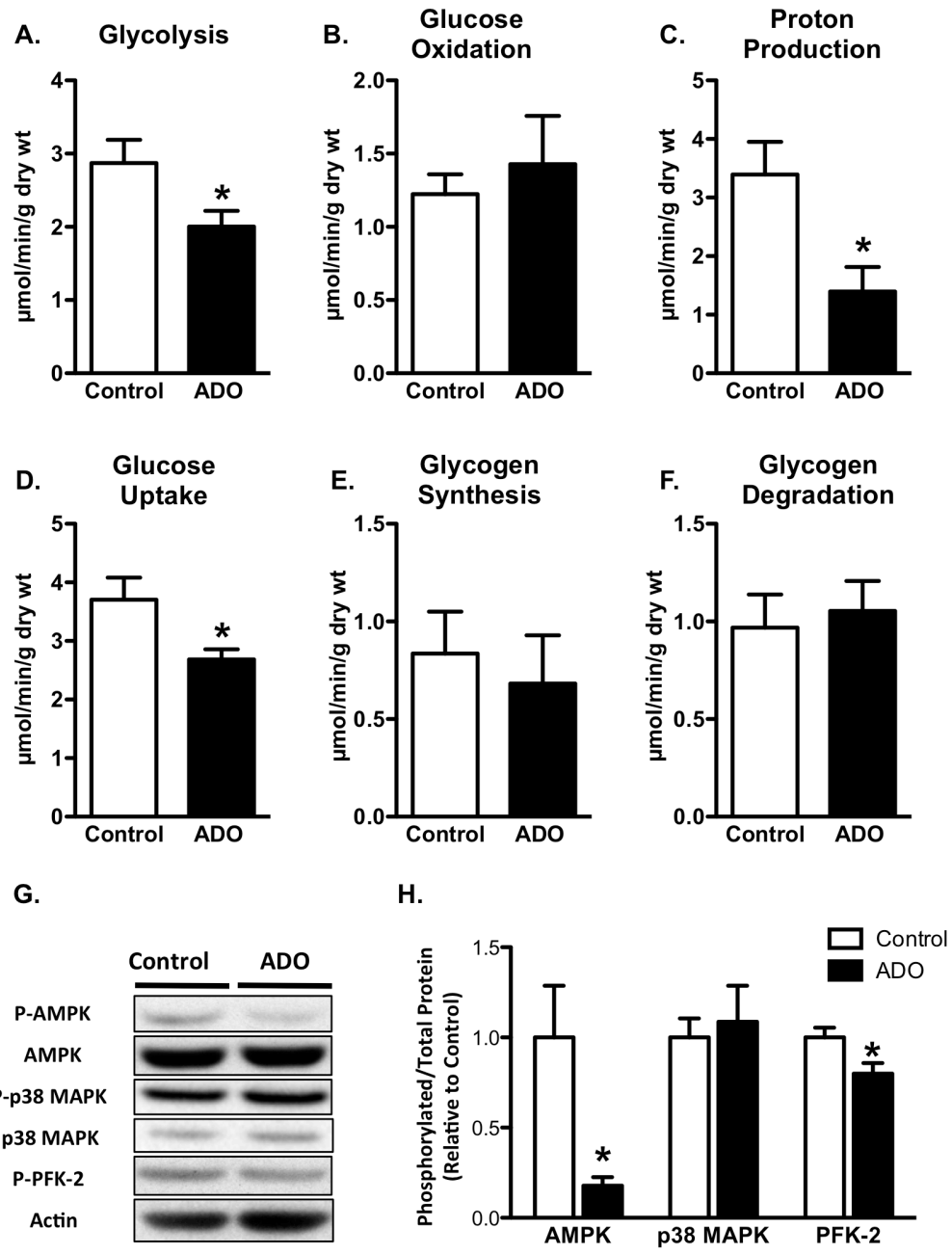

A.
Glycolysis
µmol/min/g dry wt
Control
ADO
B.
Glucose Oxidation
µmol/min/g dry wt
Control
ADO
C.
Proton Production
µmol/min/g dry wt
Control
ADO
D.
Glucose Uptake
µmol/min/g dry wt
Control
ADO
E.
Glycogen Synthesis
µmol/min/g dry wt
Control
ADO
F.
Glycogen Degradation
µmol/min/g dry wt
Control
ADO
G.
Control
ADO
P-AMPK
AMPK
P-p38 MAPK
p38 MAPK
P-PFK-2
Actin
H.
Control
ADO
Phosphorylated/Total Protein (Relative to Control)
AMPK
p38 MAPK
PFK-2

Figure 5.6: Cantharidin reverses ADO-mediated effects.

(A) Recovery of LV work during reperfusion in untreated hearts (Control, n=14), hearts treated with ADO (500 μM) 5 min prior to GI (ADO, n=12), hearts treated with cantharidin (5 μM) 15 min prior to ischemia (Can, n=6), and hearts treated with both cantharidin and ADO (Can+ADO, n=7) represented as percentage of pre-ischemic baseline values. Values for metabolic rates (μmol/min/g dry wt) during reperfusion are shown for (B) glycolysis, (C) glucose oxidation, and (D) proton production. (E) Representative immunoblots of relative amounts of phosphorylated and total protein content of AMPK and p38 MAPK in tissue homogenates from hearts frozen at the end of reperfusion. (F) Quantification of a series of immunoblots measuring the ratio of phosphorylated to total protein for P-AMPK/AMPK, P-p38 MAPK/p38 MAPK expressed relative to control values for each protein. Values are means ± SEM. *P<0.05 compared with control hearts as determined by one-way ANOVA and Bonferroni post-tests.

