University of Alberta

Myocardial Energy Substrate Metabolism at the Heart of Ischemia/Reperfusion Injury

by

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Dedication

This thesis is dedicated to my parents, Larry and Cathy Folmes, and my wife Karalyn Folmes, all of whom have supported every one of my endeavors.

Abstract

A novel mechanism to ameliorate ischemia/reperfusion (I/R) injury and improve post-ischemic functional recovery is the optimization of myocardial energy metabolism by shifting oxidative metabolism away from fatty acids to glucose. I/R is associated with an increased reliance on fatty acids as a source of mitochondrial oxidative metabolism at the expense of glucose, leading to an uncoupling of glycolysis from glucose oxidation. This uncoupling induces an accumulation of protons from the hydrolysis of glycolytically derived ATP that is associated with acidosis, sodium and calcium overload, which contributes to post-ischemic contractile dysfunction. A major regulator of myocardial energy metabolism during I/R is AMP-activated protein kinase (AMPK), although its precise role in cardioprotection during I/R has yet to be elucidated.

This thesis examines several aspects of the metabolic control of cardioprotection in I/R. Utilizing isolated working mouse hearts we demonstrate that the cardioprotective effect of insulin and its ability to inhibit AMPK is lost in the presence of a clinically relevant high concentration of fatty acids, despite having its normal downstream metabolic effects. The role of AMPK during I/R is further characterized using transgenic mouse models that up- or down-regulate AMPK activity. We demonstrate that activation of AMPK causes a greater reliance of mitochondrial oxidative metabolism on fatty acids and is not associated with cardioprotection, but may actually be detrimental to the recovery of post-ischemic function. The contribution of acidosis to I/R induced cardiac dysfunction is assessed in ssTnI mice, which have impaired recovery of post-ischemic contractile function potentially due to an increased sensitivity to calcium overload as

opposed to the resistance to acidosis. Using a proteomics approach we identified several changes in mitochondrial metabolic proteins including novel post-translational modifications in pyruvate dehydrogenase, the rate-limiting regulatory step of glucose oxidation, which are potentially important targets for the modulation of myocardial metabolism during I/R.

These studies suggest that modulation of myocardial energy substrate metabolism contributes to I/R-induce cardiac dysfunction. In particular, we demonstrate that activation of AMPK by transgenesis or by blocking the insulin suppression of AMPK with fatty acids is not cardioprotective but may be detrimental to recovery of post-ischemic contractile function.

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Abbreviations

AAR	area at risk
ACBP	acyl-CoA binding protein
ACC	acetyl-CoA carboxylase
ACE	angiotensin converting enzyme
ACS	acyl-CoA synthetase
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMI	acute myocardial infarction
AMP	adenosine monophosphate
AMPK	5'-AMP activated protein kinase
AMPKK	5'-AMP activated protein kinase kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CaMKK	calmodulin dependent kinase kinase
CAT	carnitine acetyltransferase
CBS	cystathionine β-synthase
CO_2	carbon dioxide
CoA	coenzyme A
COX	cyclooxygenase
CPT1/2	carnitine palmitoyl transferase 1/2
Cr	creatine
CT	carnitine acyltranslocase
cTnI	cardiac troponin I
DCA	dichloroacetate
Dn	dominant negative
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
$FAD/FADH_2$	flavin adenine dinucleotide
FABP	fatty acid binding protein
FAT/CD36	fatty acid translocase
FATP	fatty acid transport protein
FBPase-2	fructose 2,6-bisphosphatase
fsTnI	fast skeletal troponin I
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GIK	glucose-insulin-potassium
GLUT	glucose transporter
GPT	glutamate-pyruvate transaminase
GTP	guanosine triphosphate

H^+	proton
HIF-1α	hypoxia inducible factor-1α
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HR	heart rate
I/R	ischemia/reperfusion
IS	infarct size
IVST	intraventricular septum thickness
K_{ATP}	ATP-dependent postassium channel
KD	kinase dead
K_M	Michaelis constant
LAD	left anterior descending artery
LDH	lactate dehydrogenase
LPL	lipoprotein lipase
LV	left ventricle
LVID	left ventricular internal diameter
LVWT	left ventricular wall thickness
MCD	malonyl-CoA decarboxylase
MIF	macrophage migration inhibitory factor
MS	mass spectrometry
MS/MS	tandem mass spectrometry
Na^+	sodium ion
NAD+/NADH	nicotinamide adenine dinucleotide
$NADP/NADPH_2$	nicotinamide adenine dincleotide phosphate
NBC	sodium bicarbonate cotransporter
NCX	sodium calcium exchanger
NHE	sodium hydrogen exchanger
NMR	nuclear magnetic resonance
NOS	nitric oxide synthase
O_2	oxygen
O_2^-	superoxide radical
$^1O_2$	singlet oxygen
OH·	hydroxyl radical
$ONOO^-$	peroxynitrite
PCR	polymerase chain reaction
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
PFK-1/2	phosphofructokinase-1/2
P_i	inorganic phosphate
PKA	protein kinase A
PKC	protein kinase C
PP2A	protein phosphatase 2A
PSP	peak systolic pressure
PTMs	post-translational modifications

ROS	reactive oxygen species
SDS	sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	standard error of the mean
ssTnI	slow skeletal troponin I
TCA	tricarboxylic acid cycle
TG	triglyceride
TOF	time of flight
VLDL	very-low-density lipoprotein
V_{max}	fractional velocity
WPW	Wolff-Parkinson-White syndrome
Wt	wildtype

CHAPTER 1.

Introduction

Chapter 1.

Introduction

Myocardial Ischemia/Reperfusion Injury

Cardiovascular disease is the leading cause of mortality in Canadian society, accounting for 78,942 deaths (approximately 36 % of total deaths) in 1999.[1] Of these deaths, ischemic heart disease comprises a major portion, approximately 20 % of the total deaths in Canada. However, due to the advent of improved therapeutic modalities, there has been a marked decline in the percentage of deaths attributed to cardiovascular disease (14 % and 16 % decline in men and women, respectively). Therefore the number of Canadians living with cardiovascular disease has increased. Taken together, cardiovascular disease imposes a massive economic burden on Canadian society (over 18.4 billion dollars in 1999).[1]

Myocardial ischemia occurs when there is an imbalance between the oxygen (O_2) requirement of the contracting muscle and the O_2 supply to the heart by the coronary vasculature. The classical treatment of ischemic heart disease consists of strategies that correct this imbalance by either increasing O_2 delivery or decreasing O_2 demand. This includes invasive procedures such as coronary artery bypass grafting and percutaneous coronary interventions, as well as pharmacological thrombolysis, which re-establish flow

to the ischemic myocardium. However, restoration of flow is associated with a sequelae of events that can induce contractile dysfunction and cell death, which has been termed reperfusion injury.[2] The cellular and molecular mechanisms of ischemia/reperfusion (I/R) injury are multifaceted and are manifested as both reversible and irreversible injury, ranging from cardiac stunning and arrhythmias to cardiomyocyte death in the form of apoptosis and necrosis. Alternative pharmacological means to treat ischemic disease include agents that either improve hemodynamics or reduce O_2 demand. The group consists of organic nitrates, Ca^{2+} channel blockers, and angiotensin converting enzyme (ACE) inhibitors, which improve hemodynamics by decreasing preload and systemic vascular resistance, as well as β-adenoreceptor antagonists that reduce O_2 demand due to their negative chronotropic and inotropic action. Despite these treatment options the number of deaths from ischemic heart disease is remaining constant [1], emphasizing the importance of the development of novel therapeutic strategies, such as improving the efficiency of O_2 utilization in the ischemic and reperfused heart. One such approach is the use of metabolic modulation to optimize myocardial energy metabolism and reduce the damage resulting from an acute coronary event. However, the mechanism by which myocardial energy substrate metabolism is regulated in both an aerobic setting and during I/R is still incompletely understood and requires further examination, which will be the goal of this thesis. Thus this introduction will give an overview of I/R injury prior to a discussion on the major metabolic pathways in the heart and their regulation. A key regulator of myocardial energy substrate metabolism is the AMP-activated protein kinase (AMPK), therefore special emphasis will be placed on its downstream signaling

pathways that modulates myocardial energy substrate metabolism and its role in eliciting cardioprotection from I/R injury.

Mechanisms of Ischemia/Reperfusion Injury

Myocardial ischemia occurs when coronary blood flow and O_2 supply is inadequate to meet the O_2 requirements of the heart. This compromised coronary flow results in various chemical, electrical and mechanical changes to the ischemic zone of the myocardium. This includes a reduction in the production of adenosine triphosphate (ATP) from oxidative metabolism, thus making the heart reliant on the anaerobic production of ATP form glycolysis.[3, 4] The supply of ATP is insufficient to meet the energy demands of the heart, therefore ischemia ultimately results in a depletion of high-energy phosphates, leading to a rapid loss of contractile function and dysregulation of ionic homeostasis.[3, 4]

There is no single defined mechanism for I/R injury, instead it appears to be a multifactorial disease. Potential contributing mechanisms include: modifications in myocardial energy substrate metabolism and substrate supply to the heart; dysregulation of ionic homeostasis and intracellular Ca^{2+} overload; proteolysis of the contractile proteins; production of reactive oxygen species (ROS); induction of inflammatory and immune responses including the activation and infiltration of neutrophils and activation of the complement; induction of myocyte death (apoptosis, necrosis and autophagy); opening of the mitochondrial permeability transition pore (mPTP) and dissipation of the mitochondrial membrane potential and release of pro-apoptotic mediators; further impaired coronary flow due to an increase in thromboresistance and the no reflow

phenomenon; nitric oxide synthesis and signaling; and ventricular remodeling.[3-7] Taken together the above mechanisms of I/R injury are not mutually exclusive, and most likely I/R injury is dependent on the interaction among many of these pathways. Due to the content of this thesis the role of the dysregulation of ionic homeostasis and Ca^{2+} overload and of modifications in substrate supply and myocardial energy substrate metabolism will be highlighted.

Dysregulation of Ionic Homeostasis and Ca^{2+} Overload

The uncoupling of glucose metabolism during I/R contributes to myocardial acidosis and subsequent Na^+ and Ca^{2+} overload, as will be discussed in later sections. In addition, the production of ROS, oxygen-derived free radicals or precursor molecules that are highly unstable, can interfere with the function of several membrane ion exchange mechanisms and membrane ion channels, which compromises the maintenance of ionic homeostasis.[8] The Na^+/K^+ ATPase is essential for maintaining resting membrane potential due to its electrogenic nature by extruding three Na^+ ions in exchange for two K^+ ions. Therefore, ROS induce inactivation of Na^+/K^+ ATPase results in the inability of the cardiomyocyte to extrude Na^+ contributing to eventual Na^+ overload, which activates the reverse mode of the Na^+/Ca^{2+} exchanger (NCX).[9, 10] Na^+ may also accumulate due to late or persistent Na^+ current.[11, 12] NCX extrudes three Na^+ in exchange for one Ca^{2+}, which eventually contributes to the I/R-induce Ca^{2+} overload. Ca^{2+} overload is further exacerbated as ROS can also impair the activity of Ca^{2+} ATPase, which is responsible for the reuptake of Ca^{2+} into the sarcolemmal reticulum following myocyte contraction.[13]

Elevated levels of Ca^{2+} can trigger various detrimental processes, including the activation of phospholipases and proteases, modification of mitochondrial permeability and induction of cardiomyocyte cell death.[14] Several phospholipase isoforms can be directly activated by Ca^{2+} leading to the hydrolysis of phospholipids that adversely affects the stability, fluidity and integrity of membranes and can compromise membrane transporters.[14] This also increases cytosolic free fatty acids, which can exacerbate ischemic injury by increasing intracellular acidosis [15], as well as Ca^{2+} overload by activating voltage-dependent Ca^{2+} channels [16], by inducing ROS production from uncoupled mitochondria [17] and by modifying myocardial metabolism. I/R also activates Ca^{2+} dependent proteases (calpains) via autocatalytic proteolysis, leading to the proteolytic degradation of many intracellular proteins. Targets of calpains include the contractile proteins troponin I and troponin T [18, 19], Ca^{2+} handling proteins including the Ca^{2+}-ATPase, ryanodine receptor and phospholamban [20] and apoptotic signaling proteins including Bcl-2, Bcl-XL, Bax and caspase-3.[21] Cytosolic Ca^{2+} overload can contribute to elevated mitochondrial Ca^{2+} levels, which are also associated with opening of the mPTP, which resides in the inner mitochondrial membrane and has a size exclusion of 1.5 kDa.[14, 22] Opening of the pore will disrupt mitochondrial homeostasis leading to the dissipation of membrane potential, the loss of mitochondrial proteins and ATP depletion as the F_1F_0-ATPase may begin to hydrolyze ATP to try to maintain membrane potential.[14, 22]

Severity of Myocardial Ischemia/Reperfusion Injury

There is a continuum of the severity of ischemic heart disease, which can range from angina to permanent occlusion of one of the coronary vessels. The most common manifestation is angina pectoris, which is due to obstruction of the large coronary vessels by stenosis, or by vasospasm, reduced coronary flow reserve or microvascular dysfunction.[4] This results in decreased blood flow to the heart and impairs the ability to increase coronary flow in response to a rise in demand (demand-induced ischemia), thus supplying inadequate amount of O_2 for maintenance of energy homeostasis.[4] Acute myocardial infarction may occur due to a more severe impairment of blood flow resulting from large atherosclerotic plaques, from a thrombosis caused by platelet aggregation at an unstable plaque that ruptured, embolic phenomena or severe vasospasm.[4] Chronic ischemia due to permanent occlusion of one of the coronary vessels results in infarction and cell death, with eventual cardiac remodeling in an attempt to compensate for the loss of cardiac muscle.[4] Each of these forms of ischemic heart disease can be characterized as reversible or irreversible I/R, which will be discussed in more detail in the following sections.

Reversible Ischemia/Reperfusion Injury

Brief periods of transient myocardial ischemia, less than 15 min in the heart, result in reversible I/R injury, beyond which irreversible injury occurs.[23] Reversible I/R injury is defined as the mechanical dysfunction that persists after reperfusion in the absence of irreversible injury (myocyte death). This type of injury can manifest as either dysregulation of ionic homeostasis promoting a pro-arrhythmic phenotype or as

myocardial stunning.[24, 25] Myocardial stunning refers to the mechanical dysfunction that persists after reperfusion in the absence of irreversible cardiomyocyte damage in the presence of normal or near-normal coronary flow.[26] It may require hours to days following the relief of ischemia for the full restoration of cardiac function in the post-ischemic but viable myocardium.[2, 26] Chronic myocardial stunning may occur in patients who experience repetitive episodes of I/R that may ultimately lead to myocardial hibernation, which is characterized by an impaired left ventricular function at rest due to reduced coronary blood flow that can be restored to normal if the O_2 supply/demand relationship is favorably altered.[27, 28] Myocardial stunning can occur in many aspects of ischemic heart disease, including following spontaneous reperfusion in patients with unstable angina or following high-risk coronary artery bypass grafting, which may lead to hemodynamic instability and ultimately can contribute to the morbidity and mortality associated with ischemic heart disease.[29]

Irreversible Ischemia/Reperfusion Injury

As ischemic duration increases irreversible damage (myocyte death) occurs in the myocardium resulting in areas of infarction, the size of which depends on many factors including the severity and duration of the ischemic insult prior to reperfusion. Classically, myocyte death has been considered to be dependent on two processes, either necrosis and/or apoptosis, however recently autophagic cell death has emerged as an alternative form of cell death.[7] Necrosis is an unregulated process of cell death, characterized by cell swelling followed by membrane rupture producing cellular debris that induces an inflammatory response.[3] In contrast, both apoptosis and autophagic cell

death are forms of organized energy-requiring programmed cell death.[7] Apoptotic cell death is characterized by cell shrinkage, membrane blebbing, nuclear condensation, DNA fragmentation and activation of caspases.[7] Eventually cells undergoing apoptosis are broken into small apoptotic bodies which are removed either by macrophages or taken up by neighboring cells in order to prevent an inflammatory response. Autophagy is a normal process in the heart by which dysfunctional cytosolic components are removed via a lysosomal degradative pathway, which may act as a protective response during I/R.[7] However, autophagic cell death is implicated in contributing to cardiac dysfunction in the failing heart as autophagosomes are increased during the progression of this disease.[7] It appears that overall cardiomyocyte death following I/R is most likely a spectrum of the three forms of cell death depending on the length and severity of the ischemic insult. Myocardial infarction from cell death spreads through the myocardial wall from the subendocardium to the subepicardium in what is termed the wave front phenomenon.[3] The greater the degree of flow reduction, the faster the wave front advances contributing to wall motion abnormalities.[3] Post-infarction remodeling occurs in order to compensate for the impaired ventricular function, but it may ultimately lead to adverse cardiac hypertrophy of the remaining viable myocardium, left ventricular dilatation and interstitial fibrosis progressing to heart failure and death.[3]

Myocardial Energetics

Due to the small energy reserve (approximately 20 to 30 μmol/g dry wt of ATP) and the need to meet the high energy demands of contraction and ionic homeostasis

(approximately 200 μmol/g dry wt/min), the heart must produce a constant and abundant supply of ATP (between 3.5 and 5 kg ATP per day).[1] Based on these observations, the entire heart ATP pool is turned over at least four times per min. Therefore without continuous ATP production, the myocardial energy pool would be exhausted in less than a min. Normally in the well-perfused heart maintenance of ATP levels is dependent upon oxidative phosphorylation, which consists of the transport of protons across the inner mitochondrial membrane by the electron transport chain coupled with ATP synthesis. However during myocardial ischemia, oxidative metabolism is inhibited due to the lack of O_2 to act as the final electron acceptor of the electron transport chain. Therefore other mechanisms are required to maintain ATP levels.

Three mechanisms to maintain ATP levels during metabolic stress include glycolysis, the creatine kinase and adenylate kinase reactions.[30] The stimulation of glycolysis during myocardial ischemia is important in order to produce an anaerobic source of ATP by substrate level phosphorylation. However a number of studies demonstrate that if glycolysis is not coupled to glucose oxidation, then hydrolysis of glycolytically produced ATP leads to a net production of protons, as will be further discussed in the following sections.[31, 32] Phosphocreatine (PCr) is an alternative source of energy for the heart (approximately 30 – 50 μmol/g dry wt), and is of particular importance during times of increased metabolic demand, as ATP levels can be maintained by the creatine kinase reaction which couples the breakdown of phosphocreatine to creatine (Cr) with the synthesis of ATP:

$$ATP + H_2O \leftrightarrow ADP + Pi + H^+$$

$$ADP + PCr + H^+ \leftrightarrow Cr + ATP$$

In addition, adenylate kinase removes excess ADP produced as a result of phosphoryl group transfers from ATP, producing ATP and AMP (which can be utilized to simulate glycolysis):

$$2\ ADP \leftrightarrow ATP + AMP$$

Myocardial Energy Substrate Metabolism

Fatty Acid Metabolism

Circulating Lipids and Fatty Acid Uptake

In healthy individuals circulating plasma fatty acid levels range from 0.2 to 0.8 mM during the course of a day. However, in pathological states, which are associated with a metabolic stress, such as diabetes and ischemia, circulating fatty acid levels can far exceed these normal levels (>1.0 mM).[33] Plasma fatty acid concentration is dependent on their release from triglycerides (TGs) in the adipocytes, which is based on the balance of enzymatic activity of hormone-sensitive lipase for TG degradation and glycerolphosphate acyltransferase for TG synthesis.[33] As the name implies, hormone-sensitive lipase is regulated by hormones, including catecholamines, which activate the enzyme, and insulin, which inhibits the enzyme. Thus pathological states such as ischemic heart disease, which increases β-adrenergic stimulation, results in elevated plasma fatty acid concentration.[33] In addition, heparin administration during myocardial infarction contributes to elevated circulating fatty acids, as heparin releases lipoprotein lipase (LPL) from its heparan sulfate proteoglycan linkage to endothelia cells, thus increasing circulating LPL that can act upon lipoproteins releasing free fatty acids.[34-36] These

fluctuations in plasma fatty acid levels are important, as the rate of fatty acid uptake into the cardiomyocyte is very dependent on the concentration of nonesterified fatty acids supplied in the coronary vasculature.[37, 38] In the plasma they are found in their nonesterified form bound to high affinity binding sites on albumin, or contained as TGs within TG-rich lipoproteins, chylomicrons and very-low-density lipoproteins (VLDL), all of which contribute to myocardial fatty acid metabolism.[39] Hauton *et al.* demonstrate that TG from VLDL or chylomicrons could support cardiac function; however they are of secondary importance when in the presence of fatty acid bound to albumin.[40] In addition, the presence of free fatty acids suppress utilization of TG from chylomicrons but not from VLDL. Further studies show that TG from chylomicrons are oxidized and contribute to the maintenance of mechanical function, while TG from VLDL is mostly incorporated into tissue lipids.[41]

Fatty acids are transported into the cardiomyocyte either via passive diffusion in a flip-flop mechanism [42] or by carrier-mediated processes by FAT/CD36, FABPpm and/or FATP. FAT/CD36 is a transmembrane protein abundantly expressed in fatty acid metabolizing tissues such as cardiac muscle, and appears to be the major fatty acid transporter as it can account for up to 50 % of the fatty acid uptake in the isolated heart.[43] Fractionation studies of cardiac myocytes show that under basal conditions CD36 is localized at both the sarcolemmal membrane and in intracellular stores and that contraction or an increase in energy demand can induce a translocation of CD36 from the intracellular pool to the sarcolemmal membrane, in a similar mode of regulation as the glucose transporter4 (GLUT4).[44, 45] The sarcolemmal membrane fatty acid binding protein (FABPpm) is a 40 kDa protein that is loosely associated with the sarcolemmal

membrane. The evidence supporting a role of FABPpm in fatty acid transport was initially based on an observation that treatment with an antibody raised against this protein results in a reduction in fatty acid uptake in a variety of cells.[46, 47] Fatty acid transport protein (FATP) is an integral membrane protein, which when expressed in a stable fibroblast cell line results in a 3- to 4-fold increase in long-chain fatty acid transport.[48] The cloning of this first FATP led to the discovery of a family of evolutionarily conserved proteins, encoded by at least five different genes in the mouse and displaying different tissue-specific expression patterns with FATP1 being the major isoform found in heart and skeletal muscle.[49]

Fatty Acid Transport and Activation in the Cytosol

FABPs (heart and epidermal type are expressed in the heart) are the cytoplasmic equivalent of albumin. These proteins were first identified as having a high affinity for fatty acids [50], however their role in fatty acid metabolism was only determined recently when it was shown that knockout mice of heart FABP have a 50 % reduction in rates of palmitate uptake.[51] Fatty acids are then activated by esterification to fatty acyl-CoA esters by the action of a family of fatty acyl-CoA synthetases (ACS), which differ in their chain length specificity and their subcellular location. The heart isoform of long-chain ACS is localized to the aqueous cytoplasmic face of the outer mitochondrial membrane.[52] The conversion to a more reactive CoA thioester is a prerequisite for the subsequent catabolic process. Despite the essential role that ACS plays in the catabolism of fatty acids, their regulatory role in fatty acid metabolism has yet to be elucidated.

Once activated, fatty acyl-CoA esters can bind to another cytoplasmic binding protein, acyl-CoA binding protein (ACBP). These proteins may buffer the concentration of long-chain acyl-CoA esters, which would relieve the end product inhibition of acetyl-CoA carboxylase (ACC) and adenine nucleotide translocase.[53] These long-chain fatty acyl-CoA esters can then either be esterified to TGs or phospholipids by glycerolphosphate acyltransferase or transported into the mitochondria for subsequent β-oxidation.[54, 55]

Regulation of Fatty Acyl-CoA Ester Transport into the Mitochondria

β-oxidation of fatty acids occurs primarily in the mitochondria, however a small portion also occurs in the peroxisomes.[56] Before long-chain acyl-CoA esters can undergo oxidation, they first must be transported into the mitochondrial matrix. The inner mitochondrial membrane is not freely permeable to acyl-CoA esters, most likely to maintain separate cytosolic and mitochondrial CoA pools, therefore a carnitine-dependent transport system is required.[57] This system consists of three components: carnitine palmitoyl transferase 1 (CPT1), carnitine acyltranslocase (CT) and carnitine palmitoyl transferase 2 (CPT2). First CPT1, found on the inner surface of the outer mitochondrial membrane, catalyzes the conversion of long-chain acyl-CoA ester to long-chain acylcarnitine.[58] Second, CT exchanges long-chain acylcarnitine for free carnitine across the inner mitochondrial membrane. Lastly, CPT2 regenerates long-chain acyl-CoA ester in the mitochondrial matrix. Of these three enzymes, CPT1 is the major site of regulation in controlling the uptake of fatty acids into the mitochondria.[57]

Fatty acid oxidation is tightly controlled at a number of steps, but the uptake of fatty acyl-CoA esters into the mitochondria is arguably one of the most important. CPT1 is the major site of regulation as it is strongly inhibited by malonyl-CoA, a compound that binds on the cytosolic side of the enzyme.[59, 60] The heart expresses two isoforms of CPT1 (CPT1β (82 kDa) and CPT1α (88kDa)) with the 82 kDa isoform predominating. The CPT1β isoform is 30-fold more sensitive to malonyl-CoA inhibition that the CPT1α liver isoform.[59, 61] This makes malonyl-CoA a key endogenous inhibitor of fatty acid oxidation in the heart, as malonyl-CoA levels are inversely correlated with fatty acid oxidation rates.[62-64]

The half-life of malonyl-CoA in the heart is approximately 1.25 min, making both the synthesis and degradation of malonyl-CoA important in the control of its steady state level.[65] Malonyl-CoA is synthesized by the carboxylation of acetyl-CoA by ACC [63, 66] and degraded to acetyl-CoA and carbon dioxide in the cytosol and mitochondria by malonyl-CoA decarboxylase (MCD).[67, 68] Two different isoform of ACC have been identified, a 265 kDa isoform (ACC265 or ACC α) and a 280 kDa isoform (ACC280 or ACCβ); both isoforms are expressed in the heart, although the prominent form is ACC280 and it appears to contribute most of the heart ACC activity.[69, 70] It has been speculated that ACC280 is directly involved in the regulation of fatty acid oxidation because it is found in tissue with high oxidative capacity and is in close association with the mitochondrial membrane, which would allow malonyl-CoA produced to directly inhibit CPT1 activity.[71, 72] Isolated working heart studies provide direct evidence that ACC is an important regulator of fatty acid oxidation.[62, 63, 73] ACC activity is dependent on the supply of its substrate, acetyl-CoA, however most of the acetyl-CoA in

cardiomyocytes resides in the mitochondria.[74] There is indirect evidence that acetyl-CoA can be derived from the export of mitochondrial acetyl-CoA as acetylcarnitine by the CT [75] or from citrate via the ATP-citrate lyase reaction.[76, 77]

Regulation of β-oxidation

Once acyl-CoA esters have entered the mitochondria they undergo β-oxidation, which produces NADH and $FADH_2$ for the electron transport chain and acetyl-CoA for further catabolism in the TCA cycle. β-oxidation requires four different steps as outlined in Figure 1-1, each of which is catalyzed by an enzyme with specificity for short-, medium- and long-chain acyl-CoA ester intermediates.[37] The acyl-CoA ester undergoes repeated oxidation via these reactions until only acetyl-CoA units remain. The first step of β-oxidation consists of the conversion of acyl-CoA ester to Δ^2-3-trans-enoyl-CoA in the presence of the FAD, and is catalyzed by acyl-CoA dehydrogenase. Enoyl-CoA hydratase then adds water across the double bond to produce L-3-hydroxyacyl-CoA. In the second dehydrogenation step of the pathway, L-3-hydroxyacyl-CoA dehydrogenase catalyzes the oxidation of L-3-hydroxyacyl-CoA to L-3-ketoacyl-CoA, with the involvement of NAD^+ as a cofactor. The final step of the pathway consists of the splitting of L-3-ketoacyl-CoA at the 2,3-position by 3-ketoacyl-CoA thiolase, in the presence of CoA, to produce one acetyl-CoA unit and an acyl-CoA that is 2 carbons shorter than the original acyl-CoA. This acyl-CoA feeds back to acyl-CoA dehydrogenase to start another cycle of β-oxidation, while acetyl-CoA goes to the TCA cycle for further oxidation.

β-oxidation is primarily dependent on the workload of the heart, as ratios of $NADH/NAD^+$ and acetyl-CoA/CoA decrease in response to an increase in workload.[78, 79] The decrease in acetyl-CoA/CoA ratio releases the suppression of L-3-ketoacyl-CoA thiolase activity and a decrease in matrix acetyl-CoA also activates acyl-CoA dehydrogenase.[78] A decrease in workload would result in an increase in L-3-ketoacyl-CoA, which feeds back and inhibits acyl-CoA dehydrogenase.[80] Reports in liver suggest that $NADH/NAD^+$ ratio regulates L-3-hydroxyacyl-CoA dehydrogenase in which low acetyl-CoA levels exists, however it is unknown whether this is an important site of regulation in the heart.[81]

Carbohydrate Metabolism

Glucose Uptake

The majority of glucose for cardiac metabolism is derived from the blood, and its uptake is facilitated in an energy independent mechanism (down the transmembrane glucose gradient) by glucose transporters, GLUT1, which sustains basal glucose uptake, and GLUT4, which translocates from an intracellular pool in response to insulin and contraction.[9] Both of these glucose transporters are transcriptionally and hormonally regulated and can have differing expression levels in various pathological states.[82] In the normal heart, the major factors affecting glucose uptake include the degree of cardiac workload [83], the dietary state (availability of alternative substrates) [84], circulating plasma glucose and insulin [85], catecholamines [86] and thyroid hormone.[87] The mechanisms by which these physiological signals regulates glucose uptake remain unclear, but may include modifications in the trafficking of GLUTs (including translocation to the plasma

membrane and endocytosis and recyling), expression and transcription of the transporters, substrate supply of both carbohydrates and fatty acids and their metabolism, and activity of AMPK, which will be discussed in following sections.[82] Once glucose is transported into the cell it is phosphorylated by hexokinase to glucose 6-phosphate making it impermeable to the cell membrane and removing it from the transmembrane concentration gradient. Glucose 6-phosphate has several metabolic fates including glycogen synthesis, the pentose phosphate pathway as well as glycolysis and subsequent glucose oxidation.

Glycogen Metabolism

The major storage depot for carbohydrates in the heart is glycogen, which is a polysaccharide of repeated glucose units in α1,4 and α1,6 glycosidic bonds that form large granules in the cytoplasm of the heart.[55] Although typically considered a passive storage depot, the glycogen molecule is in a constant state of turnover due to the coordinated activity of two different enzyme systems controlling glycogen synthesis and breakdown.[55] The regulation of these systems is complex and is regulated both by protein phosphorylation and allosteric mechanisms.[55] In the case of glycogen synthesis, the critical step is catalyzed by glycogen synthase and consists of the transfer of glucose 1-phosphate to the end of the glycogen chain. This process occurs when glycogen synthase is in its active dephosphorylated form, which is dependent on the activity of glycogen synthase kinase, protein kinase A (PKA) and protein phosphatase.[55] The important step of glycogenolysis, the cleavage of glucose-1-phosphate from the glycogen chain, is catalyzed by glycogen phosphorylase, either in its phosphorylated active form

(a) or by stimulation of its inactive form (b) by AMP and inorganic phosphate. The phosphorylation of glycogen phosphorylase is dependent on the activity of phosphorylase kinase (which is activated by phosphorylation by PKA and allosterically by Ca^{2+}) and protein phosphatase.[55] Therefore the combined effects of phosphorylation of glycogen synthase and phosphorylase provide an important regulatory network to control the flux of glucose into and out of glycogen stores. In the case of adrenaline or an increase in mechanical activity (with an increase in cytosolic Ca^{2+}), phosphorylation via a PKA would increase resulting in a greater rate of glycogenolysis. On the other hand, stimulation by insulin would promote glycogen synthesis via the inactivation of PKA (secondary to the activation of cyclic AMP phosphodiesterase-3B and a reduction in cyclic AMP levels) and glycogen synthase kinase and the activation of protein phosphorylase.[88-90]

Pentose Phosphate Pathway

Alternatively, glucose 6-phosphate can be metabolized by glucose-6-phosphate dehydrogenase and enter the pentose phosphate pathway. The major importance of this pathway is to produce ribose 5-phosphate, which is used to synthesize nucleic acids, and $NADPH_2$, which is primarily used to maintain glutathione in its reduced state to protect the heart from oxidative damage and for biosynthetic pathways particularly in the liver.[91] Both of these essential metabolites are produced in the oxidative branch of the pentose phosphate pathway, with the non-oxidative branch being responsible for the regeneration of glycolytic intermediates. However rates of the oxidative branch are extremely low in

the heart due to low activity of the rate-limiting enzyme, glucose-6-phosphate dehydrogenase, thus the importance of this pathway in the heart is controversial.[92]

Glycolysis

The major metabolic pathway downstream of glucose uptake and glycogenolysis is glycolysis, which consists of the catabolic metabolism of one molecule of glucose to two molecules of pyruvate with the net production of 2 ATP, as shown in figure 1-2. In the aerobically perfused heart, pyruvate is subsequently transported into the mitochondria and is oxidized, with glycolysis contributing less than 10 % of total ATP production from substrate level phosphorylation.[2] However the greater importance of glycolysis occurs when oxidative metabolism is impaired due to the limited availability of O_2 as the final electron acceptor of the electron transport chain. Under these conditions rates of glycolysis are elevated with pyruvate being converted to lactate via lactate dehydrogenase (LDH). Thus this anaerobic source of ATP is an essential pool for the ischemic heart and has been suggested by some to be specifically used for maintaining ion homeostasis.[93]

Glycolysis is a highly regulated metabolic pathway that can be stimulated by lack of O_2 supply [93], increase in cardiac work [94, 95], short-term exercise [96] and cardiac hypertrophy.[97, 98] Glycolysis is regulated at multiple steps including supply of glucose and the enzymatic activity of hexokinase, phosphofructokinase-1 (PFK-1), pyruvate kinase and glyceraldehyde 3-phophate dehydrogenase (GAPDH).

Hexokinase catalyses the first irreversible step of glycolysis as such it is a potentially important site of regulation. The heart expresses two isoforms of hexokinase

(I and II), with hexokinase II predominating in the adult heart.[99] Unlike glucokinase found in the liver, hexokinase has a low K_m, suggesting that even if glucose levels drop during strenuous exercise, the hexokinase reaction can still proceed.[55] Hexokinase is subjected to feedback regulation by it product glucose 6-phosphate, thus inhibiting flux through glycolysis.[55, 100] Elevated levels of glucose 6-phosphate occur due to the inhibition of PFK-1 such as occurs during ischemia. In addition, hexokinase activity may be regulated due to its localization, as it resides in the cytosolic fraction of the cell but can also bind to the outer mitochondrial membrane.[101] Binding of hexokinase to the mitochondrial membrane further lowers its K_m for glucose and suppresses its inhibition by glucose 6-phosphate.[101] Insulin and ischemia can induce the translocation of hexokinase to the mitochondrial membrane thus modifying its enzymatic activity.[102, 103]

PFK-1 catalyses the second regulatory site of glycolysis, which commits glucose 6-phosphate to its catabolic fate:

Fructose 6-phosphate + ATP → Fructose 1,6-bisphosphate + ADP

Basal enzymatic activity of PFK-1 is low compared to the other enzymes of glycolysis and its mass action ratio of the reactants is far removed from its equilibrium constant making it an optimal site for the control of glycolytic flux.[3] Allosteric control of PFK-1 allows for large changes in enzyme activity, as positive allosteric regulators include AMP, inorganic phosphate, fructose 6-phosphate and fructose 2,6-bisphosphate; negative allosteric regulators include ATP, PCr, protons (H^+) and citrate. Alternatively fructose 6-phosphate can be metabolized by the bifunctional enzyme phosphofructkinase-2 (PFK-2)/fructose 2,6-bisphosphatase (FBPase-2) to form fructose 2,6-bisphosphate, which is a not a glycolytic intermediate, but a potent allosteric stimulator of PFK-1.

Phosphorylation of this enzyme allows for the hormonal and workload control of glycolysis, as phosphorylation of Ser466 and/ or Ser483 by wortmannin insensitive protein kinase (insulin) [104], PKA (adrenaline) [105] and Akt (increase in workload) [94, 106] leads to the activation of the kinase domain of the enzyme resulting in elevated levels of fructose 2,6-bisphosphate and a stimulation of glycolysis.

The terminal step of glycolysis, which consists of the conversion of phosphoenolpyruvate to pyruvate coupled with the formation of ATP, catalyzed by pyruvate kinase might also contribute to the regulation of the glycolysis. Pyruvate kinase is activated in a feed forward mechanism by fructose 1,6-bisphosphate, the product of PFK-1.[107] This would allow for the upregulation of flux through PFK-1 without the accumulation of downstream glycolytic intermediates.

During extreme conditions such as total ischemia and abrupt anoxia the regulation of glycolysis can be passed from the above mechanisms to GAPDH.[3] GAPDH catalyzes the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate coupled with the reduction of NAD^+ to NADH. As the cardiomyocyte has limited amount of NAD^+, glycolysis would soon come to a halt if NAD^+ were not continuously regenerated. This is of particular important during severe ischemia, as glycolysis becomes a major contributor to total ATP production. Therefore to ensure metabolic flux through GAPDH is not restricted during ischemia, NADH is re-oxidized during the conversion of pyruvate to lactate catalyzed by LDH. In the presence of O_2, NADH does not build up and inhibit GAPDH as the NADH reducing equivalents are transported into the mitochondria by the malate-aspartate shuttle and are consumed by the electron transport chain.[55]

Metabolic Fates of Pyruvate and Glucose Oxidation

Pyruvate, the final product of glycolysis has several metabolic fates. As previously discussed in the ischemic heart, pyruvate is converted to lactate via LDH to ensure a constant supply of NAD^+ for the GAPDH reaction. Under aerobic conditions a small amount of pyruvate (3 – 6 %) can be used for anaplerosis by carboxylation to oxaloacetate or malate by pyruvate carboxylase and malic enzyme, respectively, however in the presence of a high concentration of fatty acids pyruvate carboxylation is significantly blunted.[108-112] In the well-perfused heart, the majority of pyruvate is aerobically disposed of in the mitochondria in a pathway named glucose oxidation. This process is initiated by the transport of pyruvate into the mitochondrial matrix via the monocarboxylate transporter, where it undergoes oxidative decarboxylation by the pyruvate dehydrogenase (PDH) complex yielding acetyl-CoA. This product enters the TCA cycle where it undergoes complete oxidation to carbon dioxide (CO_2). The majority of ATP from carbohydrate sources is produced during glucose oxidation.

The PDH complex catalyzes the rate-limiting regulatory step of glucose oxidation. This complex consists of multiple copies of three enzymes E1 (pyruvate dehydrogenase), E2 (dihydrolipoyl transacetylase) and E3 (dihydrolipoyl dehydrogenase), with the E1 being a tetramer consisting of 2α and 2β subunits.[55] Under basal conditions only approximately 20 % of PDH is in its active form, which allows for a large jump in flux through the complex in response to an increased workload or the presence of catecholamines, both of which would stimulate glycolysis providing addition pyruvate to the complex.[94, 95] This complex can be directly regulated by allosteric mechanisms by the products of the reaction, such as inhibition of the enzyme by an

increase in the acetyl-CoA/CoA and NADH/NAD^+ ratios. PDH is also highly regulated by phosphorylation/dephosphorylation by the PDH kinase (PDK) that phosphorylates and inactivates the complex, and PDH phosphatase (PDP) that dephosphorylates and activates the complex.[113] The kinase and phosphatase are also under allosteric regulation. The kinase is positively regulated by acetyl-CoA and NADH, and negatively regulated by pyruvate, CoA and NAD^+, while the phosphatase is positively regulated by Ca^{2+} and magnesium.[55] This feedback inhibition of PDH is important as both acetyl-CoA derived from carbohydrates and from fatty acids may activate the PDH kinase, and inactivate glucose oxidation.

The Tricarboxylic Acid Cycle

The tricarboxylic acid (TCA) cycle consists of eight enzymatic reactions that occur in the mitochondrial matrix. Acetyl-CoA from the catabolism of both fatty acids and glucose enters the cycle via a condensation reaction with oxaloacetate to form citrate. These acetyl-CoA derived carbon equivalents undergo complete oxidation to carbon dioxide, with the regeneration of oxaloacetate for the continuation of the cycle. Each turn of the TCA cycle results in the production of one GTP by substrate-level phosphorylation and three NADH and one $FADH_2$ reducing equivalents, which are subsequently oxidized by the electron transport chain to produce ATP by oxidative phosphorylation.[114]

As the TCA cycle is the major source of reducing equivalents for the electron transport chain and hence for ATP production, the activity of the cycle is exquisitely geared to the metabolic needs of the heart such that cycle activity increases with an increase in workload, and cycle activity decreases with a fall in workload. One of the

major regulatory mechanisms mediating this is the intramitochondrial $NADH/NAD^+$ ratio, which regulates isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase.[3] At the beginning of an increase in cardiac work, the $NADH/NAD^+$ ratio decreases due to an increase in energy demand, which stimulates the TCA cycle dehydrogenases, thus increasing TCA cycle activity. The stimulation of the TCA cycle by an increase in cardiac work may also be mediated by increase in cytosol and intramitochondrial Ca^{2+}, which stimulates isocitrate dehydrogenase and α-ketoglutarate dehydrogenase.[115] In addition, the activity of the TCA cycle may be limited by the rate at which ADP can be transported into the mitochondria via the ATP/ADP translocase.[3]

Oxidative Phosphorylation

Oxidative phosphorylation occurs on the inner mitochondrial membrane and consists of the oxidation of the reduced electron carriers by the electron transport chain coupled with the synthesis of ATP by ATP synthase. The electron transport chain consists of four protein complexes: I (NADH-ubiquinone oxidoreductase), II (succinate dehydrogenase), III (ubiquinone-cyctochrome c oxidoreductase), and IV (cytochrome oxidase), and two carrier proteins: ubiquinone and cytochrome c, with O_2 being the final electron acceptor.[55] Reduced electron carriers (NADH and $FADH_2$) undergo oxidation and donate their electrons via complex I and II, respectively. Through a series of oxidation/reduction reactions with decreasing standard reduction potentials, the potential energy associated with these electrons is coupled with the transport of H^+ from the mitochondrial matrix to the intermembrane space.[55] Complex I and III each transport out

4 H^+ and complex IV transports out 2 H^+, thus each NADH oxidized produces enough energy to pump a total of 10 H^+ and each $FADH_2$ produced enough energy to pump out a total of 6 H^+.[3] This produces an unequal distribution of H^+ across the inner mitochondrial membrane creating an electrochemical gradient of approximately -140 mV.[55] Acutely the electron transport chain is regulated by the availability of reduced electron carriers to donate their protons and electrons and by the availability of O_2 to act as the final electron acceptor.[55] Therefore when O_2 availability is limited, such as during myocardial ischemia, the activity of the electron transport chain is impaired due to the inability to donate electrons to O_2. This ultimately leads to impaired oxidative metabolism as the concentrations of reduced electrons carriers rise and as previously discussed increases in $NADH/NAD^+$ ratio impairs TCA cycle activity and increases in $FADH_2$/FAD and acetyl-CoA/CoA ratios can impair pyruvate and fatty acid oxidation.[3]

The proton-motive force (i.e. electrochemical gradient) developed by the electron transport chain is coupled with ATP synthesis. ATP synthase allows H^+ to flow down their electrochemical gradient across the inner mitochondrial membrane, and couples this process with the synthesis of ATP at a stoichiometric ratio of 4 H^+ per ATP.[3] Acute regulation of ATP synthesis is dependent on the maintenance of the proton-motive force and the availability of ADP, which is supplied by the adenine nucleotide translocase.[3] Therefore ATP synthesis is closely coupled with the generation of the proton-motive force by the electron transport chain and mechanisms that dissipate the proton-motive force, such as activity of the adenine nucleotide translocase, the phosphate translocase, and uncoupling proteins, impairs ATP synthesis.[55, 116]

Coordinate Regulation of Fatty Acid and Carbohydrate Metabolism

Under normal physiological conditions, ATP synthesis is dependent on the mitochondrial oxidation of acetyl-CoA, which is predominately produced from the β-oxidation of fatty acids (contributes between 60-80 % of the required ATP), with carbohydrates contributing the residual 20-40 %.[5, 54] This ratio can be modified in response to hormonal control and workload of the heart, as well as by substrate and O_2 supply to the heart. Although fatty acids have a higher ATP yield than carbohydrates (104 ATP per palmitate molecule versus 31 ATP per glucose molecule), their catabolism utilizes O_2 less efficiently, thus fatty acids require approximately 10 % more O_2 to produce an equivalent amount of ATP (P/O ratio of 2.33 for palmitate versus 2.58 for glucose).[3] As the heart always metabolizes some combination of fatty acids and carbohydrates, a 10 % difference in O_2 consumption would not be observed physiologically, however this observation may be of particular importance during times of stress when O_2 is the limiting factor for oxidative metabolism.

Rates of glucose and fatty acid oxidation are reciprocally regulated as was originally defined by Randle's group in 1963 and this observation has been coined Randle's glucose/fatty acid cycle.[117] Elevated rates of fatty acid oxidation can inhibit PDH activity via an increase in mitochondrial NADH/NAD^+ and acetyl-CoA/CoA ratios, which allosterically inhibits PDH, as well as activating the PDH kinase, which then phosphorylates and inhibits PDH. In addition, elevated rates of fatty acid oxidation also inhibit glycolysis upstream of PDH as PFK-1 is inhibited by citrate. Elevated flux through glucose oxidation also results in an increase in matrix acetyl-CoA which can inhibit fatty acid oxidation. It has been proposed that this excess acetyl-CoA is

transported out of the mitochondria by one of two mechanisms: 1) conversion to acetylcarnitine by carnitine acetyltransferase (CAT), transport across the membrane by CT, and conversion back to acetyl-CoA by a cytosolic CAT [62, 75] or 2) removal of citrate from the mitochondria by the tricarboxylic acid transporter and conversion to acetyl-CoA by the ATP-citrate lyase.[76, 77] Each of these processes increases cytosolic acetyl-CoA levels, which acts as a substrate for ACC and has the potential to accelerate the production of malonyl-CoA, and therefore suppress fatty acid oxidation.

Myocardial Energy Substrate Metabolism During Ischemia and Reperfusion

Inadequate supply of O_2 during ischemia ultimately impairs the ability of the heart to oxidatively metabolize pyruvate and fatty acids in the mitochondria to produce ATP by oxidative phosphorylation. As previously discussed, the heart has minimal ATP and PCr stores, therefore the inability to produce ATP oxidatively results in a decline in ATP levels, which can compromise contractile function and ion homeostasis.[6] Alterations in myocardial metabolism contribute to the loss of contractile function during ischemia, however as contractile function declines prior to a significant decline in ATP, other mechanisms also contribute to the loss of contractile function during ischemia.[6]

During ischemia, flux through glycolysis is elevated due its ability to produce ATP independent of O_2 via substrate level phosphorylation. Glycolysis is stimulated by an activation of PFK-1 due to an increase in the AMP/ATP ratio in the cell and a decrease in cytosolic citrate levels.[118] Supply of glucose 6-phosphate for glycolysis is also elevated due to an increase in glucose uptake via the ischemia-induced translocation

of GLUT1 and GLUT4 to the plasma membrane [119, 120] and a stimulation of glycogenolysis (which can become the primary source of glucose 6-phosphate when substrate supply is limiting during severe ischemia).[93] During mild and moderate ischemia, rates of glycolysis can be maintained, as pyruvate normally metabolized in the mitochondria is converted to lactate to preserve enough oxidized NAD^+ to sustain flux through GAPDH and glycolysis. Although this anaerobic source of ATP is beneficial in order to maintain ion homeostasis during ischemia, if the hydrolysis of glycolytically derived ATP is not coupled with pyruvate oxidation (uncoupled glucose metabolism) it can lead to an accumulation of lactate and H^+, which can further exacerbate the ischemic-induced dysregulation of ion homeostasis.[31, 32, 54, 121] Therefore this further impairs contractile function as more ATP has to be directed towards the reestablishment of ion homeostasis. During severe ischemia, when coronary flow is insufficient to remove these metabolic by-products of anaerobic glycolysis, flux through the pathway is inhibited due to the insufficient availability of NAD^+ for GAPDH and allosteric inhibition of PFK-1 by H^+.

When reperfusion of reversibly injured myocardium occurs, a rapid recovery of O_2 consumption and TCA cycle activity leads to a replenishment of the supply of ATP.[122, 123] This is associated with a recovery of mechanical function once Ca^{2+} levels have normalized. During this reperfusion period, fatty acids can provide over 90 % of the myocardium's energy requirement.[5, 54, 122] This excessive use of fatty acids is due to increased levels of circulating fatty acids as a result of a β-adrenergic stimulation of hormone sensitive lipase in the adipose tissue [33, 124] and a decrease in the cytosolic malonyl-CoA levels.[31, 32, 63, 73, 121-123] In addition, heparin administration during

myocardial infarction contributes to elevated circulating fatty acids, as heparin increases circulating LPL by releasing LPL from its heparan sulfate proteoglycan linkage to endothelia cells, which can act upon lipoproteins releasing free fatty acids.[34-36] Although much is already known about the I/R-induced modifications of myocardial energy substrate metabolism and its contribution to post-ischemic contractile dysfunction, the exact molecular and cellular mechanisms remain elusive. To better understand the effects of I/R on myocardial metabolism we utilized a functional proteomics approach to observe the global I/R-induced changes in mitochondrial protein abundance and identified several novel post-translational modifications in PDH. The results of this study are discussed in chapter 7.

The preferential oxidation of fatty acids at the expense of glycolytically derived pyruvate further aggravates the uncoupling of glucose metabolism leading to myocardial acidosis. These excess protons are removed via two Na^+ dependent mechanisms, the Na^+/H^+ exchanger 1 (NHE1), and the Na^+/HCO_3^- cotransporter (NBC1, 3 or 4).[125, 126] During reperfusion there is a large pH gradient across the sarcolemmal membrane because the extracellular pH is quickly normalized during reperfusion. This concentration gradient drives H^+/Na^+ exchange via NHE resulting in increased intracellular Na^+.[127] During ischemia, the Na^+-K^+ ATPase is inhibited due to reduced supply of ATP and a decrease in extracellular pH, aiding the rise in intracellular Na^+ levels.[24, 128] The resultant intracellular Na^+ overload leads to the activation of the reverse mode of the NCX ultimately producing the ischemia-induced Ca^{2+} overload.[127, 129, 130] This dysregulation of ion homeostasis leads to a sequelae of adverse events, including decreased cardiac pressure development,[131] the initiation of cardiac arrhythmias [132] and

decreased response of contractile proteins to Ca^{2+}.[24] If the pyruvate from glycolysis is aerobically metabolized (i.e. glucose oxidation), then there is no net production of lactate and protons as they are consumed in the mitochondria.[133, 134]

During early reperfusion, a number of ATPases are activated in order to re-establish ion homeostasis at the expense of utilizing energy. Ca^{2+} ATPase and Na^{+}-K^{+} ATPase activity quickly recovers to reestablish ion homeostasis, thereby using ATP for non-contractile purposes and reducing cardiac efficiency.[135] In addition, the heart expresses the vacuolar proton ATPase, which can also utilize ATP in order to extrude protons from the cardiomyocyte, thus reducing myocyte acidosis, at the expense of utilizing ATP.[135]

Acidosis itself may also contribute to post-ischemic contractile dysfunction via a direct effect of protons on the contractile apparatus, as in skinned fiber bundles a drop in pH produces a rightward shift in the Ca^{2+}-force curve.[136, 137] The mechanisms by which this occurs may be due to protons out-competing Ca^{2+} for its binding site on cardiac troponin (cTnC), although this cannot completely account for the effects of acidic pH.[137-139] Acidosis can also affect the protein-protein interactions that are required to transduce the Ca^{2+} binding signal [138, 140] and reduce crossbridge binding to actin, thus reducing tension independent of Ca^{2+} and impairing the strong crossbridge induced activation of thin filaments.[141] To better understand the mechanism of acidosis induced post-ischemic contractile dysfunction, we utilized a slow skeletal troponin I transgenic mouse (ssTnI) that we hypothesize should be cardioprotective in the setting of I/R due to a blunted acidosis induced desensitization of the contractile apparatus to Ca^{2+}. The results of this study are discussed in chapter 6.

Modulation of Energy Substrate Metabolism as a Therapeutic Modality in Ischemia/Reperfusion Injury

Modification of myocardial metabolism is one of the key consequences of ischemia and reperfusion that can contribute to reversible or irreversible I/R injury. An important therapeutic approach would therefore be to reduce fatty acid oxidation and improve the coupling of glucose metabolism. This would prevent the dysregulation of ion homeostasis and the associated impairment of contractile function. Several different agents have already been tested in experimental models and some are currently being used clinically. These agents can be divided into two classes: those that lower circulating free fatty acids and those that directly modify fatty acid oxidation or glucose oxidation.

Agents that lower circulating fatty acids include compounds such as β-adrenoceptor antagonists (β-blockers), glucose-insulin-potassium (GIK) infusions, and nicotinic acid. The hypothesized modes of action of these compounds is the reduction in circulating free fatty acids, which reduces fatty acid supply to the mitochondria and therefore decreases fatty acid oxidation rates and increases the aerobic disposal of pyruvate.[142]

Another approach to lower fatty acid oxidation is to directly inhibit fatty acid oxidation. This approach has been shown with a class of piperazine derivatives, including trimetazidine. Trimetazidine is an inhibitor of long-chain 3-ketoacylcoenzyme, a thiolase that reduces fatty acid oxidation and increases glucose oxidation via the Randle cycle.[143, 144] It is the first widely used anti-anginal drug with a mechanism of action that can be attributed to the inhibition of fatty acid oxidation. Agents that accelerate glucose oxidation also reduce ischemic injury. The prototype of this class, dichloroacetate

(DCA), acts by way of the inhibition of PDH kinase, thus ultimately activating PDH and glucose oxidation.[145]

Glucose-Insulin-Potassium Therapy for Ischemia/Reperfusion Injury

The cardioprotective effect of the GIK solution was originally identified by Sodi-Pallares *et al.* to reduce the electrocardiographic abnormalities associated with acute myocardial infarction (AMI) and improve early survival.[146] These beneficial effects were originally attributed to an insulin stimulation of the Na^+/K^+ ATPase activity and K^+ reuptake thus raising the threshold for ventricular arrhythmias and a stimulation of glucose uptake and glycolysis thus providing an anaerobic source of ATP production.[147] As discussed above, GIK may also exerts its cardioprotective effects via the insulin inhibition of hormone sensitive lipase, resulting in a suppressed level of circulating fatty acids and impaired fatty acid uptake and oxidation.[90, 124, 148]

There has been renewed interest in the use of GIK therapy as a metabolic treatment for acute myocardial infarction (AMI) since both a meta-analysis of previous trials and the ECLA trial showed that GIK significantly reduced proportional in-hospital mortality.[131, 149] However the results of this analysis have recently been questioned due to the CREATE-ECLA randomized controlled trial examining the effect of GIK in patients with ST-elevated myocardial infarction.[150] Patients were assigned to receive either usual care or usual care with a high-dose GIK infusion regimen. The CREATE-ECLA trial shows no efficacy of GIK therapy in any of the predefined endpoints, including mortality, cardiac arrest, and cardiogenic shock, while the recent OASIS-6 randomized controlled trial suggests that treatment with GIK therapy may actually increase mortality during the

early post-ischemic period.[150, 151] Unfortunately plasma fatty acids levels were never assessed in these studies, therefore, the insulin-mediated decrease in plasma fatty acids may not have occurred, which could possibly account for the negative results. Post hoc analyses of the CREATE-ECLA and OASIS-6 data have suggested that the lack of benefit of GIK infusion may be associated with elevated K^+ levels and a larger volume infusion in the treated group, which may contribute to the lack of benefit with GIK infusion.[151]

In order to delineate the underlying cause of insulin induced cardioprotection during I/R or the lack of protection in the case of the CREATE-ECLA/OASIS-6, we utilized isolated working mouse hearts in order to separate the direct myocardial metabolic effects of insulin from its systemic effects on circulating fatty acids. Insulin has also blunted the activation of AMPK during both aerobic perfusion and ischemia [152-154], therefore insulin may have a salutary effect in reperfusion by indirectly inhibiting fatty acid oxidation and stimulating the aerobic disposal of pyruvate, as discussed in the next section. The results of this study are discussed in Chapter 3.

AMP-Activated Protein Kinase

AMPK was originally discovered as a regulator of fatty acid and cholesterol biosynthesis.[155, 156] It is now known that AMPK acts as a key molecular regulator of energy metabolism as it functions as an "energy conservation agent" in the liver by inhibiting anabolic processes when ATP levels are depleted.[157, 158] In skeletal and heart muscle, AMPK acts as a cellular energy sensor, such that its activation is associated with

inhibition of ATP-requiring anabolic process and activation of ATP-generating catabolic processes.