Figure 5.6

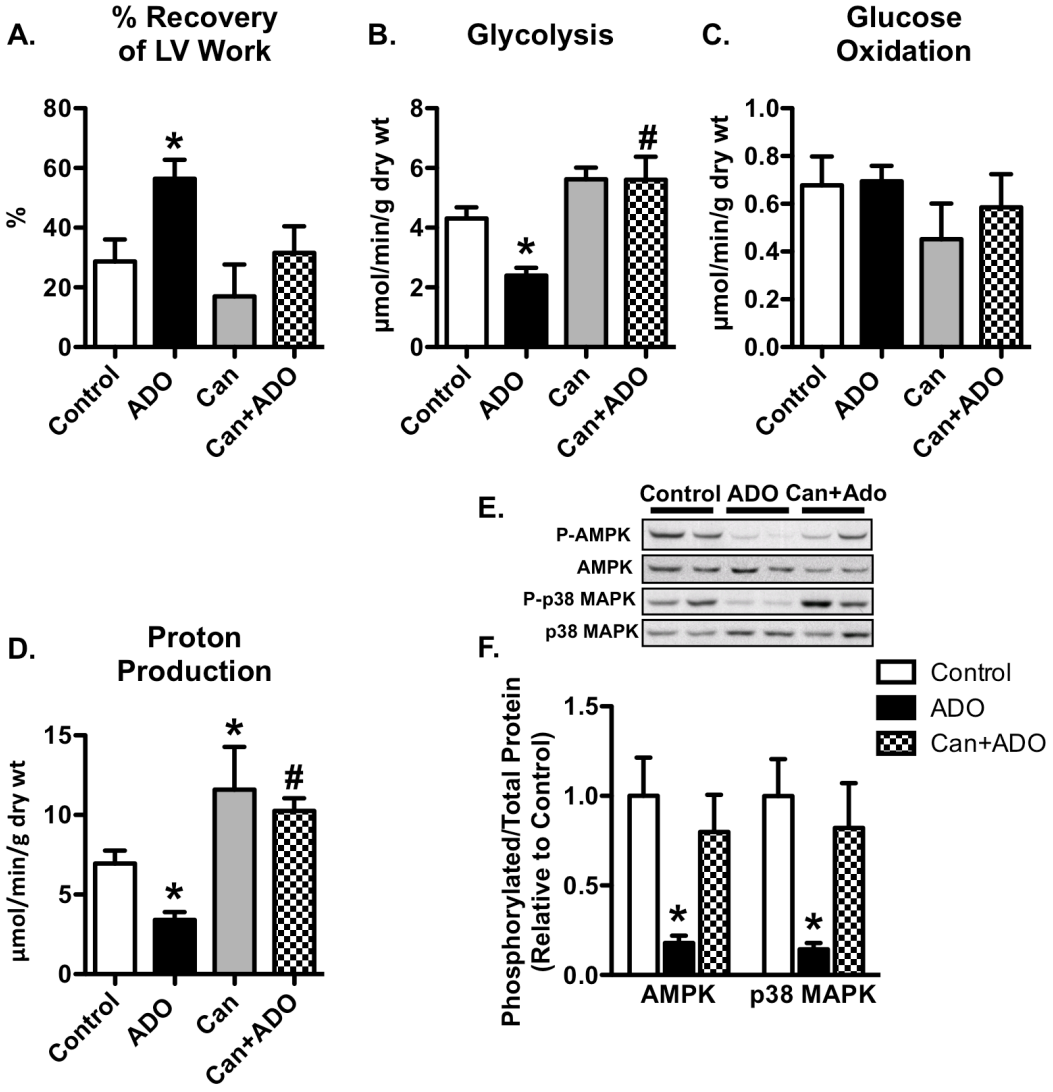

A.
% Recovery of LV Work
%
80
60
40
20
0
*
Control
ADO
Can
Can+ADO
B.
Glycolysis
µmol/min/g dry wt
8
6
4
2
0
*
#
Control
ADO
Can
Can+ADO
C.
Glucose Oxidation
µmol/min/g dry wt
1.0
0.8
0.6
0.4
0.2
0.0
Control
ADO
Can
Can+ADO
D.
Proton Production
µmol/min/g dry wt
15
10
5
0
*
*
#
Control
ADO
Can
Can+ADO
E.
Control ADO Can+Ado
P-AMPK
AMPK
P-p38 MAPK
p38 MAPK
F.
Control
ADO
Can+ADO
Phosphorylated/Total Protein (Relative to Control)
1.5
1.0
0.5
0.0
*
*
AMPK
p38 MAPK

Figure 5.7: Pharmacological inhibition of p38 MAPK during reperfusion inhibits glycolysis and enhances post-ischemic LV work.

(A) Recovery of LV work during reperfusion in untreated hearts (Control, n=14) and hearts treated with SB202190 (10 μM) at the onset of reperfusion (n=5) represented as percentage of pre-ischemic baseline values. Values for metabolic rates (μmol/min/g dry wt) during reperfusion are shown for (B) glycolysis, (C) glucose oxidation, (D), and H^+ production. (E) Representative immunoblots of relative amounts of phosphorylated and total protein content of AMPK and p38 MAPK in tissue homogenates from hearts frozen at the end of reperfusion. (F) Quantification of a series of immunoblots measuring the ratio of phosphorylated to total protein for P-AMPK/AMPK, P-p38 MAPK/p38 MAPK expressed relative to control values for each protein. Values are means ± SEM. **P*<0.05 compared with control hearts as determined by Student's t-test.

Figure 5.7

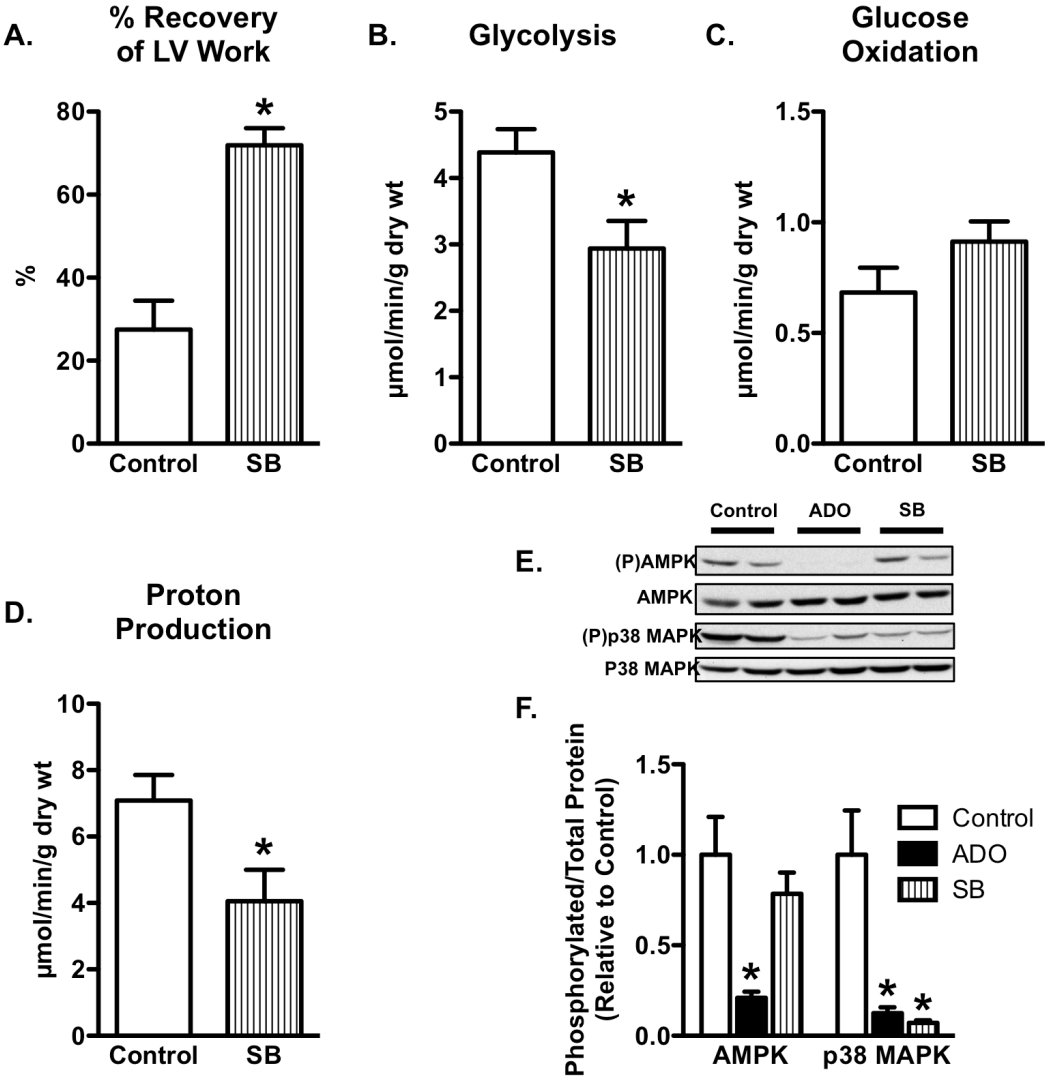

A.
% Recovery of LV Work
%
80
60
40
20
0
Control
SB
*
B.
Glycolysis
µmol/min/g dry wt
5
4
3
2
1
0
Control
SB
*
C.
Glucose Oxidation
µmol/min/g dry wt
1.5
1.0
0.5
0.0
Control
SB
D.
Proton Production
µmol/min/g dry wt
10
8
6
4
2
0
Control
SB
*
E.
Control
ADO
SB
(P)AMPK
AMPK
(P)p38 MAPK
P38 MAPK
F.
Phosphorylated/Total Protein (Relative to Control)
1.5
1.0
0.5
0.0
AMPK
p38 MAPK
Control
ADO
SB
*
*
*

Figure 5.8: Schematic diagram explaining the proposed mechanism for glycolytic inhibition by ADO.

ADO, through stimulating its receptors, activates Ser/Thr PPs to dephosphorylate and inhibit AMPK and p38 MAPK. Inhibition of AMPK results in reduced activation of PFK-2 and subsequently PFK-1 leading to inhibition of glycolysis. This results in reduced H^+ production and subsequently reduced Ca^{2+} overload and cardioprotection.

Figure 5.8:

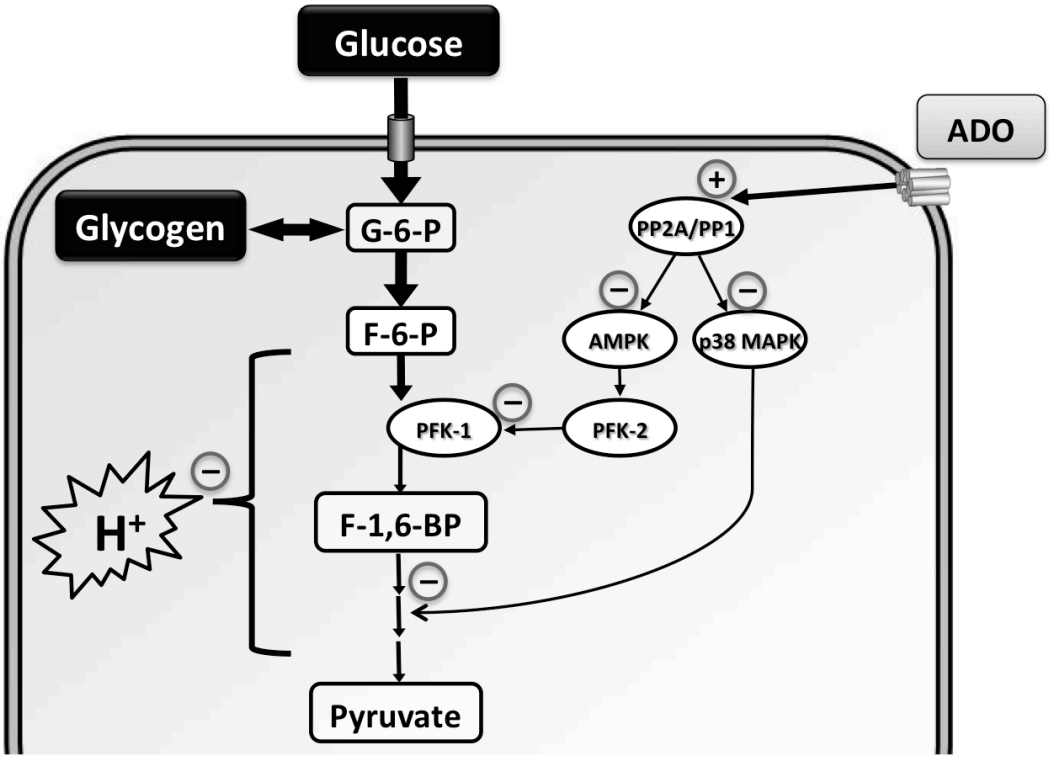

Glucose
ADO
Glycogen
G-6-P
PP2A/PP1
F-6-P
AMPK
p38 MAPK
PFK-1
PFK-2
H+
F-1,6-BP
Pyruvate

Table 5.1 Values for glycogen content in ventricular tissue

Glycogen Content (μmol/g dry wt)	Aerobic Perfusion		Ischemia-Reperfusion		
	Control	ADO	Control	ADO	Can+ADO
	n=9	*n*=8	*n*=12	*n*=11	*n*=6
Total Glycogen	147.3±8.2	138.9±7.5	69.7±11.0	110.0±5.4*	62.7±10.5$^{\#}$
Labeled Glycogen	88.9±7.5	83.5±8.7	39.9±6.1	60.2±3.4*	19.8±4.9$^{\#}$

Glycogen contents (μmol/g dry wt) were measured in heart extracts frozen after aerobic perfusion as well as after I/R. Values are means ± SEM. *P<0.05 compared with control hearts. $^{\#}P$<0.05 compared with ADO-treated hearts as determined by Student's *t*-test (aerobic) or one-way ANOVA and Bonferroni post-tests (I/R).

Table 5.2 Values for glycolytic metabolite contents in ventricular tissue

Glycolytic Metabolite	Aerobic Perfusion		Ischemia-Reperfusion			
	Control *n*=7	ADO *n*=6	Control *n*=13	ADO *n*=12	Can+ADO *n*=6	SB *n*=5
G-6-P (nmol/g dry wt)	183.1±8.2	227.5±9.0[§]	165.7±12.7	213.2±8.3*	232.2±27.7*	180.6±6.7
F-6-P (nmol/g dry wt)	39.7±3.5	54.3±3.0[§]	56.4±3.4	71.4±8.2	61.5±7.3	44.8±3.1
F-1,6-BP (nmol/g dry wt)	29.4±3.2	26.1±2.2	22.8±2.0	14.0±1.5*	20.4±2.7	32.4±5.0*
F-6-P/G-6-P	0.22±0.01	0.24±0.01	0.35±0.02	0.34±0.05	0.27±0.01	0.25±0.01*
F-1,6-BP/F-6-P	0.82±0.17	0.49±0.04	0.42±0.04	0.22±0.03*	0.40±0.13	0.77±0.19*

Glycolytic metabolite contents (nmol/g dry wt) were measured in heart extracts frozen after aerobic perfusion as well as after I/R. Values are means ± SEM. [§]$P<0.05$ compared with control aerobic hearts as determined by Student's *t*-test (aerobic). *$P<0.05$ compared with control I/R hearts as determined by one-way ANOVA and Bonferroni post-tests.

6. General Discussion and Conclusions

In Canada, every 7 minutes someone dies as a result of cardiovascular disease, which constituted in 2006 30% of all deaths. 54% of these deaths were attributed to ischemic heart diseases (446). This highlights the importance of research focused on better understanding the mechanisms underlying the pathology of ischemic heart diseases. This will facilitate the discovery of potential new targets and lead to new treatments that will reduce the mortality, morbidity and the economic burden associated with these diseases. Due to the fact that the heart is the most energy-consuming organ in the body and due to its limited energy reserves, research in the area of myocardial energy substrate metabolism has been of great importance in the understanding of a number of cardiac pathologies. Indeed, altered energy substrate metabolism contributes to the development of various cardiac pathologies including ischemic heart diseases, myocardial infarction, cardiac hypertrophy, and diabetic cardiomyopathy (447).

This thesis has investigated a number of aspects concerning alterations in glucose and glycogen metabolism and the partitioning of glucose between these pathways and their effects on myocardial I/R injury. In particular, we identified the importance of glycogen levels in influencing ischemia-induced alterations in glucose metabolism. This is an issue that is usually overlooked in studies looking at ischemia-induced changes in myocardial metabolism. We demonstrated that during acute ischemia, the heart preferentially utilizes glycogen, rather than exogenous glucose, to provide enough substrate to fuel the glycolytic pathway.

Thus, ischemia-induced activation of AMPK induces GLUT-4 translocation and stimulation of glucose uptake only under conditions when glycogen stores are depleted.

In addition, we examined a number of approaches to limit excessive myocardial glycolysis during reperfusion. As mentioned in the Introduction, reducing glycolytic rates while maintaining rates of glucose oxidation limits intracellular acidosis and reduces intracellular Ca^{2+} overload and subsequent myocardial injury. We tested the hypothesis that stimulation of glycogen synthesis will repartition G-6-P away from the glycolytic pathway leading to reduced intracellular acidosis and cardioprotection. This was achieved by pharmacological inhibition of GSK-3 during reperfusion. That study provided a novel mechanism to explain the cardioprotective effects of GSK-3 inhibitors and provided a novel approach to indirectly inhibit glycolysis and protect the heart against deleterious Ca^{2+} overload.

In the last study in this thesis, we identify a mechanism by which a well-known cardioprotective agent, ADO, inhibits glycolysis and induces cardioprotection against I/R injury. ADO activates Ser/Thr PP activity that reduces the phosphorylation of AMPK, which subsequently inhibits PFK-2 and slows glycolysis and proton production during reperfusion. In addition, we identified that inhibition of p38 MAPK is another approach that inhibits glycolysis and induces cardioprotection. However, this occurs via a different

mechanism than that of ADO. This penultimate chapter will discuss the justification and potential limitations of the methodology utilized as well as discuss the results of this thesis in the light of the current literature in addition to the main conclusions already discussed in the previous chapters.