AMPK is a heterotrimeric serine/threonine kinase, which consists of one catalytic subunit (α) and two regulatory subunits (β and γ). Each subunit has multiple isoforms (α1/α2, β1/β2 and γ1/γ2/γ3) that are products of distinct genes; the most prominent heart isoforms are the α2, β2, and γ1/2.[159-162] The α subunit contains the catalytic domain, an autoinhibitory domain and an important regulatory site, Thr172, which is phosphorylated by upstream kinases.[163, 164] The β subunit has scaffold-like properties, and contains a glycogen-binding domain and a myristoylation site in its N terminus.[165-167] The γ subunit contains 4 tandem cystathionine β-synthase (CBS) domains, which function as Bateman domains (pair of CBS) to bind either AMP or ATP.[168] There are currently two models of the formation of the AMPK heterotrimer. The first suggests that the β subunit functions as a scaffold binding both the α and γ subunits, however a recent study suggests that the α subunit binds both the β and γ subunits, with no direct interaction between the β and γ subunits.[169, 170] Irrespective of the model, AMPK acts as a metabolic sensor, which monitors the ratio of AMP/ATP. Binding of AMP via the Bateman domains on the γ subunit promotes the activation of the heterotrimer via a direct allosteric mechanism; it promotes the phosphorylation of Thr172 and may activate upstream AMPK kinases (AMPKK), and it makes the heterotrimer a poorer substrate for dephosphorylation by protein phosphatase 2C.[171, 172] Despite the discovery of the identity of several of the AMPKKs: LKB1 [173], Ca^{2+} calmodulin dependent kinase kinase (CaMKKβ1/2) [174] and TAK1 [175, 176], it appears there may be additional AMPKKs which mediate the ischemic-induced activation of AMPK.[177] Recently, Miller *et al.* demonstrated that macrophage

migration inhibitory factor (MIF) is released from the ischemic heart and can activate AMPK via a paracrine mechanism through the MIF cell-surface receptor complex (consisting of CD74 and CD44).[178] However, there is no change in either LKB1 or CaMKKβ expression in hearts subjected to I/R, so the AMPKK that plays a role in this pathway remains to be elucidated.[178]

AMP-Activated Protein Kinase Regulation of Myocardial Energy Substrate Metabolism

One of the major roles of AMPK in the heart is the phosphorylation of downstream targets in order to regulate substrate metabolism. Evidence suggests that AMPK regulates both glucose and fatty acid metabolism in the heart. As previously discussed, fatty acid uptake into the cardiomyocyte is dependent on substrate supply and the availability and activity of fatty acid transport proteins. AMPK can mediate the recruitment of LPL to the coronary lumen, leading to an increase in the availability of free fatty acids.[179] AMPK can also increase the translocation of CD36 and FATPpm to the sarcolemmal membrane, leading to stimulation of fatty acid uptake by the cardiomyocyte [180], although recent reports suggest that CD36 is almost entirely responsible for the AMPK-mediated increase in fatty acid uptake.[45, 181] AMPK also plays a key role in the regulation of fatty acid uptake into the mitochondria, due to its regulation of both enzymes that control cytosolic malonyl-CoA levels, namely ACC and MCD. Previous reports demonstrate in isolated working rat hearts that ACC co-purifies with the $AMPK_{\alpha 2}$ and can phosphorylate three serine residues in ACC265 (serines 79, 1200, and 1215), however only the phosphorylation status at Ser79 corresponds with

inactivation of the enzyme.[73, 182-184] Many studies show a correlation between increased AMPK activity, decreased ACC activity and increased fatty acid oxidation in working rat hearts.[63, 73, 185] Several studies implicate AMPK in the control of MCD activity as well. In skeletal muscle, AMPK activation is associated with an increase in MCD activity, possibly due to a phosphorylation event.[186, 187] The increase in MCD activity is also observed in H9C2 cells, however it is attributed to an increase in mitochondrial MCD expression, not to changes in phosphorylation.[188] Yet, this relationship between AMPK and MCD is not universally accepted.[189] Taken together, AMPK activation plays an important role in stimulating fatty acid oxidation by stimulating both fatty acid uptake into the cardiomyocyte and transport of fatty acyl-CoA esters into the mitochondria.

AMPK also plays important roles in the regulation of glucose metabolism in the heart. AMPK activation is associated with an increase in GLUT4 translocation and impairment in transporter endocytosis, leading to an increase in transporters localized in the plasma membrane and an increase in glucose uptake.[190-194] However this observation is not universally accepted, and may be due to the availability of glucose from glycogen.[195-197] The availability of glucose from glycogen is also under the control of AMPK, although the mechanism by which this occurs remains controversial. Carling and Hardie first suggested that AMPK can phosphorylate both glycogen synthase and phosphorylase kinase.[198] Several studies in skeletal muscle and myoblasts suggest that activation of AMPK using 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) results in an inhibition of glycogen synthase activity, although the exact mechanism is unclear but may be due to phosphorylation of glycogen synthase at Ser7.[199] However, several studies observe the opposite effects, with chronic AICAR

administration resulting in increased skeletal muscle glycogen content [200] and increased glycogen synthase and glycogen phosphorylase activity in red gastrocnemius by an indirect mechanism, possibly due to a stimulation of glucose uptake.[201] A previous report supports the finding that AICAR may activate glycogen phosphorylase, although the mechanism was not determined.[202] In addition, in isolated rat hearts, AICAR increases glycogen breakdown, without affecting the activity of glycogen synthase or glycogen phosphorylase.[203] These data must be interpreted with caution, as 5-aminoimidazole-4-carboxamide 1-D-ribofuranotide (the phosphorylated form of AICAR) may directly modify glycogen phosphorylase activity by mimicking the direct allosteric regulatory effect of AMP on this enzyme. It appears that AMPK does play a role in the regulation of glycogen metabolism, however this is no consensus as to its direct effect and mechanism of action. Downstream of glucose, AMPK can also stimulate glycolysis in the heart by the phosphorylation (Ser466) and activation of PFK-2, which increases the concentration of fructose-2,6-bisphosphate, a potent stimulator of PFK-1.[204] Therefore the activation of AMPK has the potential to elevate glucose uptake and subsequent glycolytic flux, in order to supply the heart with an anaerobic source of ATP.

AMP-Activated Protein Kinase Gamma Subunit Mutations

A mutation in $AMPK_{\gamma}$ was originally identified in purebred Hampshire pigs, which contained a R200Q mutation in $AMPK_{\gamma 3}$.[205] This loss of function mutation results in glycogen accumulation in the skeletal muscle.[205] Subsequently a variety of mutations in the $AMPK_{\gamma 2}$ (PRKAG2) gene have been identified in the hearts of patients, which result in a disease phenotype termed the PRKAG2 cardiac syndrome.[206-210] This

phenotype consists of a glycogen storage cardiomyopathy characterized by progressive conduction system disease, and ventricular pre-excitation similar to Wolff-Parkinson-White (WPW) syndrome in the presence or absence of cardiac hypertrophy. The best-characterized cardiac $AMPK_{\gamma 2}$ mutations include the R302Q (missense mutation of arginine to glutamate), the N488I (missense mutation of asparagine to isoleucine) and the R531G (missense mutation of arginine to glycine).

The R302Q mutation was originally identified in two families with a familial form of WPW associated with ventricular pre-excitation, conduction abnormalities and cardiac hypertrophy.[209] Transgenic mice with cardiac specific overexpression of this mutation recapitulate much of the human disease phenotype, consisting of increased glycogen deposition, profound cardiac hypertrophy, WPW-like conduction abnormalities including preexcitation and superventricular arrhythmia.[211] This phenotype is associated with a significant reduction in AMPK activity in the $AMPK_{\gamma 2}$ R302Q expressing hearts compared to mice overexpressing the wildtype (Wt) $_{\gamma 2}$ gene or nontransgenic controls.

The $AMPK_{\gamma 2}$ N488I mutation was originally identified in patients with cardiac hypertrophy and conduction system abnormalities.[207] Cardiac-specific expression of this gene in mice results in significant cardiac hypertrophy and a 30-fold increase in cardiomyocyte glycogen content associated with alternative atrioventricular conduction pathways consistent with WPW. In contrast to the R302Q mutation, hearts harboring the $AMPK_{\gamma 2}$ N488I mutation show increased basal $AMPK_{\alpha 1}$-associated activity and increased basal and AMP-stimulated $AMPK_{\alpha 2}$-associated activity.[212] This difference in AMPK activity is not accounted for by changes in the AMP/ATP ratio in the mutant hearts, as the ratios did not differ between groups. Interestingly, the mice expressing the

N488I mutation have higher $AMPK_{\alpha 2}$-associated activity at saturating AMP concentrations, however they are less responsive to changes in AMP and ATP concentrations. N488I mutant hearts have approximately half the total AMPK Thr172 phosphorylation as the Wt overexpressing hearts after depletion of ATP with global ischemia.[213] The concept of increased $AMPK_{\alpha 2}$ activity at basal conditions is supported by mice with the $AMPK_{\gamma 2}$ N488I mutation crossed with the $AMPK_{\alpha 2}$ Dn mutation. These double transgenic mice have normal $AMPK_{\alpha 2}$ activity and a much less severe phenotype, comparable to the mice overexpressing the $AMPK_{\gamma 2}$ Wt subunit.[214]

The R531G mutation was originally identified in another family with a familial form of WPW.[215] Cardiac-specific overexpression of this gene in the mouse also results in a progressive cardiac phenotype, which is very similar to the phenotype observed in the $AMPK_{\gamma 2}$ R302Q and N488I mice.[216] At 1 week of age these mice are essentially normal but by 4 weeks of age, they already display cardiac hypertrophy, impaired contractile function, electrical conduction abnormalities and a 40-fold increase in glycogen. Interestingly, at 1 week there was no significant difference in AMPK activity in $AMPK_{\gamma 2}$ R531G mutant mice compared to Wt mice or transgenic mice overexpressing the $AMPK_{\gamma 2}$ Wt subunit, however by 4 weeks AMPK activity was almost completely abolished in the mice with the R531G mutation. This would suggest that the effect of the mutation is not directly on AMPK activity but may be secondary to the glycogen accumulation.

Two recent studies have started to elucidate the mechanism by which mutations in the $AMPK_{\gamma 2}$ result in this PRKAG2 cardiac syndrome. Luptak *et al.* demonstrate that the $AMPK_{\gamma 2}$ N488I mutant mice have increased 2-deoxyglucose uptake, decreased glycolytic

flux of exogenous glucose and a higher reliance on fatty acid oxidation.[217] This metabolic phenotype coupled with the increased expression and activity of UDPG pyrophosphorylase and increased tissue content of glucose-6-phosphate would drive glucose towards glycogen synthesis.[217] Folmes *et al.* demonstrate in neonatal cardiomyocytes adenovirally expressing the $AMPK_{\gamma 1}$ R70Q mutation (equivalent to the $AMPK_{\gamma 2}$ R302Q mutation) that glycolysis of exogenous glucose is significantly inhibited, while glycogen synthase activity is elevated due to increased expression and reduced phosphorylation.[218] This mutation is also associated with a decrease in GSK-3β protein and an increase in nuclear factor of activated T cells activity. Taken together this pathway would also shunt glucose toward glycogen synthesis accounting for the glycogen overload associated with these mutations.[218]

Due to the lack of specific pharmacological agents that modify AMPK activity, these mutations in AMPKγ represent an important model in order to study the effect of modifying AMPK activity on myocardial metabolism and cardioprotection during I/R. In particular, we utilized the aforementioned transgenic mice harboring a R302Q mutation in $AMPK_{\gamma 2}$, a putative inactivating mutation, to determine its effect on ischemic-induced AMPK activity, AMPK regulation of myocardial energy substrate metabolism and post-ischemic contractile function. The results of this study are described in chapter 4.

AMP-Activated Protein Kinase and Cardioprotection during Ischemia/Reperfusion Injury

AMP-Activated Protein Kinase Modulation of Myocardial Metabolism during Ischemia/Reperfusion Injury

AMPK is rapidly activated during myocardial ischemia and is associated with dramatic changes in the control of glucose and fatty acid metabolism.[63, 73, 153, 191, 192, 204] This activation is thought to be an adaptive response in order to increase ATP by inhibiting energy requiring processes and stimulating energy producing processes. Indeed, much evidence now suggests that AMPK has the ability to stimulate both glucose and fatty acid metabolism in I/R. During ischemia, AMPK promotes glucose uptake via the translocation of GLUT4 to the plasma membrane, which may involve the TAB1 scaffolding protein interacting with AMPK and causing the autophosphorylation of p38 MAPK.[192, 219] In addition, as previously described, AMPK can stimulate glycolysis via indirectly activating PFK-1, thus producing an anaerobic source of ATP.[204] This stimulation of glucose utilization by ischemic-induced activation of AMPK is thought to be beneficial due to the anaerobic production of ATP. However, as mentioned, AMPK can also activate fatty acid oxidation by phosphorylating and inhibiting ACC, thus removing the malonyl-CoA inhibition of fatty acid transport into the mitochondria.[63, 73] Therefore AMPK activation has the potential to also exacerbate ischemic injury due to stimulation of fatty acid oxidation at the expense of glucose oxidation, which would increase the myocardial acid load leading to post-ischemic contractile dysfunction. [31, 32]

Thus the role of AMPK in the protection of the ischemic myocardium is still controversial.[220]

Direct Assessment of the Role of AMP-Activated Protein Kinase in Cardioprotection

Despite the wide-ranging literature characterizing the effects of ischemia on AMPK activity and the effect of ischemic-induced AMPK activation on myocardial metabolism, few studies link modifications in AMPK activity with cardiac function.[192, 221, 222] The major previous study correlating AMPK activity to reperfusion contractile function used an $AMPK_{\alpha 2}$ kinase dead (KD) transgenic mouse.[192] Russell *et al.* demonstrate that the KD mice have worse cardiac function during low-flow ischemia and reperfusion than controls, which is associated with the inability to increase glucose uptake and lactate production.[192] Associated with these metabolic and functional changes, KD hearts have an increase in cellular damage as observed by a rise in lactate dehydrogenase and creatine kinase release, and an increase in apoptotic cells. However, the KD hearts may already be compromised during the aerobic baseline period with suppressed rates of contractility and reduced glycogen content, which may predispose them to do poorly during the subsequent I/R. These hearts were perfused with low levels of fatty acids (0.4 mM), which may lessen the detrimental effect of elevated fatty acid oxidation on glucose oxidation and proton production. In addition, despite the important role of AMPK in regulating oxidative metabolism, rates of fatty acid oxidation and glucose oxidation do not change between the KD and wildtype (Wt) groups.[192] Recently, this group identified that MIF is released from the ischemic heart and can partially account for the ischemic-induced activation of AMPK. Indeed, when MIF knockout

(KO) mice are subjected to I/R in a Langendorff model, the KO hearts have impaired functional recovery during reperfusion and larger infarct sizes following *in vivo* left anterior descending artery (LAD) ligation, which the authors contribute to the inability to activate AMPK and glucose uptake.[178] However, the loss of MIF only impaired ischemic-induced AMPK activation and glucose uptake by a small margin, yet significantly impaired ischemic tolerance, suggesting that there may be alternative mechanisms by which MIF impairs ischemic tolerance.

Additional studies have observed the effect of both global ischemia and low-flow ischemia in an $AMPK_{\alpha 2}$ KO model, which abolishes $AMPK_{\alpha 2}$-associated activity but has no effect on $AMPK_{\alpha 1}$-associated activity.[221, 222] In Langendorff perfused hearts in the absence of fatty acids and insulin, global ischemia results in an increase in lactate release in Wt mice, which is blunted in the $AMPK_{\alpha 2}$ KO.[222] As previously observed in mice expressing a dominant negative $AMPK_{\alpha 2}$ (Dn) and KD mice, end diastolic pressure increases to a greater extent and rate in the $AMPK_{\alpha 2}$ KO mice, resulting in a reduction in the time to start of and greater maximal ischemic contracture.[192, 221-223] Despite these metabolic and functional observations, the absence of $AMPK_{\alpha 2}$ does not exacerbate post-ischemic contractile dysfunction.[222] This observation is further confirmed by the fact that Wt hearts subjected to low-flow ischemia in the absence of fatty acids have better-preserved ATP levels during mild ischemia and reperfusion, however this is associated with similar recovery of contractile function during reperfusion.[221] When subjected to low-flow ischemia and reperfusion in the presence of fatty acids, the $AMPK_{\alpha 2}$ knockout hearts have a slight suppression of cardiac function during the immediate reperfusion period with similar contractility during late reperfusion.[221] Unfortunately it is difficult to

determine why the addition of fatty acids to the perfusate is detrimental only to the initial functional recovery of the $AMPK_{\alpha 2}$ KO hearts as no metabolic parameters were assessed in this set of hearts.

A recent study has suggested that a single injection of metformin 18 hr prior to *in vivo* LAD ligation confers cardioprotection as observed by a reduction in infarct size and troponin-T release, as well as an improvement in ejection fraction 1 week following ischemia.[224] Metformin resulted in elevated AMPK activity and phosphorylation up to 24 hr after injection and can further increase AMPK activity over what is observed in I/R alone.[224] The activation of AMPK and downstream phosphorylation of eNOS (Ser 1177) are associated with the cardioprotective effect as the cardioprotective effect of metformin is lost when either AMPK Dn mice or eNOS-deficient mice were utilized. Metformin was also cardioprotective when utilized in a diabetic model.[224] However, oxidative metabolism was not assessed in this study or the previous studies with the Dn or $AMPK_{\alpha 2}$ KO mouse; therefore the effect of AMPK-induced modifications in myocardial oxidative metabolism on ischemic tolerance remains unclear.

The Role of AMP-Activated Protein Kinase in Adiponectin Cardioprotection

Indirect evidence that supports a beneficial effect of AMPK activation during ischemia comes from studies identifying adiponectin as a cardioprotective agent. Adiponectin KO mice develop larger infarcts, which are associated with decreased AMPK phosphorylation at Thr172 and increased TNFα expression and myocyte apoptosis.[225] Adenoviral delivery of adiponectin prior to, during or following ischemia results in a reduction in infarct size associated with an increase in AMPK

phosphorylation. This protective effect is blocked with the use of a dominant-negative AMPK, suggesting that the prosurvival mechanism is mediated by AMPK signaling.[225] However, this study could not directly link modifications in AMPK activity with ischemic cardioprotection as adiponectin may also produce this protective effect via activation of cyclooxygenase-2 (COX-2), as inhibition of this enzyme partially reversed the cardioprotective effects of adiponectin as well.[225] More recently, short-term caloric restriction has been demonstrated to be cardioprotective during ischemia and reperfusion via the adiponectin dependent activation of AMPK.[226] When hearts from caloric restricted mice are perfused in the Langendorff mode they have a marked cardioprotection, which is lost when the mice are injected with AraC (a non-selective inhibitor of AMPK) , however these hearts were perfused in the absence of a clinically relevant concentration of fatty acids. These beneficial effects of adiponectin may also be mediated by non-metabolic effects, such as reducing oxidative/nitrative stress of the heart by inhibiting inducible nitric oxide synthase (NOS) expression, which may be either an AMPK or COX-2 mediated signaling pathway.[227] Alternatively adiponectin may also be cardioprotective via activation of endothelial NOS as an increase in the nitric oxide (NO) induces perfusion of the ischemic heart.[228] Therefore further studies are require to definitively test the hypothesis that adiponectin cardioprotection occurs via the activation of AMPK and occurs in the presence of a clinically relevant concentration of fatty acids.

The Role of AMP-Activated Protein Kinase in Ischemic Preconditioning

AMPK may also play a role in ischemic preconditioning in the heart, similar to what was originally observed in the liver.[229] Ischemic preconditioning leads to an

activation of AMPK resulting in a protein kinase C (PKC) dependent upregulation of GLUT4.[230] In addition, AMPK may partially mediate preconditioning by regulating the activity and recruitment of sarcolemmal ATP-dependent potassium channels (K_{ATP}), an effect that is completely blocked in $AMPK_{\alpha 2}$ Dn cardiomyocytes.[231] Preconditioning of hearts also activates H11 kinase, which is associated with the activation of AMPK and its translocation to the nucleus where it activates hypoxia inducible factor-1α (HIF-1α) to induce a number of genes stimulating glycogenolysis, glycolysis and glucose oxidation.[232] Therefore preconditioning-induced activation of AMPK may increase the utilization of glucose, which may be beneficial to the ischemic heart, however it has not been directly determined if this activation of AMPK during ischemia itself is associated with ischemic preconditioning-induced cardioprotection. This is of particular importance, as pro-ischemic agents can confer cardioprotection by inducing ischemia and triggering the preconditioning cascade in the heart, however anti-ischemic agents like trimetazidine can impair the preconditioning inducing effects of ischemia.[233] Therefore further studies are required to delineate the role of AMPK activation during preconditioning from its role during ischemia.

The Role of AMP-Activated Protein Kinase in Alternative Models of Protection

Reduced AMPK activation has been demonstrated to be protective in a number of other models. Jaswal *et al.* have recently observed that suppressing AMPK activation in isolated working rat hearts via inhibition of p38MAPK results in the restoration of adenosine cardioprotection following transient ischemic stress.[234] The proposed mechanism of cardioprotection is due to a partial reduction of glycolysis, which

ultimately leads to a reduction in H^+ production and myocardial acidosis. Even in the absence of ischemia, H_2O_2 mediated cardiac dysfunction is associated with an increase in AMPK activity, which is partially ameliorated by the treatment with an AMPK inhibitor, compound C.[235] Similar results are observed in an *in vivo* and *in vitro* model of stroke as activation of AMPK exacerbates ischemic-induced damage while inhibition of AMPK partially ameliorates ischemic-induced damage.[236] A recent study suggests that knockout of the $AMPK_{\alpha 2}$ also confers neuroprotection from stroke.[237] These studies support the concept that activation of AMPK is not protective in pathophysiology but may actually be detrimental.

Controversy: AMP-Activated Protein Kinase, an Enemy or Ally to the Ischemic Heart?

As highlighted in a recent review, activation of AMPK during ischemia has been clearly established, however it is less clear what role this activation plays in the pathophysiology of I/R injury.[220] Based on the previous sections, direct and indirect evidence exists supporting both a cardioprotective and a detrimental effect of AMPK activation during I/R. As will be described in the subsequent chapters of this thesis, studies were undertaken to better elucidate the role of AMPK in the regulation of myocardial energy substrate metabolism and the implications this has on recovery of contractile function during reperfusion following ischemia. Due to the lack of specific pharmacological agents and the toxicity of available pharmacological agents to modify AMPK activity (unpublished data), we utilized a number of different approaches to modify AMPK activity during ischemia. Insulin was utilized to blunt AMPK activation during aerobic perfusion and ischemia, in addition we utilized two transgenic mouse

models which modify AMPK activity, an $AMPK_{\alpha 2}$ Dn mouse and a mouse expressing an $AMPK_{\gamma 2}$ R302Q mutation, a putative inactivating mutation. Using these approaches we assessed the role of AMPK in the regulation of oxidative metabolism and post-ischemic contractile function. The results of these studies are described in chapters 3-5.

Hypothesis and Objectives

General Hypothesis

AMPK is a key regulator of fatty acid and glucose metabolism. Hence the ischemic-induced activation of AMPK causes important alterations in myocardial energy metabolism including an increase in glycolysis and fatty acid oxidation at the expense of glucose oxidation, resulting in an uncoupling of glucose metabolism, which is associated with post-ischemic contractile dysfunction. Thus the AMPK-induced modification of myocardial metabolism during I/R is an important determinant of the severity of I/R injury and the degree of functional recovery during reperfusion.

Specific Hypothesis

The specific hypotheses of this thesis are described within the individual chapters pertaining to the experimental results.

Specific Aims

1. To characterize the effects of insulin on myocardial energy metabolism, AMPK activity and post-ischemic left ventricular function in isolated working mouse hearts.

2. To characterize the effects of reduced ischemic-induced AMPK activity on myocardial energy metabolism and post-ischemic left ventricular function in isolated working mouse hearts.

3. To characterize the effects of the $AMPK_{\gamma 2}$ R302Q mutation on myocardial energy metabolism, AMPK activity and post-ischemic left ventricular function in isolated working mouse hearts.

4. To characterize the effects of slow skeletal troponin I expression on myocardial energy metabolism and post-ischemic contractile function in isolated working mouse hearts and on infarct size in a left anterior descending coronary artery ligation.

5. To characterize the global I/R-induced changes in the mitochondrial proteome using a functional proteomics approach.

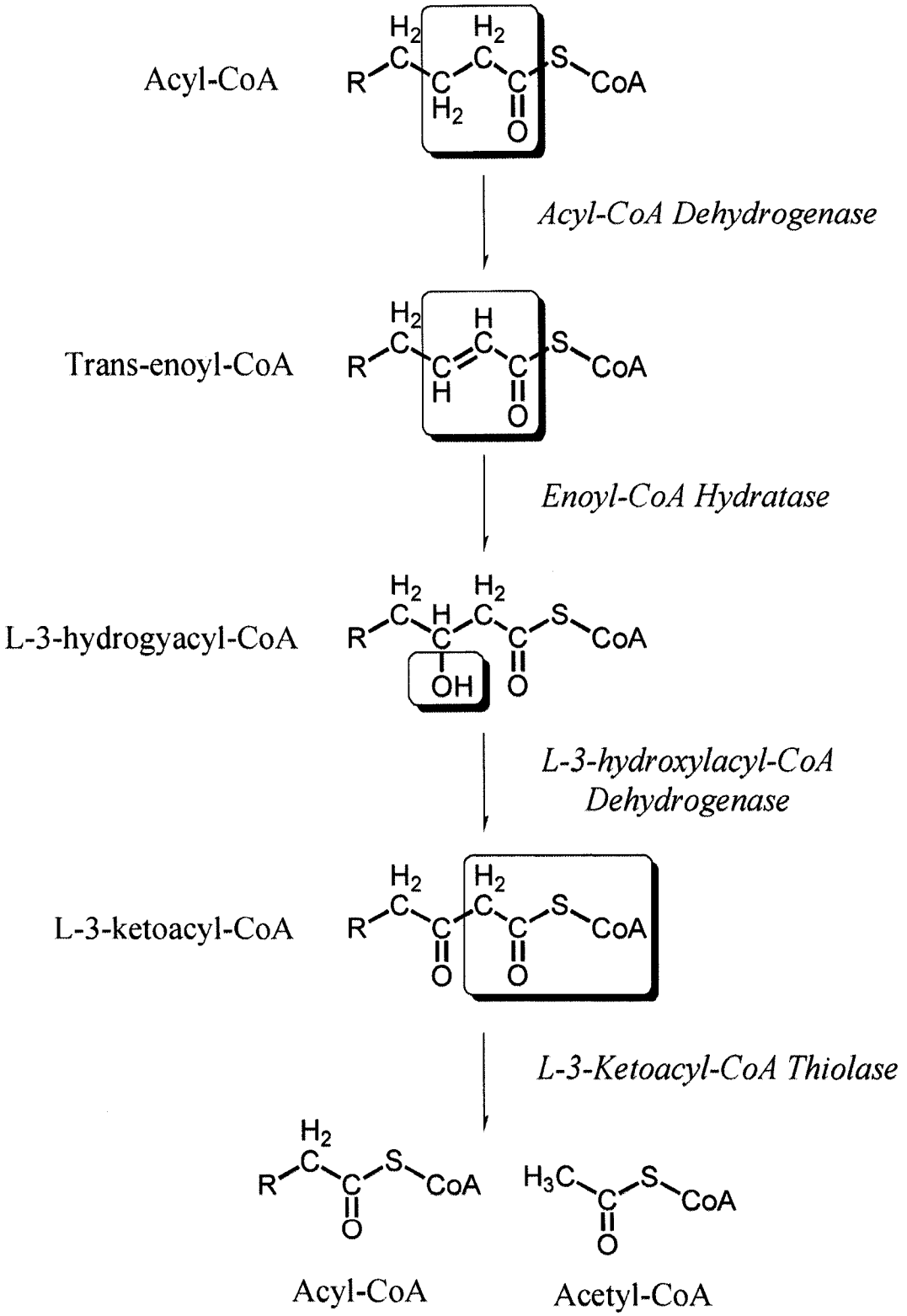


Figure 1-1: Schematic representation of the catabolism of fatty acyl-CoA esters by mitochondrial β-oxidation.

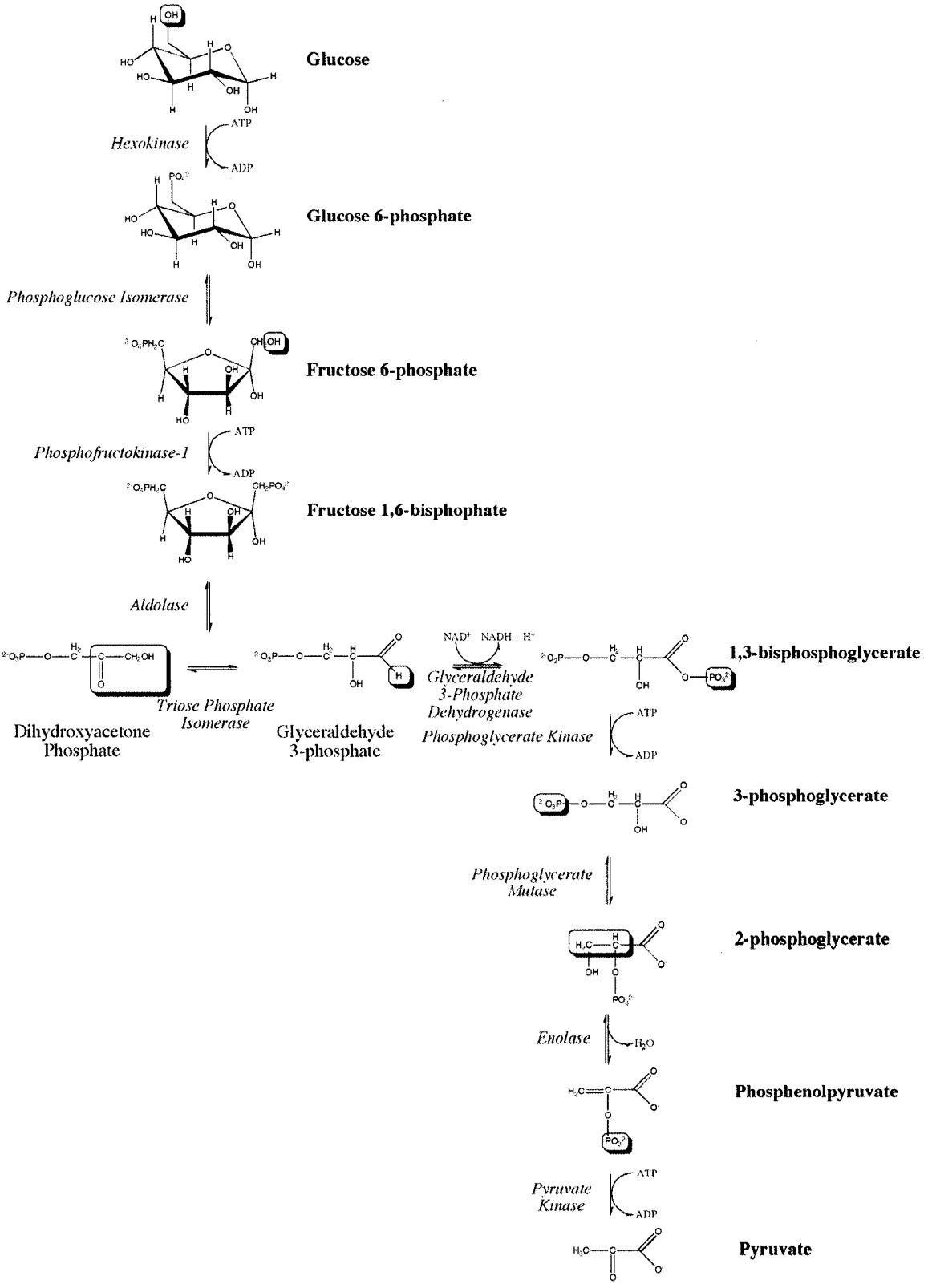


Figure 1-2: Schematic representation of the catabolism of glucose by glycolysis.

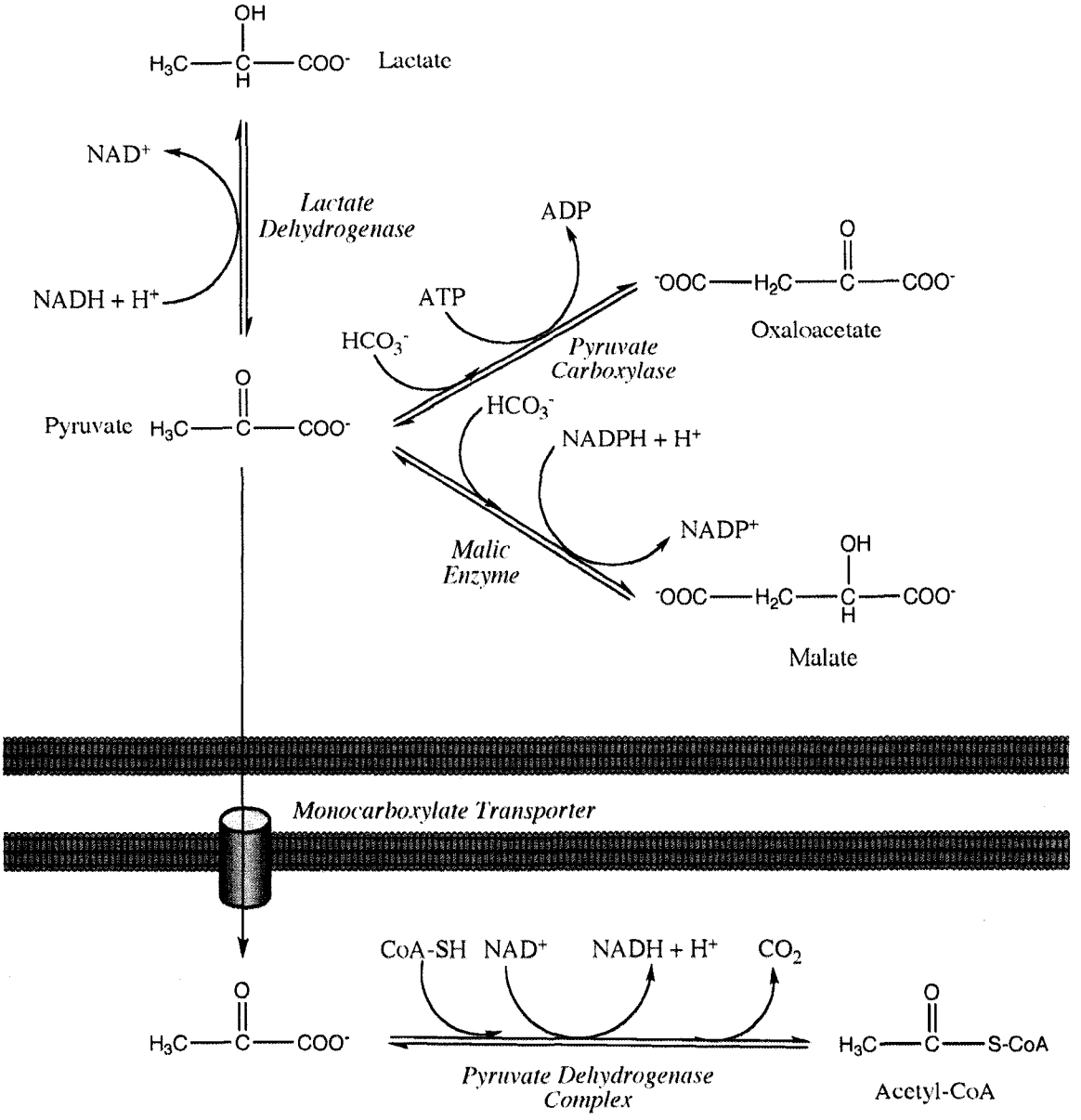


Figure 1-3: Schematic representation of the metabolic fates of pyruvate.

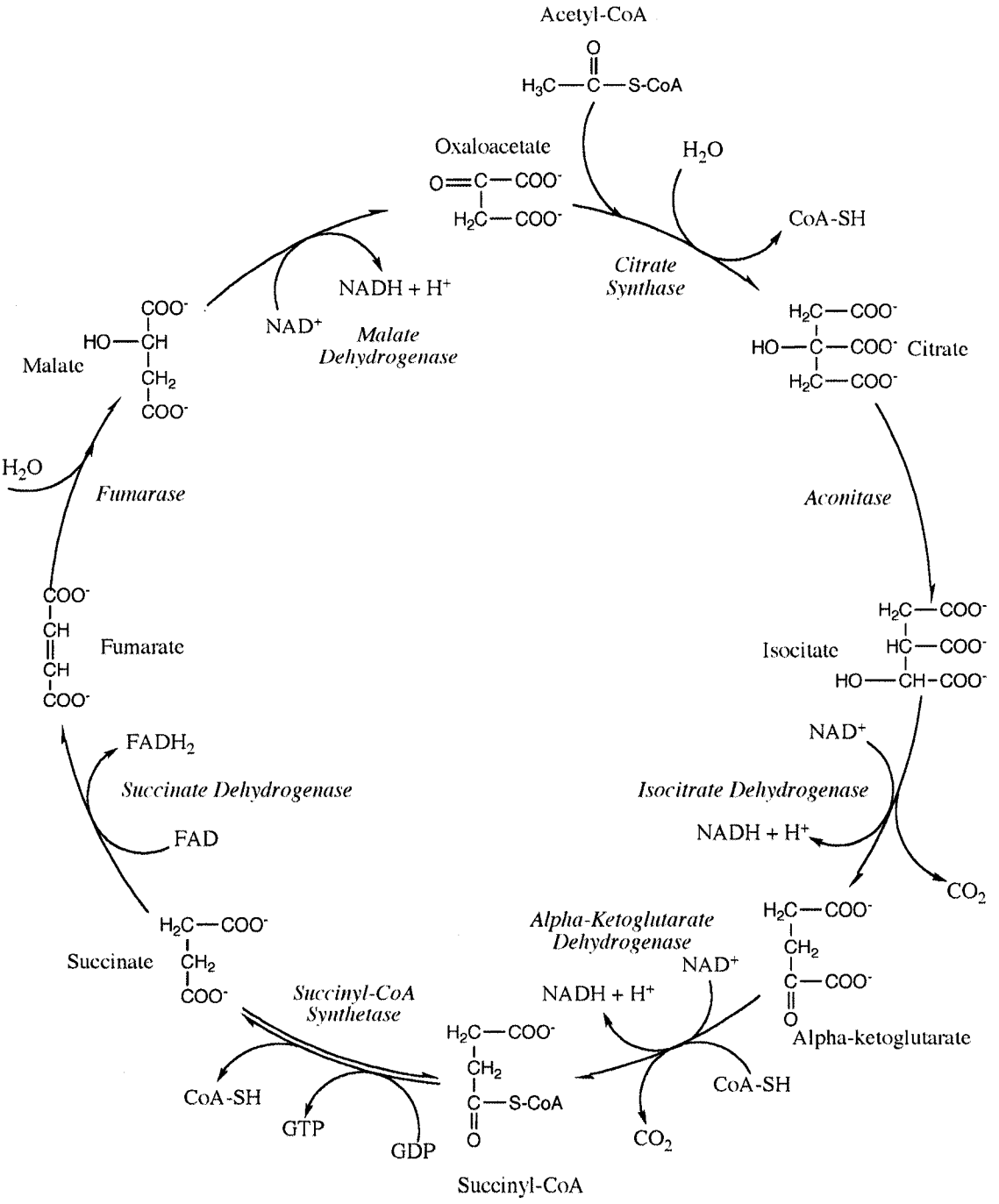


Figure 1-4: Schematic representation of the catabolism of acetyl-CoA in the tricarboxylic acid cycle.

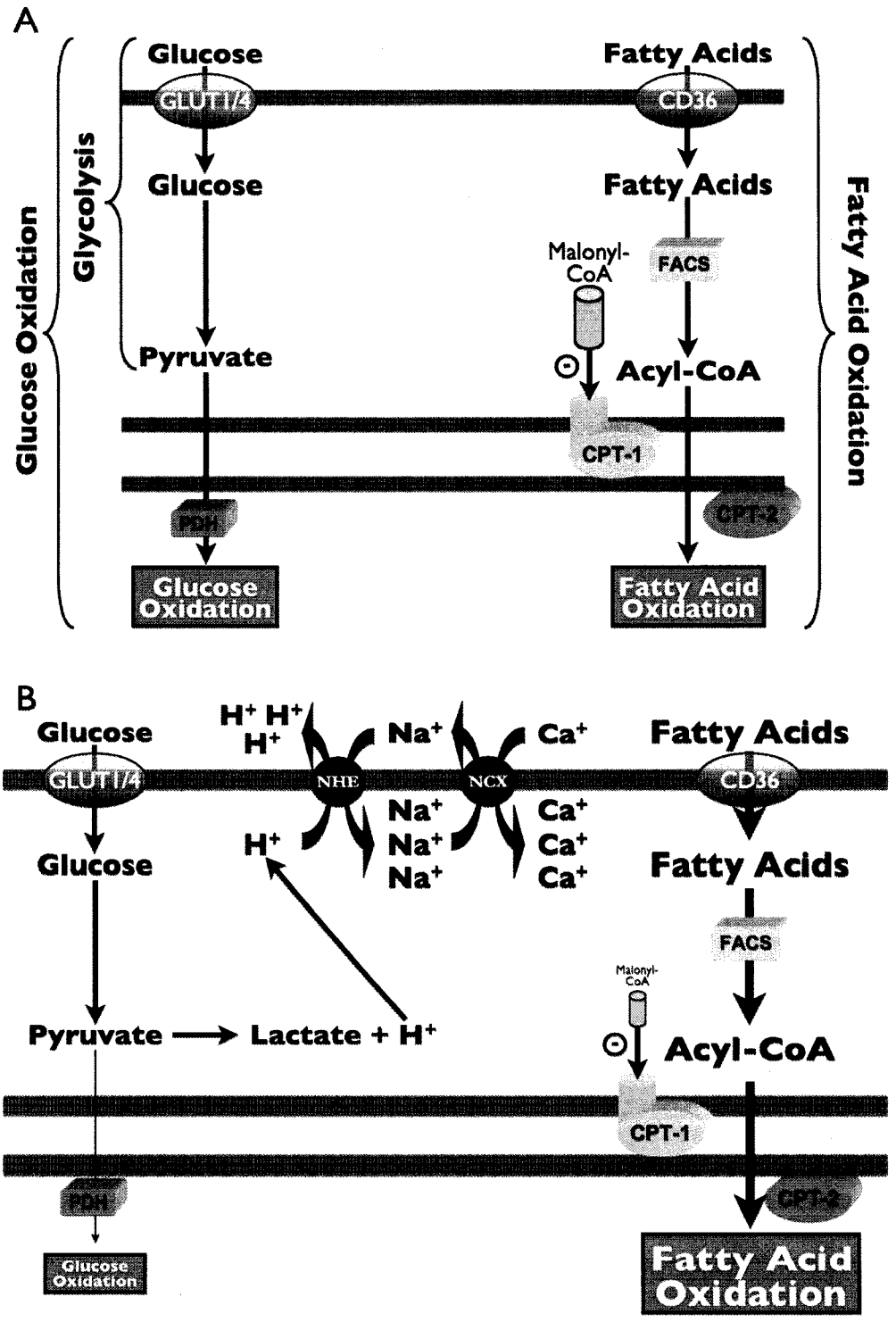


Figure 1-5: Schematic representation of A) the major metabolic pathways in the heart and B) the metabolic consequences of myocardial ischemia and reperfusion.

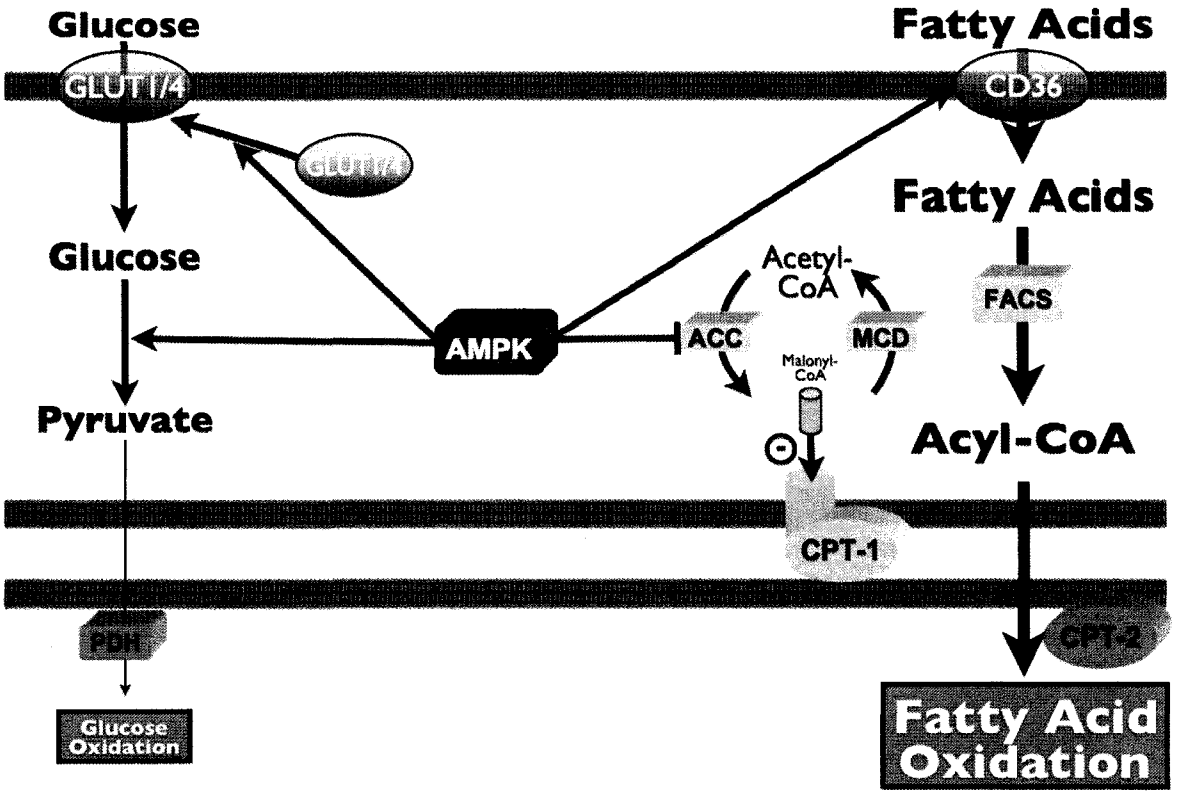


Figure 1-6: Schematic representation of metabolic effects of ischemic-induce activation of AMPK.

CHAPTER 2.

Materials and Methods

Chapter 2.

Materials and Methods

Materials

[U-^{14}C]glucose and D-[5-^{3}H]glucose were obtained from Amersham Canada Ltd (Oakville, Ontario). [9,10-^{3}H]palmitic acid and [1-^{14}C]palmitic acid were obtained from NEN Research Products (Boston, Massachusetts). Bovine serum albumin (BSA fraction V, free fatty acid free) was obtained 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™ Aqueous Counting Scintillation fluid was obtained from MP Biomedicals (Solon, Ohio). Hyamine hydroxide (1 M in methanol solution) was obtained from NEN Research Products (Boston, Massachusetts). DNeasy™ Blood and Tissue Kit was obtained from Qiagen (Mississauga, Ontario). Red Extract-N-Amp™ kit was obtained from Sigma-Aldrich (St. Louis, Missouri). Genotyping primers were obtained from Invitrogen (Burlington, Ontario). Free fatty acid assay kits were obtained from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Glucose assay kit was obtained from Sigma Diagnostics (St. Louis, Missouri). L-lactate dehydrogenase and glutamate pyruvate dehydrogenase were purchased from Roche Applied Science (Laval, Quebec). 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). For HPLC analysis of short-chain CoA esters, a Nova-Pak Supelcosil™, 4 μm particle size, C18 pre-column cartridge was purchased from Waters Company (Milford, Massachusetts) and an Adsorbosphere® C18, 3 μm particle size, 150 x 4.6 mm column was purchased from Altech Associates/Mandel Scientific (Guelph, Ontario). For HPLC analysis of long-chain CoA esters a C18 Guard Cartridge, 4 x 3 mm and a Luna, 5 μm particle size, C18(2) 100 A, 250 x 2 mm column were purchased from Phenomenex (Torrance, California). AMARA was obtained from the Alberta Peptide Institute (Edmonton, Alberta). Unifilter P81 96-well filterplates were obtained from Whatman (Florham Park, New Jersey). MicroScint PS scintillant was obtained from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). ECL Supelcosil™ Western blotting detection reagents were purchased from GE Healthcare (Piscataway, New Jersey) and Western Lightning® Chemiluminescence Reagents Plus were purchased from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario). Trans-Blot® Transfer Medium (pure nitrocellulose) was purchased from BioRad (Richmond, California). FUJI Medical X-ray films were purchased from Mandel Scientific (Guelph, Ontario). Monoclonal and polyclonal antibodies for AMPK, phospho-AMPK (Thr172), phospho-AMPK (Ser485/Ser491), Akt and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Danvers, Massachusetts). Secondary antibodies (peroxidase conjugated affinipore goat anti-mouse IgG and goat anti-rabbit IgG) were purchased through Jackson Immunoresearch Laboratories Inc. (Mississauga, Ontario). Peroxidase labeled streptavidin was purchased through Mandel

Scientific from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, Maryland). All other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri).

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.

Isolated Working Mouse and Rat Heart

Mouse and rat hearts were perfused in the working perfused mode as previously described.[238, 239] Strain of mice and any genetic modification are described in the individual chapters. Mice and rats were anesthetized with pentobarbital sodium (60 mg/Kg i.p.), and the hearts were subsequently excised and immersed in ice-cold Krebs-Henseleit bicarbonate solution (118 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 5 mM EDTA pH 7.4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$ and 5 mM glucose). The aorta was cannulated and retrograde perfusion with Krebs-Henseleit solution (37°C) was initiated at a hydrostatic pressure of 60 mmHg. Hearts were trimmed of excess tissue and the opening to the left atrium was cannulated. After equilibration in the Langendorff mode, hearts were switched to the working mode by clamping off the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow line. Oxygenated Krebs-Henseleit solution containing 0 mM (No Fat), 0.4 mM (Normal Fat) or 1.2 mM (High

Fat) palmitate bound to 3 % fatty acid free BSA (bovine serum albumin, palmitate was pre-bound to the albumin as described previously[238]), 5 mM glucose, in the presence or absence of 100 μU/mL insulin was delivered to the left atrium at a preload pressure of 11.5 mmHg. Perfusate was ejected from spontaneously beating hearts into a compliance chamber and into the aortic outflow line against a hydrostatic afterload pressure of 50 mmHg (80 mmHg for rats). The perfusate was recirculated, and pH was adjusted to 7.4 by surface gassing the perfusate in a glass oxygenator with a gas mixture containing 95 % O_2 and 5 % CO_2. At the end of the perfusion protocols the hearts were quick frozen in liquid nitrogen with Wollenberger tongs, and stored at -80 °C.

Mechanical Function Measurements in Isolated Working Mouse and Rat Hearts

Heart rate and aortic pressure (mmHg) were measured with a Gould P21 pressure transducer (Harvard Apparatus) connected to the aortic outflow line. Cardiac output and aortic flow (mL/min) were measured with Transonic T206 ultrasonic flow probes in the preload and afterload lines, respectively. Coronary flow (mL/min) was calculated as the difference between cardiac output and aortic flow. Cardiac power was calculated as the product of cardiac output times LV developed pressure (aortic systolic pressure minus preload pressure). Data were collected using an MP100 system from AcqKnowledge (BIOPAC Systems, Inc USA).

Measurement of Glycolysis, Glucose and Palmitate Oxidation

Glycolysis, glucose and palmitate oxidation were measured by perfusing hearts with [5-^{3}H/U-^{14}C]glucose and [9,10-^{3}H]palmitate, respectively.[238, 240] The total myocardial $^{3}H_2O$ production and $^{14}CO_2$ production were determined at 10 min intervals during the 30 min aerobic perfusion period and the 40 min period of reperfusion. To measure the rates of glycolysis and palmitate from ^{3}H substrate, $^{3}H_2O$ in perfusate samples was separated from [^{3}H]glucose and [^{3}H]palmitate using a vapor transfer method.[240] This method consists of adding 500 μL of water into a 5 mL scintillation vial, then placing a lidless 1.5 mL microcentrifuge tube inside the scintillation vial. A 200 μL perfusate sample is then added to the microcentrifuge tube, and the scintillation vial is capped. Scintillation vials are then stored initially at 50 °C for 24 hours and then at 4 °C for 24 hours. Following storage, the microcentrifuge tube is removed, scintillation fluid (Ecolite, ICN) is added and the vials are counted in a liquid scintillation counter. Glucose oxidation rates were determined by quantitative measurement of $^{14}CO_2$ production including $^{14}CO_2$ released as a gas in the oxygenation chamber and $^{14}CO_2$ dissolved as $H^{14}CO_3^-$ in perfusate. The gaseous $^{14}CO_2$, which exits the perfusion system via an exhaust line, was trapped in hyamine hydroxide solution. The dissolved $^{14}CO_2$ as $H^{14}CO_3^-$ was released and trapped on filter paper saturated with hyamine hydroxide in the central well of 25 mL stoppered flasks after perfusate samples were acidified by the addition of 1 mL of 9N H_2SO_4.[238]

Calculation of H^+ Production from Glucose Utilization

If glucose passes through glycolysis to lactate and the ATP so formed is hydrolyzed, a net production of 2 H^+ per molecule of glucose occurs.[32, 54] In contrast, if glycolysis is coupled to glucose oxidation, the net production of H^+ is zero. Therefore, subtracting the rate of glucose oxidation from the rate of glycolysis and multiplying by 2 determines the overall rate of H^+ production derived from glucose utilization. This calculation of H^+ production has been validated, as in a previous study in isolated working rat hearts utilizing a similar metabolic approach, the pH_i determination using ^{31}P NMR correlated well with their calculated values.[32]

Calculation of Tricarboxylic Acid Cycle Activity and ATP Production

TCA cycle activity was calculated from the rates of palmitate oxidation and glucose oxidation based on the oxidation of 1 mole of palmitate producing 8 moles of acetyl-CoA and the oxidation of 1 mole of glucose producing 2 moles of acetyl-CoA. ATP production was calculated from rates of glycolysis, glucose and palmitate oxidation based on 1 mole glucose producing 2 moles of ATP in glycolysis and 31 moles of ATP in glucose oxidation and 1 mole of palmitate producing 104 moles of ATP.

Measurement of Lactic Acid Release

Lactic acid content of 70 μL perfusate samples was determined using a coupled enzyme reaction between LDH and glutamate-pyruvate transaminase (GPT) by the appearance of NADH by means of its absorbance at 334, 340 or 365 nm. Assay was

performed in 292 μL of assay medium containing 0.2 M glycylglycine, 2 mM glutamic acid, 3.6 mM NAD, 1.6 U GPT and 5.5 U LDH.

Tissue Analysis

Frozen ventricular tissue was powdered in a mortar and pestle cooled to the temperature of liquid nitrogen. A portion of the powdered tissue was used to determine the dry wt to wet wt ratio of the ventricles. The atrial tissue remaining on the cannula was removed, dried in an oven for 24 hr at 100°C and weighed. The dried atrial weight, frozen ventricular weight and the ventricular dry to wet ratio were used to calculate the total dry wt of the heart.

Whole Cell Homogenate Preparation

Approximately 20 mg of frozen ventricular tissue was homogenized for 30 s using a Polytron® Homogenizer in 80 μL of homogenization medium containing 50 mM Tris-HCl (pH = 8 at 4 °C), 1 mM EDTA, 10 % (w/v) glycerol, 0.02 % (v/v) Brij-35, 1 mM dithiothreitol (DTT), protease inhibitors and phosphatase inhibitors. For Western Blot analysis the homogenates were centrifuged at 800 xg for 5 min at 4 °C and for AMPK activity assays the homogenates were centrifuged at 10000 xg for 20 min at 4 °C. Protein concentration was then determined in the resultant supernatants using the Bradford protein assay.[241]

AMPK Activity Assay

Activity was assayed by following the incorporation of [^{32}P]phosphate into a pseudo-substrate AMARA peptide (AMARAASAAALARRR).[177] The assay was

performed in 25 μL of assay buffer containing 40 mM HEPES-NaOH (pH = 7), 80 mM NaCl, 1 mM EDTA, 0.02 % (w/v) Brij-35, 1 mM DTT, 200 μM ATP, 5 mM $MgCl_2$, phosphatase inhibitors and protease inhibitors. The assay was performed at 30 °C for 5 min and was terminated by the addition of H_3PO_4 (1 % v/v, final concentration). 15 μL aliquots were removed and spotted on a Unifilter P81 96-well filterplate. Each well was then washed 10 times with 300 μL of 1 % (v/v) H_3PO_4, and 2 times with methanol. The filterplate was dried, and 50 μL of MicroScint PS scintillant was added to each well and was subsequently counted in a Wallac MicroBeta Trilux scintillation counter.