6.1 Experimental model and methods

6.1.1 Isolated working rat heart preparation

The isolated working rat heart preparation is a well-established experimental model that has been in use for over 40 years as an invaluable tool to study different aspects of the heart physiology and metabolism (448). This experimental model allows for the concomitant measurement of myocardial mechanical function and myocardial energy substrate metabolism under conditions of physiological preload and afterload. This is of great importance as energy substrate metabolism can be greatly influenced by the mechanical work performed by the heart and vice versa. Thus the isolated working rat heart model allows for proper characterization of myocardial substrate metabolism in the absence of the confounding effects of other organs, circulating hormones and autonomic regulation.

In studies on energy substrate metabolism, the isolated working rat heart model has a distinct advantage over isolated cell preparations as these

preparations are generally quiescent and thus the cells are under an abnormally low energy demand which results in much lower rates of substrate metabolism. In addition, the perfused working heart provides a clear advantage over isolated muscle preparations, such as papillary muscle, as these preparations depend on superfusion, which might be inadequate to supply the core of the muscle with sufficient O_2 and substrates, which likely complicates metabolic measurements.

The presence of an adequate supply of endogenous and exogenous energy substrates is fundamental to any study on energy substrate metabolism. In all of the experiments described in this thesis both 11 mM glucose and 1.2 mM palmitate were included in heart perfusates as energy substrates, and insulin (100 μU/ml) was also included in all perfusates. These conditions ensure sufficient supply of energy substrates that are required for the optimum mechanical function of the heart as well as to re-build glycogen stores that are inadvertently depleted during heart extraction. Previous work indicates that aerobic perfusion for 45 to 60 min with Krebs-Henseleit solution containing glucose, insulin and palmitate is required to replenish glycogen content to near normal physiological levels (58,148,226). Results presented in this thesis highlight the importance of controlling the status of glycogen repletion in experiments studying energy substrate metabolism. In order to simulate conditions where myocardial glycogen is depleted, hearts were perfused with substrate-free solution and paced at 420 beats per min in some perfusion protocols to deliberately deplete glycogen in an

ischemia-independent manner, so that LV mechanical function is not adversely affected. This method served as a very useful approach to help elucidate the effects of glycogen depletion *per se*, independent of other, potentially confounding, effects of ischemia.

The inclusion of a concentration of 1.2 mM of palmitate can be considered higher than physiological if compared to normal human NEFA plasma levels (0.2 – 0.8 mM) (42). However, under conditions of metabolic stress, such as during ischemia, NEFA levels increase to values as high as 2 mM (190,191). It is also noteworthy that in Sprague Dawley rats fed normal chow *ad libitum*, normal physiological NEFA levels may be as high as 1.5 mM (449).

The use of 11 mM of glucose is sometimes criticized as being higher than the normal range, if compared to fasting human plasma glucose levels. However, it has been reported that in Sprague Dawley rats, plasma glucose levels range from 8 – 10 mM during the day, which can increase to higher levels under conditions of metabolic stress, such as with uncontrolled diabetes or myocardial ischemia (450). The normal plasma insulin level in Sprague Dawley rats has been reported to be approximately 50 μU/ml (451). However, in the perfusion system, a large proportion of the insulin added to the perfusate (100 μU/ml) binds to glass as well as to plastic tubing resulting in a measured free insulin concentration of approximately 50 μU/ml (unpublished observation by Dr. Manoj Gandhi).

The crystalloid solution used for heart perfusion in the experiments described in this thesis has a much lower O_2 carrying capacity than normal blood. This results in coronary flows much higher than those observed *in vivo*. Despite this limitation, the crystalloid solution still delivers adequate O_2 to the heart as a result of the high partial pressure of oxygen (pO_2) obtained by gassing the solution with a mixture of 95% O_2 and 5% CO_2 using a thin-film glass oxygenator (452). This is evidenced by: a) reducing the perfusion solution pO_2 by gassing with a mixture containing only 70% O_2 does not impair contractile performance; b) the venous pO_2 remains relatively high indicating a sufficient reserve in O_2 availability; c) the relative stability of the mechanical function; d) isolated perfused hearts can respond to positive inotropic agents by increasing their work for sustained periods without injury (452).

In order to study recovery of mechanical function following ischemia, we utilized either global low-flow ischemia or global zero-flow ischemia to impose ischemic injury on the heart. Neither approach directly represents a corresponding clinical scenario. Acute myocardial ischemia *in vivo* usually occurs as a result of thrombotic occlusion of one of the coronary arteries resulting in reduced flow to the area of the myocardium supplied by that artery. This results in development of an ischemic zone while the remainder of the myocardium remains non-ischemic, unlike our experimental system in which the entire heart becomes ischemic. However, use of regional ischemia as a model of

I/R injury would have complicated measurement of metabolic rates, as it is not possible to discriminate between the substrate utilization in ischemic and non-ischemic zone. Thus, whole-heart ischemia is considered a better approach as the whole heart is subjected to a homogenous ischemic injury, which facilitates interpretation of the observed changes in myocardial mechanical and metabolic function. Furthermore, the use of low-flow ischemia provides an opportunity to measure changes in myocardial substrate metabolism during the ischemic period.

6.1.2 Measurement of energy substrate metabolism

The rates of energy substrate metabolism have been measured in the studies described in this thesis by following the utilization of radiolabeled substrates. [5-^{3}H]glucose, [U-^{14}C]glucose and [9,10-^{3}H]palmitate were used to measure the rates of glycolysis, glucose oxidation, and fatty acid oxidation, respectively. The details of the methods used to collect and quantify radiolabeled end products of these metabolic pathways are described in Chapter 2. In this section, the main benefits and limitations of these approaches are discussed.

Direct measurement of glycolysis

All the studies described in this thesis utilized [5-^{3}H]glucose as a tracer for determination of flux through the glycolytic pathway. $^{3}H_2O$ is liberated from [5-^{3}H]glucose at the triose phosphate isomerase reaction and the enolase reaction (453). This method has been validated by comparisons to glycolytic rates

measured by calculating the sum of lactate and pyruvate accumulation (454). [2-^{3}H]glucose can also been used to measure glycolytic rates where ^3H_2O is liberated at the phosphoglucose isomerase step. In general, rates of glycolysis calculated from detritiation of either [5-^{3}H]glucose or [2-^{3}H]glucose yield comparable results (455-457). However, the use of [2-^{3}H]glucose can result in inaccurate results under conditions where there are direct changes in the activities of the enzymes catalyzing the rate-limiting steps of the glycolytic pathway (PFK-1 and GAPDH). This is because, in case of [2-^{3}H]glucose, ^3H_2O is released upstream of these rate-limiting steps unlike when [5-^{3}H]glucose is used.

Goodwin *et al.* suggested that the use of [5-^{3}H]glucose may overestimate the actual rates of glycolysis based on the observation that the rates calculated from the detritiation of [5-^{3}H]glucose were higher than the rates calculated from the sum of the rates of lactate and pyruvate accumulation in the perfusate and the rate of glucose oxidation. The authors attributed this discrepancy to non-glycolytic detritiation of [5-^{3}H]glucose by the non-oxidative branch of the PPP (458). However, the rates of actual rates of glycolysis may have been underestimated in that study as a result of abnormally low rates of glucose oxidation. A subsequent study by Leong *et al.* shows very good correlation between the rates of glycolysis measured by the use of [5-^{3}H]glucose and the true rates of glycolysis (measured as the sum of the rates of lactate and pyruvate accumulation in the perfusate and the rates of glucose oxidation) and suggest only

a minimal contribution of PPP to measured rates during normal aerobic conditions (454).

Calculation of the rate of H^+ production from uncoupled glucose metabolism

In all the studies described in this thesis, the rate of H^+ production arising from glucose metabolism was used an index of intracellular acidosis and the degree of uncoupling between the rates of glycolysis and glucose oxidation. If glucose metabolism proceeds through glycolysis to lactate a net production of 2 H^+ occurs (459)

$$glucose + 2\ ADP + 2\ P_i \rightarrow 2\ lactate + 2\ ATP + 2\ H_2O$$

$$2\ ATP + 2\ H_2O \rightarrow 2\ ADP + 2\ P_i + 2\ H^+$$

On the other hand, if glycolysis is completely coupled with glucose oxidation, the net productions of H^+ is zero

$$glucose + 38\ ADP + 38\ P_i + 38\ H^+ + 6\ H_2O \rightarrow 6\ CO_2 + 38\ ATP + 42\ H_2O$$

$$38\ ATP + 38\ H_2O \rightarrow 38\ ADP + 38\ P_i + 38\ H^+$$

Thus, when glycolysis is not completely coupled with glucose oxidation, there is a net production of 2 H^+ for each glucose molecule that is metabolized through glycolysis and not subjected to glucose oxidation. In this thesis, H^+ production

derived from glucose metabolism is calculated by subtracting glucose oxidation rates from the rates of glycolysis and multiplying by 2 (456). This method of calculation correlates well with pH_i measurement using ^{31}P NMR spectroscopy in isolated working rat hearts (381). Thus, it provides a direct and accurate assessment of the consequences of uncoupled glucose metabolism, which is a key determinant of I/R injury that can be targeted to induce cardioprotection.

Calculation of the rates of glucose uptake

"Glucose transport" and "glucose uptake" are two terms that are used in the literature, often interchangeably despite major differences in their definition. "Glucose transport" refers to the process of transport of glucose through the GLUTs from the extracellular space to the inside of the cell, while "glucose uptake" is a broader term that refers to both the processes of glucose transport and subsequent utilization intracellularly. Indeed, increases in GLUT-4 translocation can occur in the heart without any corresponding increase in glucose uptake (460). Thus it is of great importance to distinguish between these two processes and to interpret experimental results accordingly.

Another important consideration is the approach used to assess glucose uptake, as different techniques have been reported in the literature. Glucose uptake is commonly measured using the non-metabolizable glucose analogues, 2-deoxyglucose or ^{18}FDG, but their use is subject to a number of limitations. These

analogues, similar to glucose, are taken up and phosphorylated; however, they are not further metabolized and may accumulate and slow rates of influx. The kinetic parameters of the transport and phosphorylation of these analogues are different from those of glucose, which is accounted for by the use of a correction factor, termed the "lumped constant" (LC) (461). This constant is equal to the ratio of the analogue uptake over glucose uptake and it was considered to be of constant value in the heart muscle. However, several reports have shown that the LC varies with changes in the levels of insulin (462), which was attributed to compartmentalization of hexokinase II and a differential effect of insulin on the affinity of hexokinase for glucose and 2-deoxyglucose (130). In addition, the presence of competing substrates, as well as low-flow ischemia, changes the LC (463,464). Furthermore, phosphorylated 2-deoxyglucose is a substrate of glycogen synthase (401). Thus, correct measurement of glucose uptake using these glucose analogues must utilize a LC that reflects the conditions of the experiment and must also account for the proportion of the analogue that is incorporated into glycogen.

Glucose uptake has also been assessed by the liberation of $^{3}H_2O$ from [2-^{3}H]glucose, but studies often neglect to account for incorporation of [2-^{3}H]glucose into glycogen. Thus, such measurements actually correspond to glycolytic rates rather than rates of glucose uptake. Glycogen metabolism can be affected by numerous factors including insulin and ischemia, which have a great

effect on glucose uptake. Our results show that the contribution of glycogen synthesis to the amount of glucose taken up by the cell ranges from 15% to more than 60% under different experimental conditions. Glucose uptake is influenced by glycogen availability and turnover because glycogen synthesis is a route of glucose utilization and enhances glucose uptake, whereas glycogenolysis produces endogenous G-6-P that limits glucose uptake. Glucose uptake in this thesis is measured as the sum of the rates of glycolysis and glycogen synthesis and so accounts for both of the main fates of glucose following its uptake without the need of glucose analogues. This approach provides a more accurate measure of the rates of glucose uptake and is ideally suited for measurement of glucose uptake under conditions of variable glycogen availability, such as during and following myocardial ischemia.

6.2 Pre-ischemic glycogen, AMPK, and glucose uptake

One of the main aspects of glucose metabolism that may be affected by ischemia and subsequent AMPK activation is glucose uptake. The current paradigm in the literature states that ischemia-induced activation of AMPK increases translocation of GLUT-4 to the sarcolemma and increases glucose uptake (see reviews (290,324,465)). However, this is not universally accepted, as several reports show that ischemia is not associated with increased glucose uptake (148,169,221-224,385) and AMPK activation can occur with no subsequent

changes in glucose uptake rates (225,226). These conflicting results may be attributed to the different methods used for glucose uptake measurement and the different metabolic environments in which these studies were conducted. In the study described in Chapter 3, this controversy has been addressed by following the time-dependent effects of ischemia-induced activation of AMPK on glucose uptake, glycolysis, and glycogen turnover during LFI, and under conditions of replete or depleted pre-ischemic glycogen. Importantly, the method utilized to measure glucose uptake accounts for the two major intracellular metabolic fates of glucose.

Our results provide evidence that the state of glycogen repletion is an important determinant that can influence the extent to which AMPK can influence glucose uptake and GLUT-4 translocation. In hearts with normal (replete) pre-ischemic glycogen, glucose uptake and GLUT-4 translocation are not stimulated by either short-term or long-term LFI, despite a robust activation of AMPK. On the other hand, in hearts with depleted pre-ischemic glycogen stores, LFI stimulates glucose uptake and GLUT-4 translocation after 15 min, an effect that is not observed after longer periods of LFI due to exhaustion of glycogen stores. Our results highlight the importance of pre-ischemic glycogen in modulating the effects of AMPK activation. This can be attributed to the fact that the heart preferentially utilizes glycogen during ischemia as it provides an already phosphorylated substrate for glycolysis. This is evidenced by the significant

inverse correlation between the rates of glucose uptake and glycogen degradation, which shows that these two processes complement each other to provide sufficient G-6-P for glycolysis.

The relationship between AMPK and glycogen is complex and still not completely understood. The differential consequences of AMPK activation in hearts with different glycogen contents may be attributed to a direct effect of glycogen on AMPK localization and activation. The β subunit of AMPK contains a glycogen-binding domain that enables the AMPK complex to bind to glycogen (293,294). In addition, a recent report shows that AMPK can sense the structure of the glycogen pool where highly-branched glycogen allosterically inhibits AMPK activity more than less-branched glycogen (466). We cannot exclude in our study the possible effect of the glycogen structure on AMPK-mediated effects. This might be of particular interest since the glycogen pool in our model gets depleted during the initial extraction of the heart and then replenished during the initial aerobic perfusion phase.

Another interesting observation in the study described in Chapter 3 is that AMPK is activated early during LFI despite the AMP/ATP ratio being elevated only after more prolonged LFI. Indeed, the profile of AMPK activation is more related to the increased Cr/PCr ratio, which is an earlier indicator of energy deprivation. This might be due to the fact that AMPK is sensitive to very small changes in free AMP that are not detected by HPLC measurement of tissue

homogenates (299). In addition, two recent reports indicate that AMPK is not only sensitive to AMP and ATP but also to ADP (300,301) which, like AMP, hinders the dephosphorylation of Thr-172 but, unlike AMP, does not allosterically activate AMPK. Thus, small changes in ADP/ATP ratio might also explain the discrepancy between AMPK activity and the observed AMP/ATP ratio.

One of the main findings of the current study is that the content of glycogen greatly influences myocardial metabolic pathways. This important factor should be taken into consideration when interpreting results of studies investigating myocardial metabolism. We believe that variations in glycogen content in the different experimental models contribute to some of the conflicting results observed in experimental studies on myocardial energy substrate metabolism.