Immunoblotting

Whole cell homogenates were diluted in protein sample buffer containing 62.5 mM Tris (pH 6.8), 6 M Urea, 10 % (w/v) glycerol, 2 % (w/v) sodium dodecylsulfate (SDS), 0.003 % (w/v) bromophenol blue and 5 % (v/v) 2-mercaptoethanol, and boiled for 5min. Equal amounts of proteins of were resolved by SDS polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). Samples were resolved using a Mini Trans-Blot Cell (Biorad) in protein running buffer containing 25 mM Tris (pH 8.3), 0.192 M glycine and 0.1% (w/v) SDS. Proteins were subsequently transferred to a nitrocellulose membrane using transfer buffer containg 25 mM Tris (pH 8.3), 193 mM glycine and 20 % (v/v) methanol.[242, 243] Membranes were blocked for 1 hr at room temperature with either 5 % (w/v) milk powder or bovine serum albumin dissolved in Tris-buffered saline containing 0.1 % (v/v) tween. The membranes were then incubated overnight at 4 °C in primary antibody diluted in the blocking buffer. Membranes were washed with Tris-buffer saline containing 0.1 % (v/v) tween to remove erroneous binding

and the membranes were incubated in the appropriate secondary antibody conjugated to horseradish peroxidase at room temperature for 1 hr. After washing, the protein expression was visualized using the ECL western blotting detection kit or Western Lightning Chemiluminescence Reagents Plus.

Densitometic Analysis of Western Blots

X-ray films were scanned using a GS-800 calibrated densitometer and analyzed using Quantity One software (Bio-Rad, Richmond, California) or ImageJ (National Institutes of Health, Bethesda, Maryland). A control sample was run on all gels and the relative density of the bands determined with the quantification software was standardized to the control sample. This eliminated differences in the transfer of proteins to the membrane and any differences in the time of exposure to the X-ray film.

Measurement of Adenine Nucleotides and Short-Chain CoA esters

Approximately 30 mg of frozen ventricular tissue was homogenized for 30 s using a Polytron® Homogenizer in 300 μL of 6 % (v/v) perchloric acid and 2 mM DTT. After homogenization the samples were left on ice for 30 min and then centrifuged at 12000 xg for 5 min. The resultant supernatant was split, half was ready for analysis of short-chain CoA esters by HPLC [244] and the remaining supernatant required further processing for measurement of adenine nucleotides. EGTA was added until there was a final concentration of 0.5 mM and the samples titrated to a pH to 7.6 – 7.8 using 0.5 M K_2CO_3, to ensure stability of the adenine nucleotides. Samples were left on ice for 10

min and then centrifuged at 10000 xg for 2 min and the resultant supernatant was ready for HPLC analysis.[245]

For short-chain CoA ester analysis, 100 μL of each sample was run through a Nova-Pak C18 precolumn and an Adsorbosphere C18 column on a Beckman System Gold HPLC. Flow rate was set at 1 mL/min and analyte detection occurred at an absorbance of 254 nm on a Beckman System Gold model 168 diode array detector. The mobile phase consisted of a mixture of buffer A (0.2 M NaH_2PO_4, pH 5.0) and buffer B (0.25 M NaH_2PO_4 and acetonitrile, pH 5.0). The gradient elution profile consisted of the following, 0 - 2.5 min, 97 % A 3 % B; 2.5 – 7.5 min, 97 % A 3 % B to 82 % A 18 % B; 7.5 – 15 min, 82 % A 18 % B; 15 – 18 min, 82 % A 18 % B to 63 % A 37 % B; 18 – 35 min, 63 % A 37 % B to 10 % A 90 % B; 35 – 42 min, 10 % A 90 % B.[244] Peaks were integrated using the Beckman System Gold software package.

For adenine nucleotide analysis, 100 μL of each sample was run through a Supelcosil™ LC-18-T guard cartridge and a Supelcosil™ 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 tetrabutylammoniumhydrogensulfate, 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. [245] Peaks were integrated using the Beckman System Gold software.

Measurement of Long-Chain CoA Esters

Approximately 5 mg of frozen ventricular tissue was sonicated in 200 μl of cold 100 mM KH_2PO_4 for 20 s followed by the addition of 200 μL of cold n-propanol and further sonicated for 20 s. 25 μL of saturated ammonium sulphate and 400 μL of acetonitrile were added successively to the homogenate, and vortexed for 5 min and centrifuged at 2100 xg. The top layer was transferred to a microcentrifuge tube and evaporated under nitrogen. The solid residue was mixed with 100 μL of 0.5 M chloroacetaldehyde reagent and then centrifuged at 21,000 xg for 5 min to form acyl etheno-CoA esters. The resultant supernatant was then heated at 85 °C for 20 min and stored at -20 °C until HPLC analysis.[246]

5 to 20 μL of sample was run through a C18 Guard Cartridge and a Luna column of phenylhexyl coated silica particles maintained at 40 °C on a Beckman System Gold HPLC. Flow rate was set at 0.4 mL/min and the analyte was detected using a Perkin Elmer LS-5 fluorescence spectrophotometer with an excitation wavelength of 230 nm and an emission wavelength of 420 nm. The mobile phase consisted of buffer A (90 % (v/v) acetonitrile, 1 % (v/v) acetic acid) and buffer B (90 % (v/v) acetonitrile). The gradient elution profile consisted of the following, 0 - 30 min, 80 % A 20 % B to 65 % A 35% B; 30 – 40 min, 65 % A 35 % B; 40 – 42 min, 65 % A 35 % C to 100 % B; 42 – 47 min, 100 % B. [247] Peaks were integrated using the Beckman System Gold software.

Measurement of Glycogen Content and Accumulation

Glycogen was extracted from approximately 25 mg of frozen ventricular tissue by the addition of 300 μL of 30 % (w/v) KOH followed by boiling the samples for 1 hr or

until all the tissue was dissolved. Samples were then cooled to room temperature and 200 μL of 2 % (w/v) Na_2SO_4 and 2 mL of absolute ethanol were added. The samples were then centrifuged at 800xg for 5 min and the supernatant was discarded. The resultant pellet was then washed with 2 mL of 66 % (v/v) ethanol and allowed to dry. Extracted glycogen was converted to glucose by addition of 4 M H_2SO_4 and boiling for 3 hr, following which 500 μL of 1 M MOPS was added to each sample and were titrated to a pH to 6.8 – 7.0. Samples were centrifuged at 800xg for 5 min and the resultant supernatants were used to measure total glucose content using a Sigma Diagnostics glucose analysis kit. Glycogen accumulation could be calculated based on the incorporation of either [5-^{3}H]glucose or [U-^{14}C]glucose into the glycogen pool.

Statistical Analysis

All data are presented as the mean ± S.E.M. The data were analyzed with the statistical programs GB-stat and Prism. Specific statistical tests are described in the individual chapters. Values of $P < 0.05$ were considered significant.

CHAPTER 3.

Fatty Acids Attenuate Insulin Regulation of 5'-AMP-Activated Protein Kinase and Insulin Cardioprotection after Ischemia

A version of this chapter has been published. Folmes CDL, Clanachan AS, and Lopaschuk GD. *Circ Res*. 2006;99:61-68.

My role in this work involved performing all the experiments (except those noted below) as well as writing the manuscript. Ken Strynadka and Panakkezhum Thomas performed the HPLC analysis of the adenine nucleotides, short and long-chain CoA esters.

CHAPTER 3.

Fatty Acids Attenuate Insulin Regulation of 5'-AMP-Activated Protein Kinase and Insulin Cardioprotection after Ischemia

Abstract

The cardioprotective effect of insulin during I/R is associated with a stimulation of glucose uptake and glycolysis. Although fatty acids and AMPK are regulators of glucose metabolism, it is unknown what effect insulin has on post-ischemic function and AMPK activity in the presence of high levels of fatty acid. Isolated perfused mouse hearts were perfused with Krebs-Henseleit solution containing 5 mM glucose, and 0, 0.2 or 1.2 mM palmitate, with or without 100 μU/ml insulin. During aerobic perfusion in the absence of palmitate, insulin stimulates glycolysis (7300 ± 810 vs. 4220 ± 450 nmol/g dry wt/min, n = 7 per group, $P < 0.05$) and glucose oxidation (2950 ± 290 vs. 1920 ± 240 nmol/g dry wt/min, n = 7 per group, $P < 0.05$) while inhibiting AMPK activity (63 ± 12 vs. 110 ± 14 pmol/mg/min, n = 7 per group, $P < 0.05$). In the presence of 0.2 (low fat) or 1.2 mM (high fat) palmitate, insulin stimulates the low glycolysis in the low fat group (8270 ± 730 vs. 3910 ± 460 nmol/g dry wt/min, n = 10 and 12, $P < 0.05$) and in the high fat group (5360 ± 630 vs. 2610 ± 360 nmol/g dry wt/min, n = 14 per group, $P < 0.05$).

Insulin also increases glucose oxidation in the low fat group (2840 ± 260 vs. 1640 ± 150 nmol/g dry wt/min, n = 10 and 12, $P < 0.05$) and in the high fat group (1690 ± 130 vs. 452 ± 53 nmol/g dry wt/min, n = 14 per group, $P < 0.05$) but no longer inhibits AMPK activity. During reperfusion of hearts in the absence of palmitate, insulin increases recovery of cardiac power (54 ± 10 vs. 29 ± 8 mJoule, n = 13 and 11, $P < 0.05$). This is associated with an increase in glycolysis (5280 ± 450 vs. 2680 ± 420 nmol/g dry wt/min, n = 7 per group, $P < 0.05$) and glucose oxidation (2760 ± 370 vs. 1060 ± 130 nmol/g dry wt/min, n = 13 and 11, $P < 0.05$). However, in the presence of 1.2 mM palmitate, insulin now impairs recovery of cardiac power (26 ± 5 vs. 44 ± 5 mJoule, n = 22 and 24, $P < 0.05$). During reperfusion, glucose oxidation was inhibited by high fat compared to aerobic values (740 ± 110 vs. 440 ± 50 nmol/g dry wt/min, n = 16 and 19, $P < 0.05$), but insulin-stimulated glycolysis remains high (4380 ± 430 vs. 2300 ± 200 nmol/g dry wt/min, n = 16 and 14, $P < 0.05$), resulting in increased H^+ production. In the absence of fatty acids, insulin blunts the ischemic-induced activation of AMPK (437 ± 23 vs. 112 ± 14 pmol/mg/min, n = 12 and 6, $P < 0.05$), but this effect is lost in the presence of fatty acids. We demonstrate that the cardioprotective effect of insulin and its ability to inhibit AMPK activity are lost in the presence of high concentrations of fatty acids.

Introduction

The cardioprotective effect of insulin as part of GIK solution was originally identified by Sodi-Pallares *et al.* to reduce the electrocardiographic abnormalities associated with acute myocardial infarction (AMI).[146] Recently there has been renewed interest in the use of GIK therapy as a metabolic treatment for AMI since both a meta-analysis of previous trials and the ECLA trial showed that GIK significantly reduced proportional in-hospital mortality.[131, 149] However the results of this analysis have been recently questioned due to the CREATE-ECLA randomized controlled trial examining the effect of GIK in patients with ST-elevated myocardial infarction, in which patients were assigned to receive either usual care or usual care with a high-dose GIK infusion regimen.[150] That study showed no efficacy of GIK therapy in any of the predefined endpoints, including mortality, cardiac arrest, and cardiogenic shock. Despite the fact that insulin can suppress circulating fatty acid concentrations, plasma fatty acids levels were not assessed in that study. Therefore, the normal insulin-mediated decrease in plasma fatty acids may not have occurred, which could possibly account for the negative results.

The cardioprotective effect of insulin is well established in experimental studies in which hearts are perfused in the absence of fatty acids or in the presence of a low concentration of fatty acids.[248-250] This beneficial effect may be due to either a direct positive inotropic effect of insulin, or to a metabolic effect of insulin, such as stimulation of anaerobic glycolysis to contribute to basal ATP production. In addition to insulin, AMPK is also an important regulator of glucose metabolism in the heart.[251, 252] Of

potential importance is that our laboratory and others demonstrate that insulin can inhibit the activation of AMPK during both aerobic perfusion and ischemia.[152-154] Therefore insulin may have a salutary effect in ischemia by indirectly inhibiting fatty acid oxidation and stimulating the aerobic disposal of pyruvate, thus reducing the sequelae of adverse events associated with increased H^+ production as discussed in Chapter 1.

Despite the importance of insulin, AMPK, and fatty acids in controlling glucose metabolism in the heart, little is known about their interaction during and following ischemia. Recently, Clark *et al.* showed that fatty acids attenuate the ability of insulin to inhibit AMPK activation.[253] Since fatty acids are elevated in most clinical forms of myocardial ischemia it is possible that high fatty acids overcome any beneficial effects of insulin during ischemia.[33] Therefore, we investigated the effect of insulin on AMPK activation and myocardial metabolism in the presence of graded concentrations of fatty acids. In addition, we considered the implications of these observations on AMPK activation during ischemia, and the effect on recovery of contractile function following ischemia.

Materials and 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.

Isolated Working Mouse Heart Perfusions

CD-1 mouse hearts (98 mice) were perfused in the working perfused mode as previously described in Chapter 2.[239] Hearts were perfused in the presence or absence of 100 μU/mL insulin, depending on the experimental protocol. The oxygenated Krebs-Henseleit solution contained either: no fatty acids (no fat), 0.2 (low fat) or 1.2 mM (high fat) palmitate. Spontaneously beating hearts were perfused at a constant left atrial preload pressure of 11.5 mmHg and a constant aortic afterload pressure of 50 mmHg for: 1) a 40 min aerobic perfusion, 2) a 30 min aerobic perfusion followed by 16 min (no fat) or 18 min (high fat) of global no-flow ischemia and 40 min of aerobic reperfusion, 3) a 30 min aerobic control and 4) a 30 min aerobic perfusion followed by 16 min (no fat) or 18 min (high fat) of global no-flow ischemia, for an ischemia control (please see Figure 1). At the end of the perfusion protocols the hearts were quick frozen in liquid nitrogen with Wollenberger tongs, and stored at -80 °C.

Glycolysis, glucose and palmitate oxidation were measured by quantitative collection of the $^{3}H_2O$ and $^{14}CO_2$, derived from [5-^{3}H/U-^{14}C] glucose and [9, 10-^{3}H]

palmitate, as described in Chapter 2.[134, 238] Rates of H^+ production were calculated as described in Chapter 2.

Tissue Extractions

AMPK was isolated and assayed using the AMARA peptide as described in Chapter 2. Immunoblotting was performed on cleared homogenates for total and phosphorylated AMPK and Akt as described in Chapter 2. Adenine nucleotides, long and short-chain CoA esters were extracted for HPLC analysis as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± S.E.M. The data were analyzed with the statistical program Instat 2.01 and GB-stat. Two-way repeated measures ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for cardiac power. One-way or two-way ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for the metabolic data. For the AMPK activity data, a t-test was used to determine differences between the no insulin and insulin groups at the three different time points (aerobic, ischemia, and reperfusion) and then a two-way ANOVA to determine the difference between the time points. Values of $P < 0.05$ were considered significant.

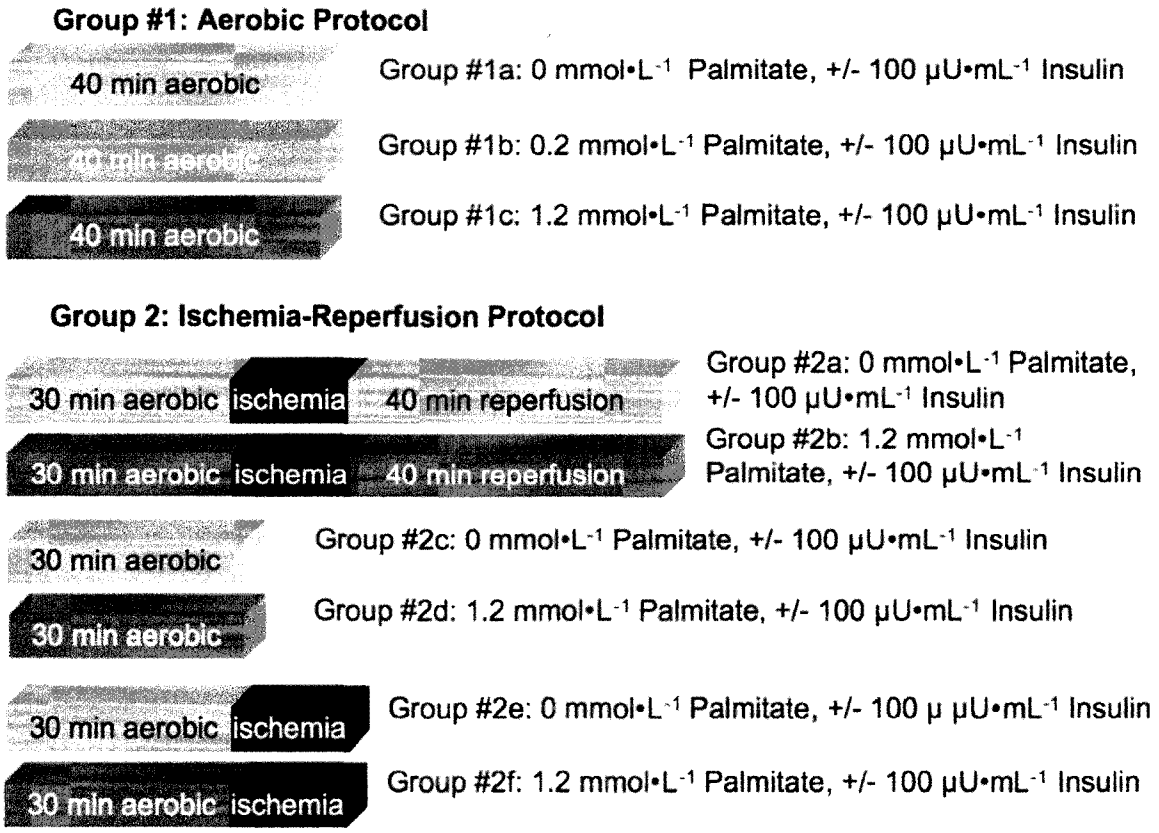


Figure 3-1: Isolated working mouse heart perfusion groups.

Results

Baseline Aerobic Values in 40 Min Aerobic Hearts Perfused in the Absence of Fatty Acids

The addition of insulin to aerobically perfused mouse hearts in the absence of fatty acids causes a significant increase in cardiac power (71 ± 4 vs. 87 ± 6 mJoule, n = 7 per group, $P < 0.05$, for the no insulin and insulin groups, respectively). Heart rate, cardiac output and coronary flow does not differ between these groups (data not shown). As expected, insulin increases glycolysis (7300 ± 810 vs. 4220 ± 450 nmol/g dry wt/min, n = 7 per group, $P < 0.05$, Figure 3-2B) and glucose oxidation (2950 ± 290 vs. 1920 ± 240 nmol/g dry wt/min, n = 7 per group, $P < 0.05$, Figure 3-2C) in these hearts. Insulin also decreases AMPK activity (63 ± 12 vs. 110 ± 14 pmol/mg/min, n = 7 per group, $P < 0.05$, Figure 3-2A) in these hearts.

Immunoblot analysis shows that insulin produces a 3-fold increase in phosphorylation of Akt at Ser473 (Ser473/total Akt: 0.82 ± 0.08 vs. 0.29 ± 0.03, n = 3 per group, $P < 0.05$, Figure 3-3A). Despite this activation of Akt, there is no downstream effect on phosphorylation of AMPK at Ser485/491 (Figure 3-3B).

Baseline Aerobic Values in 40 Min Aerobic Hearts Perfused in the Presence of Fatty Acids

The addition of insulin to perfused mouse hearts in the presence of either 0.2 or 1.2 mM palmitate does not significantly modify cardiac power, or any of the other parameters of contractile function (data not shown). Despite the lack of change in contractile function, insulin results an increase in glycolysis in the low fat group (8270 ± 730 vs. 3910 ± 460 nmol/g dry wt/min, n = 10 and 12, $P < 0.05$, Figure 3-4B) and in the high fat group (5360 ± 630 vs. 2610 ± 360 nmol/g dry wt/min, n = 14 per group, $P < 0.05$, Figure 3-2B). Insulin also increases glucose oxidation in the low fat group (2840 ± 260 vs. 1640 ± 150 nmol/g dry wt/min, n = 10 and 12, $P < 0.05$, Figure 3-4B) and in the high fat group (1690 ± 130 vs. 452 ± 53 nmol/g dry wt/min, n = 14 per group, $P < 0.05$, Figure 3-4C). However, in the presence of both low and high fat, insulin no longer inhibits AMPK activity (Figure 4A).

Immunoblot analysis also shows that in the presence of high fat, insulin produces a 2.5-fold increase in phosphorylation of Akt at Ser473 (Ser473/total Akt: 1.18 ± 0.08 vs. 0.48 ± .05, n = 3 per group, $P < 0.05$, Figure 3-3A) and there is no downstream effect on phosphorylation of AMPK at Ser485/491 (Figure 3-3B).

The Effect of Insulin and Palmitate on Myocardial Metabolism and Functional Recovery During Reperfusion Following Global No-Flow Ischemia

Ischemia and Reperfusion in the Absence of Fatty Acids

Insulin produces a small but insignificant increase in cardiac output and cardiac power and does not significantly change any other parameters of contractile function of hearts perfused in the absence of fatty acids during the first 30 minutes of aerobic perfusion (Table 3-1). During reperfusion of hearts following ischemia, insulin produces a significant increase in the recovery of cardiac power in hearts perfused in the absence of fatty acids (54 ± 10 vs. 29 ± 8 mJoule, n = 13 and 11, $P < 0.05$, Figure 3-5A and Table 3-1). Insulin effects on glucose metabolism persist during reperfusion, where increases in glycolysis (5280 ± 450 vs. 2680 ± 420 nmol/g dry wt/min, n = 7 per group, $P < 0.05$, Figure 3-6A), and glucose oxidation (2760 ± 370 vs. 1060 ± 130 nmol/g dry wt/min, n = 13 and 11, $P < 0.05$, Figure 3-6B) are observed. Glucose oxidation during reperfusion recovers to the corresponding pre-ischemic value in both the presence and absence of insulin. During reperfusion, a slight reduction in glycolysis in the insulin-treated hearts is observed, although this did not reach statistical significance ($P = 0.08$). Although H^+ production is increased by insulin during aerobic perfusion (9840 ± 1840 vs. 4280 ± 580 nmol/g dry wt/min, n = 7 per group, $P < 0.05$, Figure 3-6C), insulin has no significant effect on H^+ production during reperfusion (Figure 6C).

In hearts perfused in the absence of fatty acids and frozen immediately following ischemia, there is a dramatic increase in AMPK activity (437 ± 23 vs. 112 ± 14

pmol/mg/min, n = 12 and 6, P<0.05, Figure 3-8A) that is inhibited by insulin (357 ± 17 vs. 437 ± 23 pmol/mg/min, n = 12 per group, P < 0.05, Figure 3-8A) despite no change in the AMP/ATP ratio (Table 3-3). Insulin did increase malonyl-CoA levels (8.0 ± 1.4 vs. 2.6 ± 0.3 nmol/g dry wt, n = 6 per group, P < 0.05, Table 3-5) and decrease acetyl-CoA levels at the end of reperfusion (29 ± 2 vs. 39 ± 3 nmol/g dry wt, n = 6 per group, P < 0.05, Table 3-5) however there is no significant difference in long-chain CoAs (Table 3-7). In the absence of fatty acids, total glycogen content is low, and is unaffected by the presence of insulin (Figure 3-9).

Ischemia and Reperfusion in the Presence of a High Concentration of Fatty Acids

During the initial aerobic period, the presence of insulin does not significantly alter any parameters of contractile function (Table 3-2). However during reperfusion in the presence of high fat, the cardioprotective effect of insulin seen in the absence of fatty acids is lost, and insulin now impairs recovery of cardiac power (26 ± 5 vs. 44 ± 5 mJoule, n = 22 and 24, P < 0.05, Figure 3-5B and Table 2). During reperfusion, insulin increases glycolysis (4380 ± 430 vs. 2300 ± 200 nmol/g dry wt/min, n = 16 and 14, P < 0.05, Figure 3-7A) and glucose oxidation (740 ± 110 vs. 440 ± 50 nmol/g dry wt/min, n = 16 and 19, P < 0.05, Figure 3-7B). Unlike the no fat group, glycolysis remains elevated during reperfusion, and glucose oxidation is suppressed compared to aerobic values (740 ± 110 vs. 1690 ± 130 nmol/g dry wt/min, n = 16 and 19, P < 0.05, Figure 3-7B). As a consequence, H^+ production from glucose metabolism is increased in the insulin-treated hearts during reperfusion compared to the no insulin hearts (7020 ± 840 vs. 3660 ± 440 nmol/g dry wt/min, n = 14 per group, Figure 3-7C, P < 0.05). The addition of insulin

suppresses rates of fatty acid oxidation during both the aerobic period (350 ± 50 vs. 920 ± 70 nmol/g dry wt/min, n = 6 and 4, $P < 0.05$, Figure 3-7D) and the reperfusion period (430 ± 80 vs. 710 ± 60 nmol/g dry wt/min, n = 6 and 4, $P < 0.05$, Figure 3-7D).

In the hearts perfused with high fat, insulin does not have any effect on AMPK activity either during the aerobic perfusion or post-ischemic reperfusion (Figure 3-8B). In addition, insulin is no longer able to blunt the dramatic rise in AMPK activity that occurs during ischemia in the presence of high fat (Figure 3-8B). As observed in the no fat group, insulin decreases concentrations of acetyl-CoA (47 ± 3 vs. 91 ± 4 nmol/g dry wt, n = 6 per group, $P < 0.05$, Table 3-6) but also decreases concentrations of malonyl-CoA (8.1 ± 0.2 vs. 13.9 ± 1.7 nmol/g dry wt, n = 6 per group, $P < 0.05$, Table 3-6) (Table 3-6). In addition, insulin decreases the total concentration of long-chain CoAs (16 ± 2 vs. 25 ± 2 nmol/g dry wt, n = 16 per group, $P < 0.05$, Table 3-8). In the presence of high fat, insulin stimulates greater production of glycogen during the aerobic period, however glycogen content falls to similar levels during ischemia (71 ± 6 vs. 51 ± 6 μmol/g dry wt, n = 6 per group, $P < 0.05$, Figure 3-9).

Table 3-1. The effect of insulin on parameters of contractile function during aerobic reperfusion of ischemic hearts perfused in the absence of fatty acids.

Insulin	Aerobic Perfusion		Reperfusion	
	-	+	-	+
	(n = 13)	(n = 11)	(n = 13)	(n = 11)
Heart Rate (beats/min)	294 ± 8	296 ± 13	173 ± 8*	223 ± 16*
Peak Systolic Pressure (mmHg)	72 ± 1	73 ± 2	51 ± 6*	59 ± 6*
Coronary Flow (mL/min)	3.2 ± 0.3	3.2 ± 0.3	1.7 ± 0.4*	2.8 ± 0.6
Cardiac Output (mL/min)	11.1 ± 0.4	12.7 ± 0.5	2.9 ± 0.7*	6.1 ± 1.0*
Cardiac Power (mJoule)	89 ± 4	103 ± 6	21 ± 6*	46 ± 9*

Differences were determined using a 2-way ANOVA with a Bonferroni posthoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Table 3-2. The effect of insulin on parameters of contractile function during aerobic reperfusion of ischemic hearts perfused in the presence of high fat.

Insulin	Aerobic Perfusion		Reperfusion	
	-	+	-	+
	(n = 24)	(n = 22)	(n = 24)	(n = 22)
Heart Rate (beats/min)	327 ± 7	322 ± 11	224 ± 10*	192 ± 17*
Peak Systolic Pressure (mmHg)	66 ± 1	67 ± 1	57 ± 3*	51 ± 3*
Coronary Flow (mL/min)	3.6 ± 0.3	3.6 ± 0.2	2.6 ± 0.3*	1.5 ± 0.2*
Cardiac Output (mL/min)	11.3 ± 0.6	9.9 ± 0.5	5.8 ± 0.6*	3.2 ± 0.6*
Cardiac Power (mJoule)	83 ± 5	74 ± 4	40 ± 6*	22 ± 5*

Differences were determined using a 2-way ANOVA with a Bonferroni posthoc test.* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Table 3-3. The effect of insulin on adenine nucleotides during aerobic perfusion, ischemia and aerobic reperfusion in isolated working mouse hearts in the absence of fatty acids.

	Aerobic Perfusion		Ischemia		Reperfusion	
Insulin	-	+	-	+	-	+
(μmol/ g dry wt)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
AMP	1.7 ± 0.2	1.0 ± 0.1#	1.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.6 ± 0.1*
ADP	6.8 ± 0.7	6.9 ± 0.7	9.5 ± 0.7	8.5 ± 0.7	6.3 ± 0.3	6.8 ± 0.6
ATP	21.1 ± 1.1	23.4 ± 0.8	6.6 ± 0.7	6.2 ± 0.7	16.3 ± 1.3*	21.4 ± 0.7#
AMP/ATP	0.08 ± .01	0.04 ± .01#	0.21 ± .02	0.21 ± .01	0.09 ± .01	0.07 ± .01*
ADP/ATP	0.37 ± .01	0.36 ± .01	1.57 ± .07	1.42 ± .07	0.42 ± .03	0.32 ± .02#

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding no insulin group.

Table 3-4. The effect of insulin on adenine nucleotides during aerobic perfusion, ischemia and aerobic reperfusion in isolated working mouse hearts in the presence of High Fat.

	Aerobic Perfusion		Ischemia		Reperfusion	
Insulin	-	+	-	+	-	+
(μmol/ g dry wt)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
AMP	2.0 ± 0.1	1.7 ± 0.1	10.1 ± 0.7	7.8 ± 0.4	1.8 ± 0.1	1.5 ± 0.1
ADP	8.7 ± 0.4	6.6 ± 0.1#	11.0 ± 0.5	9.1 ± 0.3	6.3 ± 0.2*	5.5 ± 0.2*
ATP	23.6 ± 1.3	18.3 ± 0.4#	7.6 ± 0.2	7.2 ± 0.3	15.1 ± 0.6*	14.9 ± .5*
AMP/ATP	0.09 ± .01	0.09 ± .01	1.31 ± .07	1.16 ± .10	0.13 ± .01*	0.11 ± .01
ADP/ATP	0.37 ± .1	0.36 ± .01	1.44 ± .05	1.31 ± .07	0.43 ± .02	0.38 ± .02

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding no insulin group.

Table 3-5. The effect of insulin on short-chain CoAs during aerobic perfusion, ischemia and aerobic reperfusion in isolated working mouse hearts in the absence of fatty acids.

	Aerobic Perfusion		Ischemia		Reperfusion	
Insulin	-	+	-	+	-	+
(μmol/ g dry wt)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
CoA	1.8 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
Acetyl-CoA	20 ± 3	25 ± 1	38 ± 4*	50 ± 4*#	40 ± 3*	29 ± 2#
Malonyl-CoA	3.5 ± 0.5	7.7 ± 0.7#	2.9 ± 0.4	3.0 ± 0.5*	2.6 ± 0.3	8.0 ± 1.4#

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding no insulin group.

Table 3-6. The effect of insulin on short-chain CoAs during aerobic perfusion, ischemia and aerobic reperfusion in isolated working mouse hearts in the presence of High Fat.

	Aerobic Perfusion		Ischemia		Reperfusion	
Insulin	-	+	-	+	-	+
(μmol/ g dry wt)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
CoA	1.0 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Acetyl-CoA	104 ± 8	54 ± 3$^{\#}$	58 ± 3*	64 ± 3	91 ± 5	47 ± 3$^{\#}$
Malonyl-CoA	7.6 ± 0.4	7.4 ± 0.2	16.3 ± 0.8*	12.1 ± 0.3*$^{\#}$	13.9 ± 1.7*	8.1 ± 0.2$^{\#}$

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding no insulin group.

Table 3-7. The effect of insulin on long-chain CoAs during aerobic perfusion, ischemia and aerobic reperfusion in isolated working mouse hearts in the absence of fatty acids.

	Aerobic Perfusion		Ischemia		Reperfusion	
Insulin	-	+	-	+	-	+
(nmol/ g dry wt)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
16:0	1.8 ± 0.5	2.1 ± 0.4	3.7 ± 0.7	4.5 ± 0.9	2.3 ± 0.6	2.1 ± 0.1
18:1	1.9 ± 0.6	2.1 ± 0.4	4.8 ± 0.9*	6.0 ± 1.5*	2.6 ± 0.9	2.1 ± 0.1
18:0	2.1 ± 0.7	2.0 ± 0.4	7.9 ± 1.3*	9.0 ± 1.5*	2.8 ± 1.0	1.2 ± 0.2
Total	5.8 ± 1.8	6.2 ± 1.0	16.0 ± 2.8*	19.6 ± 3.8*	7.7 ± 2.5	5.4 ± 0.4

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding no insulin group.

Table 3-8. The effect of insulin on long-chain CoAs during aerobic perfusion, ischemia and aerobic reperfusion in isolated working mouse hearts in the presence of High Fat.

	Aerobic Perfusion		Ischemia		Reperfusion	
Insulin	-	+	-	+	-	+
(nmol/ g dry wt)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
16:0	23.7 ± 1.8	15.4 ± 1.4#	21.2 ± 4.4	16.9 ± 1.5	21.2 ± 1.5	13.0 ± 1.9#
18:1	1.6 ± 0.2	2.1 ± 0.4	2.2 ± 0.7	2.7 ± 0.5	0.4 ± 0.2	0.9 ± 0.2
18:0	2.2 ± 0.7	1.2 ± 0.4	19.1 ± 6.6*	16.4 ± 2.7*	3.7 ± 0.8	1.8 ± 0.5
Total	28 ± 3	19 ± 2#	43 ± 12*	36 ± 5*	25 ± 2	16 ± 2#

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding no insulin group.

Figure 3-2: Insulin inhibits AMPK activation and stimulates glycolysis and glucose oxidation in aerobic hearts perfused in the absence of fatty acids.

A) AMPK activity (n = 6 per group), B) glycolysis (n = 7 per group), and C) glucose oxidation (n = 7 per group) in isolated mouse hearts aerobically perfused in the absence of fatty acids, and in either the presence or absence of 100 μU/ml insulin. Values represent mean ± SEM. Differences were determined using a 2-way t-test.

* represents $P < 0.05$, significantly different from no insulin group.

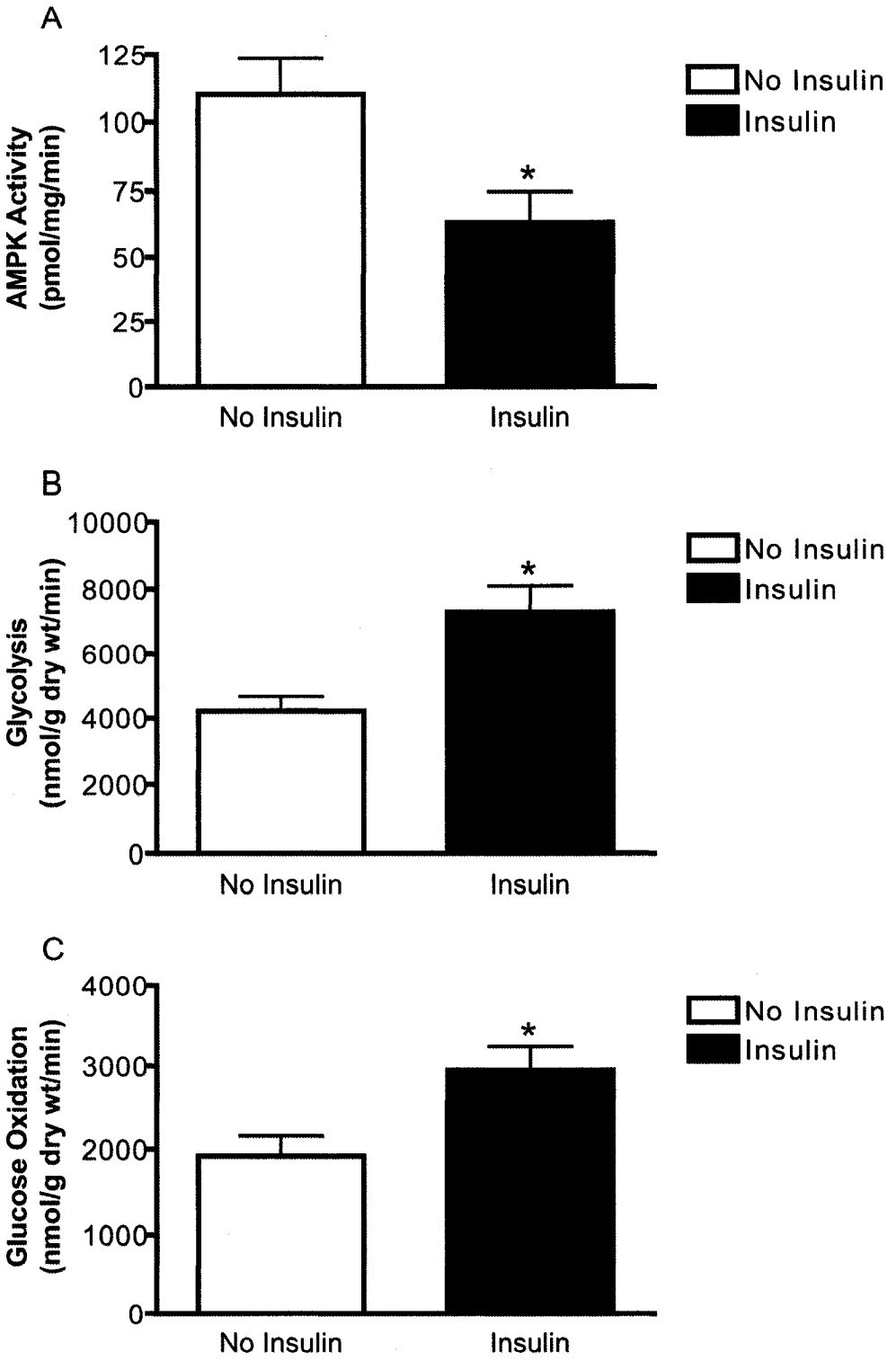

A
AMPK Activity (pmol/mg/min)
125
100
75
50
25
0
No Insulin
Insulin
*
No Insulin
Insulin
B
Glycolysis (nmol/g dry wt/min)
10000
8000
6000
4000
2000
0
No Insulin
Insulin
*
No Insulin
Insulin
C
Glucose Oxidation (nmol/g dry wt/min)
4000
3000
2000
1000
0
No Insulin
Insulin
*
No Insulin
Insulin

Figure 3-2

Figure 3-3: Insulin produces a robust increase in Ser-473 phosporylation of Akt, however has no effect on phosphorylation of AMPK on Ser-485/491.

A) Western blot of P-Ser-473 of Akt and total Akt (n = 3 per group) and B) Western blot of P-Ser-485/491 of AMPK and total AMPK (n = 6 per group).

* represents $P < 0.05$, significantly different from corresponding no insulin group.

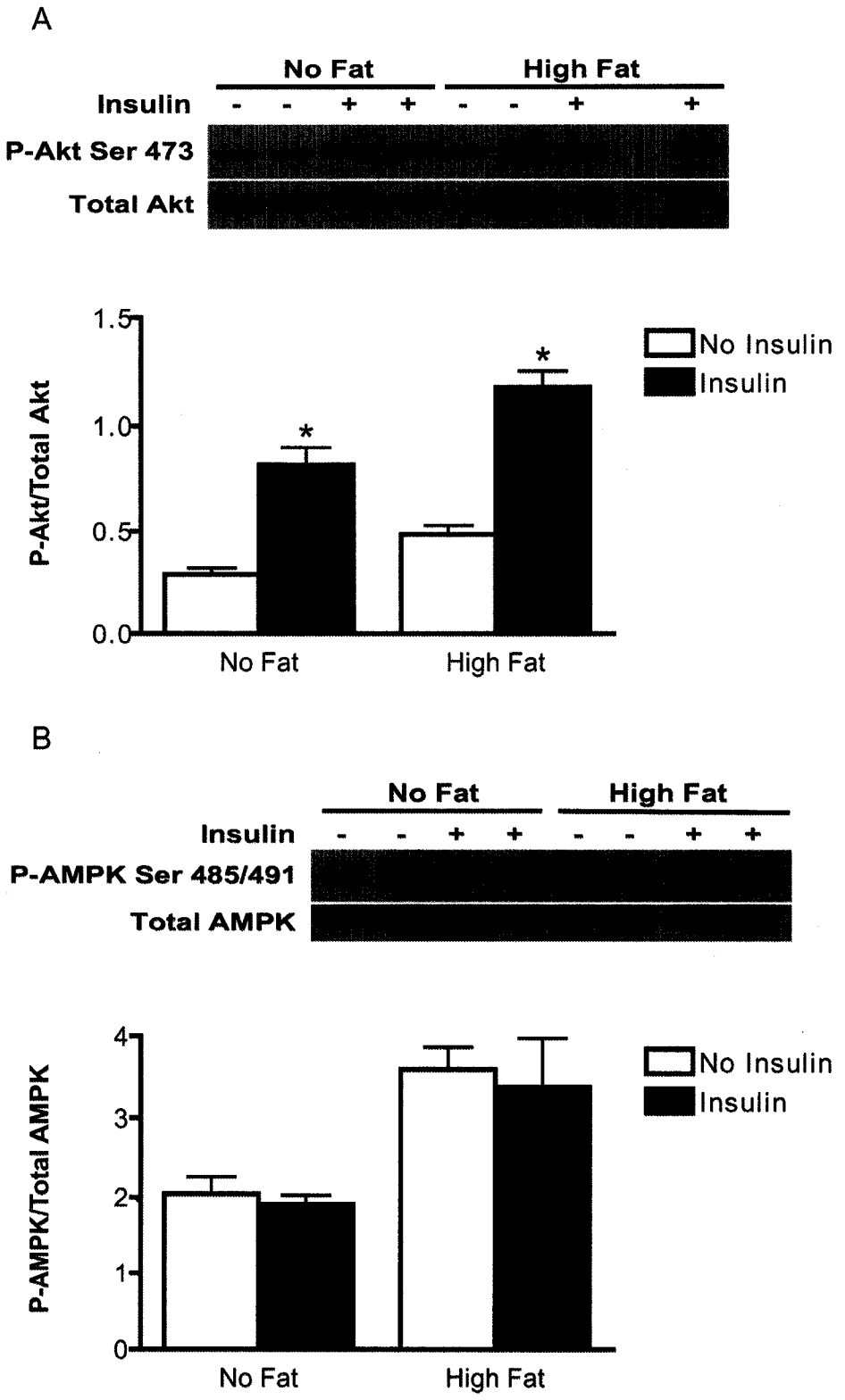

A
No Fat
High Fat
Insulin
- - + + - - + +
P-Akt Ser 473
Total Akt
P-Akt/Total Akt
1.5
1.0
0.5
0.0
*
*
No Insulin
Insulin
No Fat
High Fat
B
No Fat
High Fat
Insulin
- - + + - - + +
P-AMPK Ser 485/491
Total AMPK
P-AMPK/Total AMPK
4
3
2
1
0
No Insulin
Insulin
No Fat
High Fat

Figure 3-3

Figure 3-4: Fatty acids attenuate the insulin-induced inhibition of AMPK, but not the stimulation of glycolysis and glucose oxidation in aerobically perfused hearts.

A) AMPK activity (n = 6 per group), B) glycolysis, and C) glucose oxidation (n = 12 and n = 10 for low fat, no insulin and insulin respectively, and n = 14 and n = 14 for high fat, no insulin and insulin respectively) in isolated mouse hearts aerobically perfused in the presence of either low or high fat, and in either the presence or absence of 100 μU/ml insulin. Values represent mean ± SEM. Differences were determined using a 1-way ANOVA with a Bonferroni posthoc test.

* represents $P < 0.05$, significantly different from no insulin group.

represents $P < 0.05$, significantly different from corresponding low fat group.

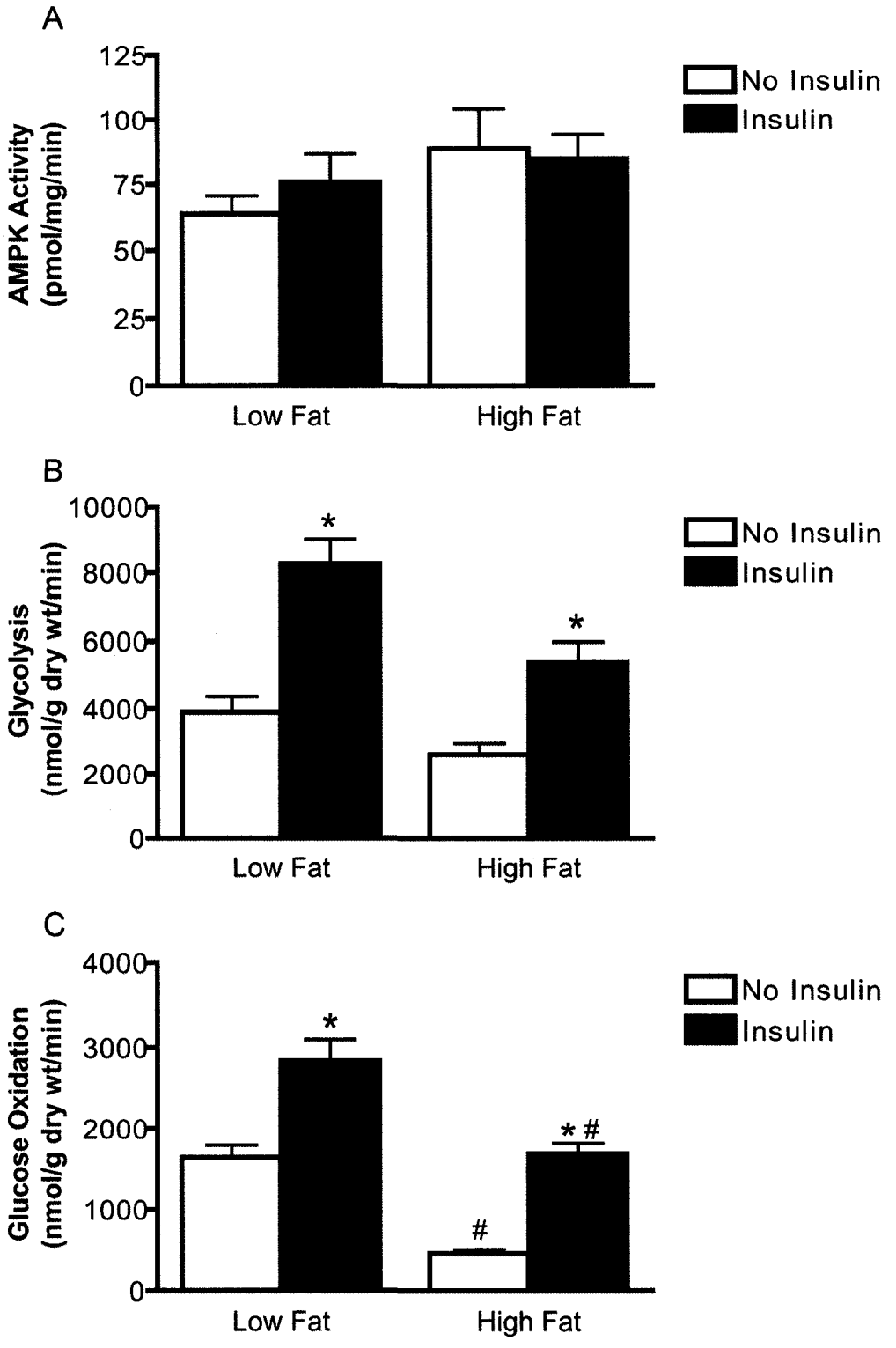

A
AMPK Activity (pmol/mg/min)
125
100
75
50
25
0
Low Fat
High Fat
No Insulin
Insulin
B
Glycolysis (nmol/g dry wt/min)
10000
8000
6000
4000
2000
0
*
*
Low Fat
High Fat
No Insulin
Insulin
C
Glucose Oxidation (nmol/g dry wt/min)
4000
3000
2000
1000
0
*
*#
#
Low Fat
High Fat
No Insulin
Insulin

Figure 3-4

Figure 3-5: The cardioprotective effect of insulin seen in the absence of fatty acids during aerobic reperfusion of ischemic hearts is lost in the presence of high fat.

A) Cardiac power during the ischemia-reperfusion protocol in isolated mouse hearts perfused in the absence of fatty acids (n = 11 and n = 13 for no insulin and insulin (100 μU/ml), respectively). B) Cardiac power during the ischemia-reperfusion protocol in isolated mouse hearts perfused in the presence of high fat (n = 24 and n = 22 for no insulin and insulin groups, respectively). Differences were determined using a 2-way repeated measures ANOVA with a Bonferroni posthoc test.

* represents $P < 0.05$, significantly different from no insulin group.

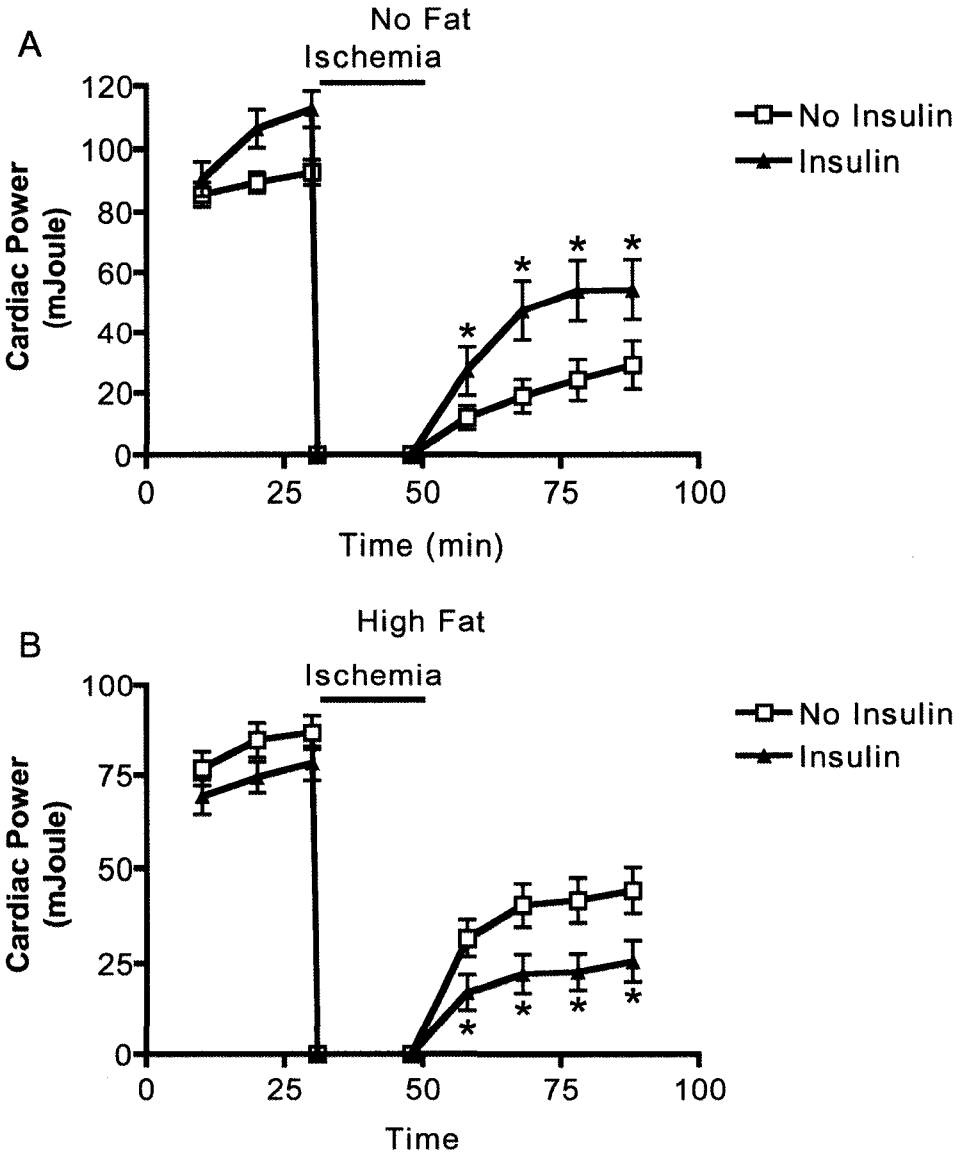

A
No Fat
Ischemia
Cardiac Power (mJoule)
120
100
80
60
40
20
0
0
25
50
75
100
Time (min)
No Insulin
Insulin
B
High Fat
Ischemia
Cardiac Power (mJoule)
100
75
50
25
0
0
25
50
75
100
Time
No Insulin
Insulin

Figure 3-5

Figure 3-6: Insulin stimulates glycolysis and glucose oxidation during aerobic reperfusion of ischemic hearts perfused in the absence of fat.

A) Glycolysis (n = 7 per group), B) Glucose oxidation (n = 11 and n = 13 for no insulin and insulin (100 μU/ml) respectively) and C) Proton production from glucose metabolism (n = 7 per group) in isolated perfused mouse hearts, before and after 16 min global no-flow ischemia. Differences were determined using a 2-way ANOVA with a Bonferroni posthoc test.

* represents $P < 0.05$, significantly different from corresponding no insulin group.

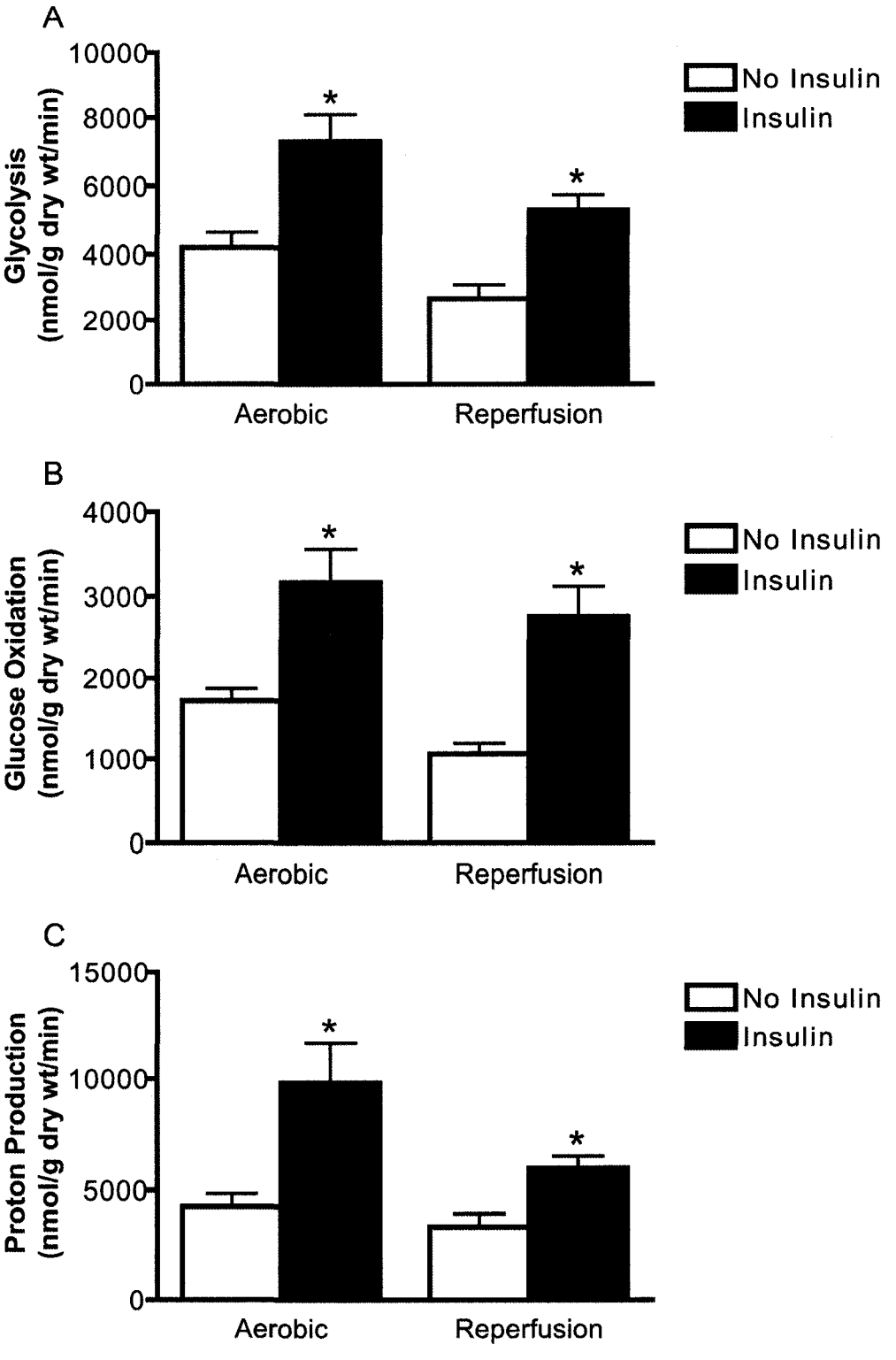

A
Glycolysis (nmol/g dry wt/min)
10000
8000
6000
4000
2000
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin
B
Glucose Oxidation (nmol/g dry wt/min)
4000
3000
2000
1000
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin
C
Proton Production (nmol/g dry wt/min)
15000
10000
5000
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin

Figure 3-6

Figure 3-7: Insulin stimulates glycolysis and glucose oxidation to a smaller extent during aerobic reperfusion of ischemic hearts perfused in the presence of high fat.