6.3 Glycolysis, H^+ production, and I/R injury

The role of glycolysis in the pathogenesis of I/R injury is controversial. Several reports demonstrate that enhancing glycolysis during ischemia improves post-ischemic recovery of mechanical function (467-473). In addition, it has been reported that post-ischemic functional recovery might require accelerated glycolytic rates during reperfusion (467,470,474). This has been attributed to the importance of glycolytically derived ATP in fueling sarcolemmal ionic pumps

(475). On the other hand, several other reports as well as results presented in Chapters 4 and 5 in this thesis suggest that partial inhibition of glycolysis during reperfusion confers cardioprotection (28,45,58,232,282,285). These conflicting results are potentially due to the different experimental models used, as well as differences in perfusate composition, most importantly the presence or absence of fatty acids. When glucose is the only energy substrate available for the heart, the heart must rely completely on glycolysis and subsequent glucose oxidation for ATP production. Under these conditions, it is expected that inhibition of glycolysis will not only limit ATP production by glycolysis, but may also limit ATP production by glucose oxidation, leading to detrimental effects on the heart. However, in the presence of fatty acids, the heart relies more on fatty acid oxidation for ATP production. During reperfusion, fatty acid oxidation recovers rapidly leading to the recovery of ATP production rates to near pre-ischemic levels (227). However, the recovered ATP production is not efficiently translated into mechanical work during reperfusion as a result of the increased utilization of ATP to correct ionic imbalances (Na^+ accumulation and Ca^{2+} overload) in the cardiomyocyte (227). The imbalance between the rates of glycolysis and glucose oxidation during reperfusion in the fatty acid perfused heart results in increased acidosis and decreased cardiac efficiency. Thus, under these conditions, partial inhibition of glycolysis will improve its coupling with glucose oxidation, reduce H^+ production, enhance cardiac efficiency, and improve post-ischemic recovery.

The studies described in Chapters 4 and 5 represent two different approaches to limit excessive glycolysis during reperfusion. Glycolysis was targeted either indirectly through repartitioning of G-6-P away from glycolysis by stimulating glycogen synthesis or directly by inhibiting rate-limiting steps in the glycolytic pathways with ADO or p38 MAPK inhibitors.

6.4 Stimulation of glycogen synthesis as a cardioprotective strategy

The study described in Chapter 4 examined the hypothesis that stimulation of glycogen synthesis will repartition G-6-P away from glycolysis leading to reduced rates of H^+ production, less Ca^{2+} overload and enhanced cardioprotection. Stimulation of glycogen synthesis was achieved by pharmacological inhibition of GSK-3 thus relieving its inhibitory effects on GS. It has been shown in several reports that inhibition of GSK-3 is implicated in cardioprotection against I/R injury (see recent reviews (467,470,474)). However, the current study is the first one to relate the metabolic consequences of GSK-3 inhibition to cardioprotection. Indeed, we found that inhibition of GSK-3 during reperfusion stimulates glycogen synthesis, inhibits glycolysis and H^+ production resulting in reduced intracellular Ca^{2+} overload. This is a novel mechanism that contributes to the cardioprotective effects of GSK-3 inhibitors, and it can participate in the previously reported mechanisms, such as inhibition of MPTP.

Several reports imply that the cardioprotective effects of GSK-3 inhibition are due to reduced opening of the MPTP. Juhaszova *et al.* showed that several cardioprotective strategies converge on inhibition of GSK-3, specifically GSK-3β, with subsequent inhibition of MPTP opening (41). However, the mechanism by which GSK-3 modulates MPTP opening is still not fully understood. The main factor that regulates the opening of the MPTP is mitochondrial matrix Ca^{2+} levels. Restoration of the mitochondrial membrane potential and the increased cytosolic Ca^{2+} concentrations at the onset of reperfusion facilitates mitochondrial uptake of Ca^{2+} by the Ca^{2+} uniporter (20). Thus, reduced intracellular Ca^{2+} as a consequence of GSK-3 inhibition could potentially contribute to reduced MPTP opening. In addition, reduced H^{+} production during reperfusion also limits MPTP opening, as it has been shown that acidosis enhances inorganic phosphate uptake into respiring mitochondria which favors MPTP opening (81). Thus, inhibition of acidosis and Ca^{2+} overload induced by GSK-3 inhibition is a new mechanism explaining the link between GSK-3 and MPTP. A limitation in our study is that we did not have any direct measurement of MPTP opening, mitochondrial function, or cell death. However, the observed enhanced glucose and palmitate oxidation in hearts treated with the GSK-3 inhibitor is a clear index of the preserved mitochondrial function in these hearts.

The GSK-3 inhibitor study also highlights the influence of glycogen levels on myocardial metabolism. Inhibition of GSK-3 only stimulates glycogen

synthesis and inhibits glycolytic rates when glycogen stores are depleted, an effect that was observed following both ischemic and non-ischemic glycogen depletion. In hearts with replenished glycogen stores, GSK-3 inhibition demonstrated no detectable changes in myocardial metabolism. This absence of effect may be due to a negative feedback mechanism that becomes activated when glycogen stores are replete, and which overrides the regulatory effects of GSK-3. This selective action in glycogen-depleted hearts is potentially beneficial clinically as the use of GSK-3 inhibition will not induce excessive glycogen accumulation, an effect that is known to induce cardiac dysfunction (476). It is also clear that the effects of GSK-3 inhibition on glycogen synthesis and glycolysis are mainly influenced by the content of glycogen rather than by ischemia because glycogen synthesis was stimulated and glycolysis was inhibited in hearts subjected to ischemic as well as to non-ischemic glycogen depletion. However, unlike hearts subjected to I/R, changes in glucose oxidation were not observed as a consequence of GSK-3 inhibition in the hearts subjected to non-ischemic glycogen depletion. This shows that the accelerated glucose oxidation observed during reperfusion is mainly a consequence of improved mitochondrial function and not a direct effect of GSK-3 inhibition.

The fact that GSK-3 inhibition protects specifically against reperfusion injury, in contrast to ischemic injury, may be of great clinical value. Our results confirm that treatment with GSK-3 inhibitors only at the onset of reperfusion

produces comparable cardioprotective properties to administration prior to the onset of ischemia. Despite the fact that we observe a significant reduction in Ca^{2+} levels during ischemia as a result of GSK-3 inhibition, it is clearly evident that the reduction in Ca^{2+} overload during reperfusion is the main underlying mechanism for the observed cardioprotection. Thus, targeting GSK-3 to ameliorate I/R injury is advantageous because of clinical feasibility of administering GSK-3 inhibitors just prior to the onset of reperfusion. Another advantage of GSK-3 inhibitors as potential therapy is that they retain their cardioprotective properties in the presence of other co-morbidities such as ageing (249), myocardial hypertrophy (409), or diabetes (477-479). On the other hand, chronic inhibition of GSK-3 raises concern about cancer development and development of cardiac hypertrophy (414). However, these risks will not be a major concern if GSK-3 inhibitors are utilized only in acute treatment as an adjunct to reperfusion strategies (417).

6.5 Mechanism of adenosine-induced inhibition of glycolysis

ADO is a well-known cardioprotective agent and several mechanisms have been proposed to explain its beneficial actions. Previous work from our group demonstrated that inhibition of glycolysis is an important contributor to ADO-induced cardioprotection (28,232,283). The mechanism responsible for this beneficial effect is still not fully understood. Inhibition of glycolysis may occur

because of reduced substrate availability due to reduced glucose uptake, reduced glycogen degradation, or increased glycogen synthesis, in addition to direct inhibition of the glycolytic pathway. In the study described in Chapter 5, we examine the mechanism of ADO-induced inhibition of glycolysis with an emphasis on the potential roles of stress-responsive protein kinases. Our results show that ADO-mediated inhibition of glycolysis occurs as a result of direct inhibition of the glycolytic pathway. ADO, through activation of protein phosphatases (PPs), reduces the phosphorylation and activation of AMPK leading to reduced activity of PFK-2. This results in less activation of PFK-1 by F-2,6-BP and inhibition of the glycolytic pathway at that rate-limiting step. These results are in agreement with a previous report that showed that ADO receptor blockade stimulates glycolytic rates by a mechanism that activates PFK-1 (280).