A) Glycolysis (n = 14 and n = 16 for no insulin and insulin (100 μU/ml), respectively), B) Glucose oxidation (n = 19 and n = 16 for no insulin and insulin, respectively), C) Proton production from glucose metabolism (n = 14 and n = 14 for no insulin and insulin respectively) and D) Palmitate oxidation (n = 4 and n = 6 for no insulin and insulin respectively) in isolated perfused mouse hearts, before and after 16 min global no-flow ischemia. Differences were determined using a 2-way ANOVA with a Bonferroni posthoc test.

* represents $P < 0.05$, significantly different from corresponding no insulin group.

represents $P < 0.05$, significantly different from corresponding aerobic group.

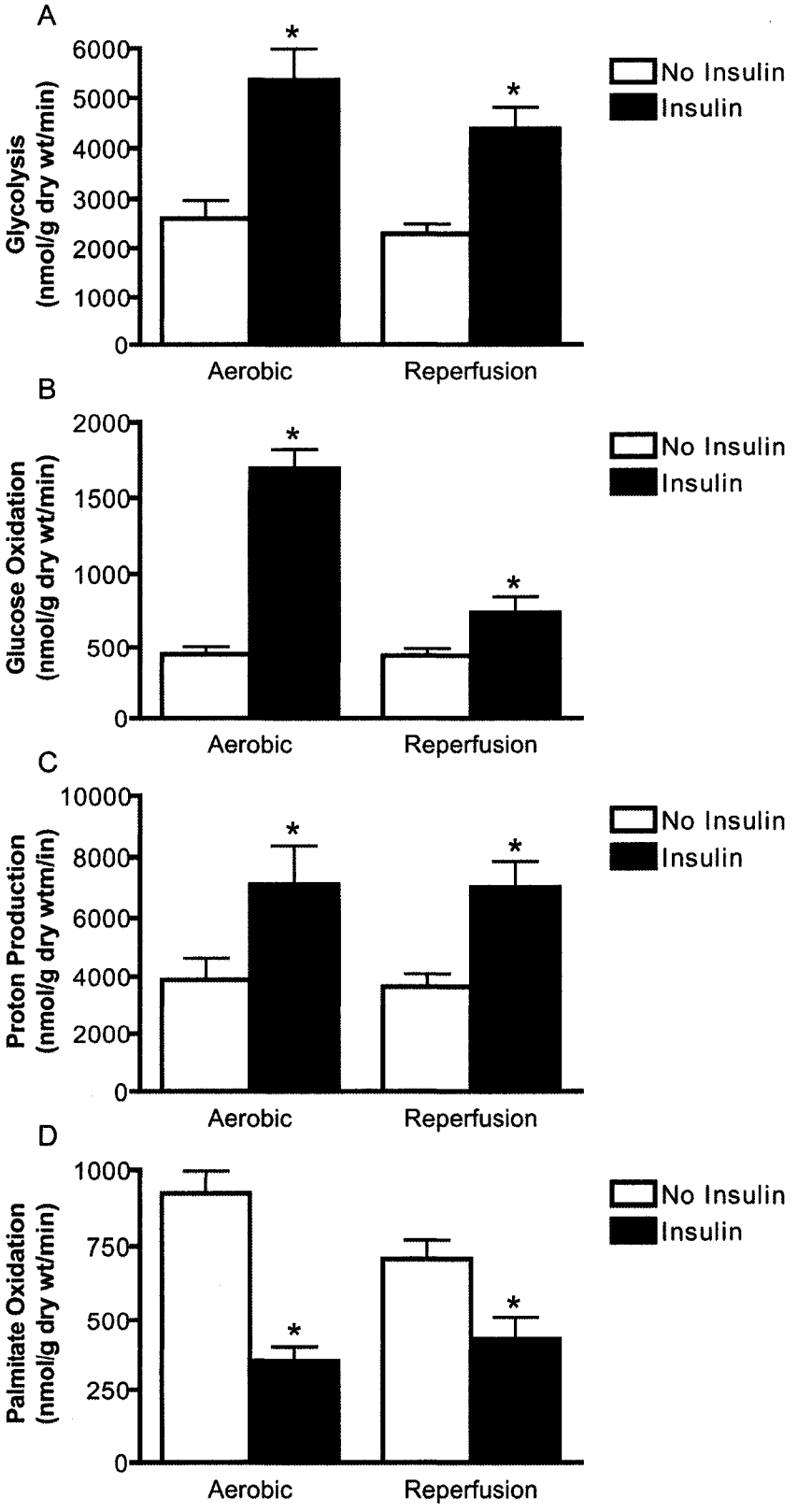

A
Glycolysis (nmol/g dry wt/min)
6000
5000
4000
3000
2000
1000
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin
B
Glucose Oxidation (nmol/g dry wt/min)
2000
1500
1000
500
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin
C
Proton Production (nmol/g dry wtm/in)
10000
8000
6000
4000
2000
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin
D
Palmitate Oxidation (nmol/g dry wt/min)
1000
750
500
250
0
*
*
Aerobic
Reperfusion
No Insulin
Insulin

Figure 3-7

Figure 3-8: Insulin blunts ischemic-induced activation of AMPK in hearts perfused in the absence of fatty acids, but this effect is attenuated in hearts perfused in the presence of high fat.

A) AMPK activity in aerobic, ischemic and reperfused (n = 6, n = 12 and n = 6, respectively) isolated mouse hearts perfused in the absence of fat. B) AMPK activity in aerobic, ischemic and reperfused (n = 6 per group) isolated mouse hearts perfused in the presence of high fat. Differences were determined using t-test to determine differences between the no insulin and insulin groups at the three different time points and a two-way ANOVA to determine the difference between the time points.

* represents $P < 0.05$, significantly different from corresponding no insulin group.

represents $P < 0.05$, significantly different from corresponding aerobic, ischemic and reperfusion group.

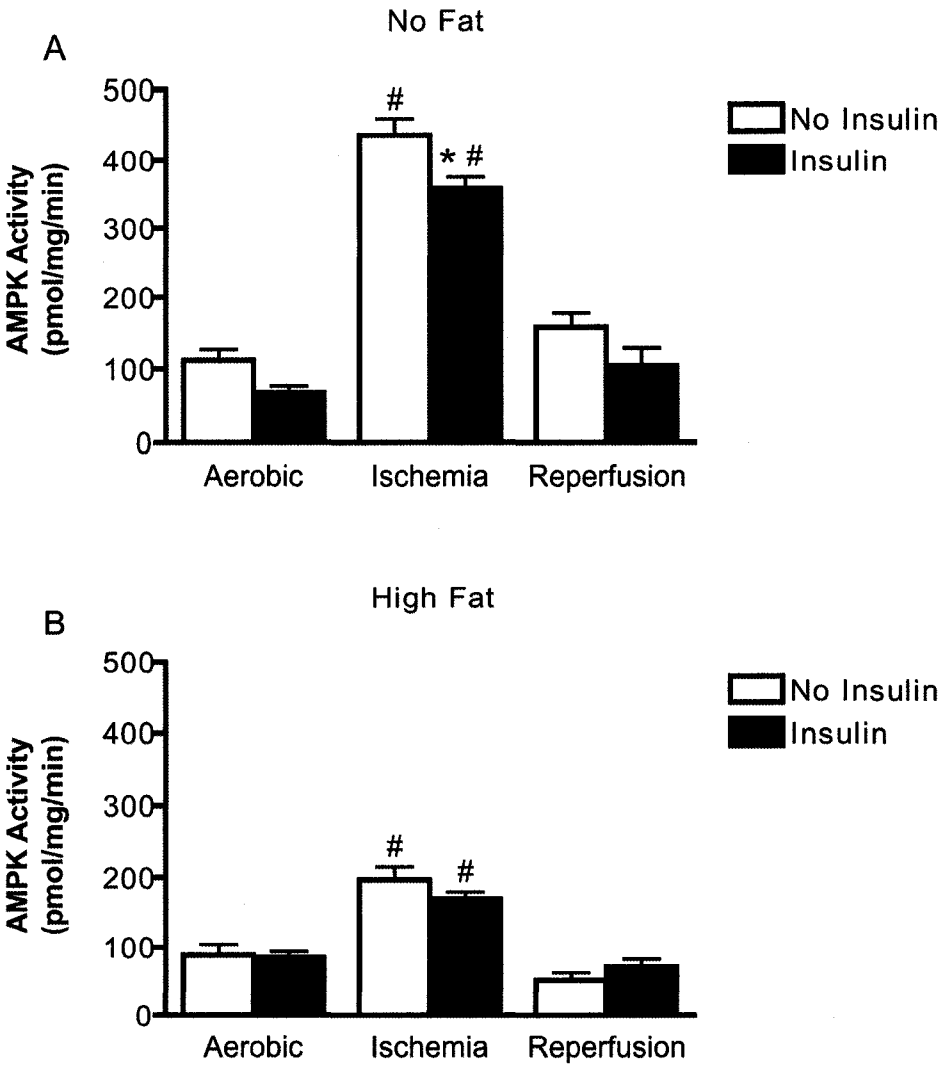

No Fat
A
AMPK Activity (pmol/mg/min)
500
400
300
200
100
0
#
* #
No Insulin
Insulin
Aerobic
Ischemia
Reperfusion
High Fat
B
AMPK Activity (pmol/mg/min)
500
400
300
200
100
0
#
#
No Insulin
Insulin
Aerobic
Ischemia
Reperfusion

Figure 3-8

Figure 3-9: Insulin increases glycogen content during aerobic perfusion in the presence of High Fat, but does not in the presence of No Fat.

A) Glycogen content (n = 6 per group) in isolated perfused mouse hearts in the absence of fat. B) Glycogen content (n = 6 per group) in isolated mouse hearts prefused in the presence of high fat.

represents $P < 0.05$, significantly different from corresponding aerobic and reperfusion group.

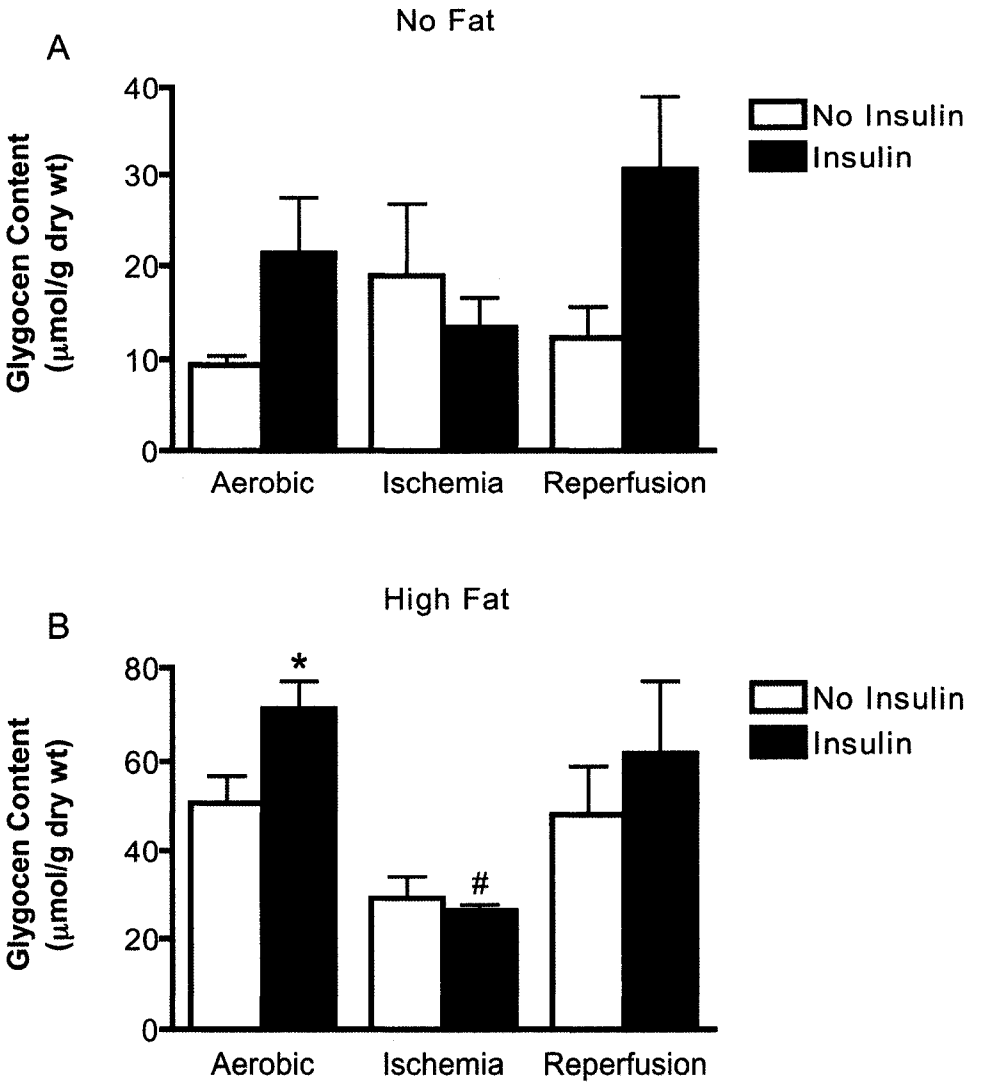

No Fat
A
Glygocen Content (µmol/g dry wt)
40
30
20
10
0
Aerobic
Ischemia
Reperfusion
No Insulin
Insulin
High Fat
B
Glygocen Content (µmol/g dry wt)
80
60
40
20
0
*
#
Aerobic
Ischemia
Reperfusion
No Insulin
Insulin

Figure 3-9

Discussion

A number of previous studies have examined the importance of substrate preference and its relationship to the recovery of cardiac function during reperfusion of severely ischemic hearts.[31, 32, 122, 134, 254, 255] While insulin, fatty acids, and AMPK all have important effects on both function and metabolism following ischemia, the interaction among these three regulators of metabolism has not been examined. In this study we used the isolated perfused mouse heart, which is very sensitive to insulin, to examine the effect of insulin and fatty acids on myocardial metabolism, AMPK activity and functional recovery during reperfusion following ischemia. Using this approach we made a number of important findings. First, the ability of insulin to inhibit AMPK activity during both aerobic perfusion and during ischemia is dependent on the presence of fatty acids in the perfusate. Second, the cardioprotective effect of insulin is lost when hearts are perfused in the presence of high fatty acid levels that are normally seen during and following clinically relevant ischemia. Third, even though insulin cannot inhibit AMPK activity in the presence of a high concentration of fatty acids, other downstream effectors of insulin are intact, including its ability to stimulate glycolysis and glucose oxidation.

A recent study by Clark *et al.* examined the effect of fatty acids on insulin inhibition of AMPK in aerobically perfused rat hearts.[253] Those authors confirm our previous observation that insulin inhibits AMPK activity.[152, 185] However, they observe that this effect is lost as fatty acid concentrations are increased. In this study we reproduce those results in the perfused mouse heart, showing that insulin inhibits AMPK

in the absence of fatty acids, but loses this ability in the presence of low (0.2 mM) or high (1.2 mM) concentrations of palmitate (Figure 3-2 and 3-4). This high fat concentration was chosen to reproduce clinically relevant conditions in humans, where ischemia results in an increased efflux of fatty acids into the serum causing a rapid elevation of circulating fatty acids. Lopaschuk *et al.* have previously characterized this response in adult patients, who had undergone an AMI and found that free fatty acid concentrations peak at 1.51 +/- 0.15 mM (compared with 0.40 +/- 0.013 mM in control subjects).[33]

A novel finding of our study is that even though palmitate attenuates the ability of insulin to inhibit AMPK, other downstream signaling pathways of insulin are intact, since insulin activation of glycolysis, glucose oxidation and phosphorylation of Ser473 of Akt (Figure 3-2, 3-3 and 3-4) are not affected by the presence of fatty acid. This suggests that, in mouse heart, there is a fatty acid dependent insulin-signaling pathway that modifies AMPK activity and a fatty acid independent insulin-signaling pathway that modifies myocardial metabolism. Previous reports suggested that long-chain acyl-CoAs modify the activation of AMPK [156, 256, 257], however in the absence of fatty acids when insulin modified AMPK activity, there is no change in the concentration of long-chain acyl-CoAs.

These observations are of particular importance as insulin inhibits the ischemic-induced activation of AMPK, which would benefit the ischemic and reperfused myocardium by decreasing fatty acid oxidation.[152-154] However, these previous studies were performed in the absence of fatty acids. During reperfusion of ischemic hearts oxidative metabolism quickly recovers, but high rates of fatty acid oxidation result in low glucose oxidation rates due to the Randle cycle.[31, 32, 117, 134] This reduction in glucose

oxidation is detrimental during reperfusion as it contributes to the severity of acidosis in the ischemic and reperfused heart. Ischemic-induced activation of AMPK contributes to the low glucose oxidation rates during reperfusion of ischemic hearts, secondary to stimulation of fatty acid oxidation.[63] Insulin may attenuate these effects by inhibiting AMPK. However, we observe that insulin only inhibits AMPK activity in hearts perfused in the absence of fatty acids. Although insulin only induces a 15% inhibition in AMPK activity at the end of ischemia, we may be missing a larger effect as Beauloye *et al.* demonstrate that ischemic-induced AMPK activation peaks after 10 min of global ischemia, and decreases with extended ischemic times and we have confirmed this in the mouse heart.[153] Therefore insulin may suppress AMPK activation to a greater extent at 10 min of ischemia where peak activation occurs.

The cardioprotective effect of insulin is well established in experimental studies.[248-250] This effect is due to either a positive inotropic effect, or to a metabolic effect of insulin, such as the stimulation of anaerobic glycolysis to maintain basal ATP production and to switch the preferred oxidative substrate to glucose, which is a more oxygen efficient substrate and does not produce the toxic intermediates of fatty acid oxidation. As seen in Figure 3-5, we confirm that insulin produces a cardioprotective effect in the absence of fatty acids, improving recovery of cardiac power by 47%. The beneficial effect of insulin during ischemia and reperfusion in the absence of fatty acids may be due to an acceleration of glycolysis, which would increase the amount of ATP being produced by the heart. H^+ production from glucose metabolism is significantly elevated by insulin in hearts perfused in the absence of fatty acids. Although not significantly different, the overall rates of proton production appear to be suppressed in

the insulin treated group compared to the aerobic period due a slight impairment of the rate of glycolysis during reperfusion. However, as cardiac function is significantly impaired during reperfusion, the rate of proton production normalized to cardiac power is significantly elevated during reperfusion compared to the aerobic period. Therefore the beneficial effects of insulin would be two-fold, by increasing ATP production by the stimulation of both glycolysis and glucose oxidation, which would outweigh the detrimental effects of elevated proton production.

A surprising finding from this study is that in the presence of high fat insulin impairs the recovery of contractile function during reperfusion. Like the no fat condition, insulin stimulates both glycolysis and glucose oxidation during the aerobic period and the reperfusion period. However, since insulin produces a greater stimulation of glycolysis than glucose oxidation, there is an increased in H^+ production from glucose metabolism during reperfusion (Figure 3-7). This may explain the detrimental effects of insulin during high fat perfusion.[31, 32] As rates of fatty acid oxidation increase, this increases intracellular concentrations of acetyl-CoA which is an allosteric inhibitor of PDH. Indeed, the failure of insulin to inhibit AMPK activity may also contribute to this phenomenon. AMPK activation may leads to an acceleration of glucose uptake thus supplying substrate for glycolysis, but also leads to phosphorylation of ACC, leading to a decrease in malonyl-CoA levels, and an acceleration of fatty acid oxidation, which suppresses glucose oxidation by the above-mentioned mechanism.

However, H^+ production during reperfusion in the high fat plus insulin group is not increased significantly from values in the no fat with insulin group, despite dramatic differences in the effect on insulin on post-ischemic contractile function. The benefits of

insulin on recovery of contractile function in the no fat hearts may be related to an increase in energy supply to these hearts. Perfusion with glucose alone renders the heart energetically starved and potentially compromised, leading to reduced glycogen stores, an increase the AMP/ATP ratio and activation of AMPK. Insulin decreases the AMP/ATP ratio during aerobic perfusion, and increases ATP at the end of reperfusion in the no fat group (Table 3-3). Therefore the cardioprotective effect of insulin may be due an increase in the insulin-stimulated glucose uptake and an improvement of overall cardiac energetics, which would results in a reduction in AMPK activity. However in the presence of high fat, insulin does not change the AMP/ATP ratio during either aerobic perfusion or during reperfusion, which correlated with the lack of effect on insulin on AMPK activity.

In contrast to previous results in rat hearts where AMPK activity remains elevated at the end of reperfusion, in the present study AMPK activity returns to baseline aerobic values by the end of reperfusion.[63] To the author's knowledge no study has addressed the time-course of AMPK activity during reperfusion following ischemia. Therefore AMPK activity may potentially normalize very quickly during reperfusion in the mouse heart, which may account for the lack of an increase in palmitate oxidation during reperfusion (Figure 3-7). This may suggest that there may be a differential effect of AMPK activation on early versus late reperfusion. Further studies are required to address the effect of AMPK activation/inhibition not only during the different time periods of reperfusion but also to differentiate the effects of AMPK during ischemia from the effects during reperfusion.

A recent study by Horman *et al.* suggests that insulin may inhibit AMPK via Akt phosphorylation of Ser485/491 on the $AMPK_{\alpha}$, which corresponds with a previous report that Akt activity negatively correlates with AMPK Thr172 phosphorylation.[154, 258] Contradictory results were obtained in the present study. The addition of insulin produces an increase in phosphorylation of Akt at Ser473, which is indicative of Akt activity. Despite this apparent activation of this insulin-signaling pathway, there is no downstream inhibition of AMPK in the no fat group. This difference may be due to the use of a supraphysiological insulin concentration of 100 nmol/L in the Horman study, compared with a more physiological concentration (0.6 nmol/L) in the present study. The only clinical study to report arterial insulin concentrations after GIK administration was a study of coronary surgery patients with Type II diabetes. High-dose GIK therapy increases insulin concentrations to 10.3 nmol/L following administration.[259]

Considerable interest has also focused on GIK in both AMI and cardiac surgery patients.[131, 150] However the recent large CREATE trial did not show GIK effectiveness. It is possible that the discrepant results may be related to differences in plasma fatty acid levels in the patient population. Despite the fact that some of the benefits of GIK have been attributed to a lowering of plasma free fatty acids, previous trials have not determined what effect GIK has on plasma fatty acids levels post-MI or post-surgery. In addition, the time of administration of GIK may play a key role in its efficacy, as the only group in the CREATE-ECLA trial with a trend to improvement was the population that received GIK prior to percutaneous coronary intervention, but this did reach statistical significance in a more recent trial.[150, 260]

Limitations

This study utilized the isolated working heart method, which has some limitations. These hearts are perfused with crystalloid perfusate, which requires relatively high rates of coronary flow due to the low O_2 carrying capacity of the perfusate. Despite this drawback, the crystalloid perfusate is capable of delivering the required amounts of O_2 due to a high partial pressure of O_2 by gassing the perfusate with carbogen (95 % O_2 and 5 % CO_2). Even gassing with 70 % O_2 is sufficient to sustain basal function and an increase in function in response to a positive inotrope without sustaining injury.[261]

Although our perfusion conditions attempt to match plasma levels of fatty acids, glucose and insulin, our perfusate composition is not completely physiological due to the lack of other hormones, adipokines, lipoproteins and carbohydrates (lactate and pyruvate). In addition we do not assess the contribution of endogenous substrates (glycogen and triglycerides) to the overall metabolic rates in these studies. This study was performed in the presence or absence of 100 μU/mL of insulin in order to reproduce previous experimental conditions in the literature.[248-250] However, it is worth noting that the condition of no insulin would never be observed physiologically. In addition, physiologically the heart is exposed to a complex milieu of fatty acids with oleate and palmitate being the most prominent. We utilized palmitate exclusively as our source of fatty acids due to a number of technical reasons (the double bond in oleate could potentially be oxidized by the protocol used to bind fatty acids to albumin and radiolabelled oleate is more expensive than palmitate). In addition, previous reports have demonstrated that overall rates of fatty acid oxidation did not differ between hearts

perfused with oleate and palmitate as their source of fat.[31, 262] Despite the similar oxidative rates from these different fatty acids, their potential to induce insulin resistance differs markedly. A number of basic and epidemiological studies have demonstrated correlation between unsaturated fatty acids, in particular palmitic acid, with insulin resistance.[263-265] In contrast, monounsaturated fatty acids such as oleate are associated with an increase in insulin sensitivity in healthy individuals and diabetic patients.[263, 266, 267] In particular, a recent study has suggested that oleate can reverse palmitate-induced insulin resistance by channeling palmitate into TG and reducing the formation of diacylglycerol and subsequent activation of PKC/nuclear factor κB pathway and by upregulating β-oxidation via peroxisome proliferator-activated receptor thus reducing lipid metabolite accumulation.[268] Therefore in the present study the use of palmitate may induce an insulin-resistant state that may modify the interpretation of the data, however insulin resistance was not examined. We acknowledge that modification of the perfusate composition and measurement of the turnover of endogenous substrates may alter the observed changes in myocardial metabolism and post-ischemic cardiac function and modify our interpretation of the data.

In summary, this study demonstrates that the cardioprotective effect of insulin and its ability to modify AMPK activity in the isolated perfused mouse heart is highly dependent on the concentration of fatty acids present in the perfusate. However, other insulin signaling pathways, such as modification of glucose metabolism are still intact in the presence of clinically relevant high concentrations of fatty acids. These findings have important implications in the use of insulin therapy for the treatment of ischemic heart disease.

CHAPTER 4.

Suppression of AMP-Activated Protein Kinase Activity Does Not Impair Recovery of Contractile Function During Reperfusion of Ischemic Hearts

My role in this work involved performing all the experiments (except those noted below) as well as writing the manuscript. Cory Wagg performed the isolated working mouse hearts and Ken Strynadka and Panakkezhum Thomas performed the HPLC analysis of the adenine nucleotides.

CHAPTER 4.

Suppression of AMP-Activated Protein Kinase Activity Does Not Impair Recovery of Contractile Function During Reperfusion of Ischemic Hearts

Abstract

Activation of AMPK is suggested to benefit the heart during I/R by increasing energy production. While AMPK stimulates glycolysis, mitochondrial oxidative metabolism is the major source of cardiac ATP production during reperfusion of ischemic hearts. Stimulating AMPK increases mitochondrial fatty acid oxidation, but this is usually accompanied by a decrease in glucose oxidation. To examine the relationship between AMPK activation and cardiac energy substrate metabolism, we measured energy metabolism in hearts from mice expressing a dominant negative $AMPK_{\alpha 2}$ subunit (AMPK Dn). Isolated working mouse hearts were subjected to 20 min global no-flow ischemia followed by 40 min of aerobic reperfusion with Krebs-Henseleit solution containing 5mM [U-^{14}C]glucose, 0.4 mM [9, 10-^{3}H]palmitate, and 100 μU/ml insulin. As expected, the AMPK Dn hearts have reduced AMPK activity at the end of reperfusion, (82 ± 9 vs. 141 ± 7 pmol/mg/min, n = 4 per group, $P < 0.05$) with no

changes in high-energy phosphates. Despite this, AMPK Dn hearts have improved recovery of function during reperfusion compared to Wt controls (15 ± 0.8 vs. 10 ± 1.4 bpm·mmHg·10^{-3}, n = 4 per group, $P < 0.05$). During reperfusion, fatty acid oxidation provides 52.6 ± 6.4 % (n = 4) of total TCA cycle in the AMPK Dn hearts compared to 55.0 ± 3.2 % (n = 4) in the Wt hearts. Since insulin can inhibit both AMPK activation and fatty acid oxidation, we also examined functional recovery from ischemia in AMPK Dn and Wt hearts perfused in the absence of insulin. In the absence of insulin, fatty acid oxidation provides a greater proportion of energy production during reperfusion in both AMPK Dn and Wt hearts (66.4 ± 9.4 % vs. 85.3 ± 4.3 %, respectively, n = 5 per group, $P < 0.05$). Functional recovery is similar in both groups, despite a decreased in ischemic-induced activation of AMPK in the AMPK Dn hearts. These data demonstrate that suppression of cardiac AMPK activity does not produce an energetically compromised phenotype and does not impair, but may in fact improve, the recovery of function following ischemia.

Introduction

Myocardial ischemia occurs when the oxygen requirement of the heart exceeds the oxygen supplied via the coronary circulation and is a devastating cause of morbidity and mortality worldwide. A contributing factor to I/R injury is the modification of myocardial energy metabolism.[5, 54, 122] The majority of the energy requirement of the normal well-perfused heart is met by the oxidation of fatty acids, with the remainder coming from the oxidation of carbohydrates.[5, 54, 122] However during ischemia, mitochondrial oxidative metabolism is suppressed and anaerobic glycolysis becomes an important source of ATP.[133] As pyruvate from glycolysis cannot be subsequently oxidized, there is a net production of deleterious byproducts (lactate and H^+ from the hydrolysis of glycolytically-derived ATP).[31, 32, 121, 269, 270]

An important regulator of myocardial energy metabolism during ischemia is AMPK.[251, 252] We and others have shown that AMPK is rapidly activated during myocardial ischemia and is associated with dramatic changes in the control of glucose and fatty acid metabolism.[63, 73, 153, 178, 192, 204, 221, 240] However the role of AMPK in the protection of the ischemic myocardium is still controversial.[220] AMPK activation during ischemia is believed to be an attempt to restore cardiac energy balance by stimulating ATP generating pathways.[220] Indeed, during ischemia, AMPK promotes glycolysis by stimulating glucose uptake [192] and indirectly activating PFK-1 [204], thus producing an anaerobic source of ATP. During reperfusion, AMPK activates fatty acid oxidation by phosphorylating and inhibiting ACC, thus removing the malonyl-CoA inhibition of fatty acid transport into the mitochondria.[63, 73] However, AMPK activation may also

exacerbate ischemic injury due to stimulation of fatty acid oxidation at the expense of glucose oxidation, which may increase the myocardial acid load and subsequent post-ischemic contractile dysfunction.[31, 32, 134]

Few studies have directly associated AMPK activity with functional recovery during reperfusion following ischemia due to the unavailability of pharmacological agents that modify AMPK activity in an isolated heart preparation.[192, 221, 222] Using an $AMPK_{\alpha 2}$ KD (K45R) transgenic mouse, Russell *et al.* demonstrate that the KD mice have worse cardiac function during aerobic perfusion, low-flow ischemia, and reperfusion, which is associated with the inability to increase glucose uptake and lactate production.[192] However, recent studies using an $AMPK_{\alpha 2}$ KO show no functional depression during reperfusion following ischemia in the absence of fatty acids or a slight depression in the immediate reperfusion period following low flow ischemia in the presence of a low concentration of fatty acids.[221, 222] Thus the role AMPK plays in cardioprotection is still controversial and may be highly dependent on substrate availability and on the balance of the effects of AMPK on glucose and fatty acid metabolism.

The purpose of the present study is to investigate the effect of modifying AMPK activity on myocardial oxidative metabolism and ischemic tolerance in an isolated working heart model. We utilized isolated working hearts from transgenic mice expressing an $AMPK_{\alpha 2}$ Dn, which reduces ischemia-induced AMPK activation and glucose uptake.[223] Due to the complex regulation of AMPK by fatty acids and insulin [240], metabolism and functional recovery in the presence and absence of insulin will be examined.

Materials and 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.

Transgenic Mice

AMPK Wt and Dn mice were a kind gift of Rong Tian (Harvard Medical School). These mice were generated as previously described, by replacing the aspartic acid at residue 157 with an alanine, which renders the catalytic subunit inactive.[223, 271] This recombinant DNA construct under the control of the α-myosin heavy chain promoter, was injected into fertilized Friend virus B-type mouse oocytes to generate the AMPK Dn mice.[223]

Isolated Working Mouse Heart Perfusions

AMPK Wt and Dn mouse hearts were perfused in the working perfused mode as previously described in Chapter 2.[239] The oxygenated Krebs-Henseleit solution contained 1.2 mM palmitate, 5 mM glucose in the presence or absence of 100 μU/mL insulin. Spontaneously beating hearts were perfused at a constant left atrial preload pressure of 11.5 mmHg and a constant aortic afterload pressure of 50 mmHg for 30 min aerobic perfusion followed by 20 min of global no-flow ischemia and 40 min of aerobic

reperfusion. At the end of the perfusion protocols, the hearts were quick frozen in liquid nitrogen with Wollenberger tongs, and stored at -80 °C.

Glucose and palmitate oxidation were measured by quantitative collection of the $^{14}CO_2$ and $^{3}H_2O$, respectively, derived from [U-^{14}C]glucose and [9, 10-^{3}H]palmitate, as described in Chapter 2.[134, 238]

Tissue Extractions

AMPK was isolated and assayed using the AMARA peptide as described in Chapter 2. Immunoblotting was performed on cleared homogenates for total and phosphorylated AMPK and ACC as described in Chapter 2. Adenine nucleotides were extracted for HPLC analysis as described in Chapter 2. Glycogen content was measured as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± S.E.M. The data were analyzed with the statistical program Instat 2.01 and GB-stat. Two-way repeated measures ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for cardiac function (rate pressure product (RPP)). One-way or two-way ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for the metabolic data and AMPK data. Values of $P < 0.05$ were considered significant.

Results

Baseline Aerobic Cardiac Function and Metabolism in Hearts perfused in the Presence of Insulin

In the presence on insulin, contractile function during the aerobic period is not significantly different between the Wt and AMPK Dn hearts as seen in Figure 4-1 and Table 2, although there is a trend towards elevated heart rate and cardiac output. AMPK Dn hearts have significantly higher rates of glucose oxidation during the aerobic perfusion (2061 ± 158 vs. 1089 ± 117 nmol/g dry wt/min, n = 4 per group, $P < 0.05$, Figure 4-2A), however there is no difference in rates of palmitate oxidation (Figure 4-2B). Therefore total acetyl-CoA production is elevated in AMPK Dn hearts (5.47 ± 0.57 vs. 3.28 ± 0.30 μmol/g dry wt/min, n = 4 per group, $P < 0.05$, Figure 4-2C).

Cardiac Function and Metabolism in Hearts Subjected to Ischemia and Reperfusion in the Presence of Insulin

When hearts were subjected to 20 min ischemia followed by 40 min of reperfusion in the presence of insulin, AMPK Dn hearts recover to a greater extent than Wt hearts (14.3 ± 0.9 vs. 9.3 ± 1.3 BPM•mmHg•10^{-3}, n = 4 per group, $P < 0.05$, Figure 4-1 and Table 1). I/R blunts glucose oxidation in the AMPK Dn group, so that glucose oxidation rates no longer differed from the Wt group (Figure 4-2A). In addition, rates of palmitate oxidation did not differ during reperfusion (Figure 4-2B). Therefore during

reperfusion overall acetyl-CoA production is not different between the groups (Figure 4-2C).

Analysis of tissue at the end of reperfusion shows that even in the presence of insulin, AMPK Dn hearts have a significant reduction in AMPK activity (81.6 ± 8.9 vs. 144.4 ± 6.5 pmol/mg/min, n = 4 per group, $P < 0.05$, Figure 4-3) and phosphorylation of its downstream target ACC (280 kDa isoform: 1.17 ± 0.17 vs. 0.61 ± 0.13 arbitrary units, n = 4 per group $P < 0.05$, Figure 4-3). Despite this change in AMPK activation, there is no significant difference in the concentration of adenine nucleotides at the end of reperfusion, however insulin did increase total glycogen content in the AMPK Dn hearts (13.6 ± 3.0 vs. 6.4 ± 0.4 μmol/g dry wt, n = 4 per group, $P < 0.05$, Table 4-3).

Baseline Aerobic Cardiac Function and Metabolism in Hearts Perfused in the Absence of Insulin

In the absence of insulin, contractile function during the aerobic perfusion period is not significantly different between the Wt and AMPK Dn hearts as seen in Figure 4-4 and Table 4-2. Although there is no difference in rates of palmitate oxidation (Figure 4-4B) between the groups, there is a trend to elevated glucose oxidation in the AMPK Dn group (p = 0.071). In addition, total TCA cycle acteyl-CoA production and percent TCA cycle production did not differ between the groups (Figure 4-5C and D).

Cardiac Function and Metabolism in Hearts Subjected to Ischemia and Reperfusion in the Absence of Insulin

Wt and AMPK Dn hearts subjected to 20 min of global ischemia and reperfused for 40 min recover to the same level of cardiac function (Figure 4-4 and Table 4-2). During reperfusion there is a trend towards elevated levels of glucose oxidation and a suppression of palmitate oxidation in the AMPK Dn hearts, however these did not reach statistical significance (Figure 4-5A and B). Although total acetyl-CoA production during reperfusion is not different between the groups (Figure 4-4C), the AMPK Dn hearts have a higher reliance on glucose as a source of acetyl-CoA (33.6 ± 9.4% vs. 14.6 ± 4.3%, n = 5 per group, $P < 0.05$, Figure 4-5D).

Analysis of tissue at the end of reperfusion show that AMPK Dn hearts have a significant reduction in AMPK activity (76.6 ± 11.1 vs. 183.8 ± 51.4 pmol/mg/min, n = 5 per group, $P < 0.05$, Figure 4-5) and phosphorylation of its downstream target ACC (280 kDa isoform: 0.96 ± 0.16 vs. 0.27 ± 0.04 arbitrary units and 265 kDa isoform: 1.27 ± 0.39 vs. 0.25 ± 0.08 arbitrary units, n = 5 per group, $P < 0.05$, Figure 4-5). Despite this change in AMPK activation, there is no significant difference in the concentration of adenine nucleotides or total glycogen content at the end of reperfusion (Table 4-3).

Table 4-1. Parameters of contractile function in isolated working mouse hearts during aerobic perfusion and reperfusion of ischemia hearts perfused in the presence of insulin

	Aerobic Perfusion		Reperfusion	
	AMPK Wt (n = 4)	AMPK Dn (n = 4)	AMPK Wt (n = 4)	AMPK Dn (n = 4)
Heart Rate (beats/min)	263 ± 18	303 ± 15	195 ± 13 *	240 ± 6 *
Peak Systolic Pressure (mmHg)	70 ± 1	69 ± 1	49 ± 3 *	55 ± 3 *
Coronary Flow (mL/min)	1.7 ± 0.2	2.2 ± 0.3	1.0 ± 0.1 *	1.4 ± 0.2 *
Cardiac Output (mL/min)	8.4 ± 0.9	11.5 ± 1.1	1.1 ± 0.3 *	3.6 ± 1.1 *
Cardiac Power (mJoule)	65 ± 6	89 ± 8	6 ± 2 *	23 ± 7 *

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Table 4-2. Parameter of contractile function in isolated working mouse hearts during aerobic perfusion and reperfusion of ischemia hearts perfused in the absence of insulin.

	Aerobic Perfusion		Reperfusion	
	AMPK Wt (n = 5)	AMPK Dn (n = 5)	AMPK Wt (n = 5)	AMPK Dn (n = 5)
Heart Rate (beats/min)	290 ± 8	278 ± 9	157 ± 47 *	185 ± 21 *
Peak Systolic Pressure (mmHg)	75 ± 1	75 ± 1	44 ± 9 *	44 ± 6 *
Coronary Flow (mL/min)	2.4 ± 0.2	2.3 ± 0.1	0.6 ± 0.4 *	0.4 ± 0.2 *
Cardiac Output (mL/min)	9.5 ± 0.6	8.1 ± 0.7	1.2 ± 0.7 *	0.8 ± 0.6 *
Cardiac Power (mJoule)	80 ± 6	74 ± 7	9 ± 5 *	6 ± 5 *

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Table 4-3. Adenine nucleotide and glycogen content at the end of reperfusion in isolated working mouse hearts.

	No Insulin		Insulin	
	AMPK Wt (n = 5)	AMPK Dn (n = 5)	AMPK Wt (n = 4)	AMPK Dn (n = 4)
AMP (μmol/g dry wt)	1.9 ± 0.6	2.3 ± 0.7	0.58 ± 0.15	0.81 ± 0.25
ADP (μmol/g dry wt)	3.9 ± 0.4	4.3 ± 0.5	2.3 ± 0.6	2.9 ± 0.6
ATP (μmol/g dry wt)	7.2 ± 1.6	6.9 ± 0.7	7.8 ± 2.1	8.3 ± 2.2
AMP/ATP	0.35 ± 0.14	0.38 ± 0.13	0.08 ± 0.02	0.10 ± 0.02
ADP/ATP	0.63 ± 0.16	0.67 ± 0.13	0.30 ± 0.03	0.36 ± 0.04
Glycogen (μmol/g dry wt)	8.4 ± 2.9	10.2 ± 2.1	6.4 ± 0.4	13.6 ± 3.0*

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding wildtype perfusion group.

Figure 4-1: AMPK Dn hearts have improved recovery in the presence of insulin.

A) Cardiac function, B) percent cardiac function and C) cardiac power in the presence of insulin (n = 4 per group) in isolated mouse hearts during aerobic perfusion and reperfusion. Values represent mean ± SEM. Differences were determined using a 2-way repeated measures ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from the wildtype group.

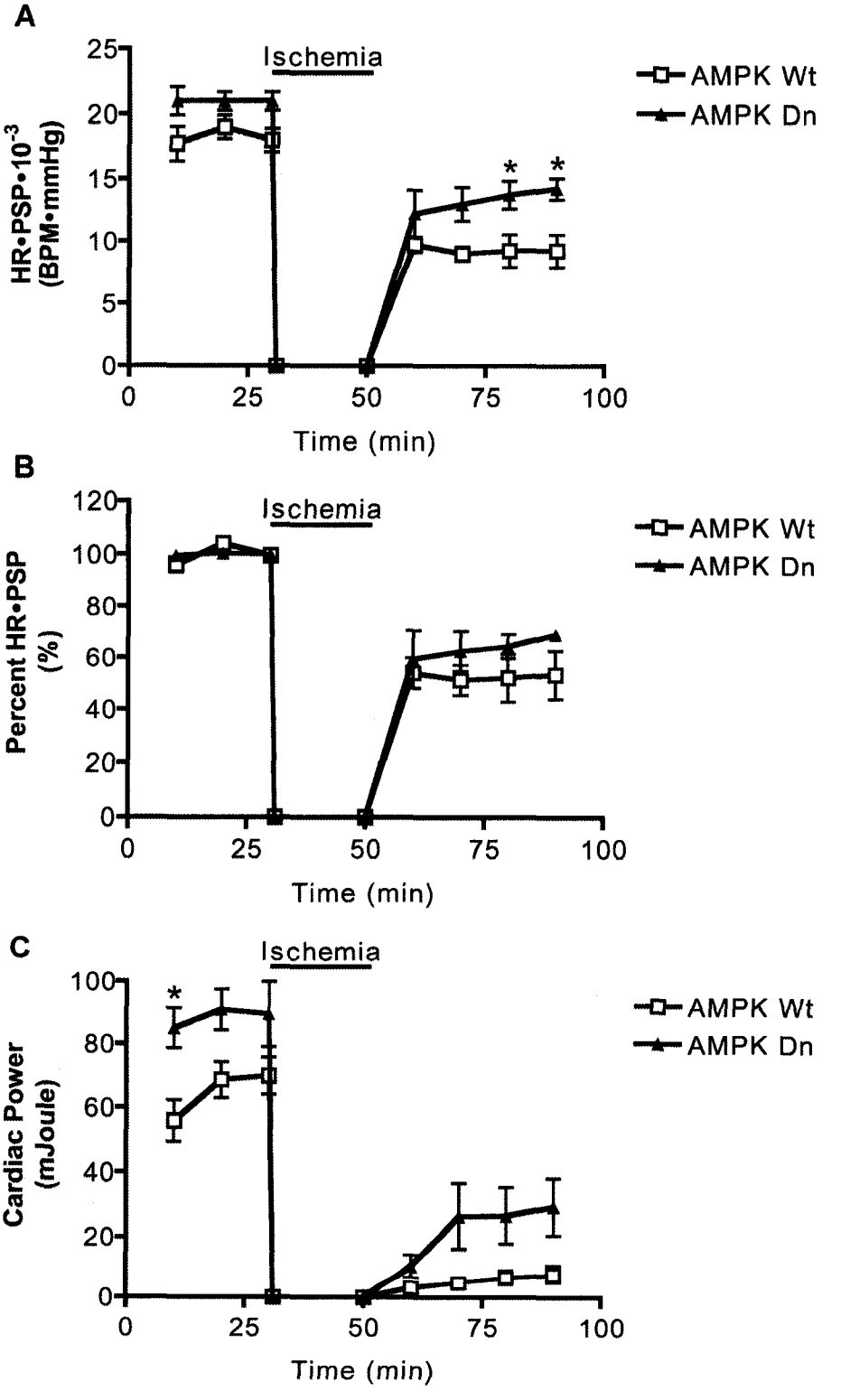

A
Ischemia
AMPK Wt
AMPK Dn
HR•PSP•10-3 (BPM•mmHg)
Time (min)
B
Ischemia
AMPK Wt
AMPK Dn
Percent HR•PSP (%)
Time (min)
C
Ischemia
AMPK Wt
AMPK Dn
Cardiac Power (mJoule)
Time (min)

Figure 4-1

Figure 4-2: Glucose oxidation and total acetyl-CoA production is elevated in AMPK Dn hearts during aerobic perfusion in the presence of insulin.

A) Glucose oxidation, B) palmitate oxidation, C) total aceyl-CoA production and D) percent TCA cycle contribution in isolated working mouse hearts subjected to I/R in the presence of insulin (n = 4 per group). Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

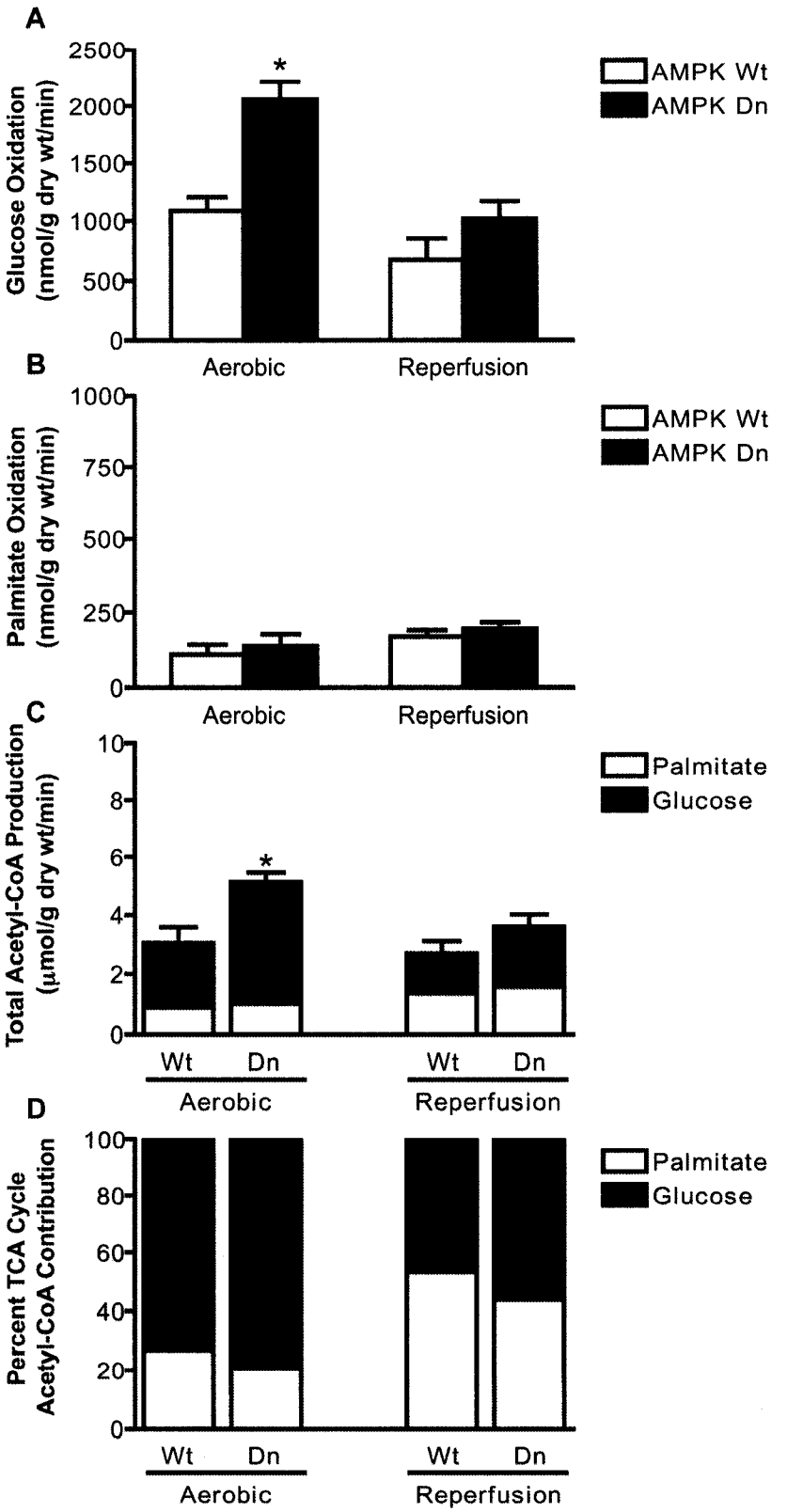

A
Glucose Oxidation (nmol/g dry wt/min)
2500
2000
1500
1000
500
0
*
AMPK Wt
AMPK Dn
Aerobic
Reperfusion
B
Palmitate Oxidation (nmol/g dry wt/min)
1000
750
500
250
0
AMPK Wt
AMPK Dn
Aerobic
Reperfusion
C
Total Acetyl-CoA Production (µmol/g dry wt/min)
10
8
6
4
2
0
*
Palmitate
Glucose
Wt
Dn
Wt
Dn
Aerobic
Reperfusion
D
Percent TCA Cycle Acetyl-CoA Contribution
100
80
60
40
20
0
Palmitate
Glucose
Wt
Dn
Wt
Dn
Aerobic
Reperfusion

Figure 4-2

Figure 4-3: Expression of AMPK Dn reduces AMPK activity at the end of reperfusion in the presence of insulin.

A) AMPK activity, and B) ACC phosporylation status in isolated working mouse hearts subjected to I/R in the presence of insulin (n = 4 per group). Values represent mean ± SEM. Differences were determined using a t-test (AMPK activity) or 2-way ANOVA with a Bonferroni post hoc test (ACC phosphorylation).

* represents $P < 0.05$, significantly different from the wildtype control group.

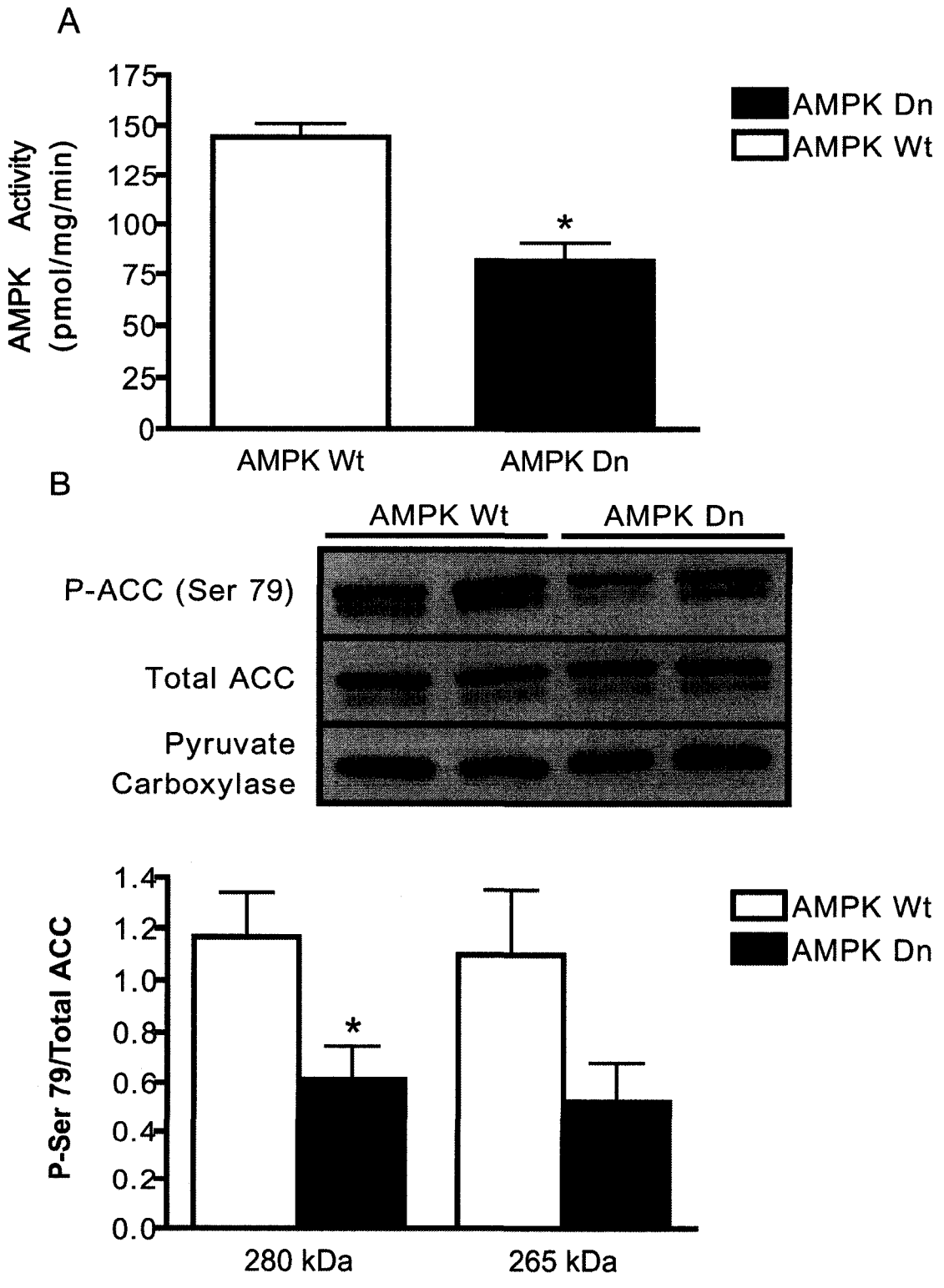

A
AMPK Activity (pmol/mg/min)
175
150
125
100
75
50
25
0
AMPK Dn
AMPK Wt
*
AMPK Wt
AMPK Dn
B
AMPK Wt
AMPK Dn
P-ACC (Ser 79)
Total ACC
Pyruvate Carboxylase
P-Ser 79/Total ACC
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
AMPK Wt
AMPK Dn
*
280 kDa
265 kDa

Figure 4-3

Figure 4-4: AMPK Dn hearts have similar post-ischemic cardiac function to Wt hearts in the absence of insulin.

A) Cardiac function B) percent cardiac function and C) cardiac power in the presence of insulin (n = 5 per group) in isolated mouse hearts during aerobic perfusion and reperfusion. Values represent mean ± SEM. Differences were determined using a 2-way repeated measures ANOVA with a Bonferroni post hoc test.

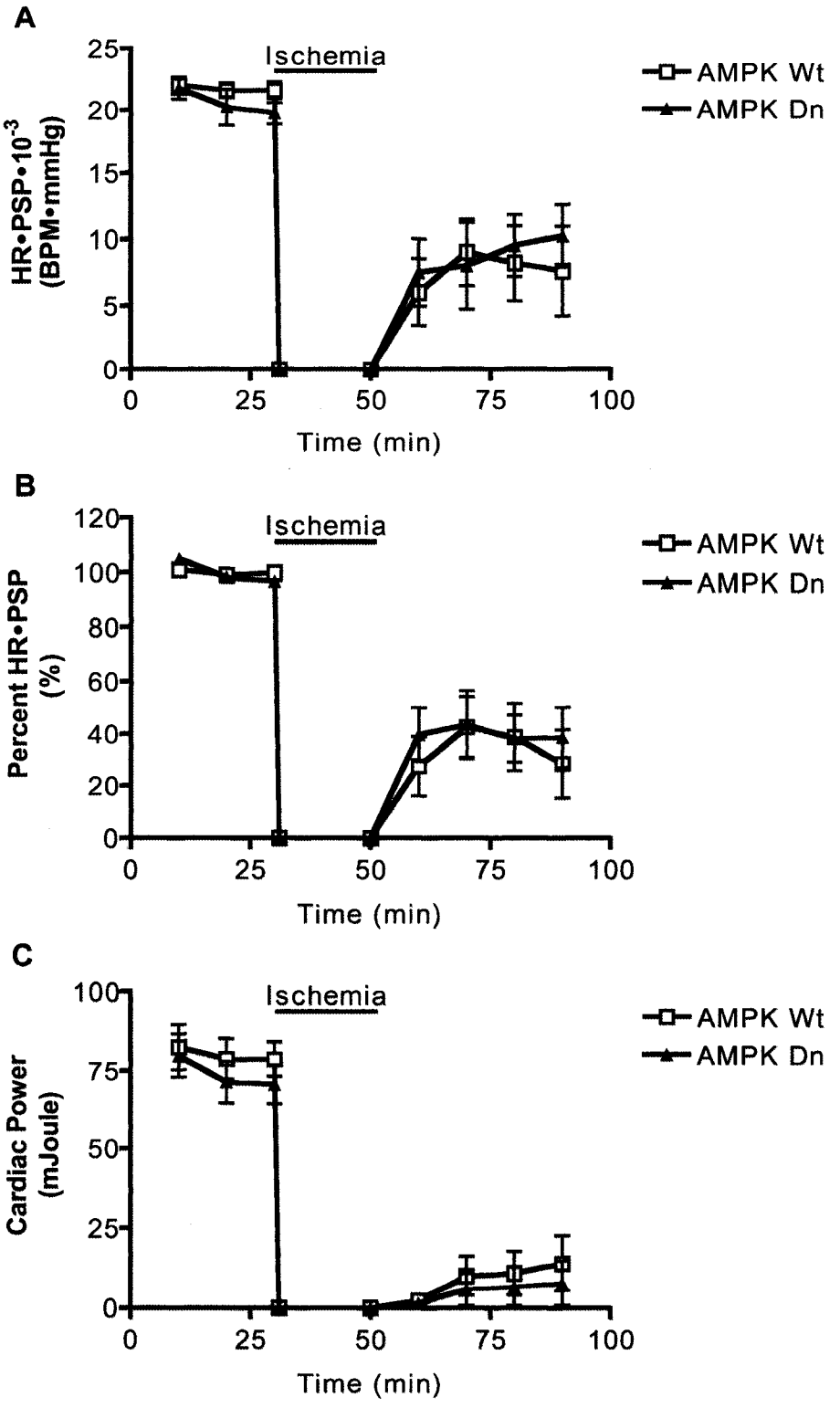

A
Ischemia
AMPK Wt
AMPK Dn
HR•PSP•10-3
(BPM•mmHg)
25
20
15
10
5
0
0
25
50
75
100
Time (min)
B
Ischemia
AMPK Wt
AMPK Dn
Percent HR•PSP
(%)
120
100
80
60
40
20
0
0
25
50
75
100
Time (min)
C
Ischemia
AMPK Wt
AMPK Dn
Cardiac Power
(mJoule)
100
75
50
25
0
0
25
50
75
100
Time (min)

Figure 4-4

Figure 4-5: AMPK Dn hearts have a greater reliance on glucose oxidation during reperfusion in the absence of insulin.

A) Glucose oxidation, B) palmitate oxidation, C) total aceyl-CoA production and D) percent TCA cycle contribution in isolated working mouse hearts subjected to I/R in the absence of insulin (n = 5 per group). Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

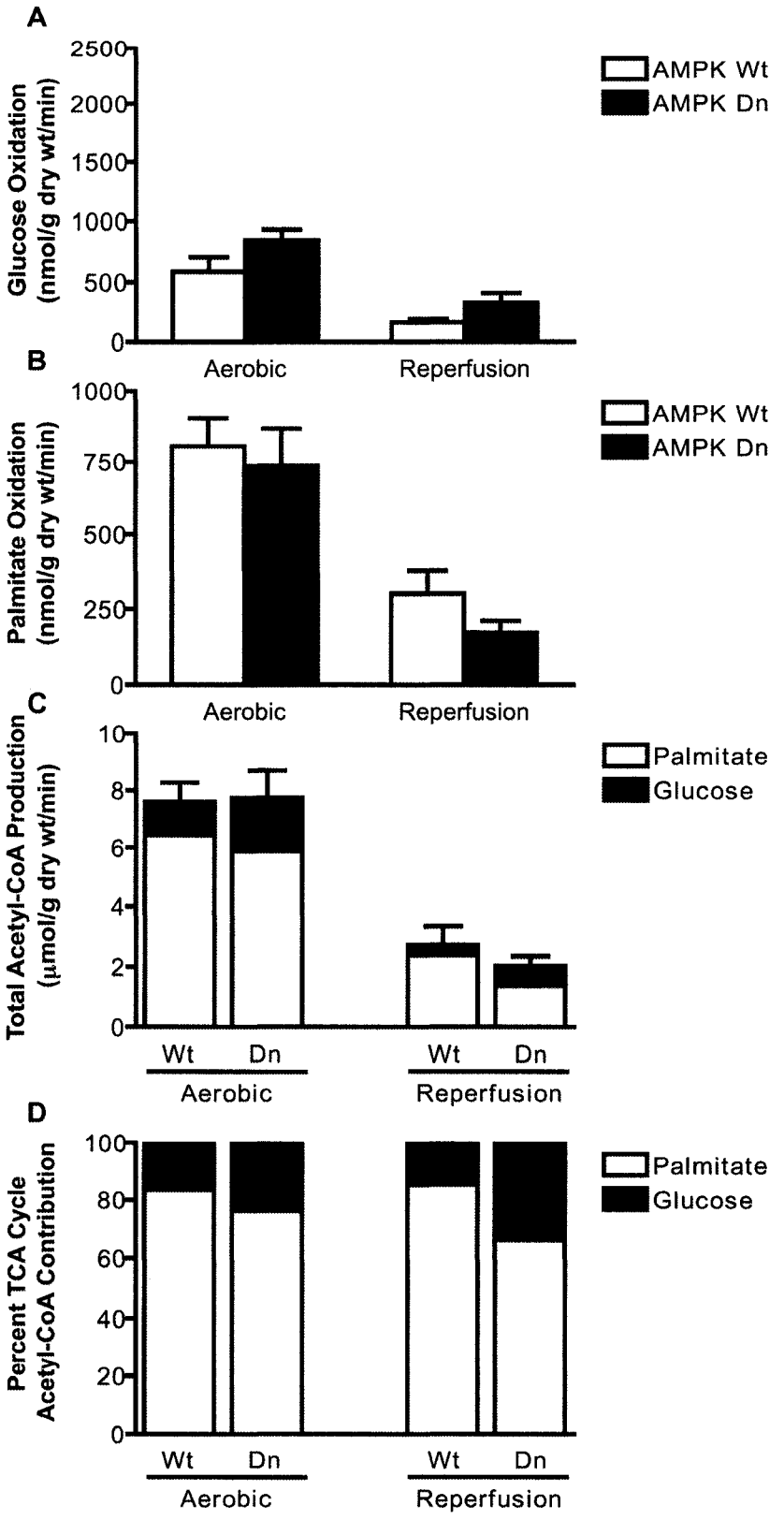

A
Glucose Oxidation (nmol/g dry wt/min)
2500
2000
1500
1000
500
0
Aerobic
Reperfusion
AMPK Wt
AMPK Dn
B
Palmitate Oxidation (nmol/g dry wt/min)
1000
750
500
250
0
Aerobic
Reperfusion
AMPK Wt
AMPK Dn
C
Total Acetyl-CoA Production (µmol/g dry wt/min)
10
8
6
4
2
0
Wt
Dn
Wt
Dn
Aerobic
Reperfusion
Palmitate
Glucose
D
Percent TCA Cycle Acetyl-CoA Contribution
100
80
60
40
20
0
Wt
Dn
Wt
Dn
Aerobic
Reperfusion
Palmitate
Glucose

Figure 4-5

Figure 4-6: Expression of AMPK Dn reduces AMPK activity at the end of reperfusion in the absence of insulin.

A) AMPK activity, and B) ACC phosporylation status in isolated working mouse hearts subjected to I/R in the absence of insulin (n = 5 per group). Values represent mean ± SEM. Differences were determined using a t-test (AMPK activity) or 2-way ANOVA with a Bonferroni post hoc test (ACC phosphorylation).

* represents $P < 0.05$, significantly different from the wildtype control group.

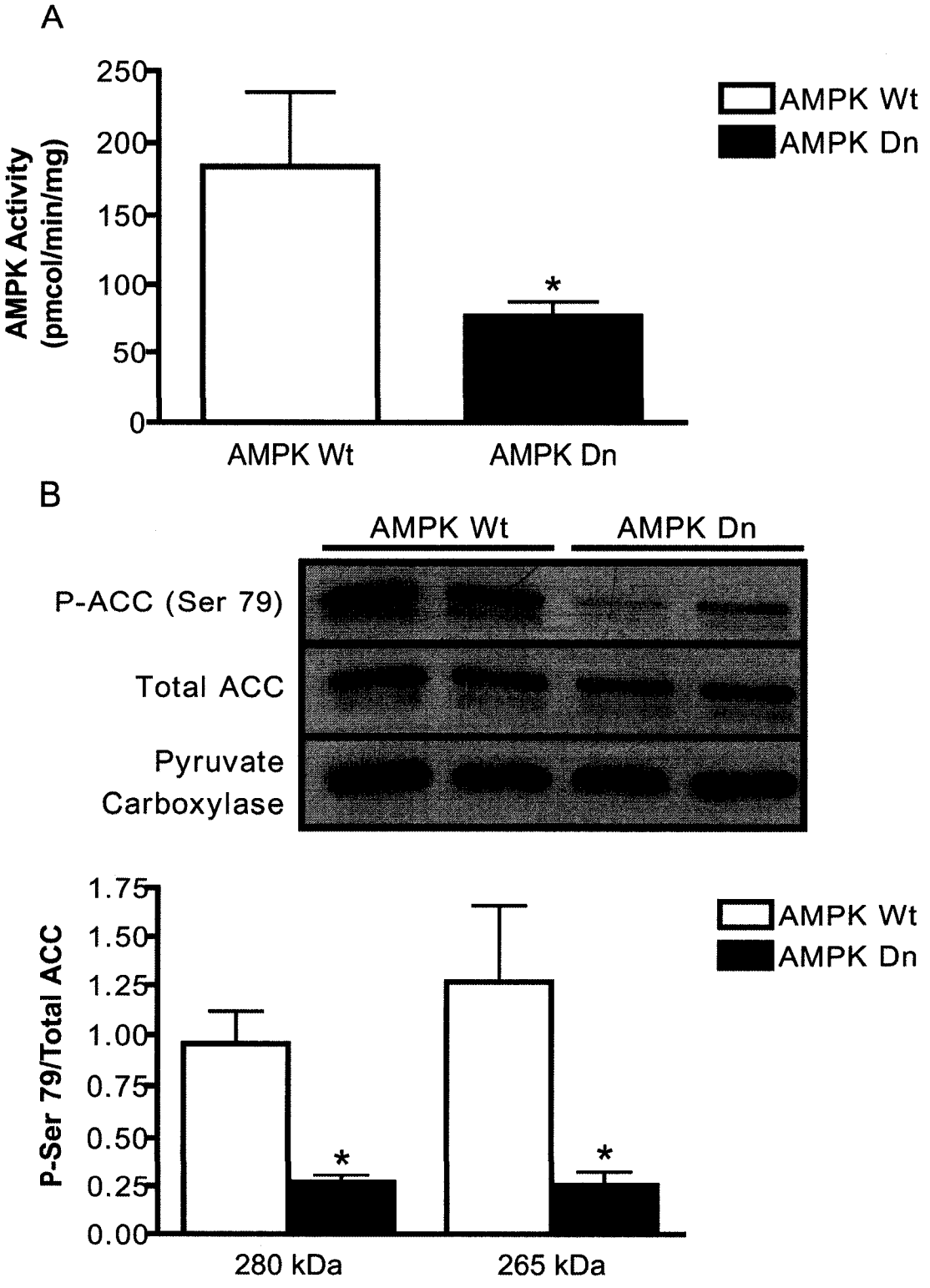

A
AMPK Activity (pmcol/min/mg)
250
200
150
100
50
0
AMPK Wt
AMPK Dn
*
AMPK Wt
AMPK Dn
B
AMPK Wt
AMPK Dn
P-ACC (Ser 79)
Total ACC
Pyruvate Carboxylase
P-Ser 79/Total ACC
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
280 kDa
265 kDa
AMPK Wt
AMPK Dn

Figure 4-6

Discussion

Only a few studies directly associate AMPK activity with functional recovery during reperfusion following ischemia, and these have reached differing conclusions.[192, 221, 222] Therefore the role that AMPK plays in the cardioprotection of the ischemic myocardium is still controversial.[220] In this study we utilized isolated working mouse hearts from AMPK Dn mice to examine the direct correlation of reduced ischemic-induced AMPK activity on myocardial oxidative metabolism and functional recovery during reperfusion following ischemia. Using this approach we made a number of important novel findings. First, AMPK Dn hearts perfused in the presence of a normal concentration of fatty acids are not functionally compromised during reperfusion following ischemia in the absence of insulin, but the blunting of ischemia-induced AMPK activation may be cardioprotective in the presence of insulin. Second, the inability to activate AMPK does not produce a metabolically or functionally compromised heart.

The AMPK Dn mice used in this study are grossly normal, with no difference in heart weight, body weight or survival up to one year.[223] The $AMPK_{\alpha 2}$-specific reduction in AMPK activity does not affect baseline cardiac function (an observation confirmed in our studies), however it does lead to a greater rise in end-diastolic pressure when those hearts are subjected to global ischemia, which is associated with a larger fall in ATP levels.[223] Unfortunately, only rates of glucose uptake were assessed in that study (with no measure of oxidative rates), and those hearts were not reperfused following ischemia. Of interest is that Xing *et al.* [223] observed that there is a slower rate of decline of pH in

the AMPK Dn hearts, which would suggest a reduced accumulation of intracellular H^+s, presumably from improved coupling between glycolysis and glucose oxidation. Although we did not directly assess rates of glycolysis in this study, we do observe that AMPK Dn hearts have a greater reliance on glucose as a source of mitochondrial acetyl-CoA production, which suggests that more pyruvate and H^+s are being consumed aerobically (Figure 4-2 and 4-4).

The major previous study correlating AMPK activity to reperfusion contractile function used an $AMPK_{\alpha 2}$ KD transgenic mouse.[192] Russell *et al.* demonstrate that the KD mice have worse cardiac function during aerobic perfusion, low-flow ischemia and reperfusion than controls, which is associated with the inability to increase glucose uptake and lactate production.[192] Related to these metabolic and functional changes, KD hearts have increased cellular damage as observed by an increase in LDH and creatine kinase release, and an increase in apoptotic cells. Interestingly, these hearts were perfused with low levels of fatty acids (0.4 mM), which may lessen the detrimental effect of elevated fatty acid oxidation on glucose oxidation and H^+ production. Surprisingly, rates of fatty acid oxidation and glucose oxidation do not change between the KD and Wt groups.[192] On the contrary, in our study we observe that AMPK Dn hearts have a greater reliance on glucose oxidation for oxidative metabolism and that functional recovery was either comparable to Wt hearts in the absence of insulin or slightly better in the presence of insulin (Figure 4-1, 4-2 and 4-4). These contradictory results may be due to the fact that the KD mutation almost completely abolishes $AMPK_{\alpha 2}$ activity and also substantially blunts $AMPK_{\alpha 1}$ activity even under baseline conditions. Therefore during ischemia, total AMPK activity in the KD hearts was lower than AMPK activity in the

basal state in Wt hearts. It is possible that there is some lower limit of AMPK activity that is required for the response of the heart to ischemia. The discrepant result may also be accounted for due to the difference in models, as we have utilized an isolated working mouse heart, which potentially has a higher workload and energy demand compared to the Langendorff hearts used in previous studies. Presumably our model would have a greater degree of stress compared to the Langendorff models; in addition, acidosis would play a more important role in our model of global no-flow ischemia, as there would be washout of glycolytic byproducts in the low-flow models.

Recent studies have examined the effect of both global ischemia and low-flow ischemia in an $AMPK_{\alpha 2}$ KO model, which has abolished $AMPK_{\alpha 2}$-associated activity but has no effect on $AMPK_{\alpha 1}$-associated activity. This model mimics to a greater extent the effects of the Dn model used in the present study.[221, 222] In Langendorff perfused hearts in the absence of fatty acids and insulin, global ischemia produces an increase in lactate release in Wt mice, which is blunted in the $AMPK_{\alpha 2}$ KO.[222] As previously observed in the Dn and KD mice, end diastolic pressure increases to a greater extent and rate in the $AMPK_{\alpha 2}$ KO mice, resulting in a decreased time to start of and greater maximal ischemic contracture.[192, 221-223] Despite these metabolic and functional observations, the absence of $AMPK_{\alpha 2}$ does not exacerbate post-ischemia contractile dysfunction.[222] This is not surprising, as previous reports suggest that ischemic contracture does not correlate with post-ischemic contractile function.[272, 273] This observation is further confirmed as Wt hearts subjected to low-flow ischemia in the absence of fatty acids have better-preserved ATP levels during mild ischemia and reperfusion, however this is associated with similar recovery of contractile function during reperfusion.[221] However when hearts are

subjected to low-flow ischemia and reperfusion in the presence of fatty acids, the $AMPK_{\alpha 2}$ KO hearts have a slight suppression of cardiac function during the immediate reperfusion period with similar contractility during late reperfusion.[221] Unfortunately it is difficult to determine why the addition of fatty acids to the perfusate is detrimental only to the functional recovery of the $AMPK_{\alpha 2}$ KO hearts as no metabolic parameters were assessed in this set of hearts. Therefore it appears that the role of AMPK during I/R is highly dependent on the substrate availability and on the balance of the effects of AMPK on glucose and fatty acid metabolism.

Reduced AMPK activation has been demonstrated to be protective in a number of other models. Jaswal *et al.* have recently observed that suppressing AMPK activation in isolated working rat hearts via inhibition of p38 MAPK results in the restoration of adenosine cardioprotection following transient ischemic stress.[234] The proposed mechanism of cardioprotection is suggested to be due to a partial reduction of glycolysis, which ultimately leads to a reduction in H^+ production and myocardial acidosis. Even in the absence of ischemia, it is demonstrated that H_2O_2 mediated cardiac dysfunction is associated with an increase in AMPK activity, which is partially ameliorated by the treatment with an AMPK inhibitor, compound C.[235] Similar results are observed in an *in vivo* and *in vitro* model of stroke as activation of AMPK exacerbates ischemia-induced damage while inhibition of AMPK partially ameliorates ischemic-induced damage.[236, 237]

An important approach to determine if AMPK activation or inhibition is cardioprotective is to use an *in vivo* model of I/R. Recently this approach was used by Banerjee *et al.*, who demonstrate that mice with a T400N mutation in the $AMPK_{\gamma 2}$, which have reduced AMPK activity at six weeks and 100-fold increase in glycogen, are more

susceptible to ischemic damage.[274] However it is most likely that the small change observed in AMPK is secondary to the large glycogen store in these mice, which is detrimental to functional recovery during reperfusion due to an increase in myocardial acidosis from anaerobic glycolysis.[275] Recently, Miller *et al.* observed that MIF is released from the ischemic heart and can partially account for the ischemic-induced activation of AMPK.[178] When MIF KO mice are subjected to I/R in an *in vivo* LAD ligation model they have larger infarct sizes, which the authors contribute to the inability to activate AMPK and glucose uptake.[178] This correlates with the KO hearts having impaired functional recovery during reperfusion in their Langendorff heart model. However the loss of MIF only impairs ischemic-induced AMPK activation and glucose uptake by a small margin, but results in significant impairment of ischemic tolerance, suggesting that there may be alternative mechanisms by which MIF impairs ischemic tolerance. Therefore further studies are required to determine the direct role of AMPK in cardioprotection *in vivo*.

It has been suggested that the inability to activate AMPK would lead to an energetically compromised heart. Previous studies have supported this concept as during normoxic perfusion, $AMPK_{\alpha 2}$ KOs have significantly decreased total glycogen content, which is further decreased during global ischemia compared to Wt hearts.[222] In addition, global ischemia results in a large increase in the AMP/ATP ratio in those hearts, which is also observed during low-flow ischemia.[221, 222] However, we show using direct assessment of oxidative metabolism that the AMPK Dn hearts have similar if not elevated levels of TCA acetyl-CoA production compared to Wt hearts. In addition, at the end of reperfusion AMP/ATP ratios are similar between groups, which agrees with the

observed ratios at the end of reperfusion in the $AMPK_{\alpha 2}$ KO mice.[221, 222] Of interest is that glycogen levels in the AMPK Dn hearts are elevated at the end of reperfusion in the presence of insulin, which suggests that the mutation may be shunting glucose towards glycogen synthesis. This correlates well with a recent study, which demonstrates that reduced AMPK activity during reperfusion results in an elevated content of glycogen at the end of reperfusion.[234]

A novel observation is that suppression of AMPK activity during ischemia results in cardioprotection in the presence of insulin but has no effect on functional recovery during reperfusion in the absence of insulin. We have previously shown that even at low concentrations of fatty acid, the ability of insulin to inhibit AMPK is blocked, although other insulin signaling pathways remain functional, which is associated with impaired functional recovery during reperfusion in the presence of a high concentration of fatty acids.[240] We have also observed that the addition of insulin in the perfusate does not affect AMPK activity in the present study, therefore the beneficial effects of insulin in the AMPK Dn hearts must be due to a non-AMPK related pathway. It is plausible that insulin stimulation of glycolysis and glucose oxidation overrides the ability of AMPK to modify cardiac metabolism.

Limitations

This study utilized the isolated working heart method, which has some limitations as previously discussed in Chapter 3 and Chapter 8. In this study, $AMPK_{\alpha 1}$- versus $AMPK_{\alpha 2}$-associated activity is not separated out, thus total activity and phosphorylation status of AMPK was reported. This limits our ability to delineate isoform specific effects

of modifying AMPK activity during I/R. In addition, enzyme activities where determined under V_{max} conditions; therefore any changes in enzyme activity related to modifications in K_m would not be detected. Despite the relatively small standard errors in the data in this chapter a potential limitation of this study is the small number of animals utilized in each group due to the limited availability of the AMPK Dn mice. Although not significantly different, the Wt hearts perfused in the presence of insulin have slightly lower cardiac function (cardiac output, cardiac power and coronary flow) compared to the AMPK Dn hearts, which could potentially partially account for their poor recovery of function during reperfusion. When recovery of cardiac function is expressed as a percent of baseline aerobic function, functional recovery is not different between groups, suggesting that inhibition of AMPK is not detrimental to post-ischemic contractile function but has a neutral effect. Another potential limitation is that the calculated values for acetyl-CoA production are based only on metabolic rates of exogenously provided radiolabelled substrates, which does not take into account metabolic rates of endogenous substrates, such as glycogen or TGs. As AMPK has potential roles in the regulation of endogenous substrates, the metabolic rates of these substrates may differ between groups and could potentially modify of the distribution of acetyl-CoA production from carbohydrates and fat.

In summary, this study demonstrates that suppression of AMPK activity during ischemia and reperfusion does not produce an energetically compromised heart and does not impair recovery of contractile function during reperfusion. These findings have important implications for the potential role of AMPK as a therapeutic target to modulate myocardial energy metabolism during I/R injury.

CHAPTER 5.

Cardiac Specific Expression of the AMPK$_{\gamma 2}$ R302Q Mutation Causes Increased Ischemic-Induced AMPK Activation and Impairs Recovery of Contractile Function During Reperfusion

My role in this work involved performing all the experiments (except those noted below) as well as writing the manuscript. Cory S. Wagg performed the isolated working mouse heart perfusions.

CHAPTER 5.

Cardiac Specific Expression of the $AMPK_{\gamma 2}$ R302Q Mutation Causes Increased Ischemic-Induced AMPK Activation and Impairs Recovery of Contractile Function During Reperfusion

Abstract

Mutations in the γ subunit of AMPK can result in dramatic glycogen overload and cardiac hypertrophy. However there is controversy over whether these mutations activate or inhibit AMPK. To examine the effect of the R302Q mutation on AMPK activity after ischemic stress, we subjected isolated working hearts from mice with the transgenic (TG) mutation of R302Q (TG-Mut), transgenic mice overexpressing the wildtype $AMPK_{\gamma 2}$ subunit (TG-Wt), and FVB control mice to a 30 min aerobic perfusion, 20 min of global no-flow ischemia, and 40 min of aerobic reperfusion with Krebs-Henseleit solution containing 5 mM [5-^{3}H/U-^{14}C]glucose, 1.2 mM [9,10-^{3}H]palmitate prebound to 3 % albumin, and 100 μU/ml insulin. During aerobic perfusion, glycolytic rates are not significantly different in TG-Mut hearts compared with either TG-Wt or FVB hearts. However, there is an increase in lactate production during the aerobic period in the TG-Mut compared with TG-Wt and FVB hearts (190 ± 19 vs. 89 ± 16, and 72 ± 46 μmol/g

dry wt, respectively, n = 6 per group, $P < 0.05$) due to an increase in breakdown of endogenous glycogen stores which are elevated in the TG-Mut hearts (382 ± 63 vs. 67 ± 4, and 32 ± 3 μmol/g dry wt, respectively, n = 6 per group, $P < 0.05$). Despite the high glycolytic rates in the TG-Mut hearts, glucose oxidation rates during aerobic perfusion are suppressed in both the TG-Mut and TG-Wt groups compared with FVB hearts (320 ± 40, 470 ± 40 vs. 990 ± 130 nmol/g dry wt/min, respectively, n = 11, 10 and 8, $P < 0.05$), which was associated with elevated rates of palmitate oxidation in both of these groups compared to FVB hearts (620 ± 50, 900 ± 110, vs. 280 ± 10 μmol/g dry wt/min, respectively, n = 6, 5 and 4, $P < 0.05$). Similar metabolic trends were observed during reperfusion. AMPK activity (465 ± 50 vs. 320 ± 23, and 134 ± 13 pmol/min/mg, respectively, n = 6 per group, $P < 0.05$) and AMPK phosphorylation (Thr172/Total AMPK ratio: 0.80 ±0.11 vs. 0.50 ± 0.07, and 0.33 ± 0.05, respectively, n = 6 per group, $P < 0.05$) measured at the end of reperfusion are elevated in the TG-Mut compared to TG-Wt and FVB hearts. Acetyl-CoA carboxylase phosphorylation (Ser79/Total ACC ratio: 265 kDa: 2.17 ± 0.16 vs. 1.36 ± 0.35, and 0.69 ± 0.1, respectively, 280 kDa: 0.72 ± 0.04 vs. 0.39 ± 0.13, and 0.22 ± 0.03, respectively, n = 6 per group, $P < 0.05$) is also elevated in the TG-Mut group. Despite the activation of AMPK in the TG-Mut hearts, they have reduced recovery of cardiac function during reperfusion compared to TG-Wt and FVB hearts (5.5 ± 1.8 vs. 11.5 ± 0.8 and 10.9 ± 1.2 bpm•mmHg, respectively, n = 11, 10 and 8, $P < 0.05$). We demonstrate that the R302Q mutation can result in an increase in ischemia-induced AMPK activation, but is not associated with improved recovery of contractile function during reperfusion.

Introduction

Myocardial ischemia occurs when the oxygen requirement of the heart exceeds the oxygen supplied via the coronary circulation and is a devastating cause of morbidity and mortality worldwide. A contributing factor to I/R injury is modification of myocardial energy metabolism.[5, 54, 122] A majority of the energy requirement of the normal well-perfused heart is met by the oxidation of fatty acids, with the remainder coming from the oxidation of carbohydrates.[5, 54, 122] However during ischemia, mitochondrial oxidative metabolism is suppressed and anaerobic glycolysis becomes an important source of ATP.[133] As pyruvate from glycolysis cannot be subsequently oxidized, there is the potential for the net production of deleterious byproducts (lactate and H^+ from the hydrolysis of glycolytically-derived ATP).[31, 32, 121, 269, 270]

An important regulator of myocardial energy metabolism during ischemia is AMPK.[251, 252] We and others have shown that AMPK is rapidly activated during myocardial ischemia and is associated with dramatic changes in the control of glucose and fatty acid metabolism.[63, 73, 153, 178, 192, 204, 221, 240] However the role of AMPK in the protection of the ischemic myocardium is still controversial.[220] AMPK activation during ischemia is believed to be an attempt to restore cardiac energy balance by stimulating ATP generating pathways.[220] Indeed, during ischemia, AMPK promotes glycolysis by stimulating glucose uptake [192] and indirectly activating PFK-1 [204], thus producing an anaerobic source of ATP. During reperfusion, AMPK activates fatty acid oxidation by phosphorylating and inhibiting ACC, thus removing the malonyl-CoA inhibition of fatty acid transport into the mitochondria.[63, 73] However, AMPK activation may exacerbate

post-ischemic contractile function due to stimulation of fatty acid oxidation at the expense of glucose oxidation, which would increase the myocardial acid load and Na^+ and Ca^{2+} overload.[31, 32, 134] As discussed in Chapter 2, only a few studies have directly associated AMPK activity with functional recovery during reperfusion, and these have reached different conclusions.[192, 221, 222] Therefore the question of whether AMPK is an enemy or an ally to the ischemic heart is still controversial.[220]

Transgenic models that modify AMPK activity include mice that harbor a mutation in $AMPK_{\gamma 2}$. Mutations in $AMPK_{\gamma}$ were originally identified in purebred Hampshire pigs, which contained a R200Q mutation in $AMPK_{\gamma 3}$. This loss of function mutation results in glycogen accumulation in the skeletal muscle of these pigs.[205] Subsequently, a variety of mutations in the $AMPK_{\gamma 2}$ gene have been identified in the hearts of patients [206, 207, 209, 210, 215] which result in a disease phenotype termed the PRKAG2 cardiac syndrome.[208] One of these mutations in $AMPK_{\gamma 2}$ results in the replacement of arginine 302 for glutamine, and was originally identified in two families with a familial form of WPW associated with ventricular preexcitation, conduction abnormalities and cardiac hypertrophy.[209] Transgenic mice with cardiac specific overexpression of this mutation recapitulate much of the human disease phenotype, consisting of increased glycogen deposition, profound cardiac hypertrophy, and WPW-like conduction abnormalities including preexcitation and supraventricular arrhythmia.[276] This phenotype is associated with a significant reduction in AMPK activity in the $AMPK_{\gamma 2}$ R302Q expressing hearts compared to mice overexpressing the Wt $AMPK_{\gamma 2}$ gene or nontransgenic controls.[211] Recently, Banerjee *et al.*, demonstrate that mice having a T400N mutation in the $AMPK_{\gamma 2}$, which results in a 50 % reduction in AMPK

activity at six weeks and 100-fold increase in glycogen, are more susceptible to ischemic damage in an *in vivo* model of LAD occlusion and reperfusion.[274] However, metabolic measurements were not performed in that study making it difficult to interpret the role of AMPK inhibition.

The goal of this study is to better understand the role of AMPK in the regulation of myocardial metabolism and functional recovery during reperfusion following ischemia. Mice overexpressing $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q were perfused as isolated working hearts to examine the correlation of AMPK activity with myocardial metabolism and functional recovery during reperfusion following global ischemia.

Materials and 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.

Transgenic Mice

Transgenic mice overexpressing either $AMPK_{\gamma 2}$ (TG-Wt) or $AMPK_{\gamma 2}$ R302Q (TG-Mut) were a kind gift of M. Gollob and R. Roberts (Ottawa Heart Institute). Animals were constantly outbred with FVB mice and housed in a controlled environment with a 12:12 hr light-dark cycle. Transgenes consisted of the αMHC promoter, PRKAG2 Wt or mutant gene and the 3'UTR human growth hormone and was injected into fertilized FVB mouse oocytes, as previously described.[276]

Genotyping

DNA was extracted from tail biopsies using the REDExtract-N-AMP Tissue PCR kit from Sigma (St. Louis, Missouri) and the human PRKAG2 gene was amplified using the following primers forward (5'-CTC GAG GCC GCC ACC ATG GCA TAC CCA TAC GAC GTC CCA GAC TAC GCT CCG CTC CTG GAC GG-3') and reverse (5'-TCT AGA CGG CGG TCA CTC CGT TTC TGT CTC CTT TTG TTT GGC ACC TGC TGG -3') to generate a 1626 bp gene product which was confirmed on agarose gel. This gene product was further amplified for sequencing using genotyping primers, forward (5'- GGG CAT CAG GTT TTT CTC TCC CGC TCC A-3') and reverse (5'- CCA TAG

GTG ATT TAT AGT ATC TAT GTA G-3') to produce a 571 bp fragment.[276] Fragment size was confirmed on an agarose gel, and the band of interest was cut out and purified using a Novagen SpinPrep Gel DNA kit (Mississaga, Ontario). 100 ng of each PCR product was sent for sequencing at the Molecular Biology Services Unit, University of Alberta using the reverse genotyping primer (Figure 1).

Isolated Working Mouse Heart Perfusions

FVB (strain control), TG-Wt and TG-Mut mouse hearts were perfused in the working mode as previously described in Chapter 2.[239] The oxygenated Krebs-Henseleit solution contained 1.2 mM palmitate, 5 mM glucose and 100 μU/mL insulin. Spontaneously beating hearts were perfused at a constant left atrial preload pressure of 11.5 mmHg and a constant aortic afterload pressure of 50 mmHg for the I/R protocol, which consisted of 30 min aerobic perfusion, 20 min of global no-flow ischemia and 40 min reperfusion. At the end of the perfusion protocol the hearts were quick frozen in liquid nitrogen with Wollenberger tongs, and stored at -80 °C.

Glycolysis, glucose oxidation and palmitate oxidation were measured by quantitative collection of the $^{3}H_2O$ and $^{14}CO_2$, derived from [5-^{3}H/U-^{14}C]glucose and [9, 10-^{3}H]palmitate, as described in Chapter 2.[134, 238] Lactate release into the perfusate was measured as described in Chapter 2.

Tissue Extractions

AMPK was isolated and assayed using the AMARA peptide as described in Chapter 2. Immunoblotting was performed on cleared homogenates for total and

phosphorylated AMPK and ACC as described in Chapter 2. Glycogen content was measured as described in Chapter 2.

Statistical Analysis

All data are presented as the mean ± S.E.M. The data were analyzed with the statistical program Instat 2.01 and GB-stat. Two-way repeated measures ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for cardiac function. Two-way ANOVA with a Bonferroni post-hoc test or if variances were different by a Bartlett's test, a nonparametric Kruskal-Wallis test with a Dunn's multiple comparison test were used to evaluate the statistical significance of differences among groups for the metabolic data, western blot data and adenine nucleotide data. Values of $P < 0.05$ were considered significant.

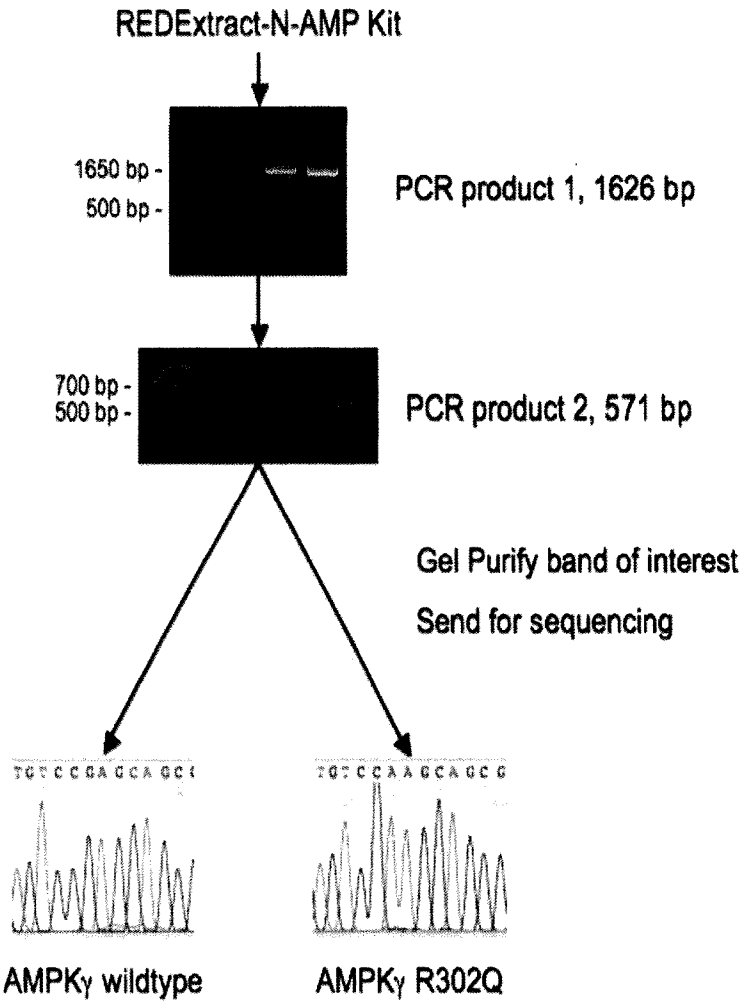


Figure 5-1: Genotyping strategy for mice overexpressing $AMPK_{\gamma 2}$ or the $AMPK_{\gamma 2}$ R302Q mutation.

Results

The Effect of Overexpression of $AMPK_{\gamma 2}$ and $AMPK_{\gamma 2}$ R302Q on Baseline Aerobic Cardiac Function and Metabolism

As previously reported, the TG-Mut hearts have profound cardiac hypertrophy compared to either the TG-Wt or FVB hearts (heart weight/body weight ratio of 0.21 ± 0.02 vs. 0.13 ± 0.01 and 0.11 ± .01, respectively, n = 11, 10 and 8, $P < 0.05$, Figure 5-2A). At the beginning of the aerobic perfusion period, cardiac function is slightly depressed in the TG-Mut hearts compared to the FVB hearts ($HR \bullet PSP \bullet 10^{-3}$ of 15.4 ± 1.7 vs. 21.1 ± 0.7 $bpm \bullet mmHg \bullet 10^{-3}$, n = 11, 10 and 8, $P < 0.05$). However, at the end of the aerobic period cardiac function does not differ among the groups (Figure 5-2B). Heart rate is significantly lower in the TG-Mut hearts compared to FVB hearts (202 ± 24 vs. 297 ± 5 bpm, $P < 0.05$, n = 11 and 8, Table 5-1), while coronary flow (3.8 ± 0.4 vs. 2.1 ± 0.4 mL/min, $P < 0.05$, n = 11 and 10 Table 5-1) is significantly elevated in TG-Mut hearts compared to TG-Wt hearts.

During the aerobic period, overexpression of either $AMPK_{\gamma 2}$ R302Q or $AMPK_{\gamma 2}$ does not affect the rate of glycolytic flux of exogenous glucose or H^+ production (Figure 5-3A and D), however glucose oxidation is significantly decreased compared to FVB hearts (320 ± 40, 470 ± 40, vs. 990 ± 130 nmol/g dry wt/min, n = 11, 10 and 8, $P < 0.05$, Figure 5-3B), which is associated with an increase in fatty acid oxidation in the TG-Mut and TG-Wt compared to the FVB hearts (620 ± 50 and 900 ± 110 vs. 280 ± 10 nmol/g dry wt/min, n = 6, 5 and 4, $P < 0.05$, Figure 5-3C). Despite there being no change in glycolytic flux of exogenous glucose, there is a significant increase in the release of

lactate into the perfusate in the TG-Mut hearts compared to the TG-Wt and FB hearts (190 ± 19 vs. 89 ± 16 and 72 ± 46 μmol/g dry wt, n = 6 per group, $P < 0.05$, Figure 5-4A) suggesting that glycogenolysis and glycolytic flux of this endogenous source of glucose is elevated in the TG-Mut hearts. Indeed glycogen content assessed in non-perfused hearts demonstrated that the TG-Mut hearts have significantly higher glycogen compared to TG-Wt or FVB hearts (107 ± 15 vs. 29 ± 3 and 3 ± 1 μmol glucosyl units/g wet weight, n = 4 per group, $P < 0.05$, personal communication Dr. Jason R. B. Dyck). TG-Wt hearts have significantly higher total TCA cycle acetyl-CoA production compared to FVB or TG-Mut hearts (7.8 ± 0.9 vs. 4.5 ± 0.5 and 5.5 ± 0.4 μmol/g dry wt/min, n = 6, 5 and 4, $P < 0.05$, Figure 5-5A). Both TG-Mut and TG-Wt have a much higher reliance on palmitate oxidation as a source of TCA acetyl-CoA (90 ± 1 and 88 ± 2 vs. 50 ± 3 %, n = 6, 5 and 4, $P < 0.05$, Figure 5-5B).

The Effect of Overexpression of $AMPK_{\gamma 2}$ and $AMPK_{\gamma 2}$ R302Q on Myocardial Metabolism and Functional Recovery During Reperfusion Following Global No-Flow Ischemia

During reperfusion following 20 min of global no-flow ischemia TG-Mut hearts have a suppressed recovery of contractile function compared to TG-Wt or FVB hearts (HR•PSP•10^{-3} of 5.5 ± 1.8 vs. 11.5 ± 0.8 and 10.9 ± 1.2 bpm•mmHg•10^{-3}, n = 11, 10 and 8, $P < 0.05$, Figure 5-2B). TG-Mut hearts have a reduced HR compared to TG-Wt or FVB hearts (100 ± 26 vs. 169 ± 13, 197 ± 8 bpm, n = 11, 10 and 8, $P < 0.05$, Table 5-1) and a reduced PSP compared to TG-Wt hearts (44 ± 5 vs. 60 ± 2 mmHg, $P < 0.05$, n = 11 and 10, Table 5-1). As observed during the aerobic period, rates of glycolytic flux of

exogenous glucose and H^+ production does not differ among the groups (Figure 5-3A and D) and lactate appearance in the perfusate also does not differ, likely due to maximal ischemia-induced stimulation of glycogenolysis and glycolysis of endogenous glucose (Figure 5-3B). Despite this, at the end of reperfusion glycogen levels are still significantly elevated in the TG-Mut group compared to the TG-Wt and FVB hearts (382 ± 63 vs. 67 ± 4, 32 ± 3 μmol/g dry wt, n = 6 per group, $P < 0.05$, Figure 5-4C). At the end of reperfusion, TG-Mut hearts have elevated AMP levels compared to FVB heart (3.9 ± 1.0 vs. 0.9 ± 0.1, μmol/g dry wt, n = 6 per group, $P < 0.05$, Table 5-2), and elevated ADP levels compared to TG-Wt and FVB hearts (6.2 ± 0.3 vs. 4.4 ± 0.3, 3.5 ± 0.3 μmol/g dry wt, n = 6 per group, $P < 0.05$, Table 5-2) with no significant changes in ATP levels. Therefore AMP/ATP ratios are also elevated in the TG-Mut hearts compared to the TG-Wt and FVB hearts (0.77 ± 0.21 vs. 0.09 ± 0.01 and 0.08 ± 0.01, n = 6 per group, $P < 0.05$, Table 5-2).

Similar metabolic trends are observed during reperfusion as seen during the aerobic period, although palmitate oxidation is accelerated in the TG-Wt hearts compared to the FVB hearts (500 ± 110 vs. 170 ± 30 nmol/g dry wt/min, n = 5 and 4, $P < 0.05$, Figure 5-3C). Therefore, TG-Wt hearts also have significantly higher total TCA cycle acetyl-CoA production compared to FVB hearts (5.5 ± 1.2 vs. 2.7 ± 0.5, n = 5 and 4, $P < 0.05$, Figure 5-5A).

Previous reports suggest that the $AMPK_{\gamma 2}$ R302Q mutation leads to a reduction in AMPK activity.[211] Measurement of AMPK activity in non-perfused hearts demonstrates a reduction in AMPK activity in the TG-Mut hearts and TG-Wt compared FVB hearts (208 ± 8 and 311 ± 53 vs. 668 ± 42 pmol/min/mg, n = 4 per group, $P < 0.05$, personal

communication with Dr. Jason R. B. Dyck). However when AMPK activity was assessed in hearts at the end of reperfusion, TG-Mut hearts have significantly higher AMPK activity compared to either TG-Wt or FVB hearts (465 ± 50 vs. 320 ± 23 and 134 ± 14 pmol/min/mg, n = 6 per group, $P < 0.05$, Figure 5-6A). Western blotting confirmed this activation of AMPK (P-AMPK (Thr172)/total AMPK: 0.80 ± 0.11 vs. 0.51 ± 0.07 and 0.33 ± 0.05, n = 6 per group, $P < 0.05$, Figure 5-6B) and phosphorylation of its downstream target ACC (P-ACC(Ser 79)/total ACC, 265 kDa: 2.17 ± 0.16 vs. 1.36 ± 0.35 and 0.69 ± 0.1, 280 kDa: 0.72 ± 0.04 vs. 0.39 ± 0.13 and 0.22 ± 0.03, n = 6 per group, $P < 0.05$, Figure 5-7A and C). Western blotting also suggests that there is more $AMPK_{\alpha}$ in the TG-Mut hearts compared to TG-Wt and FVB hearts (Total AMPK/Actin: 0.94 ± 0.06 vs. 0.62 ± 0.03 and 0.62 ± 0.09, n = 6 per group, $P < 0.05$, Figure 5-6B) and more ACC in TG-Mut and TG-Wt hearts (Total ACC/pyruvate carboxylase, 265 kDa: 0.20 ± 0.02 and 0.17 ± 0.03 vs. 0.05 ± 0.01, 280 kDa: 0.41 ± 0.04, 0.40 ± 0.04 vs. 0.28 ± 0.02, n = 6 per group, $P < 0.05$, Figure 5-7A and B).

Table 5-1. The effect of overexpression of $AMPK_{\gamma 2}$ (TG-Wt) or $AMPK_{\gamma 2}$ R302Q (TG-Mut) on parameters of contractile function during aerobic perfusion and reperfusion of ischemic hearts.

	Aerobic Perfusion			Reperfusion		
	FVB (n = 8)	TG-Wt (n = 10)	TG-Mut (n = 11)	FVB (n = 8)	TG-Wt (n = 10)	TG-Mut (n = 11)
Heart Rate (beats/min)	297 ± 5	264 ± 8	202 ± 24*	197 ± 8	169 ± 13	100 ± 26*#
Peak Systolic Pressure (mmHg)	70 ± 2	73 ± 1	80 ± 3	54 ± 4	60 ± 2	44 ± 5#
Coronary Flow (mL/min)	2.7 ± 0.4	2.1 ± 0.4	3.8 ± 0.4#	2.1 ± 0.3	1.1 ± 0.2	1.6 ± 0.6
Coronary Flow (mL/min/g dry wt)	85 ± 13	67 ± 9	61 ± 4	65 ± 9	34 ± 7	25 ± 9
Cardiac Output (mL/min)	9.8 ± 0.5	8.4 ± 0.4	9.9 ± 1.0	3.1 ± 0.5	2.4 ± 0.5	2.1 ± 0.8
Cardiac Power (mJoule)	77 ± 6	68 ± 4	90 ± 7#	19 ± 5	18 ± 4	13 ± 6

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding FVB group.

represents $P < 0.05$, significantly different from corresponding TG-Wt group.

Table 5-2. The effect of overexpression of $AMPK_{\gamma 2}$ (TG-Wt) or $AMPK_{\gamma 2}$ R302Q (TG-Mut) on adenine nucleotide at the end of reperfusion.

	FVB (n = 6)	TG-Wt (n = 6)	TG-Mut (n = 6)
AMP (μmol/g dry wt)	0.9 ± 0.1	1.2 ± 0.1	3.9 ± 1.0*
ADP (μmol/g dry wt)	3.5 ± 0.3	4.4 ± 0.3	6.2 ± 0.3*#
ATP (μmol/g dry wt)	12.1 ± 0.9	14.1 ± 1.1	9.8 ± 1.9
AMP/ATP	0.08 ± 0.01	0.09 ± 0.01	0.77 ± 0.21*#
ADP/ATP	0.29 ± 0.02	0.32 ± 0.01	0.71 ± 0.17*#

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test, or if variances were different by a Bartlett's test, a nonparametric Kruskal-Wallis test with a Dunn's multiple comparison test was performed.

* represents $P < 0.05$, significantly different from FVB.

represents $P < 0.05$, significantly different from TG-Wt.

Figure 5-2: Overexpression of $AMPK_{\gamma 2}$ R302Q impairs recovery of cardiac post-ischemic function.

A) Heart weight/body weight ratio, B) HR*PSP and C) cardiac power in isolated mouse hearts during aerobic perfusion and reperfusion of ischemic hearts (n = 8, 10, 11 for FVB, TG-Wt and TG-Mut, respectively). Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test for heart weight/body weight ratio and a 2-way repeated measures ANOVA with a Bonferroni post hoc test for contractile function.

* represents $P < 0.05$, significantly different from FVB group.

represents $P < 0.05$, significantly different from TG-Wt group.

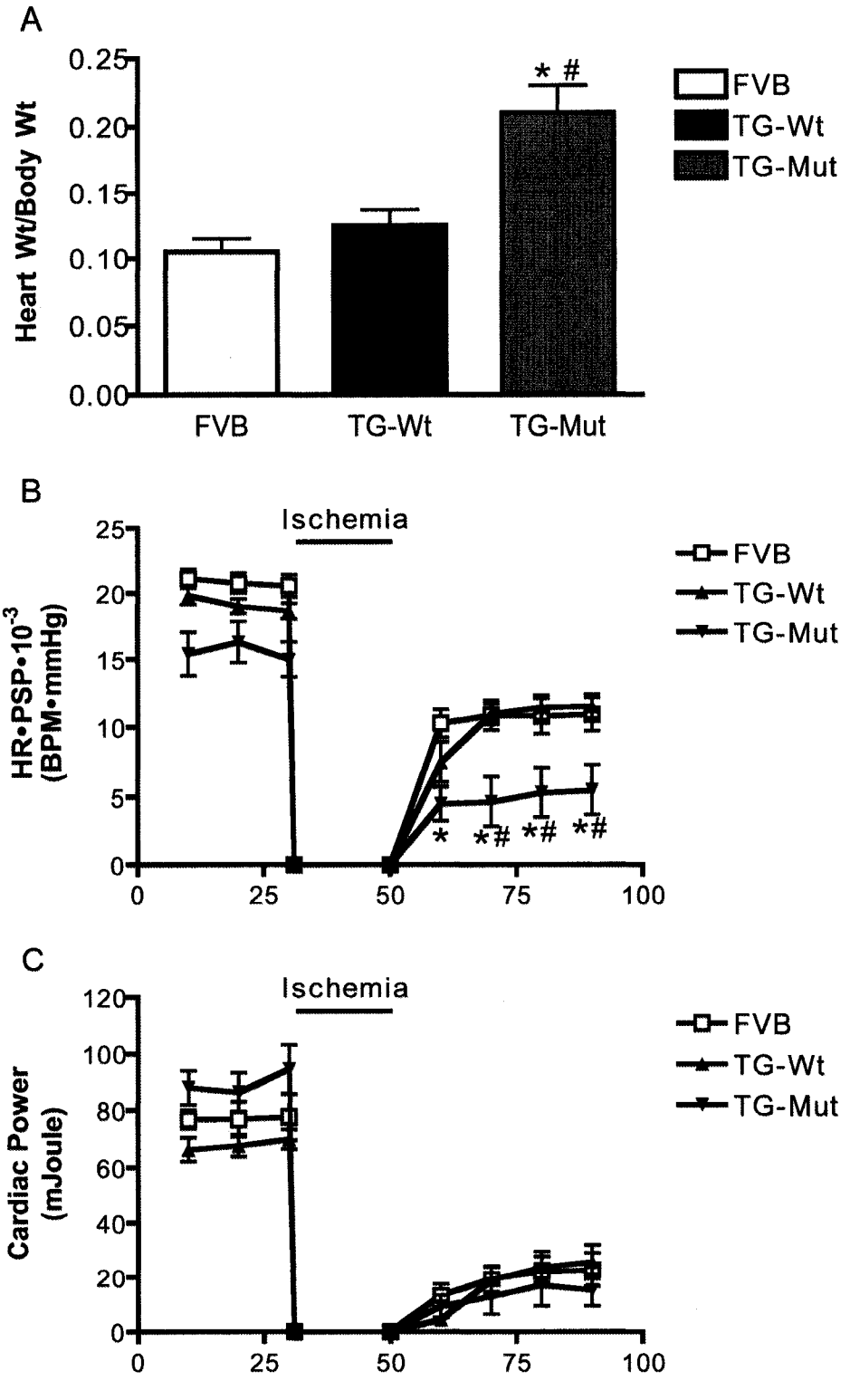

A
Heart Wt/Body Wt
0.25
0.20
0.15
0.10
0.05
0.00
* #
FVB
TG-Wt
TG-Mut
FVB
TG-Wt
TG-Mut
B
Ischemia
HR•PSP•10^-3 (BPM•mmHg)
25
20
15
10
5
0
0
25
50
75
100
*
*#
*#
*#
FVB
TG-Wt
TG-Mut
C
Ischemia
Cardiac Power (mJoule)
120
100
80
60
40
20
0
0
25
50
75
100
FVB
TG-Wt
TG-Mut

Figure 5-2

Figure 5-3: Overexpression of $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q increases cardiac palmitate oxidation at the expense of glucose oxidation.

A) Glycolytic flux (n = 4, 5, 5 for FVB, TG-Wt and TG-Mut, respectively), B) glucose oxidation (n = 8, 10, 11 for FVB, TG-Wt and TG-Mut, respectively), C) palmitate oxidation (n = 4, 5, 6 for FVB, TG-Wt and TG-Mut, respectively) and D) H^+ production (n = 4, 5, 5 for FVB, TG-Wt and TG-Mut, respectively) in isolated working mouse hearts during aerobic perfusion and reperfusion of ischemic hearts. Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from FVB group.

represents $P < 0.05$, significantly different from TG-Wt group.

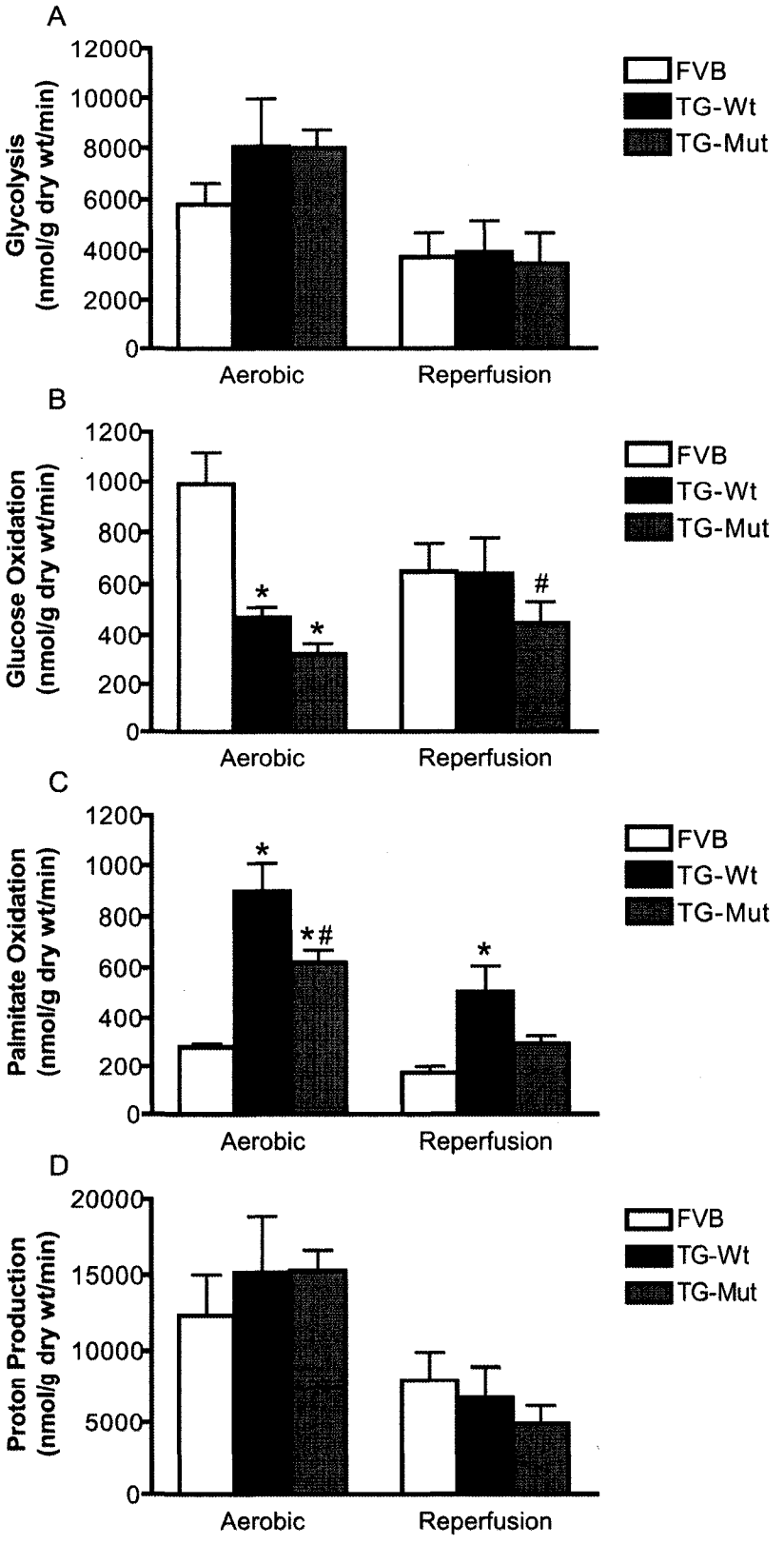

A
Glycolysis (nmol/g dry wt/min)
12000
10000
8000
6000
4000
2000
0
Aerobic
Reperfusion
FVB
TG-Wt
TG-Mut
B
Glucose Oxidation (nmol/g dry wt/min)
1200
1000
800
600
400
200
0
*
*
#
Aerobic
Reperfusion
FVB
TG-Wt
TG-Mut
C
Palmitate Oxidation (nmol/g dry wt/min)
1200
1000
800
600
400
200
0
*
*#
*
Aerobic
Reperfusion
FVB
TG-Wt
TG-Mut
D
Proton Production (nmol/g dry wt/min)
20000
15000
10000
5000
0
Aerobic
Reperfusion
FVB
TG-Wt
TG-Mut

Figure 5-3

Figure 5-4: Overexpression of AMPK$_{\gamma 2}$ R302Q increases cardiac glycolytic flux of endogenous substrates during aerobic perfusion due to increased glycogen stores.