AMPK is regarded to be an energy-sensing enzyme as it is activated by AMP and ADP and inactivated by ATP. Thus, from an energetic point of view, ADO is expected to stimulate, rather than inhibit, AMPK activity as endogenous ADO production increases during energy depletion by the action of 5'NT on AMP. However, our results show that ADO inhibits AMPK activation by stimulating its dephosphorylation by Ser/Thr PPs. Previous reports have shown that ADO activates PP2A via increased carboxymethylation in the cardiomyocyte (433) and that PP2A is one of the phosphatases that can dephosphorylate and inactivate AMPK (314). This can be explained from an energetic point of view in

which ADO, that is produced downstream of elevated ADP and AMP levels, potentially acts as a negative feedback regulator to limit excessive AMPK activation.

This study provides further evidence for the beneficial effects of limiting AMPK activation during reperfusion. This contrasts with the current understanding in the literature that AMPK activation is a beneficial adaptive mechanism that should be enhanced to ameliorate I/R injury (see reviews (465,480-482)). During ischemia, glycolysis is the only source of ATP that can maintain cardiomyocyte function. Thus, AMPK-mediated effects on glycolysis and glucose uptake are expected to be beneficial to the heart as it leads to higher rates of ATP production and better function of the ion pumps during ischemia. However, during reperfusion, oxidative ATP production resumes rapidly due to rapid recovery of fatty acid oxidation, while contractile function remains depressed (227). AMPK-mediated stimulation of fatty acid oxidation and glycolysis exaggerates the uncoupling between glycolysis and glucose oxidation during reperfusion. ADO does not limit AMPK activation during ischemia, while it causes a marked inhibition of AMPK activity during reperfusion. Thus, ADO inhibits only the detrimental effects of AMPK during reperfusion but does not affect the potentially beneficial effects of AMPK during ischemia. Furthermore, inhibition of AMPK by ADO is not a consequence of the improved recovery

during reperfusion as ADO is able to produce similar inhibition of AMPK in aerobically perfused hearts independent of changes in LV work.

Similar to AMPK, the role of p38 MAPK in the pathology of I/R injury is controversial. Several reports implicate activation of p38 MAPK as an important contributor to the cardioprotective effects of ischemic preconditioning, while on the other hand, other reports show that inhibition of p38 MAPK confers cardioprotection (342). Our results support the later view in which inhibition of p38 MAPK is associated with ADO-induced cardioprotection and pharmacological inhibition of p38 MAPK during reperfusion enhances post-ischemic recovery of LV mechanical function. It is interesting to note that p38 MAPK inhibition also results in inhibition of glycolysis and H^+ production during reperfusion. p38 MAPK is known to play an important role in stimulating myocardial glucose uptake (371-373). However, the observed glycolytic inhibition as a consequence of p38 MAPK inhibition, is not due to changes in glucose uptake as the myocardial content of G-6-P is not affected. Inhibition of glycolysis occurred at a step distal to PFK-1 in the glycolytic pathway as indicated by the accumulation of F-2,6-BP in hearts treated with p38 MAPK inhibitor. Determination of the exact site of glycolytic inhibition due to p38 MAPK inhibition during reperfusion deserves further investigation as it may represent a new target for cardioprotection, and may synergise with other inhibitors that act at other sites in the glycolytic pathway.

6.6 Conclusions

In conclusion, the studies described in this thesis demonstrate that regulation of glucose and glycogen metabolism is an important determinant of the extent of myocardial I/R injury. Several approaches that modulate myocardial glycolytic rates either directly or indirectly, significantly influence post-ischemic recovery of LV mechanical function. In addition, this thesis highlights the importance of the control of the availability of glycogen as an important energy store as it can have a great influence on rates of myocardial metabolism. Specifically, the studies described in this thesis demonstrate that:

1. Pre-ischemic glycogen content is an important determinant factor controlling ischemia-induced changes in glucose metabolism. In hearts with replenished glycogen stores, ischemia-induced activation of glycogenolysis supplies sufficient substrate for glycolysis without the need to stimulate glucose uptake. Ischemia-induced activation of AMPK results in accelerated glucose uptake only under conditions where pre-ischemic glycogen is depleted.
2. Stimulation of glycogen synthesis during reperfusion via inhibition of GSK-3 repartitions G-6-P away from the glycolytic pathway. The reduced rate of glycolysis lessens H^+ production from glucose metabolism and subsequently reduces intracellular Ca^{2+} overload, which limits LV mechanical dysfunction

in early reperfusion and may contribute to improvements in mitochondrial function and cell viability.

3. ADO-induced cardioprotection is mediated by activation of Ser/Thr PP activity that reduces the phosphorylation of AMPK, which subsequently inhibits PFK-2 and slows glycolysis and proton production during reperfusion. This study provides further evidence in favor of the benefit of limiting excessive AMPK activation, glycolysis and proton production during reperfusion.

Taken together, these studies highlight the importance of glucose and glycogen metabolism in the pathogenesis of I/R injury and present a number of targets that may be useful to manipulate the balance between glycolysis and glucose oxidation and thereby limit myocardial I/R injury.

7. Future Aims

7.1 Pentose phosphate pathway and I/R injury

In this thesis, we investigated the two main pathways of glucose metabolism in the heart, namely glycolysis and glycogen synthesis, and their relation to I/R injury. Most of the G-6-P produced in the cardiomyocyte fluxes through these two pathways, with only a small proportion consumed by other routes, such as the pentose phosphate pathway (PPP). Although the PPP operates at relatively low rates in the heart under normal conditions, changes in G-6-P availability as well as changes in G-6-P dehydrogenase (G-6-PDH) activity can affect flux through PPP. Such alterations in PPP flux may affect the antioxidant status of the cardiomyocyte, especially under conditions of increased oxidative stress, such as during reperfusion. Changes in antioxidant status arise because one of the main products of the PPP is NADPH, which is the main cofactor required for the regeneration of GSH from GSSG. Also, GSSG activates G-6-PDH and stimulates flux through PPP (173). Indeed, changes in G-6-PDH activity and PPP flux can affect GSH/GSSG ratios and inhibition of G-6-PDH depletes GSH and induces LV contractile dysfunction following myocardial ischemia (483,484). Thus, it is plausible that enhancing flux through PPP will improve the antioxidant status of the cardiomyocyte and protect against I/R injury. Indeed, benfotiamine, a transketolase activator that directs glucose to PPP, enhances recovery following myocardial infarction and improves antioxidant status (485). Also, it was shown

previously that PPP flux is accelerated by reduced utilization of G-6-P by other pathways such as glycolysis (486,487). In this thesis, we proposed a number of different approaches that inhibit glycolysis, including ADO as well as GSK-3 and p38 MAPK inhibitors. It will be of great interest to examine the possible role of PPP in the cardioprotection elicited by these agents, especially by ADO, as this agent inhibits glycolysis without inducing changes in glycogen synthesis. Thus, it is highly possible that ADO-mediated inhibition of glycolysis directs more G-6-P towards PPP leading to protection against the adverse effects of ROS that are released during reperfusion.

7.2 Genetic modulation of PFK-2

The results presented in Chapter 5 show that ADO inhibits glycolysis through inhibition of AMPK and subsequent inhibition of PFK-2. This results in slower rates of H^+ production and cardioprotection and suggests that PFK-2 may be a useful target to inhibit glycolysis and cause cardioprotection. In order to further examine this hypothesis, it is required to directly modulate PFK-2 activity, either by pharmacological agents or by genetic manipulation. To date, there are no pharmacological agents that are able to directly activate or inhibit PFK-2 activity. However, transgenic mouse models with altered PFK-2 activities have been developed. Donti *et al.* have developed a transgenic mouse model with cardiac-specific overexpression of kinase deficient PFK-2 (488). These hearts

have reduced F-2,6-BP levels leading to reduced activity of PFK-1 and impaired insulin-mediated stimulation of glycolysis. These hearts develop hypertrophy, increased fibrosis, and reduced cardiomyocyte contractility as compared to wild-type hearts. On the other hand, the same group developed another transgenic mouse with cardiac-specific overexpression of phosphatase-deficient PFK-2 (489). These hearts have higher levels of F-2,6-BP and increased glycolytic rates. They also develop hypertrophy and increased fibrosis and are not protected against *in vivo* I/R injury. Unfortunately, glucose metabolism in these PFK-2 gain-of-function and PFK-2 loss-of-function hearts was measured only during non-working Langendorff perfusion and with glucose as the sole exogenous energy substrate. It will be of great interest to study these two models under conditions of physiological energy supply and demand, and in the presence of appropriate concentrations of fatty acids. Such models will also provide an important mechanistic insight into the targets responsible for ADO-induced cardioprotection.