A) lactate production during aerobic perfusion and B) reperfusion and C) glycogen content at the end of reperfusion in isolated working mouse hearts (n = 6 per group). Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from FVB group.

represents $P < 0.05$, significantly different from TG-Mut group.

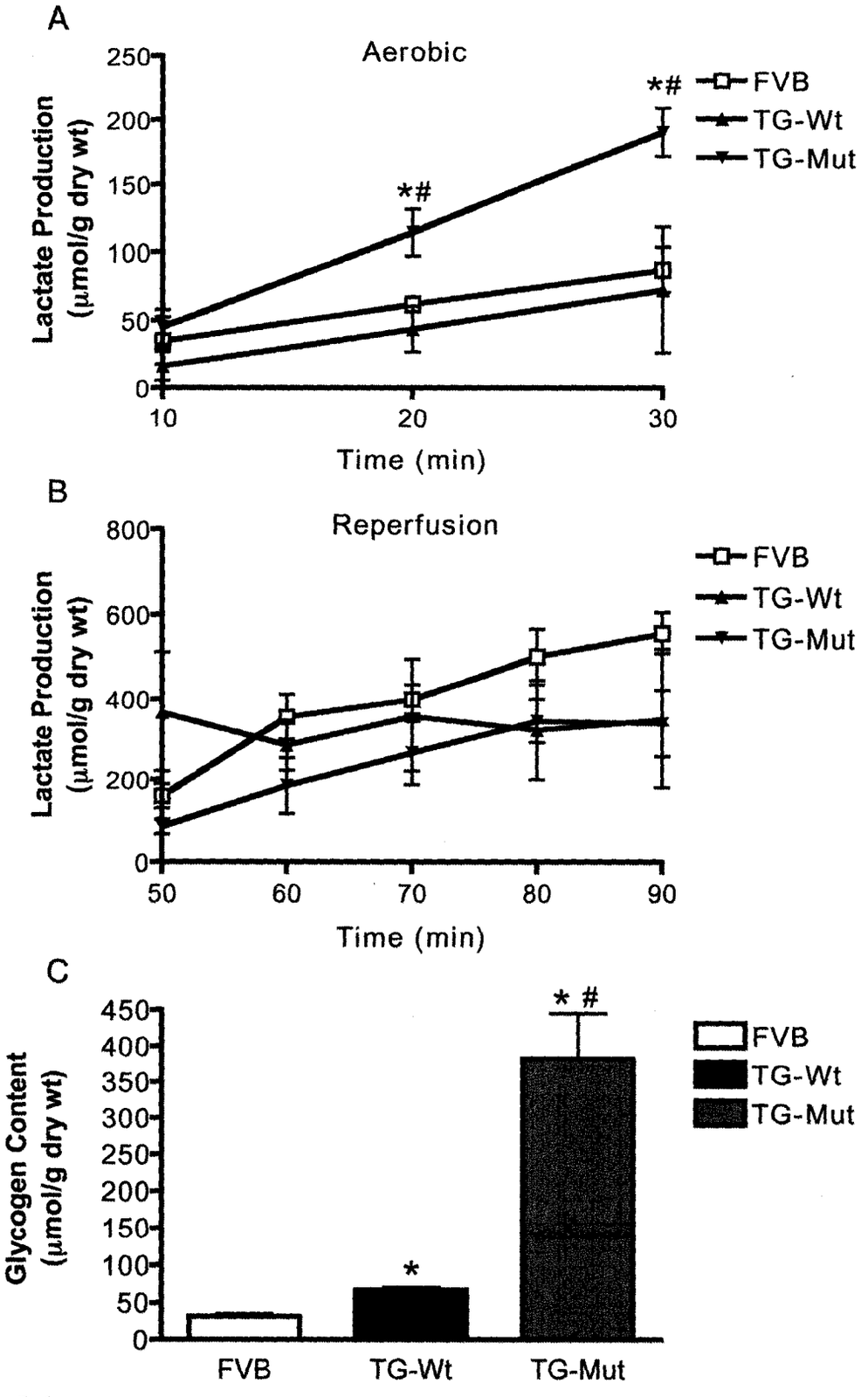

A
Aerobic
Lactate Production (µmol/g dry wt)
250
200
150
100
50
0
10
20
30
Time (min)
*#
*#
FVB
TG-Wt
TG-Mut
B
Reperfusion
Lactate Production (µmol/g dry wt)
800
600
400
200
0
50
60
70
80
90
Time (min)
FVB
TG-Wt
TG-Mut
C
Glycogen Content (µmol/g dry wt)
450
400
350
300
250
200
150
100
50
0
*
* #
FVB
TG-Wt
TG-Mut
FVB
TG-Wt
TG-Mut

Figure 5-4

Figure 5-5: Overexpression of $AMPK_{\gamma 2}$ and $AMPK_{\gamma 2}$ R302Q increases the reliance of hearts on palmitate oxidation as a source of TCA cycle acetyl-CoA production.

A) Total acetyl-CoA production and B) percent TCA cycle acetyl-CoA contribution in isolated working mouse hearts during aerobic perfusion and reperfusion of ischemic hearts (n = 4, 5, 6 for FVB, TG-Wt and TG-Mut, respectively). Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from FVB group.

represents $P < 0.05$, significantly different from TG-Wt group.

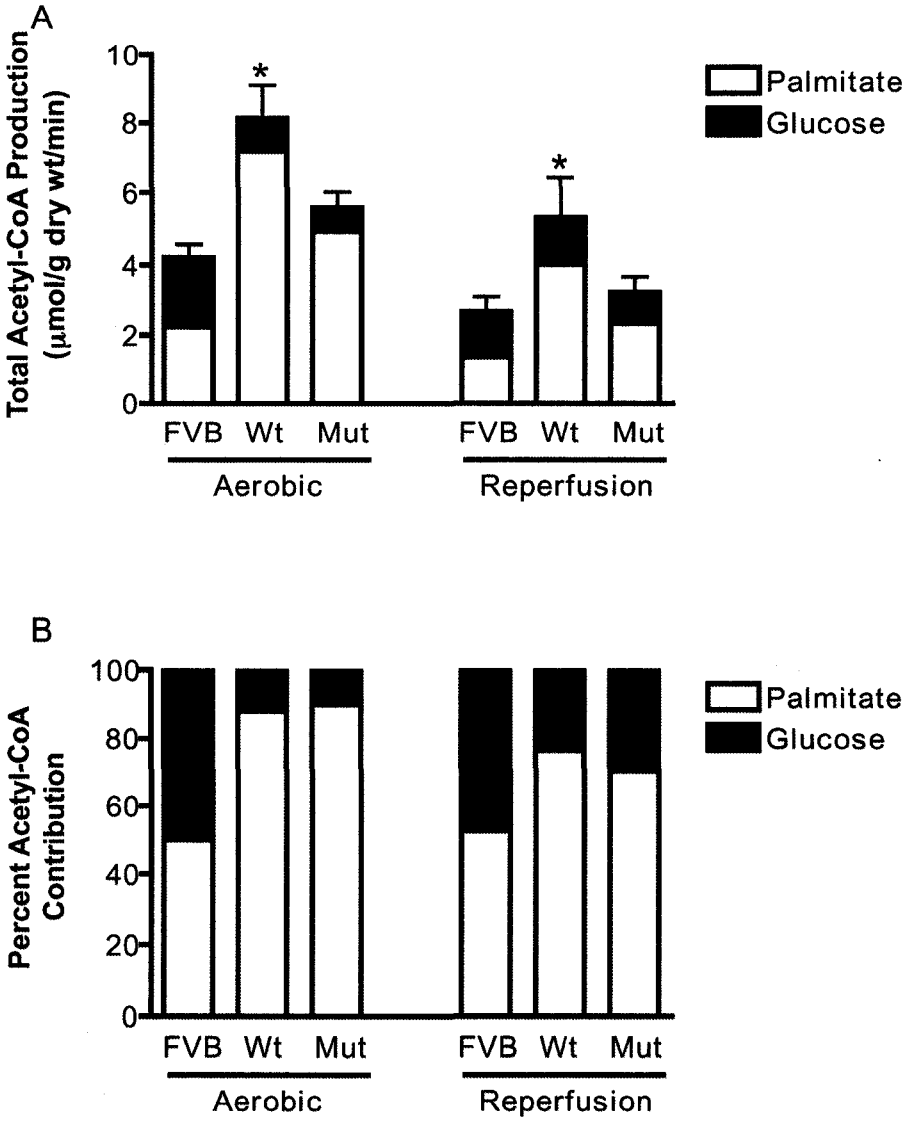

A
Total Acetyl-CoA Production
(µmol/g dry wt/min)
10
8
6
4
2
0
*
*
Palmitate
Glucose
FVB Wt Mut
FVB Wt Mut
Aerobic
Reperfusion
B
Percent Acetyl-CoA
Contribution
100
80
60
40
20
0
Palmitate
Glucose
FVB Wt Mut
FVB Wt Mut
Aerobic
Reperfusion

Figure 5-5

Figure 5-6: Overexpression of $AMPK_{\gamma 2}$ and $AMPK_{\gamma 2}$ R302Q results in increased cardiac AMPK activity during reperfusion.

A) AMPK activity and B) representative Western blot and quantification for P-AMPK (Thr 172), total AMPK and actin in isolated working mouse hearts at the end of reperfusion of ischemic hearts (n = 6 per group). Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from FVB group.

represents $P < 0.05$, significantly different from TG-Wt group.

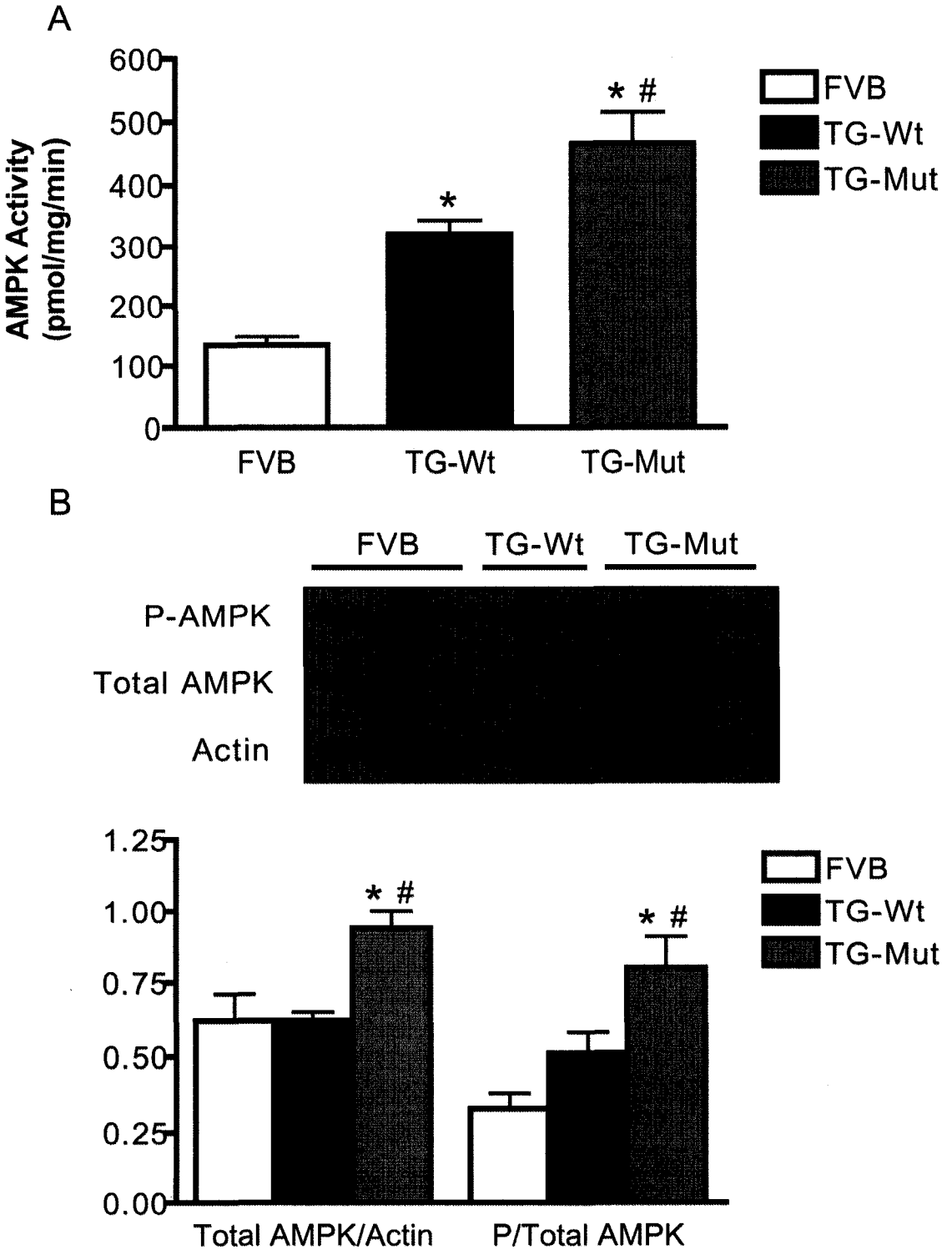

A
AMPK Activity (pmol/mg/min)
600
500
400
300
200
100
0
* #
*
FVB
TG-Wt
TG-Mut
FVB
TG-Wt
TG-Mut
B
FVB
TG-Wt
TG-Mut
P-AMPK
Total AMPK
Actin
1.25
1.00
0.75
0.50
0.25
0.00
* #
* #
FVB
TG-Wt
TG-Mut
Total AMPK/Actin
P/Total AMPK

Figure 5-6

Figure 5-7: Overexpression of $AMPK_{\gamma 2}$ R302Q increases phosphorylation of cardiac ACC.

A) Representative Western blots of P-ACC (Ser79), total ACC, and pyruvate carboxylase, B) quantification of total ACC/pyruvate carboxylase ratio and C) quantification of P-ACC (Ser79)/total ACC ratio in isolated mouse hearts during aerobic perfusion and reperfusion of ischemic hearts (n = 6 per group). Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from FVB group.

represents $P < 0.05$, significantly different from TG-Wt group.

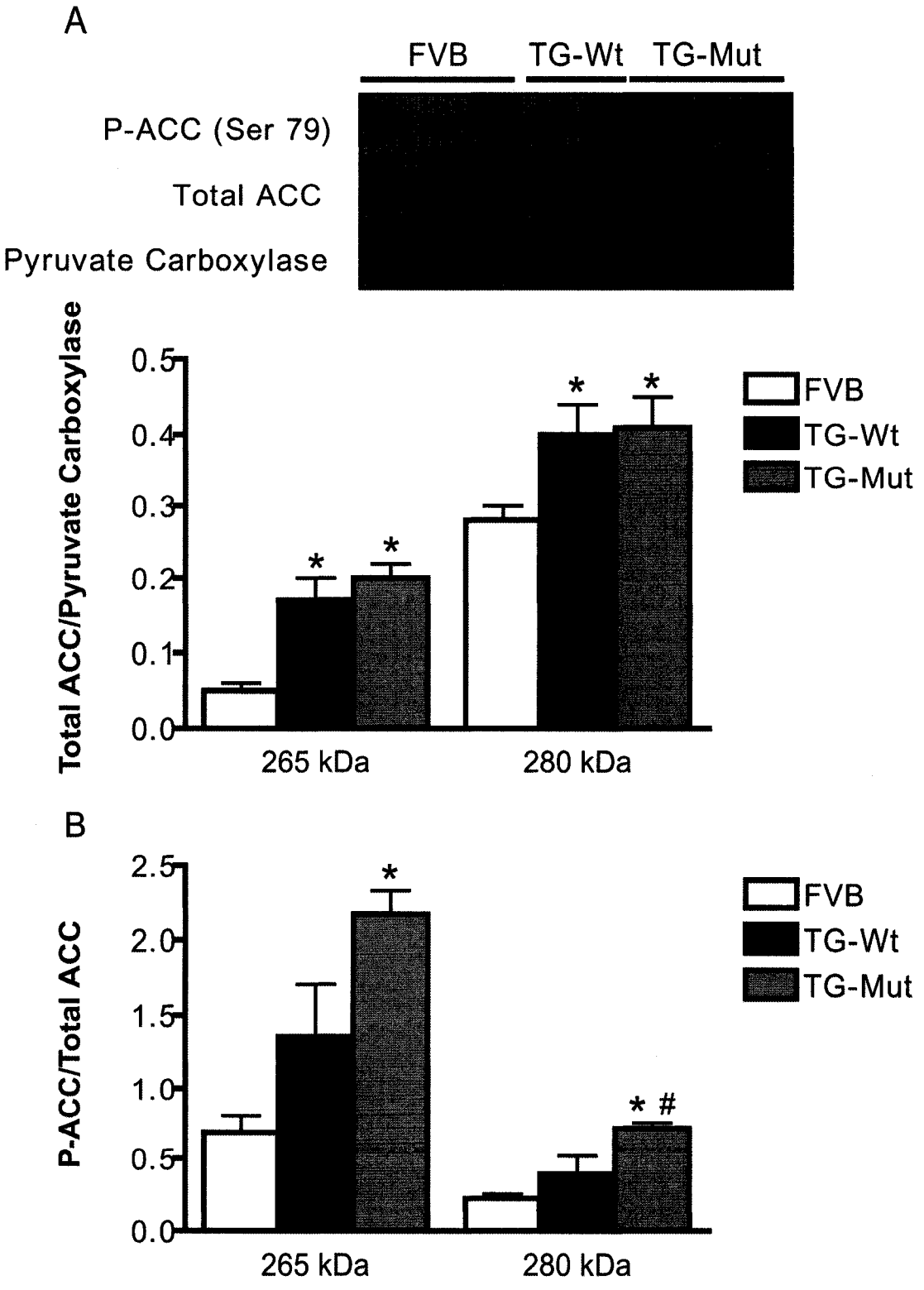

A
FVB
TG-Wt
TG-Mut
P-ACC (Ser 79)
Total ACC
Pyruvate Carboxylase
Total ACC/Pyruvate Carboxylase
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
*
265 kDa
280 kDa
FVB
TG-Wt
TG-Mut
B
P-ACC/Total ACC
2.5
2.0
1.5
1.0
0.5
0.0
*
* #
265 kDa
280 kDa
FVB
TG-Wt
TG-Mut

Figure 5-7

Discussion

Only a few studies have directly associated AMPK activity with functional recovery during reperfusion following ischemia, and these have reached differing conclusions.[192, 221, 222] Therefore, whether AMPK activation is cardioprotective in the ischemic and reperfused myocardium is still controversial.[220] In the present study we utilized isolated working hearts from mice overexpressing either $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q to examine the direct effect of increased ischemic-induced AMPK activity on myocardial oxidative metabolism and functional recovery during reperfusion following ischemia. Using this approach we made a number of important findings. First, overexpression of either $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q in the heart results in elevated AMPK activity following ischemia. This contrasts previous reports, which suggest this mutation is a loss of function mutation.[276] Indeed, in non-perfused hearts, AMPK activity is decreased in the $AMPK_{\gamma 2}$ R302Q hearts (personal communication, Dr. Jason R. B. Dyck). Second, this increase in AMPK activity is associated with a higher reliance on palmitate as a source of oxidative metabolism at the expense of glucose oxidation. Third, $AMPK_{\gamma 2}$ R302Q hearts have impaired recovery of cardiac function during reperfusion following global ischemia despite elevated AMPK activity. Fourth, overexpression of either $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q does not affect rates of glycolysis of exogenous glucose but expression of $AMPK_{\gamma 2}$ R302Q increases glycolysis of endogenous glucose, mostly likely due to an increase in glycogen stores.

Much controversy has surrounded the effect of the $AMPK_{\gamma}$ mutations on AMPK activity. A previous report suggests that when CCL13 cells are transfected with cDNA

encoding $AMPK_{\gamma 2}$ R302Q or R531G and placed under anoxic conditions, they have reduced AMPK activity and phosphorylation compared to cells transfected with $AMPK_{\gamma 2}$.[277] In addition, cells harboring either of these mutations are less responsive to changes in AMP concentration, suggesting that these mutations in the CBS domains interfere with adenine nucleotide binding.[277] Those observations are confirmed in transgenic mice harboring these mutations, as basal AMPK activity is almost completely abolished, however activity was not determined when these hearts were subjected to metabolic stress such as anoxia or ischemia.[216, 276] We show that I/R results in a significant stimulation of AMPK activity and phosphorylation in hearts harboring the R302Q mutation. This suggests that either the AMPK complexes containing the mutation are more sensitive to phosphorylation by the upstream AMPKKs or may be more sensitive to a rise in AMP levels, which are significantly elevated in the TG-Mut hearts. Experiments are currently ongoing to determine if AMPK activity is suppressed during aerobic perfusion and whether this activation of AMPK is simply due to ischemia or if reperfusion is required. This observation contrasts findings seen in transgenic mice overexpressing the $AMPK_{\gamma 2}$ N488I mutation, where these mice have high basal AMPK activity and phosphorylation and are not responsive to ATP depletion due to myocardial ischemia.[212, 213] This difference in response to ischemia may be due to the location of the mutation; unlike the R302Q mutation, the N488I mutation does not reside in a CBS domain, but resides between CBS domain 3 and 4, thus this mutation may have different effects on adenine nucleotide binding.[210]

The major previous study correlating AMPK activity to reperfusion contractile function used an $AMPK_{\alpha 2}$ KD transgenic mouse.[192] Russell *et al.* demonstrated that the

KD mice have worse cardiac function during aerobic perfusion, low-flow ischemia, and reperfusion, which is associated with the inability to increase glucose uptake and lactate production.[192] Related to these metabolic and functional changes, KD hearts have increased cellular damage as observed by increases in LDH and creatine kinase release, and an increase in apoptotic cells. Interestingly, these hearts were perfused with low levels of fatty acids (0.4 mM), which may lessen the detrimental effect of elevated fatty acid oxidation on glucose oxidation and H^+ production. Surprisingly, rates of fatty acid oxidation and glucose oxidation do not change between the KD and Wt groups.[192] Recently, this group reported that MIF is released from the ischemic heart and can partially account for the ischemic-induced activation of AMPK. Indeed, when MIF knockout (KO) mice are subjected to I/R in a Langendorff model, the KO hearts have impaired functional recovery during reperfusion and have larger infarct sizes following *in vivo* LAD ligation, which the authors contribute to the inability to activate AMPK and increase glucose uptake.[178] However, the loss of MIF only impairs ischemic-induced AMPK activation and glucose uptake by a small margin, yet results in significant impairment of ischemic tolerance, suggesting that there may be alternative mechanisms by which MIF impairs ischemic tolerance.

We demonstrate that overexpression of either $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q produces a significant increase in palmitate oxidation at the expense of glucose oxidation (Figure 5-3), such that nearly 90 % of the acetyl-CoA produced in these hearts originated from palmitate (Figure 5-5). Previous studies have demonstrated that a higher reliance on fatty acid oxidation as a source of oxidative metabolism is associated with impaired functional recovery during reperfusion [31, 32], which is also observed in the present study,

as hearts expressing $AMPK_{\gamma 2}$ R302Q have impaired functional recovery during reperfusion. Impaired glucose oxidation during I/R may lead to the poor recovery of contractile function because if the pyruvate from accelerated glycolysis is not subsequently oxidized, there is the net production of both lactate and H^+ from the hydrolysis of glycolytically-derived ATP.[269, 270] This H^+ production is a major contributor to the acidosis that is associated with ischemia.[269] The intracellular acidosis can lead to a sequelae of adverse events as previously discussed in Chapter 2. If glycolytically-derived pyruvate is aerobically metabolized (i.e. glucose oxidation), the net production of H^+ is zero as they are consumed by the TCA cycle.[134, 269, 270] Indeed, during severe ischemia overall mitochondrial oxidative metabolism is inhibited due to a decrease in oxygen supply to the heart, which leads to a competition between fatty acids and glucose as a source of residual TCA cycle acetyl-CoA.[117, 278] Overexpression of $AMPK_{\gamma 2}$ or $AMPK_{\gamma 2}$ R302Q results in severe aerobic uncoupling of glucose metabolism. However this uncoupling does not lead to contractile dysfunction, as there is sufficient coronary flow to remove the excess H^+. Overall rates of proton production is not elevated during reperfusion compared to the aerobic period, however as cardiac function is significantly impaired during reperfusion, the rate of proton production normalized to cardiac power is significantly elevated during reperfusion compared to the aerobic period.

Recently, Banerjee *et al.*, demonstrated that mice with a T400N mutation in $AMPK_{\gamma 2}$ have a 50 % reduction in AMPK activity at six weeks of age and a 100-fold increase in glycogen content. These mice are more susceptible to ischemic damage in an *in vivo* model of 30 min ligation of the LAD followed by 48 hr of reperfusion.[274] However, it is difficult to interpret these data for a number of reasons: the results are not

compared to mice overexpressing the $AMPK_{\gamma 2}$ Wt, there is a significant degree of glycogen accumulation in these mice and no metabolic measurements were performed. Basal AMPK activity in $AMPK_{\gamma 2}$ T400N mice is also approximately 50 % lower than Wt mice, however overexpression of the $AMPK_{\gamma 2}$ subunit alone can lead to a similar decrease in AMPK activity [216, 276] making it difficult to interpret the effect of the mutation on AMPK activity. In addition, previous work on ischemic tolerance and glycogen content suggests that high glycogen content may be cardioprotective during short periods of ischemia by providing a source of glycolytic ATP generation, although during prolonged ischemia, high pre-ischemic glycogen content may be detrimental to cardiac function due to accelerated H^+ production and subsequent Na^+ and Ca^{2+} overload.[275] As post-ischemic glycogen was not measured, there is no way to determine the extent of glycogen depletion and thus we cannot rule out a role of metabolic byproducts leading to the decrease in ischemic tolerance rather than impaired AMPK activity.

An unexpected finding of this study is that increased AMPK activity due to overexpression of $AMPK_{\gamma 2}$ and $AMPK_{\gamma 2}$ R302Q is not associated with a stimulation of glycolysis from exogenous glucose. Normally, AMPK activation is associated with a simulation of glycolysis via an increase in glucose uptake and the phosphorylation and activation of PFK-2, resulting in the increased production of the potent activator of PFK-1, fructose-2,6-bisphosphate.[204] However this result should be interpreted with caution, as a limitation of our model is that only glycolytic flux from exogenous radiolabelled glucose was measured in our system. Normally when glycogen levels are similar among perfusion groups this would give a good estimation of total glycolytic flux, however in the case of the present study where glycogen content is over 30-fold higher in one of the

groups, it may result in a dramatic underestimation of glycolytic flux from endogenous substrates. In the present study we measured total lactate efflux from these hearts in order to estimate the glycolytic flux from endogenous substrates, and as expected total lactate efflux is significantly accelerated in the $AMPK_{\gamma 2}$ R302Q hearts. This would suggest that total glycolytic flux of both exogenous and endogenous substrates is accelerated in these hearts. This correlates well with a recent study where Luptak *et al.* demonstrates that glycolytic flux of exogenous glucose is suppressed in $AMPK_{\gamma 2}$ N488I expressing hearts measured by ^{13}C-lactate output, however when glycogen stores are pre-labeled with ^{13}C-glucose, ^{13}C-lactate output was accelerated in mutant hearts.[217]

Limitations

This study utilized the isolated working heart method, which has some limitations as previously discussed in Chapter 3 and Chapter 8. In this study, $AMPK_{\alpha 1}$- versus $AMPK_{\alpha 2}$-associated activity is not separated out, thus total activity and phosphorylation status of AMPK was reported. This limits our ability to delineate isoform specific effects of modifying AMPK activity during I/R. In addition, enzyme activities where determined under V_{max} conditions; therefore any changes in enzyme activity related to modifications in K_m would not be detected.

In summary, this study demonstrates that overexpression of the $AMPK_{\gamma 2}$ R302Q mutation results in elevated I/R-induced AMPK activity that is associated with a higher reliance on palmitate as a source of mitochondrial oxidative metabolism. This activation of AMPK activity is associated with impaired recovery of contractile function during reperfusion following global no-flow ischemia. These findings have important

implications for the role of AMPK as a therapeutic target to modulate myocardial energy metabolism during I/R injury.

CHAPTER 6.

Transgenic Expression of Slow Skeletal Troponin I in Cardiac Muscle Impairs Recovery of Post-Ischemic Contractile Function

My role in this work involved performing all the experiments (except those noted below) as well as writing the manuscript. Ken Strynadka and Panakkezhum Thomas performed the HPLC analysis of the adenine nucleotides, Donna Beker and Sandra Kelly performed the echocardiography, and Wei Wang performed the LAD ligations.

CHAPTER 6.

Transgenic Expression of Slow Skeletal Troponin I in Cardiac Muscle Impairs Recovery of Post-Ischemic Contractile Function

Abstract

Post-ischemic acidosis due to the uncoupling of glucose metabolism is correlated with cardiac dysfunction, however the contribution of the direct effect of acidosis on the contractile apparatus is unknown. We tested the hypothesis that transgenic mice expressing a more acid-resistant slow skeletal isoform of troponin I (ssTnI) would be protected in the setting of I/R. *In vivo* function was assessed using transthoracic echocardiography. ssTnI animals have similar *in vivo* cardiac function displaying similar treadmill exercise time and distance, as well as similar systolic echocardiographic indices, however they have slight diastolic dysfunction as indicated by longer isovolumic relaxation time (21.6 ± 1.5 vs. 14.6 ± 1.0 ms, n = 9 and 7, $P < 0.05$) and pulmonary artery acceleration time (24.2 ± 1.0 vs. 19.7 ± 1.0 ms, n = 9 and 7, $P < 0.05$). To examine the effect of ssTnI expression on recovery of post-ischemic function and myocardial energy substrate metabolism, we subjected isolated working mouse hearts from ssTnI and Wt

littermate mice to a 30 min aerobic perfusion, 18 min global no-flow ischemia and 40 min aerobic reperfusion with Krebs-Henseleit solution containing 5 mM [5-^{3}H/U-^{14}C]glucose, 1.2 mM [9,10-^{3}H]palmitate, and 100 μU/ml insulin. Mechanical function and rates of glycolysis, glucose oxidation and fatty acid oxidation do not differ between ssTnI and Wt hearts, however H^{+} production from uncoupled glucose metabolism is accelerated in the ssTnI hearts (13770 ± 1500 vs. 9870 ± 1080 nmol/g dry wt/min, n = 6 and 8, $P < 0.05$). Recovery of cardiac power during reperfusion is impaired in the ssTnI hearts (2 ± 0.7 vs. 18 ± 5 mJoule, n = 16 and 17 respectively, $P < 0.05$). At the end of reperfusion, ATP content is significantly lower in ssTnI hearts (4.8 ± 0.8 vs. 11.8 ± 2.7 μmol/g dry wt, n = 6 per group, $P < 0.05$). As there is no difference in AMP content, ssTnI hearts have an elevated AMP/ATP ratio (0.67 ± 0.10 vs. 0.36 ± 0.13, n = 6 per group, $P < 0.05$). Despite the elevation in AMP/ATP ratio, the phosphorylation status of AMPK and ACC does not differ between groups. ssTnI hearts also have increased infarct size when subjected to *in vivo* LAD ligation and reperfusion (area of infarct/area at risk; 40 ± 3 vs. 26 ± 3, n = 6 per group, $P < 0.05$). Although ssTnI and Wt mice exhibit similar aerobic function *in vivo* and *in vitro*, our results demonstrate that the cardiac expression of an acid-resistant ssTnI is not cardioprotective but, unexpectedly, impairs recovery of contractile function during reperfusion.

Introduction

Myocardial ischemia occurs when the oxygen requirement of the heart exceeds the oxygen supplied via the coronary circulation and is a devastating cause of morbidity and mortality worldwide. A contributing factor to I/R injury is modification of myocardial energy substrate metabolism.[5, 54, 122] A majority of the energy requirement of the normal well-perfused heart is met by the oxidation of fatty acids, with the remainder coming from the oxidation of carbohydrates.[5, 54, 122] However during ischemia, mitochondrial oxidative metabolism is suppressed and anaerobic glycolysis becomes an important source of ATP.[133] As pyruvate from glycolysis cannot be subsequently oxidized, there is a net production of deleterious byproducts (lactate and H^+s from the hydrolysis of glycolytically-derived ATP) which has been correlated with post-ischemic cardiac dysfunction.[31, 32, 121, 269, 270] However, the mechanism by which myocardial acidosis leads to suppression of post-ischemic cardiac contractile function is unknown.

Several mechanisms may account for the suppressed recovery of post-ischemic contractile function. One of these mechanisms is that energy may be required to resume normal cellular ion homeostasis during reperfusion, which would reduce the amount available for contractile function and hence reduce cardiac efficiency. As discussed in Chapter 1, a downstream effect of I/R-induced acidosis is the intracellular accumulation of Na^+ due to the extrusion of H^+s by NHE/NBC.[125-127, 129] Na^+ may also accumulate by two other processes; the inability to remove Na^+ due to the inactivation of Na^+-K^+ ATPase because of a reduced supply of ATP, a decrease in extracellular pH and inactivation by ROS, as well as due to persistent or late Na^+ current.[9-12, 24, 128]

Subsequently this accumulation of Na^+ may lead to Ca^{2+} overload due to the activation of the reverse mode of NCX.[127, 129, 130] Ca^{2+} overload may be further exacerbated by the inability to reuptake Ca^{2+} back into the sarcolemmal reticulum due to the ROS induced inhibition of Ca^{2+} ATPase.[13] Several ATP requiring pumps are essential to deal with the dysregulation of ion homeostasis during reperfusion: the Na^+-K^+ ATPase, the vacuolar H^+ ATPase and the Ca^{2+} ATPase, all of which use ATP for non-contractile purposes thus reducing cardiac efficiency.[135]

An alternative mechanism by which acidosis may impair post-ischemic contractile function is via a direct effect of H^+s on the contractile apparatus. In skinned fiber bundles a decrease in pH (acidosis) produces a rightward shift in the Ca^{2+}-force curve.[136, 137] The mechanisms by which this occurs may be due to H^+s out-competing Ca^{2+} for its binding site on cardiac troponin (cTnC), although this cannot completely account for the effects of acidic pH.[137-139] Acidosis can also affect the protein-protein interactions that are required to transduce the Ca^{2+} binding signal [138, 140] and reduce crossbridge binding to actin, thus reducing tension independent of Ca^{2+} and impairing the strong crossbridge-induced activation of thin filaments.[141]

In order to better elucidate the mechanism of I/R-induced cardiac dysfunction we utilized a slow skeletal troponin I (ssTnI) transgenic mouse where the normal adult cTnI is quantitatively replaced with ssTnI in the heart.[279] The ssTnI isoform is normally expressed in embryonic and early postnatal life and is replaced by cTnI within the first two weeks of life in the mouse. The importance of the ssTnI isoform is that it is more resistant to acidosis than the adult isoform, such that there is less change in Ca^{2+} sensitivity due to a decrease in pH and it shifts the Ca^{2+}-tension relationship to the right

(indicating that the contractile apparatus is more sensitive to Ca^{2+}).[138, 279-285] Therefore if the detrimental effects of I/R injury is dependent on the acidosis-induced changes in the contractile apparatus, expression of ssTnI should be cardioprotective. Alternatively, if sensitivity to Ca^{2+} is a more important determinant of I/R-induced cardiac dysfunction, then ssTnI expression should be detrimental to functional recovery during reperfusion as there would be less acidosis-induced desensitization of the contractile apparatus and would be more susceptible to pathological Ca^{2+} overload leading to contractile dysfunction. A recent report has suggested that ssTnI is cardioprotective in a Langendorff model of I/R, however that study did not correlate post-ischemic cardiac function with myocardial metabolism or cardiac energetics.[286] Thus we hypothesize that hearts expressing ssTnI should be cardioprotective in the setting of I/R due to a blunted acidosis-induced desensitization of the contractile apparatus to Ca^{2+}.

Materials and 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.

Transgenic Mice

Transgenic mice expressing ssTnI were a kind gift of R. John Solaro (University of Illinois at Chicago). Animals were constantly outbred with CD-1 mice and housed in a controlled environment with a 12:12 hr light-dark cycle. The transgene consisted of the insertion of the ssTnI gene into the pMHC poly(A) vector and was injected into CD-1 single cell embryos, as previously described.[279] Experiments were performed on mice of two different ages due to the availability of mice, young mice (18 weeks of age) and old mice (8 – 9 months of age).

Genotyping

DNA was extracted from tail biopsies using the DNeasy Blood and Tissue Kit from Qiagen (Mississauga, Ontario). The ssTnI genotype was identified by the expression of both the αMHC promoter and the ssTnI gene. αMHC was amplified from extracted DNA with PCR using the following primers: forward (5'-GAG TTT GAG TGA CCG CGT CTT AAG G-3') and reverse (5'-TCG CAT GCC TGC AGA GCT CTA GAG T-3') to generate a 546 bp gene product that was confirmed on an agarose gel. ssTnI was also amplified from extracted DNA with PCR using the following primers:

forward (5'-GAT GAG GAG CGC TAT GAT ATC GAA GC-3') and reverse (5'-GCT GCA ATA AAC AAG TTG GCC ATG G-3') to generate a 474 bp gene product that was confirmed on an agarose gel (Figure 6-1).

In Vivo Echocardiography Assessment of Cardiac Function

Transthoracic echocardiography was performed on isoflurane-anesthetized mice (3.5 % to induce anesthesia and 1 - 3 % to maintain anesthesia) with a Vevo 770 High Resolution Imaging system equipped with a 30-MHz transducer (VisualSonic, Toronto, Canada), as previously described.[287, 288] Left ventricular internal dimension (LVID), intraventricular septum thickness (IVST) and left ventricular wall thickness (LVWT) were all obtained from M-mode images. All measurements were done from leading edge to leading edge according to the American Society of Echocardiography guidelines.[289-291] Percent left ventricular fractional shortening and percent ejection fraction was automatically calculated by the VisualSonic's analysis packaged based on the following equation, $(LVID_{diastole}-LVID_{systole}/LVID_{diastole})*100$ and $((LVID_{diastole})^2-(LVID_{systole})^2/(LVID_{diastole})^2)*100$, respectively.[289-291] Left ventricular isovolumic relaxation time was measured as the time interval between the end of left ventricular outflow (closing of the aortic valve) and the beginning of the mitral inflow (opening of the mitral valve) and pulmonary artery acceleration time was measured from the onset of pulmonary flow to its peak.[289, 292]

Graded Exercise Test

Exercise capacity was utilized to ensure the ssTnI mice displayed no overt cardiac dysfunction in response to an increase in workload and stress.[293] Tests were performed on a calibrated animal treadmill (Columbus Instruments, Columbus, Ohio) with incremental increases in belt speed until the animals exhibited signs of exhaustion (as defined by the lesser amount of time of the mouse spending more than 15 seconds on the shock grid, or the mouse spending more than 50 % of the time on the shock grid), as previously described.[294]

Isolated Working Mouse Heart Perfusions

CD-1 and ssTnI mouse hearts were perfused in the working perfused mode as previously described in Chapter 2.[239] The oxygenated Krebs-Henseleit solution contained either no fatty acids and no insulin (no fat/no insulin) or 1.2 mM palmitate and 100 μU/mL insulin (high fat/insulin). Spontaneously beating hearts were perfused at a constant left atrial preload pressure of 11.5 mmHg and a constant aortic afterload pressure of 50 mmHg for 30 min aerobic perfusion followed by 16 min (no fat/no insulin) or 18 min (high fat/insulin) of global no-flow ischemia and 40 min of aerobic reperfusion. At the end of the perfusion protocols, the hearts were quick frozen in liquid nitrogen with Wollenberger tongs, and stored at -80 °C.

Glycolysis, glucose and palmitate oxidation were measured by quantitative collection of the $^{3}H_2O$ and $^{14}CO_2$, respectively, derived from [5-^{3}H/U-^{14}C]glucose and [9, 10-^{3}H]palmitate, as described in Chapter 2.[134, 238] Rates of H^+ production were calculated as described in Chapter 2.

In a subset of isolated working mouse hearts, myocardial oxygen consumption was measured from the partial oxygen pressure of the perfusate using a "flow-thru" pH microelectrode (Microelectrodes, Inc, Bedford, New Hampshire) in the preload line and partial oxygen pressure of the coronary effluent by insertion of a FOXY-AL300 fiber-optic probe (Ocean Optics, Dunedin, Florida) into the pulmonary trunk.[295]

Tissue Extractions

Immunoblotting was performed on cleared homogenates for total and phosphorylated AMPK and ACC as described in Chapter 2. Adenine nucleotides were extracted for HPLC analysis as described in Chapter 2.

In Vivo Left Anterior Descending Coronary Artery Ligation and Reperfusion

Mice were placed under inhaled anesthesia (1-3% isoflurane) and intubated using a 14 or 20 gauge polyethylene catheter and ventilated (300 μL per breath at a rate of 140 breaths per minute) with 100% oxygen (containing 1-3% isoflurane) using a small animal ventilator. Throughout the procedure and for 18 hours following the mice are place on a heating plate set at 37 °C. A left thoracotamy was performed in the fifth intercostal space and the pericardium was opened to expose the left ventricle. The LAD under the tip of the left atrial appendage was temporarily ligated using a 2-0 silk suture. After 1/2 an hour of LAD ligation, the suture knot was cut to allow for reperfusion. After the onset of reperfusion, the muscle and skin was closed in layers with 6-0 silk suture and mice were allowed to recover using buprenorphine as an analgesic (0.05 mg/Kg). The sham-

operated group underwent the same procedure, except their LADs were not ligated. After 24 hours of reperfusion, mice were anesthetized with pentobarbital sodium (60 mg/Kb i.p.) and hearts were removed and perfused in the Langendorff mode with oxygenated Krebs-Henseleit solution containing 5 mM glucose. The LAD was religated and hearts were perfused with 1 % Evans blue to delineate the area at risk (AAR) and subsequently frozen. Hearts were then sliced and stained with 1 % triphenyltetrazolium chloride for 20 min at 37 °C to allow for measurement of infarct size (IS), which was quantified using ImageJ (National Institutes of Health, Bethesda, Maryland).

Statistical Analysis

All data are presented as the mean ± S.E.M. The data were analyzed with the statistical program Instat 2.01 and GB-stat. Two-way repeated measures ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for cardiac power. One-way or two-way ANOVA with a Bonferroni post-hoc test was used to evaluate the statistical significance of differences among groups for the metabolic data. Values of $P < 0.05$ were considered significant.

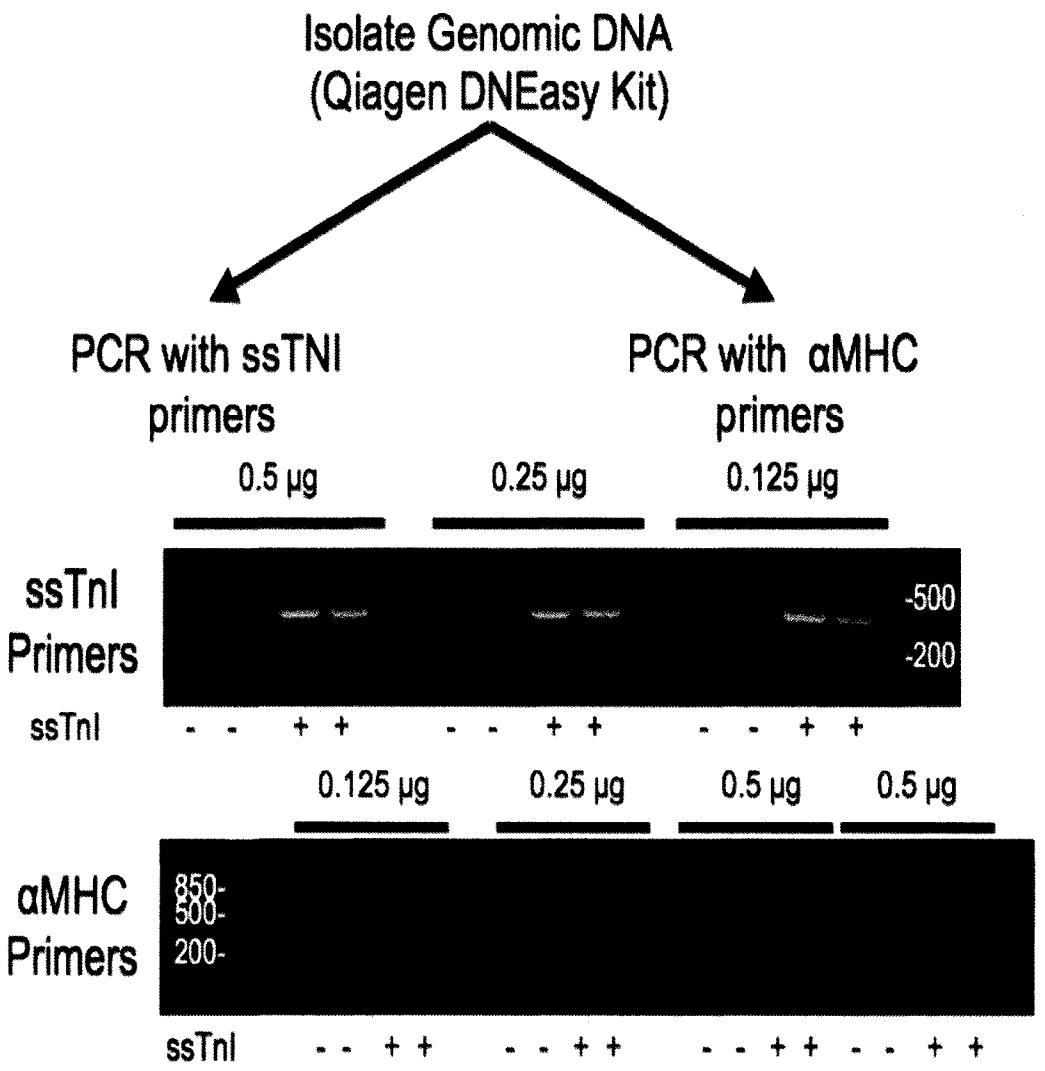


Figure 6-1: Genotyping strategy for mice expressing ssTnI.

Results

The Effect of ssTnI Expression in Old Mice

In Vivo Cardiac Function

Mice with cardiac specific transgenic expression of ssTnI are grossly normal with no overt phenotype. *In vivo* echocardiography shows that the hearts of these mice are structurally and functionally normal during systole, although they demonstrate some diastolic dysfunction as displayed by decreased isovolumic relaxation time (21.6 ± 1.5 vs. 14.6 ± 1.0 ms, n = 7 and 9, $P < 0.05$, Table 6-1) and pulmonary artery acceleration time (24.2 ± 1.0 vs. 19.7 ± 1.0 ms, n = 7 and 9, $P < 0.05$, Table 6-1). In addition, expression of ssTnI does not modify the ability of animals to respond to a treadmill exercise test, as exercise distance and time are not different between groups (Figure 6-2).

The Effect of Expression of ssTnI on Aerobic Cardiac Function and Metabolism in Isolated Working Hearts

In the presence of high fat and insulin, expression of ssTnI does not affect aerobic cardiac contractile function (Figure 6-3 and Table 6-2). In addition, ssTnI expression does not modify rates of glycolysis, glucose or palmitate oxidation, however as there are opposing trends in glycolysis and glucose oxidation, there is a significantly higher rate of H^+ production in the ssTnI mice (13770 ± 1500 vs. 9870 ± 1080 nmol/g dry wt/min, n = 6 and 8, $P < 0.05$, Figure 6-3D).

In the absence of fat and insulin, HR is significantly impaired in ssTnI mice (190 ± 30 vs. 283 ± 9 bpm, n = 4 and 7, $P < 0.05$, Table 6-3), although cardiac power, peak

systolic pressure and cardiac output does not differ significantly (Figure 6-3B and Table 6-3). In contrast to the previous group, ssTnI expression significantly decreases glucose oxidation (380 ± 47 vs. 960 ± 170 nmol/g dry wt/min, $P < 0.05$, n = 4 and 7, Figure 6-5B,) but does not modify glycolysis or H^+ production in the absence of fatty acids (Figure 6-5A and C).

The Effect of Expression of ssTnI on Cardiac Function and Metabolism During Reperfusion Following Global No-Flow Ischemia in Isolated Working Hearts

In the presence of high fat and insulin, ssTnI expression significantly impairs the recovery of cardiac power during reperfusion following ischemia (2 ± 0.7 vs. 18 ± 5 mJoule, n= 16 and 17, $P < 0.05$, Figure 6-3A and Table 6-2), which is associated with impaired PSP (18 ± 2 vs. 48 ± 5 mmHg, n= 16 and 17, $P < 0.05$, Table 6-2) and cardiac output (1.0 ± 0.3 vs. 2.7 ± 0.6 mL/min, n= 16 and 17, $P < 0.05$, Table 6-2). During reperfusion, rates of glycolysis, glucose oxidation, palmitate oxidation and H^+ production do not differ between the groups (Figure 6-4). However, at the end of reperfusion, ATP levels are impaired in the ssTnI hearts (4.8 ± 0.8 vs. 11.8 ± 2.7 μmol/g dry wt, n = 6 per group, $P < 0.05$, Table 6-4), which results in a significant increase in the AMP/ATP ratio (0.67 ± 0.10 vs. 0.36 ± 0.13, n = 6 per group, $P < 0.05$, Table 6-4). Despite these changes in adenine nucleotides, the phosphorylation status of AMPK and its downstream target ACC are not different between groups (Figure 6-6).

In the absence of fat and insulin, recovery of contractile function during reperfusion does not differ between groups (Figure 6-3B and Table 6-3). During

reperfusion, rates of glycolysis, glucose oxidation and H^+ production do not differ between the groups (Figure 6-5).

The Effect of Expression of ssTnI on Infarct Size Following In Vivo Left Anterior Descending Coronary Artery Ligation and Reperfusion

Following 30 min of LAD ligation and 24 hours of reperfusion the area at risk (AAR) of heart slices does not differ between groups (Figure 6-7B) suggesting a similar degree of ischemia is induced in both groups. Despite this, hearts expressing ssTnI have a significantly greater infarct size (IS) (IS/AAR; 40 ± 3 vs. 26 ± 3, n = 6 per group, $P < 0.05$, Figure 6-7C), suggesting that ssTnI hearts are less resistant to an ischemic insult.

The Effect of ssTnI Expression in Young Mice

Experiments were also carried out utilizing young mice in order to confirm that the results observed in the old ssTnI mice were not due to aging or aging-induce compensatory changes in these transgenic mice.

In Vivo Cardiac Function

In vivo echocardiography shows that the hearts of these mice were structurally and functionally normal (Table 6-5).

The Effect of Expression of ssTnI on Baseline Cardiac Function and Metabolism in Isolated Working Hearts

In the presence of high fat and insulin, expression of ssTnI significantly impairs HR (201 ± 16 vs. 263 ± 9 bpm, n = 7 and 9, $P < 0.05$, Table 6-6) but does not significantly affect aerobic cardiac power or cardiac efficiency (Figure 6-8 and Table 6-6). In addition, ssTnI expression does not modify rates of glycolysis, glucose oxidation, palmitate oxidation, or H^+ production (Figure 6-9).

In the absence of fat and insulin, expression of ssTnI significantly impairs cardiac output (7.1 ± 0.5 vs. 9.3 ± 0.2 mL/min, n = 3 per group, $P < 0.05$, Table 6-7) and cardiac power (59 ± 4 vs. 72 ± 4 mJoule, n = 3 per group, $P < 0.05$, Table 6-7). ssTnI expression does not modify rates of glycolysis, glucose oxidation, or H^+ production (Figure 6-10).

The Effect of Expression of ssTnI on Cardiac Function and Metabolism During Reperfusion Following Global No-Flow Ischemia in Isolated Working Hearts

In the presence of high fat and insulin, ssTnI expression significantly impairs the recovery of cardiac power during reperfusion following ischemia (1 ± 0.3 vs. 15 ± 7 mJoule, n = 7 and 9 respectively, $P < 0.05$, Figure 6-8A), however cardiac efficiency does not differ between the groups (Figure 6-8B). During reperfusion, rates of glycolysis, glucose oxidation, palmitate oxidation and H^+ production do not differ between the groups (Figure 6-9). In the absence of fat and insulin, recovery of contractile function during reperfusion does not differ between groups (Figure 6-10C and Table 6-7). During reperfusion, rates of glycolysis, glucose oxidation and H^+ production do not differ between the groups (Figure 6-5).

Table 6-1. *In vivo* echocardiography analysis of old mice

	Wildytype (n = 9)	ssTNI (n = 7)
Heart Rate (bpm)	537 ± 18	498 ± 24
% Ejection Fraction	61 ± 2	64 ± 4
% Fractional Shortening	33 ± 1	35 ± 3
Intraventricular Septum Thickness (mm)		
Systole	1.14 ± 0.09	1.07 ± 0.09
Diastole	0.74 ± 0.05	0.67 ± 0.04
Left Ventricular Internal Dimension (mm)		
Systole	2.76 ± 0.17	2.84 ± 0.17
Diastole	4.11 ± 0.20	4.33 ± 0.13
Left Ventricular Posterior Wall Thickness (mm)		
Systole	1.13 ± 0.04	1.18 ± 0.08
Diastole	0.85 ± 0.04	0.80 ± 0.06
Isovolumic Relaxation Time (ms)	14.6 ± 1.0	21.6 ± 1.5*
Pulmonary Artery Acceleration Time (ms)	19.7 ± 1.0	24.2 ± 1.0*

Differences were determined using a t-test.

* represents $P < 0.05$, significantly different from corresponding wildtype group.

Table 6-2. The effect of ssTnI expression on parameters of contractile function in old mice during aerobic perfusion and reperfusion following 18 min of global ischemia in the presence of high fat and insulin.

	Aerobic Perfusion		Reperfusion	
	Wildtype (n = 17)	ssTNI (n = 16)	Wildtype (n = 17)	ssTNI (n = 16)
Heart Rate (beats/min)	294 ± 10	283 ± 11	210 ± 19*	254 ± 15*
Peak Systolic Pressure (mmHg)	71 ± 1	71 ± 1	48 ± 5*	18 ± 2*#
Coronary Flow (mL/min)	3.0 ± 0.2	3.0 ± 0.2	1.5 ± 0.2*	1.0 ± 0.3
Cardiac Output (mL/min)	10.3 ± 0.3	9.0 ± 0.3#	2.7 ± 0.6*	1.0 ± 0.3*#

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding wildtype group.

Table 6-3. The effect of ssTnI expression on parameters of contractile function in old mice during aerobic perfusion and reperfusion following 16 min of global ischemia in the absence of fat and insulin.

	Aerobic Perfusion		Reperfusion	
	Wildytype (n = 7)	ssTNI (n = 4)	Wildtype (n = 7)	ssTNI (n = 4)
Heart Rate (beats/min)	283 ± 9	198 ± 30[#]	224 ± 13*	278 ± 15*
Peak Systolic Pressure (mmHg)	68 ± 2	73 ± 2	17 ± 1*	17 ± 5*
Coronary Flow (mL/min)	3.1 ± 0.3	4.3 ± 0.3	0.3 ± 0.05*	0.5 ± 0.2*
Cardiac Output (mL/min)	8.9 ± 0.8	9.5 ± 1.1	0.4 ± 0.02*	0.5 ± 0.2*

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

[#] represents $P < 0.05$, significantly different from corresponding wildtype group.

Table 6-4. The effect of ssTnI expression on adenine nucleotides in old mice at the end of aerobic reperfusion in the presence of high fat and insulin.

	Wildytype (n = 6)	ssTNI (n = 6)
AMP (μmol/g dry wt)	3.0 ± 0.6	2.9 ± 0.2
ADP (μmol/g dry wt)	6.4 ± 0.8	3.9 ± 0.2
ATP (μmol/g dry wt)	11.8 ± 2.7	4.8 ± 0.8*
AMP/ATP	0.36 ± 0.13	0.67 ± 0.10*
ADP/ATP	0.63 ± 0.11	0.88 ± 0.09

Differences were determined using a t-test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Table 6-5. *In vivo* echocardiography analysis of young mice.

	Wildytype (n = 3)	ssTNI (n = 4)
Heart Rate (bpm)	407 ± 24	420 ± 10
% Ejection Fraction	67 ± 3	66 ± 3
% Fractional Shortening	37 ± 2	36 ± 2
Intraventricular Septum Thickness (mm)		
Systole	1.13 ± 0.03	1.25 ± 0.05
Diastole	0.76 ± 0.03	0.84 ± 0.03
Left Ventricular Internal Diameter (mm)		
Systole	2.86 ± 0.25	2.67 ± 0.12
Diastole	4.50 ± 0.25	4.17 ± 0.21
Left Ventricular Posterior Wall Thickness (mm)		
Systole	1.34 ± 0.11	1.23 ± 0.13
Diastole	0.92 ± 0.07	0.80 ± 0.05
Isovolumic Relaxation Time (ms)	17.3 ± 3.2	25.1 ± 3.0
Pulmonary Artery Acceleration Time (ms)	21.7 ± 1.0	21.4 ± 1.8

Differences were determined using a t-test.

Table 6-6. The effect of ssTnI expression on parameters of contractile function in young mice during aerobic perfusion and reperfusion following 18 min of global ischemia in the presence of high fat and insulin.

	Aerobic Perfusion		Reperfusion	
	Wildtype (n = 9)	ssTNI (n = 7)	Wildtype (n = 9)	ssTNI (n = 7)
Heart Rate (beats/min)	263 ± 9	201 ± 16$^{\#}$	195 ± 8*	193 ± 10*
Peak Systolic Pressure (mmHg)	71 ± 1	75 ± 1	37 ± 8*	20 ± 2*$^{\#}$
Coronary Flow (mL/min)	2.3 ± 0.3	2.1 ± 0.2	1.1 ± 0.4*	0.7 ± 0.3
Cardiac Output (mL/min)	9.2 ± 0.6	7.5 ± 0.6	2.4 ± 0.9*	0.7 ± 0.3*

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

$^{\#}$ represents $P < 0.05$, significantly different from corresponding wildtype group.

Table 6-7. The effect of ssTnI expression on parameters of contractile function in young mice during aerobic perfusion and reperfusion following 16 min of global ischemia in the absence of fat and insulin.

	Aerobic Perfusion		Reperfusion	
	Wildytype (n = 3)	ssTNI (n = 3)	Wildtype (n = 3)	ssTNI (n = 3)
Heart Rate (beats/min)	254 ± 10	197 ± 56	251 ± 52*	301 ± 68*
Peak Systolic Pressure (mmHg)	70 ± 1	74 ± 4	19 ± 8*	20 ± 2*
Coronary Flow (mL/min)	3.5 ± 0.4	2.8 ± 0.5	0.1 ± 0.01*	0.3 ± 0.1*
Cardiac Output (mL/min)	9.3 ± 0.2	7.1 ± 0.5#	0.1 ± 0.01*	0.3 ± 0.1*

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

represents $P < 0.05$, significantly different from corresponding wildtype group.

Figure 6-2: Expression of ssTnI in old mice does not affect treadmill exercise capacity.

A) Treadmill exercise time and B) treadmill exercise distance (n = 6 per group). Values represent mean ± SEM. Differences were determined with a t-test.

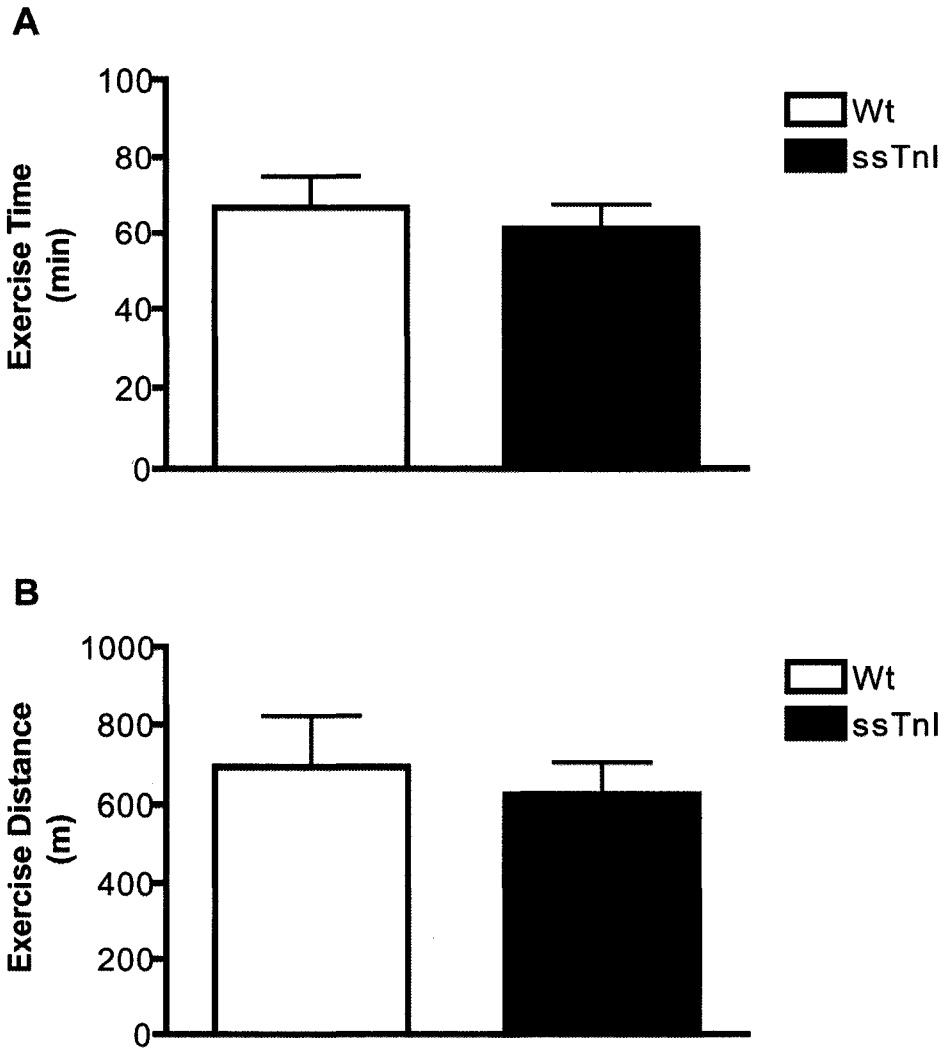

A
100
80
60
40
20
0
Exercise Time (min)
Wt
ssTnI
B
1000
800
600
400
200
0
Exercise Distance (m)
Wt
ssTnI

Figure 6-2

Figure 6-3: Expression of ssTnI in old mice impairs post-ischemic contractile function in the presence of high fat and insulin, but has no effect in the absence of fat and insulin.

A) Cardiac power (n = 17 and 16 for Wt and ssTnI repectively) in isolated mouse hearts perfused in the presence of high fat and insulin subjected to 18 min of global ischemia, and B) cardiac power (n = 9 and 7 for Wt and ssTnI respectively) in isolated mouse hearts perfused in the absence of fat and insulin subjected to 16 min of global ischemia. Values represent mean ± SEM. Differences were determined using a 2-way repeated measures ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from wildtype group.

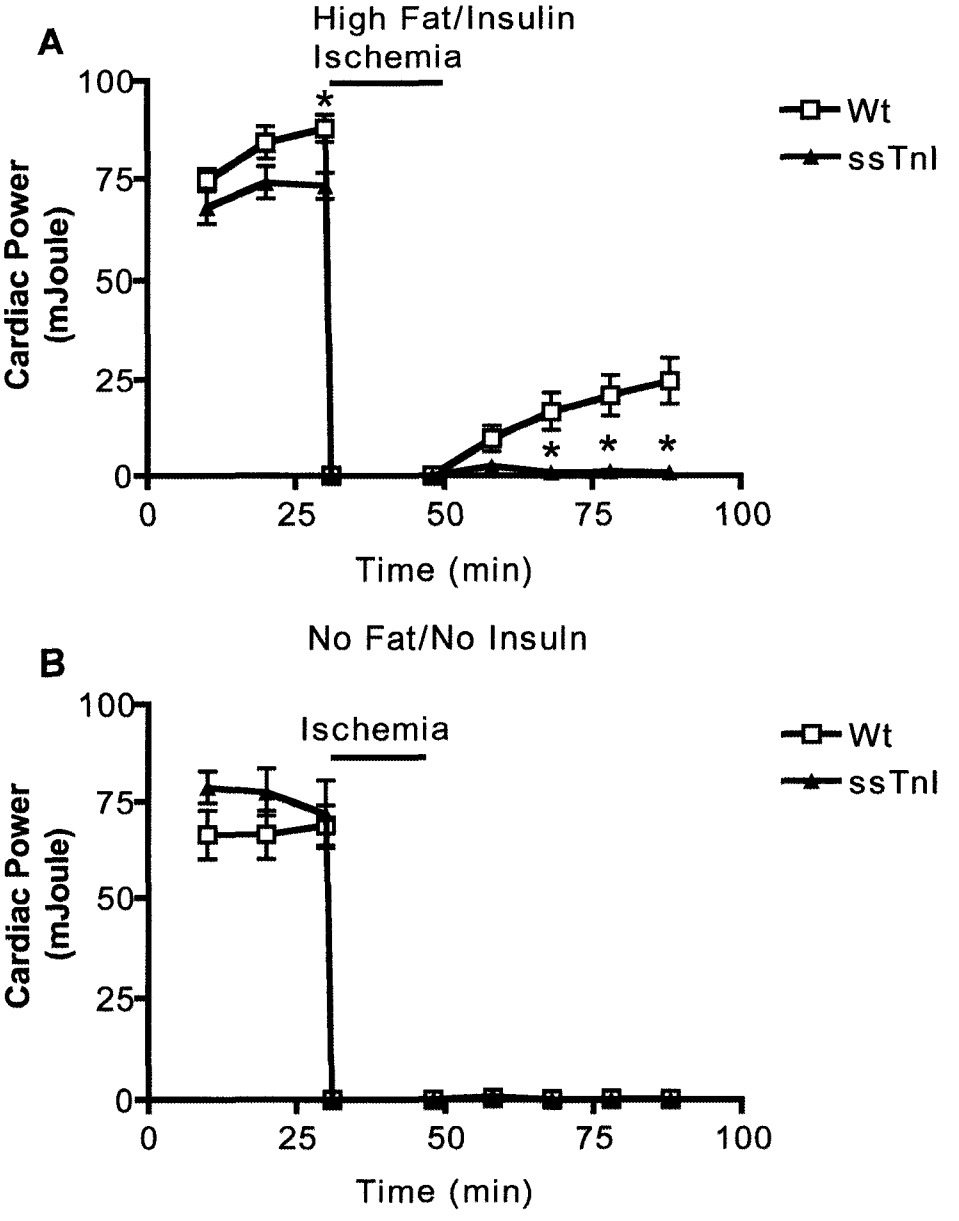

A
High Fat/Insulin
Ischemia
Cardiac Power (mJoule)
100
75
50
25
0
0
25
50
75
100
Time (min)
Wt
ssTnI
B
No Fat/No Insuln
Ischemia
Cardiac Power (mJoule)
100
75
50
25
0
0
25
50
75
100
Time (min)
Wt
ssTnI

Figure 6-3

Figure 6-4: Expression of ssTnI in old mice results in an increase in proton production in the presence of high fat and insulin.

A) Glycolysis (n = 17 and 16 for Wt and ssTnI respectively), B) glucose oxidation (n = 8 and 6 for Wt and ssTnI respectively), C) palmitate oxidation (n = 9 and 10 for Wt and ssTnI respectively) and D) proton production (n = 8 and 6 for Wt and ssTnI respectively) in isolated mouse hearts perfused in the presence of high fat and insulin. Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding wildtype group.

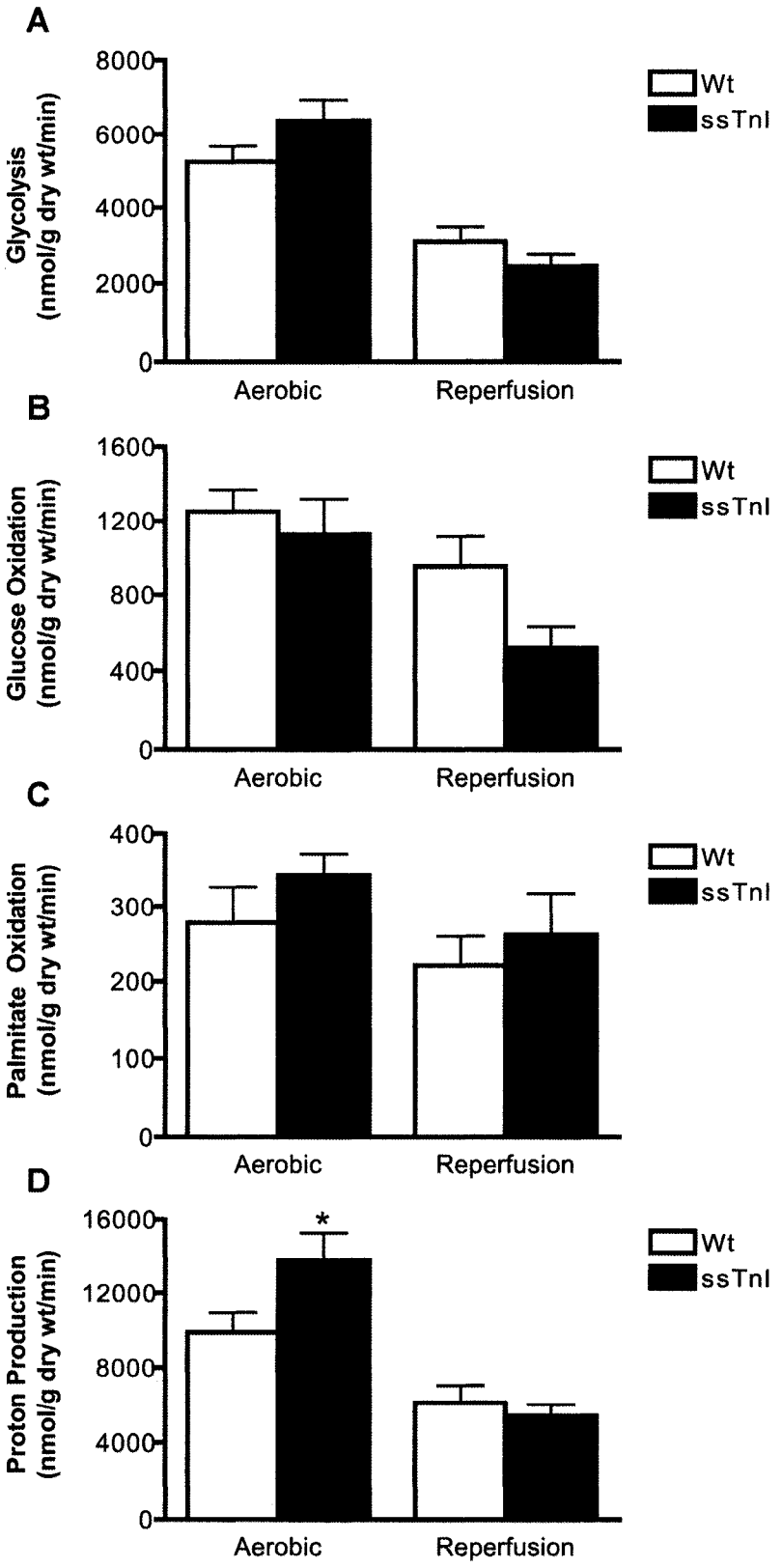

A
Glycolysis (nmol/g dry wt/min)
8000
6000
4000
2000
0
Aerobic
Reperfusion
Wt
ssTnI
B
Glucose Oxidation (nmol/g dry wt/min)
1600
1200
800
400
0
Aerobic
Reperfusion
Wt
ssTnI
C
Palmitate Oxidation (nmol/g dry wt/min)
400
300
200
100
0
Aerobic
Reperfusion
Wt
ssTnI
D
Proton Production (nmol/g dry wt/min)
16000
12000
8000
4000
0
*
Aerobic
Reperfusion
Wt
ssTnI

Figure 6-4

Figure 6-5: Expression of ssTnI in old mice impairs glucose oxidation in the absence of fat and insulin.

A) Glycolysis, B) glucose oxidation and C) proton production (n = 7 and 4 for Wt and ssTnI respectively) in isolated mouse hearts perfused in the absence of fat and insulin. Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding wildtype group.

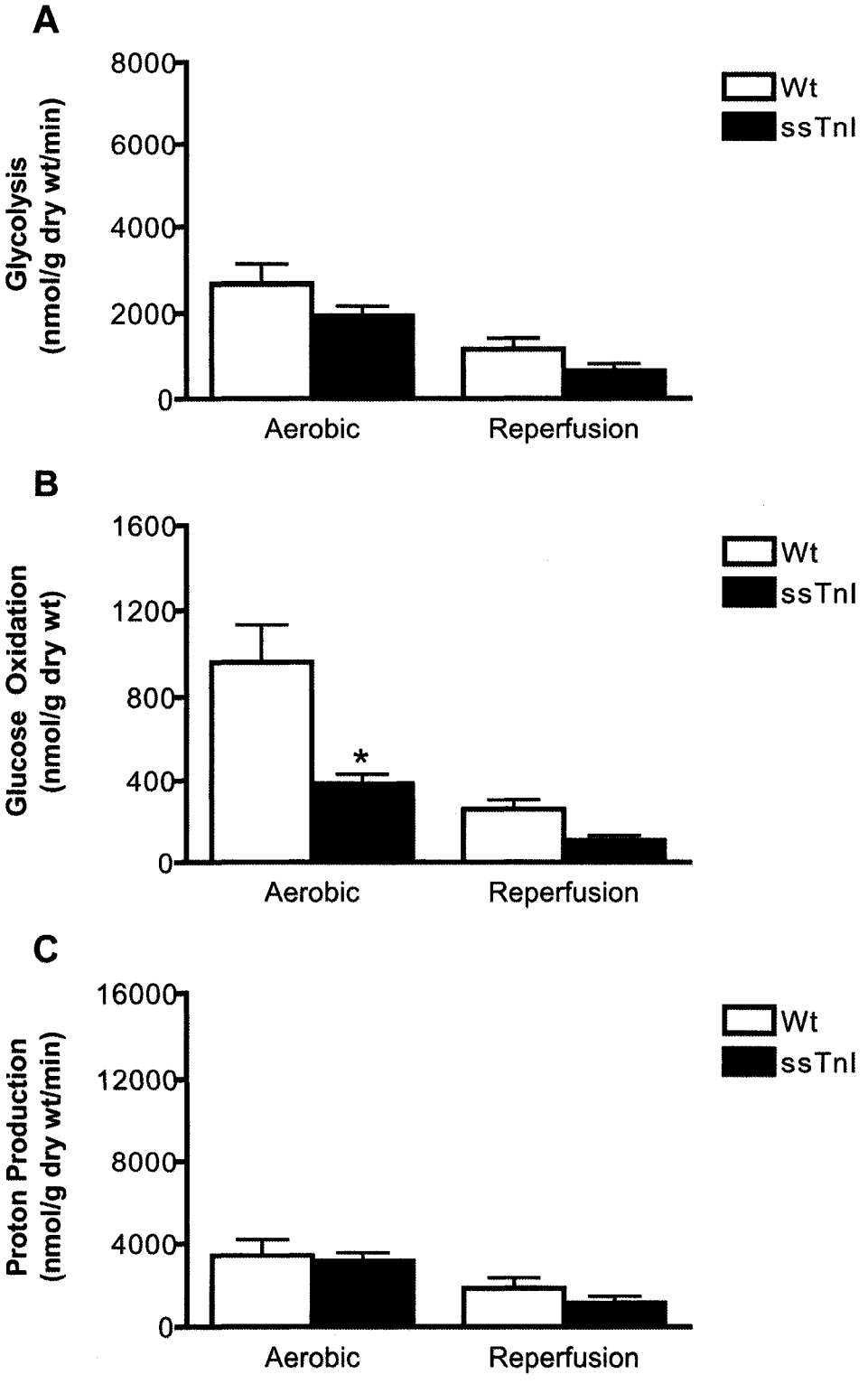

A
Glycolysis (nmol/g dry wt/min)
8000
6000
4000
2000
0
Aerobic
Reperfusion
Wt
ssTnI
B
Glucose Oxidation (nmol/g dry wt)
1600
1200
800
400
0
*
Aerobic
Reperfusion
Wt
ssTnI
C
Proton Production (nmol/g dry wt/min)
16000
12000
8000
4000
0
Aerobic
Reperfusion
Wt
ssTnI

Figure 6-5

Figure 6-6: Expression of ssTnI in old mice does not modify phosphorylation of AMPK or ACC.

A) Representative Western blot and quantification of P-AMPK(Thr172)/total AMPK ratio and B) representative Western blot and quantification of P-ACC(Ser79)/total ACC ratio (n = 6 per group) in isolated mouse hearts perfused in the presence of high fat and insulin. Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

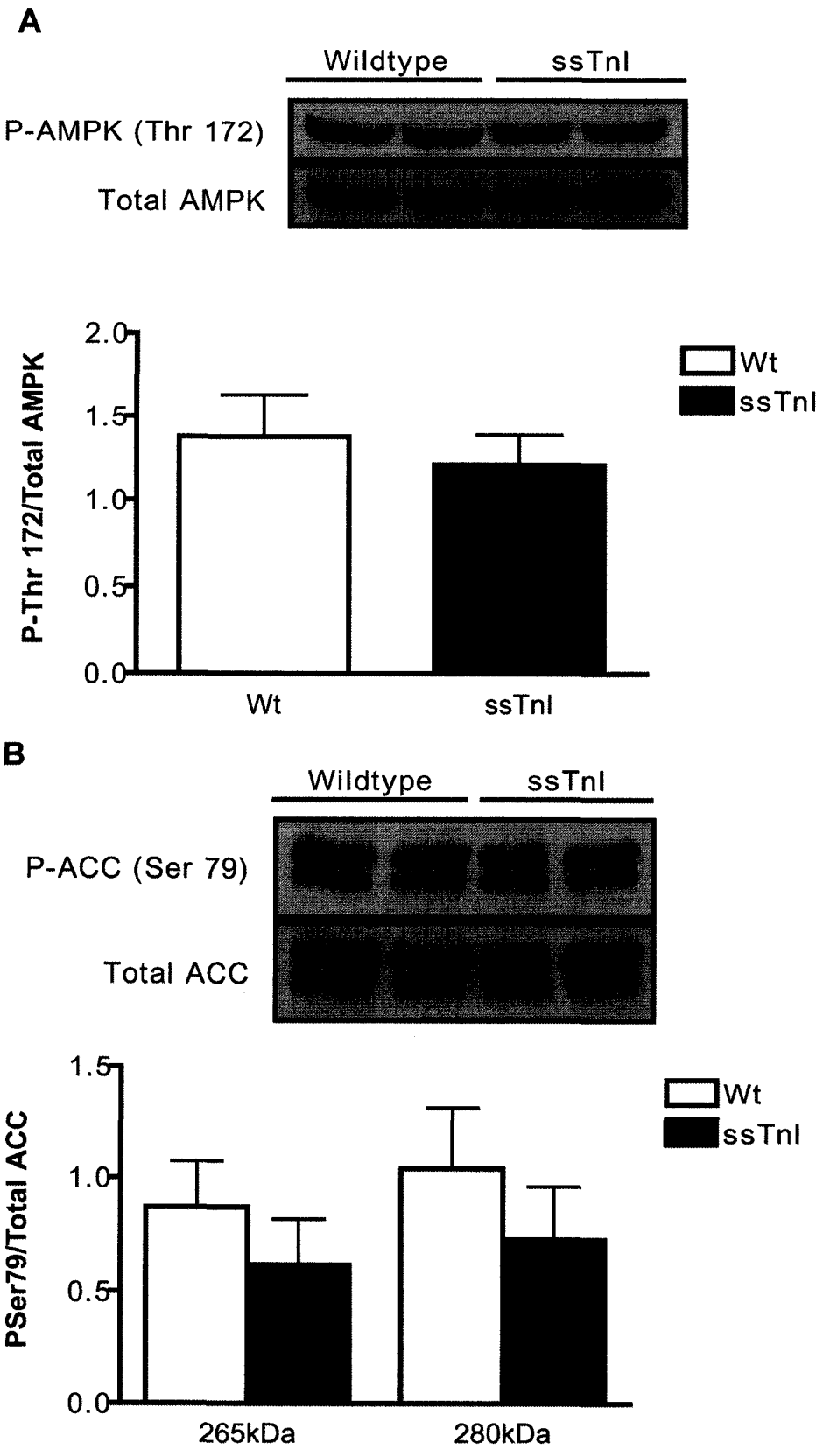

A
Wildtype
ssTnI
P-AMPK (Thr 172)
Total AMPK
P-Thr 172/Total AMPK
2.0
1.5
1.0
0.5
0.0
Wt
ssTnI
Wt
ssTnI
B
Wildtype
ssTnI
P-ACC (Ser 79)
Total ACC
PSer79/Total ACC
1.5
1.0
0.5
0.0
265kDa
280kDa
Wt
ssTnI

Figure 6-6

Figure 6-7: Expression of ssTnI in old mice increases infarct size.

A) Representative images of heart slices showing area at risk and infarct, B) quantification of area at risk and C) quantification of infarct size/ area at risk (n = 6 per group) following 30 mins LAD ligation and 24 hours reperfusion. Values represent mean ± SEM. Differences were determined using a t-test.

* represents $P < 0.05$, significantly different from wildtype group.

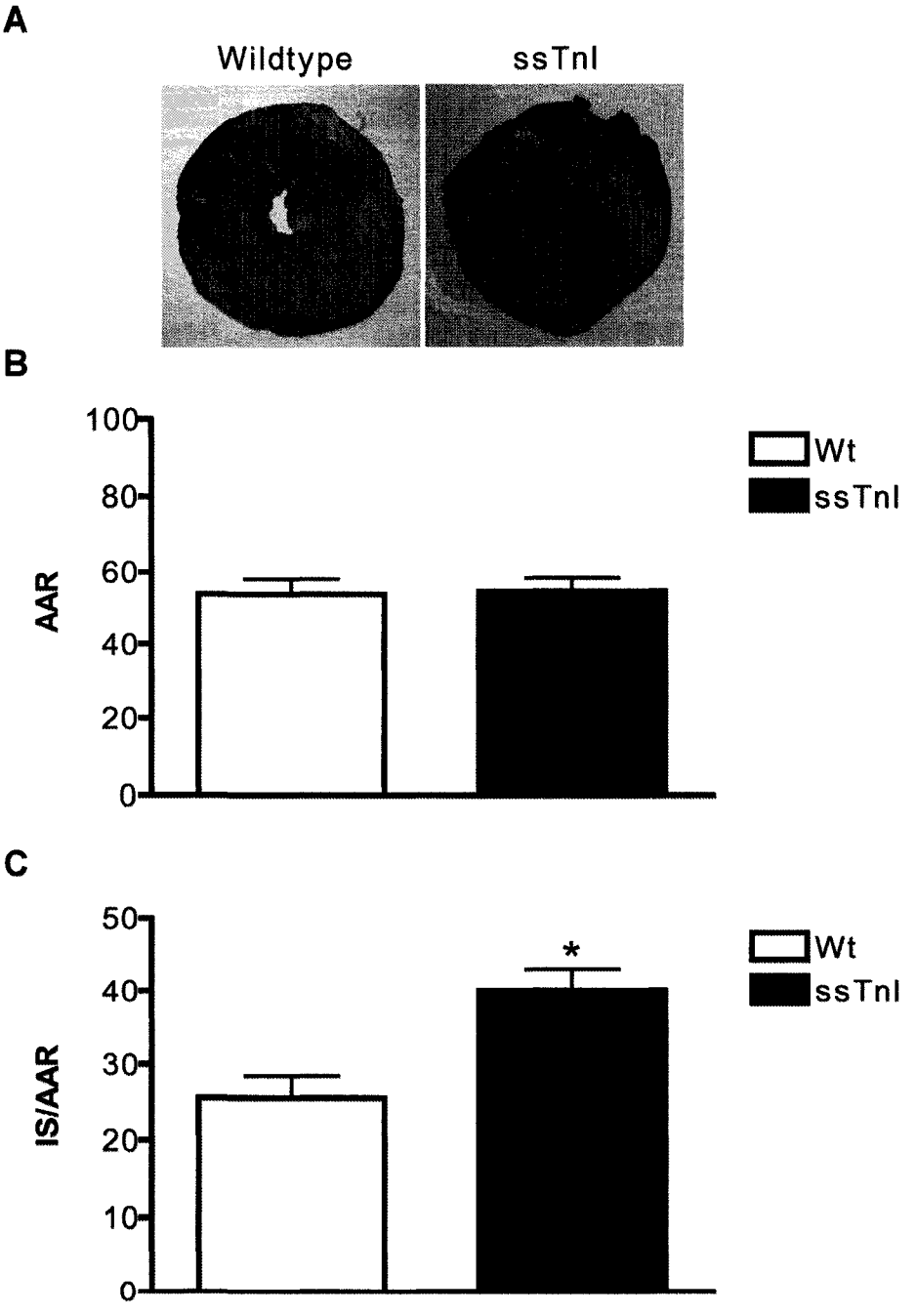

A
Wildtype
ssTnI
B
100
80
60
40
20
0
AAR
Wt
ssTnI
C
50
40
30
20
10
0
IS/AAR
*
Wt
ssTnI

Figure 6-7

Figure 6-8: Expression of ssTnI in young mice impairs post-ischemic contractile function in the presence of high fat and insulin, but has no effect in the absence of fat and insulin.

A) Cardiac power and B) cardiac efficiency (n = 9 and 7 for Wt and ssTnI repectively) in isolated mouse hearts perfused in the presence of high fat/insulin subjected to 18 min global ischemia and C) cardiac power (n = 3 per group) in isolated mouse hearts perfused in the absence of fatty acids subjected to 16 min global ischemia. Values represent mean ± SEM. Differences were determined using a 2-way repeated measures ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from wildtype group.

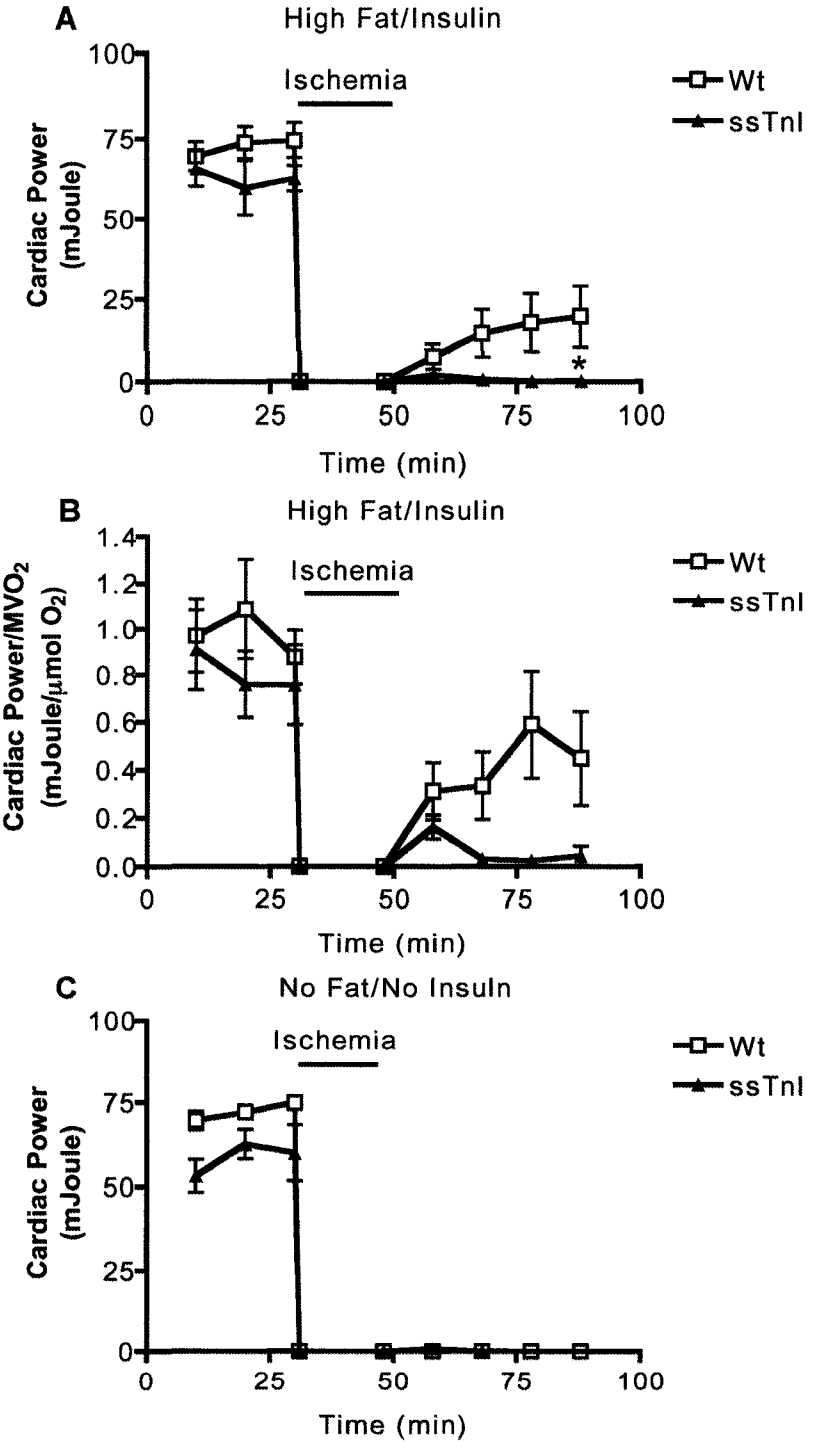

A
High Fat/Insulin
Ischemia
Wt
ssTnI
Cardiac Power (mJoule)
100
75
50
25
0
0
25
50
75
100
Time (min)
*
B
High Fat/Insulin
Ischemia
Wt
ssTnI
Cardiac Power/MVO2 (mJoule/µmol O2)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
25
50
75
100
Time (min)
C
No Fat/No Insuln
Ischemia
Wt
ssTnI
Cardiac Power (mJoule)
100
75
50
25
0
0
25
50
75
100
Time (min)

Figure 6-8

Figure 6-9: Expression of ssTnI in young mice does not affect myocardial metabolism in the presence of high fat and insulin.

A) Glycolysis, B) glucose oxidation, C) proton production (n = 9 and 7 for Wt and ssTnI respectively) in isolated mouse hearts perfused in the presence of high fat and insulin. Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

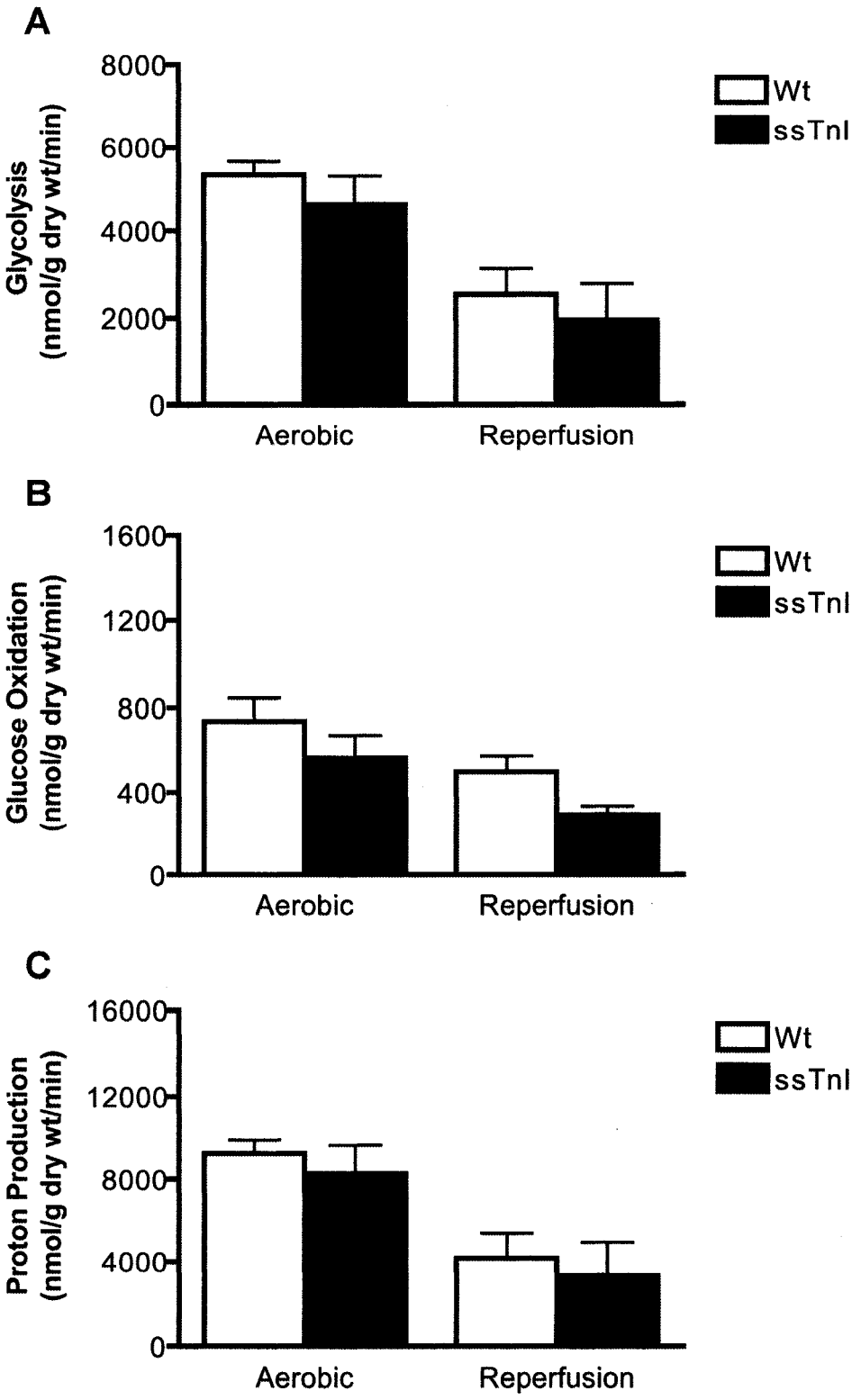

A
Glycolysis (nmol/g dry wt/min)
8000
6000
4000
2000
0
Aerobic
Reperfusion
Wt
ssTnI
B
Glucose Oxidation (nmol/g dry wt/min)
1600
1200
800
400
0
Aerobic
Reperfusion
Wt
ssTnI
C
Proton Production (nmol/g dry wt/min)
16000
12000
8000
4000
0
Aerobic
Reperfusion
Wt
ssTnI

Figure 6-9

Figure 6-10: Expression of ssTnI in young mice does not affect myocardial metabolism in the absence of fat and insulin.

A) Glycolysis, B) glucose oxidation, C) proton production (n = 3 per group) in isolated mouse hearts perfused in the presence of high fat/insulin. Values represent mean ± SEM. Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

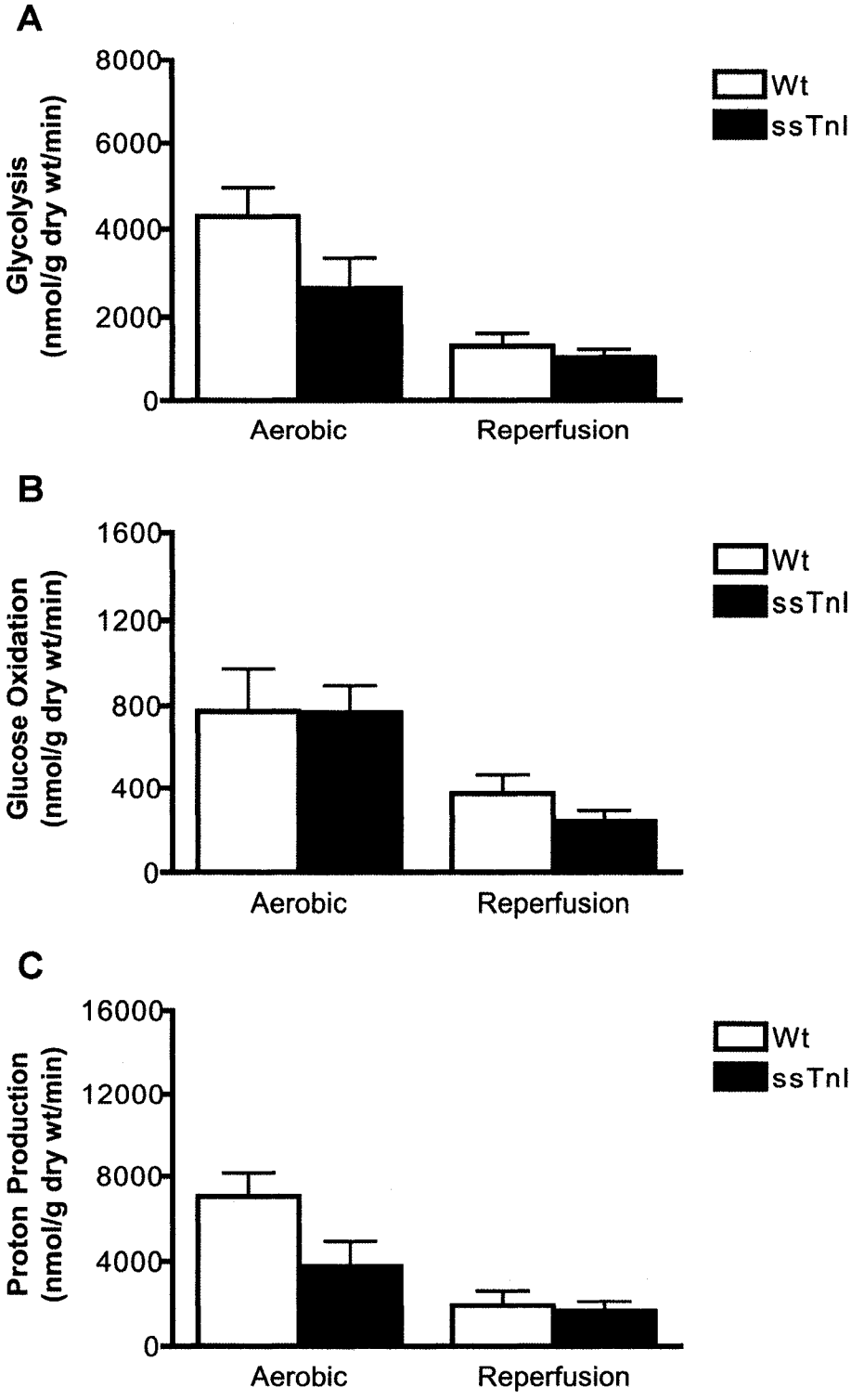

A
8000
6000
4000
2000
0
Glycolysis
(nmol/g dry wt/min)
Wt
ssTnI
Aerobic
Reperfusion
B
1600
1200
800
400
0
Glucose Oxidation
(nmol/g dry wt/min)
Wt
ssTnI
Aerobic
Reperfusion
C
16000
12000
8000
4000
0
Proton Production
(nmol/g dry wt/min)
Wt
ssTnI
Aerobic
Reperfusion

Figure 6-10

Discussion

A number of previous studies have examined the importance of substrate preference and its relationship to myocardial acidosis and the recovery of cardiac function during reperfusion of severely ischemic hearts.[31, 32, 122, 134, 254, 255] However, few studies have examined the mechanism by which myocardial acidosis produces post-ischemic contractile dysfunction. In this study we utilized the isolated working mouse heart, to examine the effect of transgenic replacement of cTnI with ssTnI on myocardial metabolism and functional recovery during reperfusion following ischemia. Using this approach we made a number of important findings. First, expression of ssTnI impairs post-ischemic contractile function in the isolated working mouse heart in the presence of high fat and insulin. Second, this impaired contractile function is not associated with changes in myocardial metabolism, although there is an increase in aerobic H^+ production in the ssTnI mice. Third, *in vivo* I/R is associated with an increase in myocardial infarction in the ssTnI mice. Fourth, we confirmed previous results that ssTnI have impaired diastolic dysfunction *in vivo*.[279, 296] Taken together, this data would suggest that the Ca^{2+} sensitivity of the contractile apparatus is a more important determinant of I/R-induce contractile dysfunction than acidosis-induced desensitization of the contractile apparatus.

Uncoupling of glucose metabolism during I/R leads to a sequelae of events as discussed in Chapter 1, which can include myocardial acidosis and subsequent Na^+ and Ca^{2+} overload.[31, 32] Depending on the length and severity of ischemia, intracellular pH can decrease from physiological (7.4 to 7.6) to between 5.5 and 6.8.[32, 297, 298] This

suppression of pH is associated with a significant accumulation of Na^+, which rises from 10 - 15 mM to values up to 30 mM in the mouse, and Ca^{2+} that rises from between 10 - 100 nM during diastole to the micromolar range, which correlates with post-ischemic contractile dysfunction.[14, 24, 299] However, it is difficult to assess the direct consequences of acidosis versus Na^+ or Ca^{2+} overload on post-ischemic contractile dysfunction. Mechanisms that increase H^+ production, such as by increasing the concentrations of fatty acids, causes a delayed in the recovery of intracellular pH and contractile function during reperfusion.[32] However, an increase in fatty acids also increases ischemic-induced Na^+ overload and impairs the recovery of intracellular Na^+ concentrations.[298] In addition, many studies have demonstrated a cardioprotective effect of pharmacologically inhibiting NHE or NCX and reducing Na^+ and Ca^{2+} overload, thus suggesting that these ions are more important in contractile dysfunction that acidosis.[300-302] Despite these observations, H^+ have a direct depressive effect on the Ca^{2+} sensitivity of skinned muscle fibers, suggesting that may be a contributing factor to I/R-induced cardiac dysfunction.[281]

H^+s have a differential depressive effect on Ca^{2+} sensitivity of skinned muscle fibers from different tissues (cardiac>fast skeletal>slow skeletal) [281], and many studies have now demonstrated that expression of ssTnI in place of cTnI, shifts the Ca^{2+}-tension relationship to the right (indicating that the contractile apparatus is more sensitive to Ca^{2+}) and markedly blunts the acidosis-induced decline in tension.[138, 279-285] Using mutational analysis, Westfall and Metzger have identified that ssTnI His132 confers a dominant effect on the resistance to acidosis, while Val134 and Asn141 may provide a partially redundant effect.[303] Indeed, performing the reverse mutation, where the equivalent amino acid in cTnI was mutated from an Ala162 to His, restores the Ca^{2+}

sensitivity of ATPase activity to similar level as ssTnI at acidic conditions, but not at neutral pH.[304] However, when this mutation is expressed in isolated cardiomyocytes, the beneficial effect is also observed at neutral pH.[305] These studies suggest that the mechanism of action is due to the pH sensitivity of the His residue, which resides adjacent to a switch peptide that can bind to the N-terminal of cTnC or actin, depending on the Ca^{2+} binding state of a single regulatory site.[306] The side chain of His would be deprotonated and hydrophobic at neutral pH and protonated and hydrophilic at acidic pH. Therefore during acidosis, the increase in positive charge on His would weaken the charge interactions with Arg and Lys residues in TnC or strengthen interactions with Glu and Asp residues in TnC.[304] This would force the TnC into the open confirmation, thus it would maintain contractile activation under acidic conditions.[305]

Serveral studies using the ssTnI transgenic mouse, have observed that hearts are protected under pathological conditions. Urboniene *et al.* demonstrate that ssTnI hearts have preserved LV systolic function when subjected to *in vivo* respiratory hypercapnia due to the resistance to acidosis.[307] In addition, Arteaga *et al.* demonstrate that ssTnI mice are cardioprotected during I/R in a Langendorff model.[286] Surprisingly, in contrast to these previous studies, mice expressing ssTnI are not cardioprotected in the setting of ischemia, but are actually shown to be detrimental to the recovery of contractile function in the presence of fatty acids, and to have no effect on functional recovery in the absence of fatty acids. Arteaga *et al.* suggested that the cardioprotective effects of ssTnI expression are attributed to blunting of the I/R-induced decrease in cross bridge dependent activation of the thin filaments, which is associated with better preservation of maximum tension and Ca^{2+} sensitivity in the ssTnI mice.[286] This may be associated with

increased economy of tension development as the Ca^{2+} activated ATPase rates of myofilaments in loaded skinned fiber bundles are approximately 20 % lower in the ssTnI mice.[308] When hearts are perfused from these mice and ATP levels were measured using NMR, there was no difference in ATP content during the aerobic period, however, the ssTnI hearts showed better-preserved ATP content during the first 10 min of ischemia. Interestingly, ATP content did not differ between ssTnI and Wt hearts in the subsequent 10 min of ischemia, and reperfusion data was not presented.[309] In contrast, we observe that ATP content is significantly reduced in ssTnI mice at the end of reperfusion (Table 6.4).

A number of technical considerations may account for the differences in functional recovery associated with the ssTnI mice. Spontaneously beating working hearts were utilized in the present study; while the previous study was performed in paced Langendorff perfused mouse hearts. Our model may have a higher workload and energy demand compared to the Langendorff hearts, and also a greater degree of stress and myocardial acidosis. Despite this, as Wt and ssTnI hearts were perfused under the same conditions, they should have similar levels of myocardial acidosis or slightly elevated levels in the ssTnI hearts in the presence of fatty acids (Figure 6-4), therefore expression of ssTnI should still be cardioprotective. However, it is possible that the working hearts would have a greater degree of pathological Ca^{2+} overload during I/R due to their greater energy demand. Although many studies have associated post-ischemic contractile dysfunction with intracellular Ca^{2+} overload, the exact mechanism of action is still unknown, but may include contracture (i.e. impaired diastolic relaxation), activation of Ca^{2+}-dependent proteases (calpains) and proteolysis of myofibril protein (such as TnI),

mitochondrial dysfunction, impaired efficiency of oxygen utilization and may ultimately lead to myocardial necrosis/apoptosis.[14, 18, 19, 21, 22] Therefore it stands to reason, if ssTnI hearts are more sensitive to Ca^{2+} under physiological Ca^{2+} concentrations, they may also be more sensitive to pathological Ca^{2+} concentrations thus accounting for the impaired post-ischemic contractile function in our working heart model.

Another significant difference between the present study and the previous studies is the use of energy substrates in the perfusate, as Arteaga *et al.* used 10 mM glucose alone, while we used 5 mM glucose, 1.2 mM palmitate and 100 μU/mL of insulin.[286] We have previously shown that the presence or absence of fatty acids could markedly change the use of insulin as a cardioprotective agent, so the choice of substrates could have important effects on post-ischemic recovery of contractile function.[240] Indeed, when hearts are perfused in the absence of fatty acids and insulin, functional recovery is poor in both ssTnI and Wt hearts. These hearts may be functionally compromised, as 5 mM glucose in the absence of insulin may be insufficient to meet the energetic needs of the heart.

We also demonstrate that ssTnI mice are more susceptible to irreversible I/R injury, as they have increased infarct size when subjected to *in vivo* LAD occlusion and reperfusion (Figure 6-7). Ca^{2+} is thought to induce irreversible I/R injury via a number of mechanisms including the activation of phospholipases leading to changes in membrane biomechanics that can compromise membrane transporter function, activation of proteases (calpains) leading to the proteolytic degradation of a number of intracellular proteins including contractile proteins, Ca^{2+} handling proteins and apoptotic signaling proteins, and opening of the mPTP and dissipation of the mitochondrial membrane

potential.[14, 26] These events ultimately lead to myocardial cell death either via apoptosis or necrosis.[7] However the role of ssTnI in the above events has not been examined. An attractive hypothesis would be that ssTnI is more susceptible to proteolytic cleavage by Ca^{2+} activated proteases, however no difference in I/R-induced troponin cleavage was found in a previous study with the ssTnI mice.[286] Interestingly, we confirmed previous results that ssTnI expressing hearts have diastolic dysfunction *in vivo*.[279, 296] As I/R induces diastolic dysfunction as well, the pre-existing diastolic dysfunction in the ssTnI mice may be exacerbated when subjected to I/R, resulting in impaired ventricular filling and cardiac output that may partially account for their poor functional recovery during reperfusion and increase in infarct size.[4] Further studies are required to elucidate the mechanism by with ssTnI expression leads to increased I/R-induced damage.

An alternative model used to study the interaction of improved myofilament Ca^{2+} sensitivity during acidosis and post-ischemic contractile function is a transgenic mouse that expresses the reverse mutation, where the equivalent amino acid in cTnI was mutated from an Ala162 to His.[305] Cardiomyocytes isolated from these animals have Ca^{2+} tension relationships similar to cardiomyocytes expressing ssTnI. These mice have significantly improved systolic function *in vivo* as assessed by echocardiography and conductance micromanometry and in isolated Langendorff hearts. The transgenic hearts have better preserved cardiac function when subjected to acidosis in isolated hearts or during *in vivo* hypoxic challenge.[305] In a Langendorff model of I/R transgenic hearts have significantly improved recovery of LV developed pressure and recovers PCr levels to nearly 100 % of baseline suggesting that these animals are energetically more economical.[305] In addition, the transgenic mice subjected to *in vivo* LAD occlusion for 30 min have significantly

better stroke work and ejection fraction during the ischemic event, however data was not presented during the following 24 hours of reperfusion and there is no significant difference in infarct size following reperfusion. Although these data are very interesting, it is difficult to interpret the I/R data in lieu of two important compensatory changes observed in the transgenic mice; NCX expression is reduced by 71 % in transgenic mice and both baseline and peak Ca^{2+} concentrations are lower in the transgenic mice.[305] Both of these observations may contribute to a reduced Ca^{2+} overload during I/R and hence less post-ischemic cardiac dysfunction. Indeed many studies have associated suppressed NCX expression with cardioprotection. Heterozygous NCX knockout mice that have a 50 % reduction in NCX protein and current density have significantly improved functional recovery during reperfusion in a Langendorff model of I/R and significantly smaller infarct size following *in vivo* LAD ligation and reperfusion.[310, 311] In addition, mice with cardiac specific NCX ablation in 80 – 90 % of cardiomyocytes have significantly improved functional recovery during reperfusion associated with slower decline of ATP and a reduction in Na^{+} accumulation during ischemia and improved recovery of ATP and PCr during reperfusion.[312] Overexpression of NCX exacerbates I/R injury only in male mice.[313] Therefore caution is required to interpret the I/R data associated with the cTnI A162H mutation, as its cardioprotective effect may be attributed to compensatory changes observed in Ca^{2+} handling rather than the mutation itself.

Many of the observations described in this study are first demonstrated in old mice (8 – 9 months of age) due to their availability. To ensure that these observations are not simply dependent on aging-induced declines in myocardial function and fatty acid oxidation or aging-induce compensatory changes due to the transgene expression, we

confirmed these observations in younger mice.[294, 314-317] Baseline cardiac function and metabolism are similar in young and old mice, although there may be a slight decline in glucose oxidation in the old mice, and cardiac power recovered to a similar extent independent of the age of the animals.

Limitations

This study utilized the isolated working heart method, which has some limitations as previously discussed in Chapter 3 and Chapter 8. In addition, the ssTnI mice have slightly impaired diastolic function *in vivo*, however diastolic function is difficult to monitor in our isolated working heart model. Therefore the poorer function observed in the ssTnI mice may be partially associated with preexisting diastolic dysfunction. Another limitation is the very poor functional recovery of both wildtype and ssTnI hearts when subjected to I/R in the absence of fatty acids and insulin, which is not surprising based on the results of Chapter 3. However, this poor functional recovery in both groups makes it difficult to assess the role of ssTnI in cardioprotection under these particular conditions.

In summary, this study demonstrates that the expression of ssTnI does not confer cardioprotection from I/R injury, but may actually exacerbate cardiac dysfunction during reperfusion in the presence of insulin and a high concentration of fatty acids. These observations support the fact that the Ca^{2+} sensitivity of the contractile apparatus is a more important determinant of I/R-induce contractile dysfunction than acidosis induced-desensitization of the contractile apparatus.

CHAPTER 7.

Novel O-palmitolylated Beta-E1 subunit of Pyruvate Dehydrogenase is Phosphorylated During Ischemia/Reperfusion Injury

A version of this chapter has been submitted for publication. Folmes CDL, Sawicki G, Masson G, Barr AJ, and Lopaschuk GD. Submitted to *Proteomics*.

My role in this work involved performing all the experiments (except those noted below) as well as writing the manuscript. Grzegorz Sawicki performed the 2-D gel experiments and part of the data analysis, Grant Masson provided metabolism data from a similar set of perfused hearts and Amy Barr isolated the mitochondria from the perfused hearts.

CHAPTER 7.