7.3 Synergism by modulating different steps in glucose metabolism

A number of agents improve the coupling between myocardial glycolysis and glucose oxidation and have been found to be cardioprotective. In this thesis, we have shown that GSK-3 inhibitors, ADO, as well as p38 MAPK inhibitors

inhibit glycolysis and are cardioprotective against I/R injury. However, they inhibit glycolysis by distinct mechanisms. In addition, it was shown previously that stimulation of glucose oxidation by activating PDH either directly by dichloroacetate (228) or indirectly by reducing fatty acid oxidation such as by trimetazidine (231) also confer cardioprotection. All these agents act to improve the coupling of glycolysis with glucose oxidation and thereby reduce H^+ production and lessen subsequent Ca^{2+} overload. It is plausible that combinations of these agents will produce synergistic cardioprotection. Indeed, previous work from our group shows that the combination of ADO with dichloroacetate is superior to either of these agents alone and almost completely inhibits H^+ production (282). It will be of great interest to study the potential synergism of action between GSK-3 inhibitors, which inhibit glycolysis indirectly, and ADO or p38 MAPK inhibitors, which inhibit the glycolytic pathway directly. Also, the combination of ADO with a p38 MAPK inhibitor is expected to inhibit glycolysis synergistically as these agents target different steps in the glycolytic pathway. Furthermore, these studies would provide important information about the extent of glycolytic inhibition that is needed to induce maximal cardioprotection as excessive inhibition of glycolysis could potentially limit glucose oxidation and reduce ATP production.

7.4 TIGAR and myocardial glycolysis

In this thesis we investigated different approaches that limit excessive glycolysis during reperfusion and elicit cardioprotection. TIGAR (TP53-induced glycolysis and apoptosis regulator) is a newly discovered molecule that was found to play a role in regulation of glycolysis (490). TIGAR shares functional sequence similarities with the phosphatase domain of PFK-2 which degrades F-2,6-BP. Indeed, TIGAR lowers F-2,6-BP levels and inhibits glycolysis at the step catalyzed by PFK-1. This inhibition of glycolysis redirects glucose towards the PPP resulting in increased production of NADPH and a decrease in intracellular ROS (490). TIGAR expression is induced by p53, which is a well-known tumor suppressor protein but there is no evidence to date of any post-translational regulation of TIGAR (490). In a recent report, Kimata *et al.* showed that prolonged hypoxia induces TIGAR expression in a p53-depndant manner in neonatal rat cardiomyocytes (491). They also showed that the prolonged activation of p53 and TIGAR in cardiomyocytes results in increased apoptosis, whereas knockdown of p53 or TIGAR with siRNA increases glycolysis and reduces myocyte apoptosis. These experiments were performed in neonatal rat cardiomyocytes and were focused mainly on the effects of longer periods of hypoxia on p53 and TIGAR. Their results suggest that prolonged TIGAR activation is detrimental to cardiomyocytes. However, it will be of great interest to investigate the role of TIGAR in modulating acute myocardial I/R injury under

conditions of physiological workload and energy demand. Further understanding of the regulation of TIGAR and the development of new research tools to investigate its role in myocardial I/R injury will help determine its possible involvement in regulating glycolysis and cardioprotection.

7.5 Role of glycogen particles in modulating metabolic signaling

Glycogen is stored in the cardiomyocyte in the form of granules, which appear as bead-like structures or β-particles when visualized using transmission electron microscopy (492). The function of these granules is not limited to just storing carbohydrates but they also non-covalently bind to several proteins and thus act as a molecular scaffold that can unite different proteins involved in glycogen metabolism. Meyer *et al.* first demonstrated the association between glycogen and proteins in 1970 (493). Thus, glycogen granules are not only carbohydrates storage sites but they also act as an independent organelle-like structures termed "glycosomes" (494). Interestingly, proteins bound to the glycosome are mainly proteins that are directly involved in glycogen metabolism. They include glycogen synthase and glycogen phosphorylase, as well as glycogenin, branching enzyme, debranching enzyme, and glycogen targeting subunits of PP1 (492). However, it is interesting to note that AMPK also binds to glycogen (293,294). As most of the studies investigating the role of glycosomes

have been conducted in skeletal muscles or hepatocytes, there is currently a paucity of information in the literature regarding the role of glycosomes in regulating metabolic signaling in the heart. Early histological experiments have shown that cardiac tissue has two populations of glycosomes, one is a free form located in the cytoplasm and another, which is bound to subcellular structures (495). We have shown in this thesis, in support of previous work (148), that glycogen is in continuous turnover in the heart and that the balance between glycogen synthesis and degradation is greatly influenced by the available energy substrates as well as by the myocardial energy demand. In addition, we have shown that glycogen is rapidly degraded during ischemia, as it is the preferred source of glucose during periods of energetic stress. Thus, it will be interesting to follow the changes in glycogen structure that occurs during periods where glycogen is depleted and resynthesized such as during and following ischemia. In addition, it will be of great value to investigate the role of glycogen-bound proteins in conditions with pathological glycogen accumulation, such as PRKAG mutations. These changes in the proteins associated with the glycosomes could have major subsequent effects on myocardial metabolic signaling.

7.6 Optimizing glucose metabolism to treat heart failure

In Western society, improved therapy, as well as improved primary and secondary prevention against cardiovascular risk factors, has resulted in marked

improvements in the number of patients surviving following myocardial ischemia. However, there has been a concomitant increase in the prevalence of heart failure where it is considered a leading cause of morbidity and mortality, as well as an enormous economic burden, in developed countries (496). Heart failure is a complex clinical condition that usually develops following myocardial infarction or following systemic conditions, such as diabetes or hypertension (496). Together with the characteristic neurohormonal changes that accompany heart failure, it is well established that it is also associated with distinct alterations in myocardial energy substrate metabolism. The hypertrophied heart demonstrates a metabolic profile more similar to the fetal heart. Specifically, it demonstrates accelerated glycolytic rates which are accompanied by increased expression of GLUT1 and increased activity of the glycolytic enzymes (497-500). This occurs in the absence of a corresponding increase in glucose oxidation (499) resulting in mismatched glucose metabolism, increased H^+ production, and decreased myocardial efficiency. Several treatments that stimulate glucose oxidation either directly, such as by dichloroacetate, or indirectly by inhibiting fatty acid oxidation, such as with trimetazidine, enhance myocardial efficiency and ameliorate the mechanical dysfunction during heart failure (501).

In this thesis, we proposed a number of approaches to limit excessive glycolysis during reperfusion. The application of these approaches in the setting of heart failure can potentially reduce H^+ production and improve myocardial

efficiency. Our discovery that ADO inhibits glycolysis through inhibition of AMPK may provide a mechanism to explain the beneficial effects of ADO in heart failure. Activation of the different subtypes of ADO receptors exerts anti-remodeling and anti-hypertrophic effects in different experimental models of heart failure (502-505). Furthermore, inhibition of p38 MAPK produces favorable effects to limit myocardial hypertrophy and remodeling (506-508) which may also be potentially attributed to glycolytic inhibition and reduced H^+ production. Thus, it will be of great value to investigate the role of glycolytic inhibition in protection against and treatment of heart failure.

Finally, the studies described in this thesis provided a better understanding of the role of glucose and glycogen metabolism in the pathology of myocardial I/R injury and provided evidence for a number of potential targets that may be exploited pharmacologically to ameliorate myocardial damage. However, the work described here also raised many questions that are yet to be answered. Future investigation of myocardial glucose and glycogen metabolism in the context of myocardial I/R injury as well as in other cardiac pathologies, such as heart failure and diabetic cardiomyopathy, may potentially provide the basis of development of novel therapies and strategies to treat or prevent the development of these conditions.

8. References

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