Novel O-palmitolylated Beta-E1 subunit of Pyruvate Dehydrogenase is Phosphorylated During Ischemia /Reperfusion Injury

Abstract

During and following myocardial ischemia glucose oxidation rates are low and fatty acid oxidation dominates as the source of mitochondrial oxidative metabolism, which is associated with impaired contractile function during reperfusion. To determine the mechanism of this decrease in glucose oxidation, a proteomics approach was utilized. 2-dimensional (2-D) gel electrophoresis performed on mitochondria from working rat hearts subjected to ischemia/reperfusion identified 32 changes in protein levels compared to aerobic controls. Of the five protein spots with the greatest change in abundance, two are increased, long chain acyl-CoA dehydrogenase and ATP synthase (α subunit), while two are decreased, NADH-ubiquinone oxidoreductase (24 kDa subunit) and ATP synthase (D subunit). Two forms of pyruvate dehydrogenase (βE1 subunit), the rate-limiting enzyme for glucose oxidation, differing in isoelectric point, are also identified. The protein level of the more acidic form of PDH was reduced during reperfusion, while

the more basic form remains unchanged. The more acidic isoform was found to have a novel O-palmitoylation, while both isoforms exhibited I/R-induced phosphorylation. *In silico* analysis identified the putative kinases as the insulin receptor kinase for the more basic form and PKC_{ζ} or PKA for the more acidic form. These modifications of PDH are associated with a decrease in glucose oxidation (290 ± 10 vs. 490 ± 50 nmol/g dry wt/min, n = 6 per group, $P < 0.05$) during reperfusion with no significant change in palmitate oxidation. As PDH is an important regulatory enzyme that modulates the rates of glucose oxidation, these post-translational modifications may be important therapeutic targets to optimize metabolism for the treatment of ischemic heart disease.

Introduction

Myocardial ischemia occurs when the oxygen requirement of the heart exceeds the oxygen supplied via the coronary circulation and is a devastating cause of morbidity and mortality worldwide. Although many studies have examined I/R injury and possible therapeutic strategies, the exact molecular and cellular mechanisms that lead to the pathophysiology of this disease have remained elusive. Proteomics is a powerful experimental approach, which allows for the observation of global changes in protein abundance and has been used to identify post-translational modifications (PTMs) of these proteins.[318, 319] Using this approach several studies have looked at the I/R- and preconditioning-induced changes of the heart proteome using 2-D gel electrophoresis.[320-324] Interestingly, many of the identified proteins have important functions in myocardial energy substrate metabolism, which suggests an important role for alterations in metabolism as a contributor to the pathophysiology of I/R injury.

The majority of the heart's essential catabolic machinery resides in the mitochondria. Despite the fact that the human heart mitochondrial proteome has been resolved and approximately 50 % of the identified proteins are involved in metabolism [325], only one study has specifically examined the I/R-induced global changes in the mitochondrial proteome.[324] In this study we used a proteomics approach to observe the I/R-induced changes in abundance of mitochondrial proteins, one of which was identified as the βE1 subunit of PDH, a key regulatory enzyme that directly modulates the rates of glucose oxidation. We subsequently demonstrate that this subunit contains novel PTMs.

These PTMs may lead to the changes in enzyme activity, and are therefore potentially important therapeutic targets for optimizing myocardial metabolism during reperfusion.

Materials and 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.

Isolated Working Rat Hearts

Sprague-Dawley rat hearts were perfused in the working perfused mode as previously described in Chapter 2. [238] Hearts were perfused in the presence 100 μU/mL insulin, 5 mM glucose and 1.2 mM palmitate. Spontaneously beating hearts were perfused at a constant left atrial preload pressure of 11.5 mmHg and a constant aortic afterload pressure of 80 mmHg for the I/R protocol, which consisted of 35 min aerobic perfusion, 25 min of global no-flow ischemia and 40 min reperfusion. Another series of hearts underwent 100 min of aerobic perfusion to serve as a time-matched aerobic control group. These hearts were immediately cut down after the perfusion protocol and mitochondria were isolated using a differential centrifugation protocol.

Glucose and palmitate oxidation were measured by quantitative collection of the $^{14}CO_2$ and $^{3}H_2O$ and, derived from [U-^{14}C] glucose and [9, 10-^{3}H] palmitate, as described in Chapter 2.[134, 238]

Isolation of Mitochondria

After the perfusions, the hearts were quickly rinsed in ice-cold 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 10 mM Tris, pH 7.5 (MSE buffer). The atria were

removed and the ventricles minced. A 20 % (w/v) tissue suspension in MSE buffer was adjusted to pH 7.4 to 7.5. The suspension was homogenized with a Polytron homogenizer for two 10 s periods at a setting of 45. The homogenate was centrifuged at 480 xg for 5 min, and the supernatant (S1) was filtered through cheesecloth and centrifuged at 10000 xg for 30 min. The pellet (Pi), from the 480 x g spin, was suspended in MSE buffer and homogenized again under the same settings and centrifuged at 480 xg for 5 min. The supernatant (S2) was filtered through cheesecloth and was centrifuged at 10000 xg for 30 min. The S2 pellet was combined with the S1 pellet suspended using MSE buffer and centrifuged at 10000 xg for 30 min. The resulting pelleted mitochondria (S3) was suspended in a urea solution in a 1:2 (w/v) ratio and used for 2D electrophoresis.[326]

Preparation of Mitochondrial Extracts

Protein samples for 2-D electrophoresis were prepared at room temperature by mixing one part of the mitochondria pellet with two parts (w:v) of rehydration buffer (8 mol/L urea, 4 % CHAPS, 10 mmol/L DTT, 0.2 % Bio-Lytes 3/10 (BioRad, Richmond, California)). Samples were sonicated twice for 5 s and centrifuged for 10 min at 10,000 xg at room temperature to remove any insoluble particles. Protein content of the mitochondrial extract in rehydration buffer was measured using the BioRad protein assay.

Two-Dimentional Polyacrylamide Gel Electrophoresis

400 μg of mitochondria extract protein was applied to 11 cm immobilized pH gradient strip with linear pH gradient from 5 to 8 (IPG, BioRad, Richmond, California), and equilibrated for 16-18 hr at 20°C in rehydration buffer. For isoelectrofocusing, the

BioRad Protean isoelectrofocusing cell was used with the conditions described previously.[327] The second dimension of electrophoresis was carried out using 8-16 % acrylamide Criterion precast gradient gels (BioRad, Richmond, California). To minimize variations in resolving proteins during the second dimension run, all gels were run simultaneously using a Criterion Dodeca Cell (BioRad, Richmond, California). After separation, proteins were detected using Coomassie Brilliant Blue R-250 (BioRad, Richmond, California). Developed gels were scanned using a calibrated GS-800 densitometer (BioRad, Richmond, California). Quantitative analysis of spot intensity from 2-D electrophoresis was measured using PDQuest 7.1 software (BioRad, Richmond, California). Only protein spots with a relative intensity between 10-100 arbitrary units were considered for analysis. Using these criteria for protein resolution and staining, we were able to obtain high reproducibility to analyze both a single protein from the same sample run in different gels and for a specific protein spot from different heart samples.

Mass Spectrometry

Protein spots were manually excised from the 2-D gel. Subsequently, in-gel trypic digests were analyzed using a Bruker Ultraflex TOF/TOF (time of flight) mass spectrometer. Digests were applied to an AnchorChip plate (Bruker, East Milton, Ontario) as previously described.[328] Mass spectra (MS) and tandem mass spectra (MS/MS) were then obtained in an automated fashion using the AutoXecute function. Typically 100 shots were accumulated to generate a peptide mass fingerprint. The 5 most intense peaks were then selected to have MS/MS performed where 400 shots on average were used to generate a MS/MS profile. Peaklist generation and peak picking was

performed using Flexanalysis 2.2 SNAP (sophisticated numerical annotation procedure), with default setting for peaklist generation. For PMF data the S/N threshold = 6, resolution > 6000, they were externally calibrated using 8 peptides with m/z ranging from 757.39 to 3147.47 and tryptic autolytic peptides are excluded. Acceptance criteria for MS/MS was at least 2 matching peptides with a score $P < 0.05$. The resultant MS or MS/MS data was searched using the MASCOT version 2.2 (www.matrixscience.com) search engine against NCBInr (2007) and Swiss-Prot (2007) databases. Specified 68,229 protein sequences from NCBInr for Rattus Norvegicus was used for identification of the proteins. The Mowse scoring algorithm was used for justification of protein identification.[329]

Calculation of the Theoretical Masses of PDHβ Peptides Generated by Enzymatic Cleavage

PeptideMass (http://au.expasy.org/tools/peptide-mass.html) was used to calculate the mass of trypsin-digested peptides from the sequence of PDHβ. For calculation of theoretical peptide masses we assumed: 1) incomplete protein digestion (1 missed cleavage level) and ± 0.2 Da mass tolerance, 2) mandatory alkylation and reduction of cysteines with iodoacteamide and 3) variable oxidation of methionine. Only peptides bigger than 500 Da were considered.

Examination of Experimental Peptide Mass Fingerprinting for Post-Translational Modifications

FindMod tool was used to find potential PTMs (http://au.expasy.org/tools/findmod/) in the experimental peptides of PDHβ. 22 types of PTMs were considered with the assumption that up to 3 PTMs in one peptide can exist.

Chemical Verification of O-Palmitoylation and Phosphorylation

Experimental peptides with identified (*in silico*) palmitoylation were incubated with 0.1 M KOH for 4 hr at room temperature [330] and analysed by MS/MS. Experimental peptides with identified (*in silico*) phosphorylation were incubated with 0.5 unit of alkaline phosphatase in 50 mM bicarbonate solution for 120 min at 0.1 M KOH for 4 hr at 30°C [330] and analysed by MS/MS.[331]

Prediction of Kinases that Phosphorylate $PDH_{\beta E1}$

PDH phosphorylating kinases were predicted using phosphorylation consensus sequences identified with Scansite (http://scansite.mit.edu/) and NetPhosK 1.0 Sever (http://www.cbs.dtu.dk/services/NetPhosK/).

Statistical Analysis

Data are shown as mean ± SEM. Functional data were analyzed using ANOVA with the Bonferroni post hoc test. Analysis of the protein spots in the 2-D electrophoresis experiments and metabolic data were performed using unpaired t-tests. A value of $P < 0.05$ was considered statistically significant.

Results

Cardiac Function and Metabolism in Hearts Subjected to Aerobic Perfusion and Ischemia/Reperfusion

Hearts were perfused either for 100 min aerobically or for 35 min aerobically followed by 25 min of global ischemia and 40 min of reperfusion. The functional recovery of hearts subjected to I/R was significantly reduced compared to hearts aerobically perfused for 100 min (15.3 ± 4. vs. 71.5 ± 7.6 mL/mmHg/min, n = 9 per group, $P < 0.05$, Figure 7-1B and Table 7-1). Glucose oxidation rates are decreased in reperfused hearts compared to the glucose oxidation rates in time-matched aerobic control hearts (290 ± 10 vs. 490 ± 50 nmol/g dry wt/min, n = 6 per group, $P < 0.05$, Figure 7-1C). Despite this reduction in glucose oxidation rates, palmitate oxidation rates do not differ between the reperfusion group and the aerobic controls (Figure 7-1D).

2-D Electrophoresis and Identification of Proteins

2-D electrophoresis identified 32 I/R-induced changes in protein abundance and we chose to identify the five spots with the greatest change in abundance (Figure 7-2A, 7-2B and Table 7-2). Two of these protein abundances are increased with I/R and are identified as long chain acyl-coenzyme A dehydrogenase and ATP synthase (α subunit). Three of these protein abundances are decreased with I/R and are identified as NADH-ubiquinone oxidoreductase (24 kDa subunit), ATP synthase (D subunit) and PDH (βE1 subunit). In addition, the protein spot adjacent to spot 2 is also identified as the PDH (βE1 subunit) (Figure 7-3B and Table 7-3). For the remainder of this study we

concentrated on post-translational changes induced in PDH due to the importance of this enzyme in the regulation of myocardial glucose oxidation. Both aerobic and I/R groups express two molecular forms of $PDH_{\beta E1}$ (which we identified as a more acidic form and more basic form based on their isoelectric point) but only the protein level of the more acidic form is decreased in I/R (Figure 7-3B).

Protein Mass Fingerprints and Post-Translational Modifications of PDH

Proteins were excised from the gels and subjected to trypsin digestion followed by MS analysis to produce PMFs of both the more acidic and more basic form of $PDH_{\beta E1}$ (Figure 7-4A). Comparison of trypsin digested peptides of the $PDH_{\beta E1}$ from control hearts using the FindMod tool shows that the more acidic form is O-palmitoylated (peptide 1493.486 Da, Figure 4A and B). This unique PTM was confirmed by KOH treatment of the peptides (Figure 7-4A), which led to the disappearance of the O-palmitoylated peptide and the appearance of a new peptide (1240.931 Da).

Upon comparison of the PMFs from the more acidic form of $PDH_{\beta E1}$ from the aerobic and I/R hearts, two new peptides with masses 984.579 Da and 2397.998 Da are detected as being potentially phosphorylated peptides (Figure 7-5). However upon treatment with alkaline phosphatase, only the smaller peptide was removed (Figure 7-5). Comparison of the PMFs from the more basic form of $PDH_{\beta E1}$ from the aerobic and I/R hearts detected that I/R triggers phosphorylation of the more basic form (peptide 1922.068, Figure 7-6A and B). This peptide peak disappeared after treatment with alkaline phosphatase (Figure 7-6A). In addition, the isoelectric point of the more basic

form of $PDH_{\beta E1}$ is decreased in I/R hearts (Figure 7-7B), further supporting the theory that this peptide was phosphorylated.

Identification of Putative Phosphorylation Sites and Kinases

Phosphorylation sites are determined by examining the sizes of trypsin digested protein fragments with the FindMod tool, and the kinases are determined using Scansite and the NetPhosK 1.0 Sever. Using this analysis we identified Ser16 of the more acidic $PDH_{\beta E1}$ to be a possible site of phosphorylation with the putative kinase being protein kinase C_ζ (PKC_ζ) or protein kinase A (PKA) (Table 7-4). For the more basic $PDH_{\beta E1}$ we identified Tyr132 as the most probable site of phosphorylation with the putative kinase being the insulin receptor kinase (Table 7-4).

Table 7-1. Parameters of contractile function during aerobic reperfusion of ischemic hearts perfused in the absence of fatty acids.

	0 - 35 min		65 – 100 min	
	Aerobic (n = 9)	I/R (n = 9)	Aerobic (n = 9)	I/R (n = 9)
Heart Rate (beats/min)	241 ± 11	239 ± 9	243 ± 10	156 ± 14 *
Peak Systolic Pressure (mmHg)	127 ± 4	128 ± 3	124 ± 3	71 ± 10 *
Coronary Flow (mL/min)	50 ± 5	51 ± 3	47 ± 6	16 ± 3 *
Cardiac Output (mL/min)	62 ± 3	58 ± 2	59 ± 2	17 ± 3 *

Differences were determined using a 2-way ANOVA with a Bonferroni post hoc test.

* represents $P < 0.05$, significantly different from corresponding aerobic perfusion group.

Table 7-2. Mass Spectrometry identification of protein spots using the Mascot Search Engine.

Protein Spot (#)	Probability Based Mowse Score* Threshold ($P < 0.05$)	Observed Score	Peptide Matched (n)	Sequence Coverage (%)	Identification
1	58	141	17	44	NADH-ubiquinone oxidoreductase (24 kDa subunit)
2	32	108	3	10	Pyruvate dehydrogenase (βE1 subunit)
3	58	127	13	34	ATP synthase (D subunit)
4	58	127	13	34	Long-chain acyl-CoA dehydrogenase
5	58	87	12	41	ATP synthase (α subunit)

* -10 log(P) where P is the probability that the observed match is a random event.

Table 7-3. Mass Spectrometry identification of protein spots corresponding to the βE1 subunit of PDH using the Mascot Search Engine.

Protein Spot (#)	Probability Based Mowse Score* Threshold ($P < 0.05$)	Observed Score	Peptide Matched (n)	Sequence Coverage (%)	Identification
1 Aerobic	32	108	3	10	Pyruvate dehydrogenase (βE1 subunit)
2 Aerobic	69	167	21	59	Pyruvate dehydrogenase (βE1 subunit)
1 I/R	31	85	2	13	Pyruvate dehydrogenase (βE1 subunit)
2 I/R	69	151	21	59	Pyruvate dehydrogenase (βE1 subunit)

* -10 log(P) where P is the probability that the observed match is a random event.

Table 7-4. Identification of phosphorylation sites and putative kinases for the βE1 subunit of PDH using Scansite and NetPhosK 1.0 Server.

Isoform Of PDH	Amino Acid	Scansite		NetPhosK 1.0 Server	
		Kinase	Score*	Kinase	Score
More Acidic	S16	PKCζ	0.61	PKA	0.57
More Acidic	S70			PKA	0.53
More Basic	T130	PKC			
More Basic	Y131				
More Basic	Y132	INSR	0.72	INSR	0.55
More Basic	S134				

* Threshold 0.5 (range 0-1)

Figure 7-1. Ischemia and reperfusion reduces cardiac work compared to aerobic control isolated working hearts.

A) Experimental protocol of hearts subjected to I/R, B) cardiac work of the heart after during reperfusion (n = 9 per group), C) Rates of glucose oxidation (n = 6 per group) and D) rates of palmitate oxidation (n = 6 per group) in isolated working rat hearts subjected to aerobic perfusion or I/R. Values represent the mean. Differences were determined using a t-test.

* represents $P < 0.05$, significantly different from aerobic value.

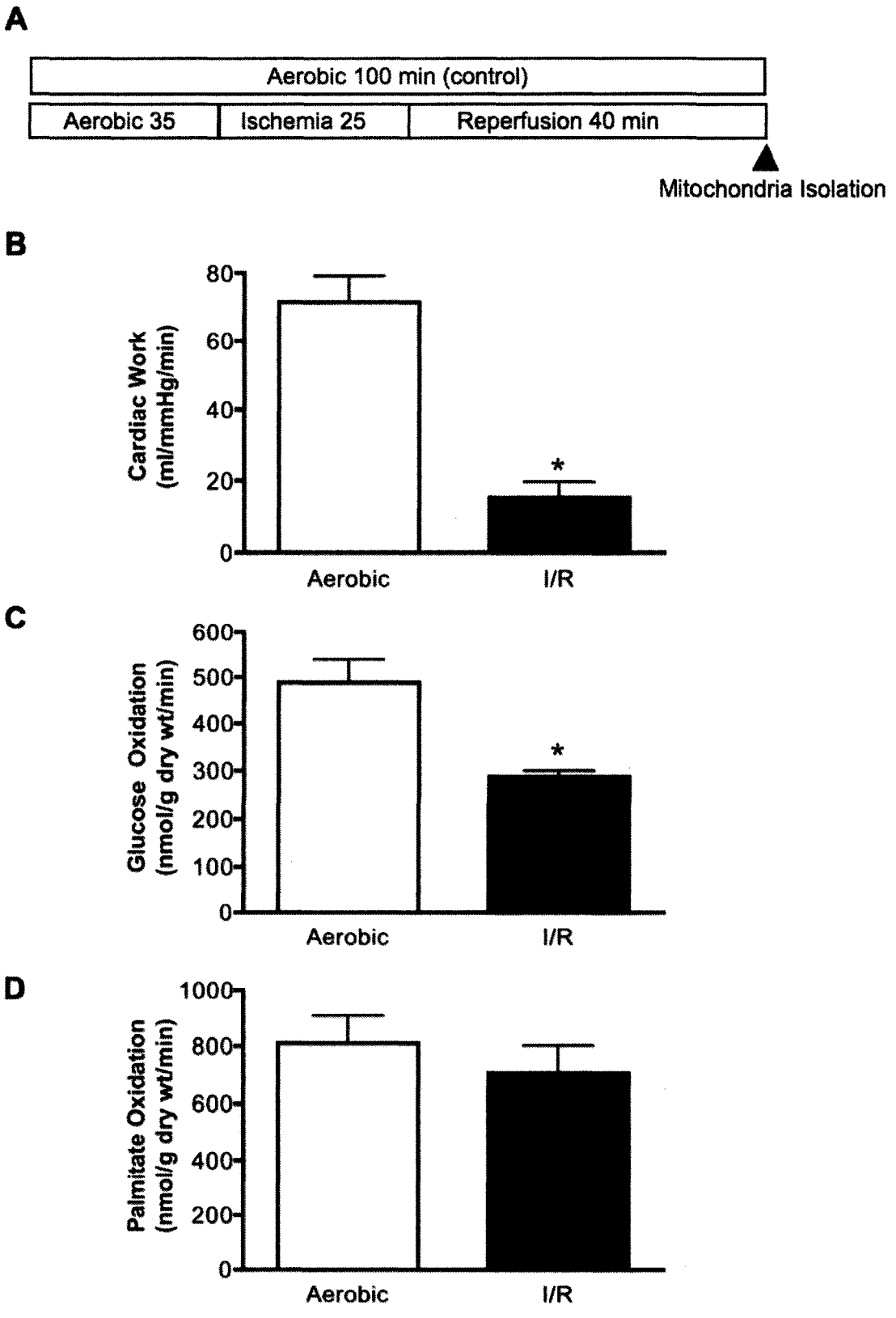

A
Aerobic 100 min (control)
Aerobic 35
Ischemia 25
Reperfusion 40 min
Mitochondria Isolation
B
Cardiac Work (ml/mmHg/min)
80
60
40
20
0
*
Aerobic
I/R
C
Glucose Oxidation (nmol/g dry wt/min)
600
500
400
300
200
100
0
*
Aerobic
I/R
D
Palmitate Oxidation (nmol/g dry wt/min)
1000
800
600
400
200
0
Aerobic
I/R

Figure 7-1

Figure 7-2. 2-D electrophoresis identifies five cardiac mitochondrial proteins that are modified following ischemia and reperfusion.

A) Representative 2-D electrophoresis of heart mitochondria indicating spots picked for identification and B) quantification of the indicated spots (n = 3 per group). Values represent the mean ± SEM. Differences were determined using a t-test.

* represents $P < 0.05$, significantly different from aerobic value.

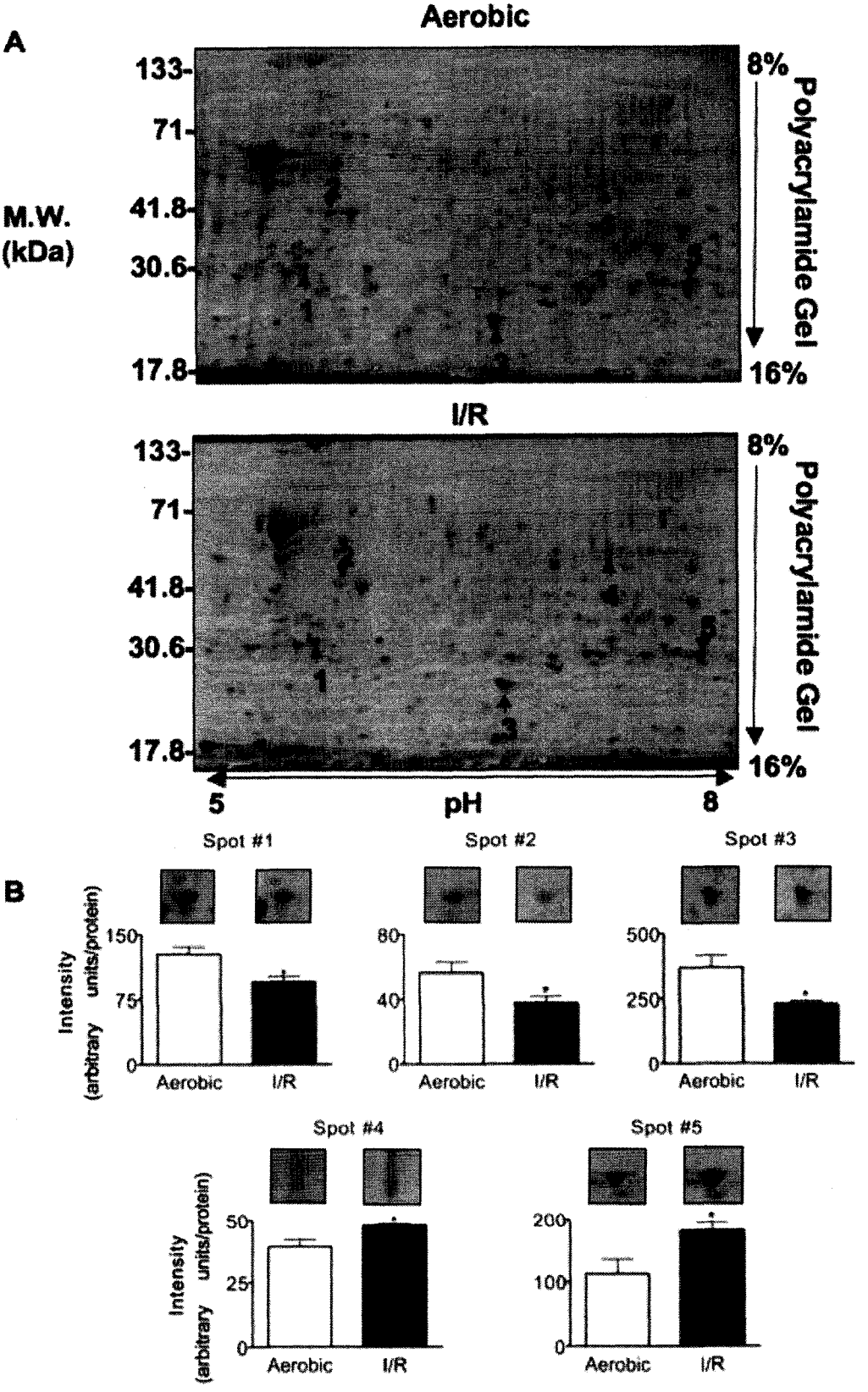
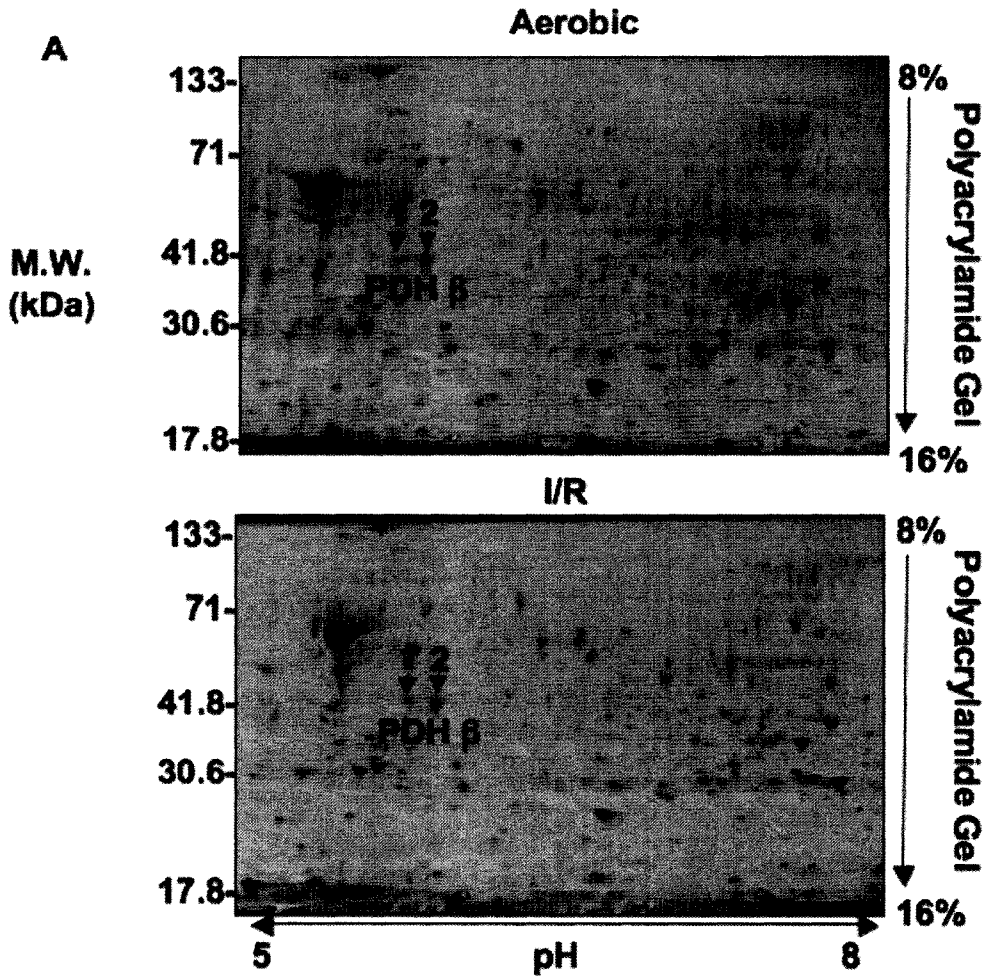

A
Aerobic
M.W. (kDa)
133-
71-
41.8-
30.6-
17.8-
8%
16%
Polyacrylamide Gel
I/R
5
pH
8
B
Spot #1
Spot #2
Spot #3
Spot #4
Spot #5
Intensity (arbitrary units/protein)
Aerobic
I/R

Figure 7-2

Figure 7-3. 2-D electrophoresis identifies two forms of the $PDH_{\beta E1}$ βE1.

A) Representative 2-D electrophoresis of heart mitochondria indicating spots that are identified as $PDH_{\beta E1}$ and B) quantification of spots identified as $PDH_{\beta E1}$ (n = 3 per group). Values represent the mean ± SEM. Differences were determined using a t-test.

* represents $P < 0.05$, significantly different from aerobic value.


A
Aerobic
133-
71-
41.8-
30.6-
17.8-
M.W.
(kDa)
1
2
PDH β
8%
16%
Polyacrylamide Gel
I/R
133-
71-
41.8-
30.6-
17.8-
1
2
PDH β
8%
16%
Polyacrylamide Gel
5
pH
8

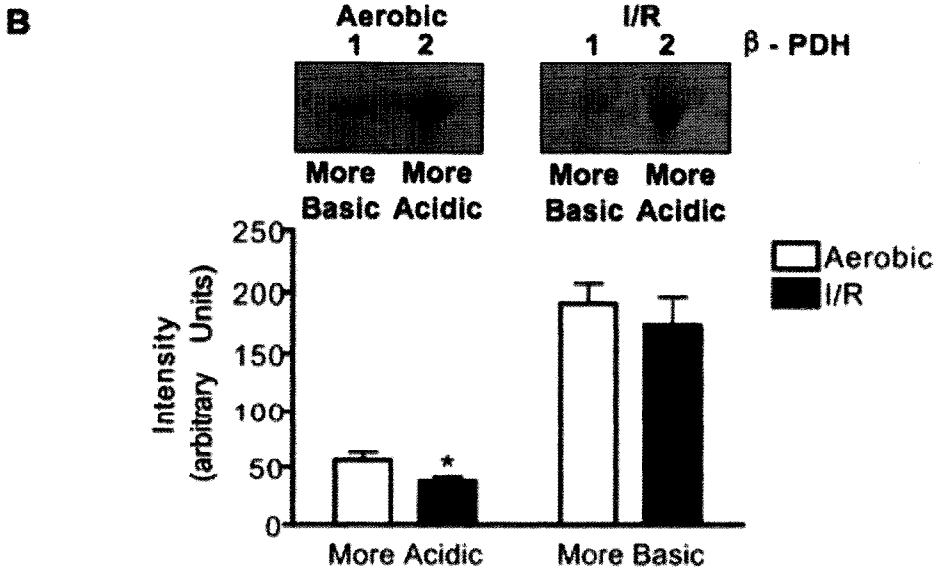

B
Aerobic
1 2
I/R
1 2
β - PDH
More Basic
More Acidic
More Basic
More Acidic
Intensity (arbitrary Units)
250
200
150
100
50
0
*
Aerobic
I/R
More Acidic
More Basic

Figure 7-3

Figure 7-4. Peptide mass fingerprints from aerobic heart mitochondria showing the novel O-palmitoylation of the more acidic form of $PDH_{\beta E1}$.

A) PMFs of the more basic and more acidic forms of $PDH_{\beta E1}$, and the PMF of the more acidic form of $PDH_{\beta E1}$ after potassium hydroxide treatment (0.1M KOH room temperature for 4 hours) and B) the potential site of O-palmitoylation of the more acidic form of $PDH_{\beta E1}$.

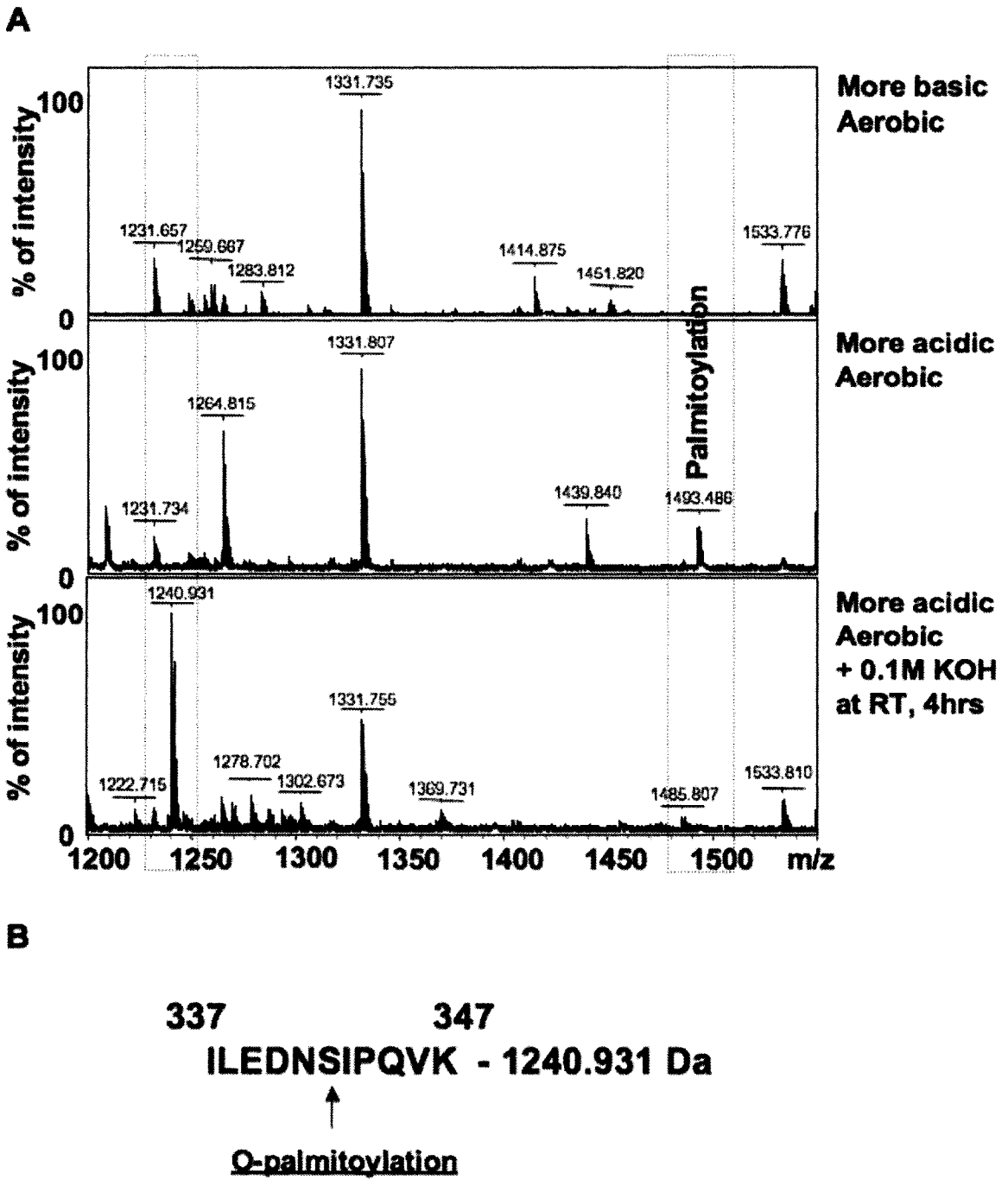

A
% of intensity
100
0
1331.735
1231.657
1259.667
1283.812
1414.875
1461.820
1533.776
More basic
Aerobic
1331.807
1264.815
1231.734
1439.840
1493.486
Palmitoylation
More acidic
Aerobic
1240.931
1331.755
1278.702
1222.715
1302.673
1369.731
1485.807
1533.810
More acidic
Aerobic
+ 0.1M KOH
at RT, 4hrs
1200
1250
1300
1350
1400
1450
1500
m/z
B
337
347
ILEDNSIPQVK - 1240.931 Da
O-palmitoylation

Figure 7-4

Figure 7-5. Peptide mass fingerprints of the more acidic form of $PDH_{\beta E1}$ showing possible phosphorylation.

A) PMFs of the more acidic form of $PDH_{\beta E1}$ from aerobic and I/R heart mitochondria and the PMF after alkaline phosphatase treatment (2 hrs at 30°C in 50 mM NH_4HCO_3) and

B) the potential sites of phosphorylation of the more acidic form of $PDH_{\beta E1}$.

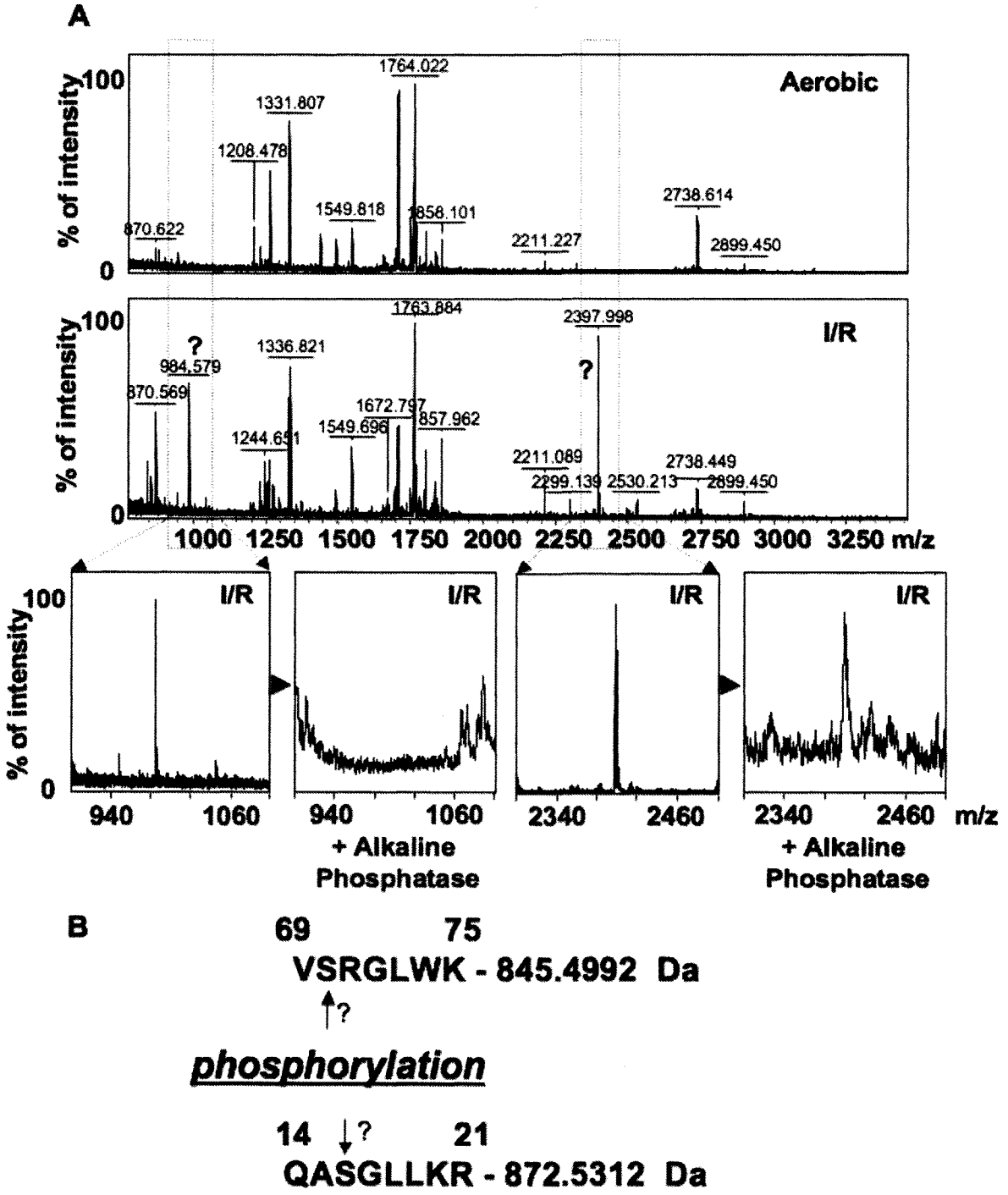

A
Aerobic
% of intensity
100
0
1764.022
1331.807
1208.478
1549.818
1858.101
870.622
2211.227
2738.614
2899.450
I/R
1763.884
2397.998
1336.821
984.579
870.569
1672.797
1549.696
857.962
1244.691
2211.089
2299.139
2530.213
2738.449
2899.450
1000 1250 1500 1750 2000 2250 2500 2750 3000 3250 m/z
940 1060
2340 2460
m/z
+ Alkaline Phosphatase
B
69 75
VSRGLWK - 845.4992 Da
phosphorylation
14 21
QASGLLKR - 872.5312 Da

Figure 7-5

Figure 7-6. Peptide mass fingerprints of the more basic form of $PDH_{\beta E1}$ showing possible phosphorylation.

A) PMFs of the more basic from of $PDH_{\beta E1}$ from aerobic and I/R heart mitochondria and the PMF after alkaline phosphatase treatment (2 hrs at 30°C in 50 mM NH_4HCO_3) and

B) the potential sites of phosphorylation of the more basic form of $PDH_{\beta E1}$.

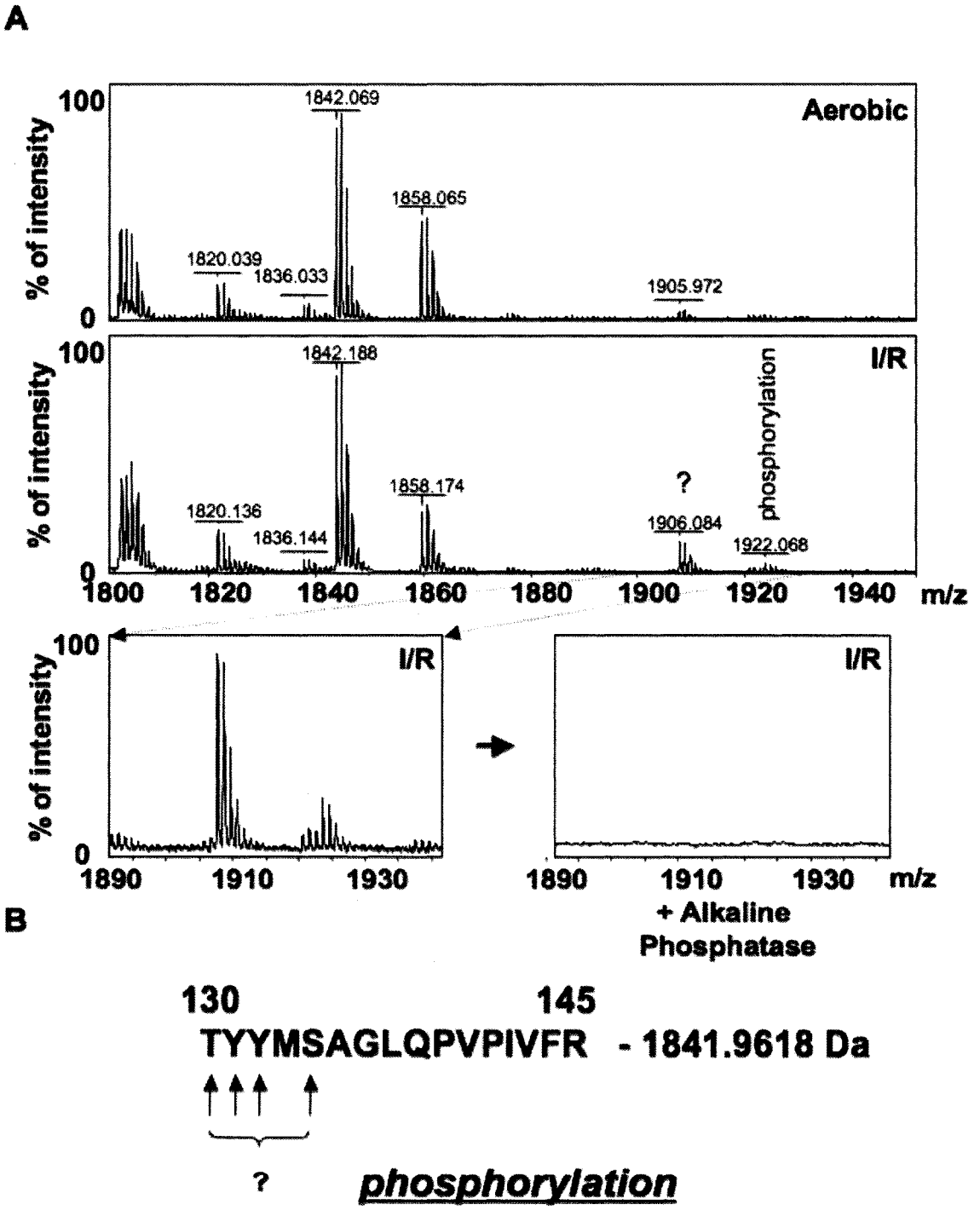

A
100
% of intensity
0
1842.069
Aerobic
1858.065
1820.039
1836.033
1905.972
100
% of intensity
0
1842.188
I/R
phosphorylation
?
1858.174
1820.136
1906.084
1836.144
1922.068
1800
1820
1840
1860
1880
1900
1920
1940
m/z
100
% of intensity
0
I/R
I/R
1890
1910
1930
1890
1910
1930
m/z
+ Alkaline
Phosphatase
B
130
145
TYYMSAGLQPVPIVFR - 1841.9618 Da
?
phosphorylation

Figure 7-6

Figure 7-7. Identification of putative kinases for the phosphorylation of $PDH_{\beta E1}$.

A) The phosphorylation potential of amino acids in the more basic form of $PDH_{\beta E1}$, B) isoelectric point of the more basic form of $PDH_{\beta E1}$ and C) the predicted secondary structure surrounding the putative phosphorylation sites.

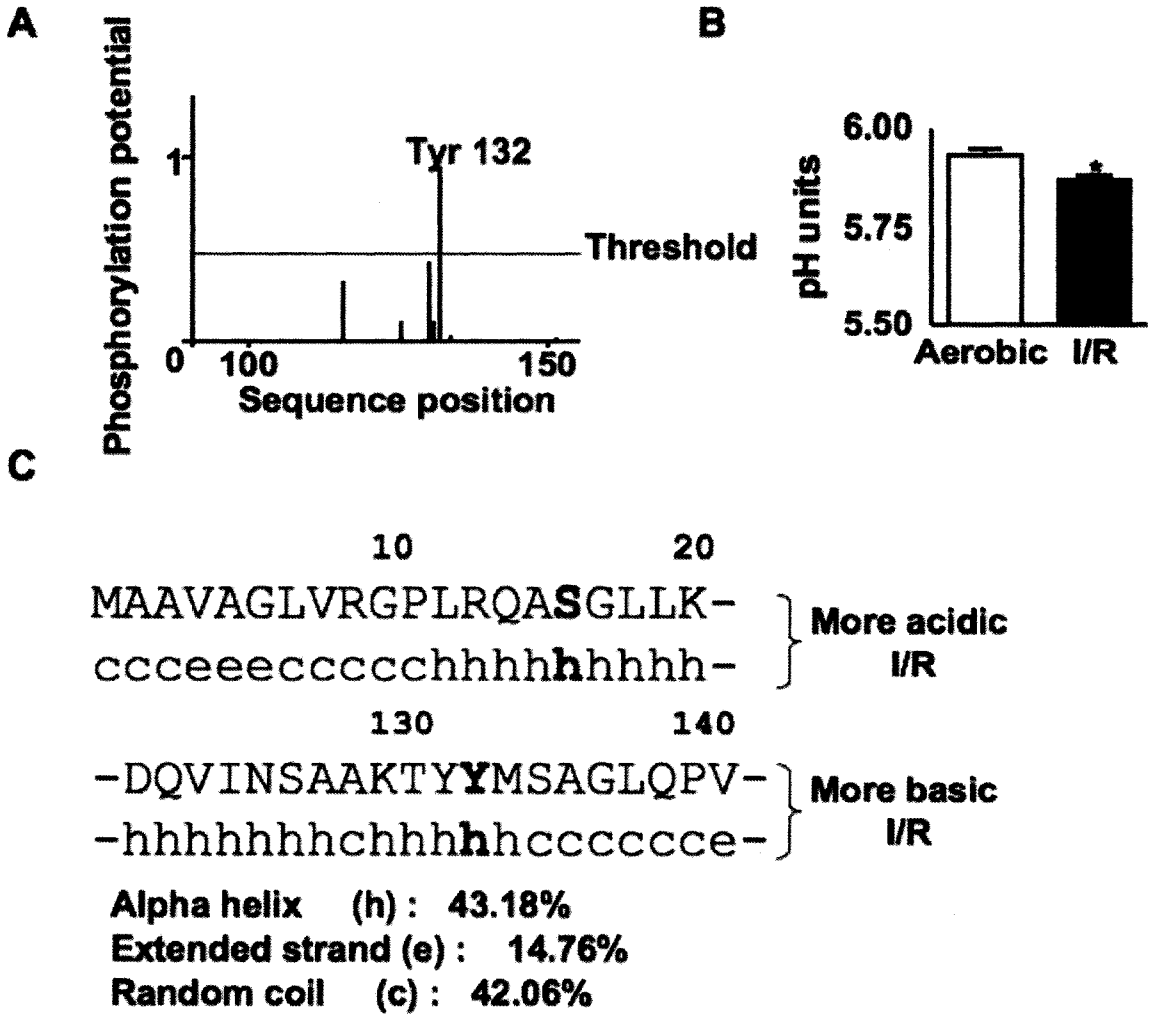

A
Phosphorylation potential
1
0
Tyr 132
Threshold
100
150
Sequence position
B
pH units
6.00
5.75
5.50
Aerobic
I/R
C
10
20
MAAVAGLVRGPLRQASGLLK-
ccceeeccccchhhhhhhhhh-
More acidic
I/R
130
140
-DQVINSAAKTYYMSAGLQPV-
-hhhhhhhchhhhhccccccce-
More basic
I/R
Alpha helix (h) : 43.18%
Extended strand (e) : 14.76%
Random coil (c) : 42.06%

Figure 7-7

Discussion

Although there are several reports describing the mitochondrial proteome, only one previous study has attempted to provide a picture of more global changes in protein abundance induced by I/R.[324] Using a proteomics approach we identified 32 protein spots, which are either up or down regulated by I/R. Our research focus was on the proteins with the largest changes in abundance, and all of the identified proteins corresponded to proteins with metabolic functions. The proteins with an increase in abundance included long chain acyl-coenzyme A dehydrogenase and ATP synthase (α subunit), while proteins with a decrease in abundance included NADH-ubiquinone oxidoreductase (24 kDa subunit), ATP synthase (D subunit) and PDH (βE1 subunit). Interestingly, 2-D gels from I/R hearts have shown up to 10 proteins that are simply isoelectric point variants of other identified spots.[322] This, coupled with the fact that we identify two spots as $PDH_{\beta E1}$, suggests that PTMs may play a crucial role in the protein changes induced by I/R.

As PDH plays a critical role in the regulation of myocardial glucose oxidation we endeavored to better characterize the two molecular forms of $PDH_{\beta E1}$, which are identified using 2-D electrophoresis. The major difference between the two forms of $PDH_{\beta E1}$ is a O-palmitoylation PTM to the more acidic form of $PDH_{\beta E1}$. In addition, I/R triggered phosphorylation of both forms of $PDH_{\beta E1}$. Using *in silico* methods the putative kinase that phosphorylates the more basic form is identified as the insulin receptor kinase while PKC_{ζ} or PKA are identified for the more acidic form.

The mitochondrial PDH complex catalyzes the decarboxylation of pyruvate to acetyl-CoA, which is then further oxidized in the tricarboxylic acid cycle. This complex consists of multiple copies of three enzymes (E1 (pyruvate dehydrogenase), E2 (dihydrolipoyl transacetylase) and E3 (dihydrolipoyl dehydrogenase), with the E1 being a tetramer consisting of 2α and 2β subunits. This complex can be directly regulated by allosteric mechanisms by the products of the reaction, such as inhibition of the enzyme by acetyl-CoA.[55] The complex is also highly regulated by phosphorylation/dephosphorylation by the PDK and PDP, which are part of the PDH complex.[332] However, this regulatory mechanism has only been documented for the α chains. Phosphorylation of PDH at three specific serine residues (site 1 (Ser-264), site 2 (Ser-271) and site 3 (Ser-203)) in the α subunit of E1 results in the complete inactivation of the enzyme.[333-335] PDK4 is the major isoform expressed in the heart, although PDK1 and PDK2 have also been detected in this tissue.[336-338] These kinases are also subjected to short-term regulation by metabolites, including inhibition by pyruvate (a substrate of PDH) and activation by acetyl-CoA and NADH (both products of the PDH complex).[339] For an in-depth overview of PDKs and PDH regulation please see the excellent review by Sugden and Holness.[332] Despite the significant work on phosphorylation of the α subunit of E1, no information is available on the phosphorylation of the $PDH_{\beta E1}$.

Many studies have looked at the phosphorylation of the $PDH_{\alpha E1}$ and extensive work has been performed correlating phosphorylation of PDH by the PDKs with activity; however to our knowledge no one has previously reported phosphorylation of $PDH_{\beta E1}$. Evidence shows that the degree of phosphorylation of PDH is correlated with pyruvate flux through PDH.[332] However the importance of phosphorylation over allosteric control

of PDH during reperfusion of previously ischemic hearts is controversial. Previous reports show that myocardial I/R results in a conversion of 45 % of the total PDH to an inactive form during the first few minutes reperfusion, slowly returning to control levels as reperfusion continued.[340] This observation is reproduced by Churchill *et al*, who show a 65 % reduction in PDH activity in response to global ischemia, with a slight recovery of activity with reperfusion.[341] This is analogous to our data where we see a 35 % decrease in glucose oxidation during reperfusion compared to time-matched aerobic controls, which is a measure of pyruvate flux through PDH. Our data suggests that novel phosphorylation events on $PDH_{\beta E1}$ may account for this decrease in glucose oxidation observed during reperfusion. However it is unknown what direct effect these phosphorylation events have on the PDH complex and its activity.

Interestingly, *in silico* methods identified the putative kinase that phosphorylates the more acidic form as PKC_ζ or PKA and while the insulin receptor kinase was identified for the more basic form. The identification of PKC_ζ is particularly intriguing, as a recent study reported that translocation of PKC_δ to the mitochondria is associated with an inhibition of PDH activity, although they did not assess the ability of PKC_ζ to translocate.[341] PKC_δ inhibits PDH activity by the direct binding of PKC_δ to PDK2, thus increasing its activity. Our studies would suggest that PKC might also directly phosphorylate the $PDH_{E1\beta}$ and exert its effects directly on PDH activity. An alternative hypothesis may be that phosphorylation at this site by PKA stimulates PDH activity, as previous reports suggest that treatment of isolated hearts with cAMP analogues or epinephrine (which signals via cAMP) preferentially increase glucose oxidation via an activation of PDH.[342, 343] This hypothesis would also be consistent with phosphorylation

and activation of PDH by insulin receptor kinase. Previous studies have shown that insulin can activate glucose oxidation [240], however there is little evidence for a role of insulin in acutely regulating PDH activity. There is some suggestion that insulin may activate PDH phosphatase 2, leading to a dephosphoryation and activation of PDH.[344] Alternatively, *in vivo* studies of insulin infusion show that PDK4 mRNA levels are decreased after 5 hr, however whether this occurs with acute insulin administration is unknown.[345] As the existence of phosphorylation sites on $PDH_{\beta E1}$ may have very important implications on the regulation of PDH activity, further research is required to determine the effect of these phosphorylation events on PDH activity, and the ability of the putative kinases to phosphorylate these sites *in vitro* and *in vivo*.

The major difference between the two forms of $PDH_{\beta E1}$ identified using *in silico* analysis and confirmed biochemically, is a novel O-palmitoylation PTM of the more acidic form of $PDH_{\beta E1}$. To our knowledge this is the first report of this modification in a mitochondrial protein. This finding may be of functional significance, as only protein levels of the O-palmitoylated form of $PDH_{\beta E1}$ are decreased during I/R in the heart. The O-palmitoylation of this enzyme is particularly novel as very few studies have reported the existence of O-acylated peptides. One report observes the O-palmitoylation at a threonine residue in an insect toxin PLTX-II, with the base removal of this palmitoyl ester resulting in a loss of biological activity by at least two orders of magnitude.[346] A more recent report observes that a serine residue on ghrelin contains an O-octanoyl moiety, and this modification is essential for its biological activity.[347] Due to the scarcity of observations involving the O-acylation of peptides, no work has been performed to date on the biological role of this modification. However, much is known about the S-

and N-palmitoylation of proteins (as reviewed by Smotrys and Linder).[348] Palmitoylation of proteins tend to promote the membrane association of soluble proteins and target proteins to lipid rafts. As PDH is reported to be localized to the inner mitochondrial membrane in cardiac tissue [349], we hypothesize that palmitoylation of PDH results in its recruitment to the inner mitochondrial membrane, whereby it could interact with the monocarboxylate transport (the transporter required for pyruvate entry into the mitochondria). If this were the case, then a reduction in the O-palmitoylated form of PDH during I/R would result in reduced mitochondrial inner membrane localization of PDH, which may account for the reduction in pyruvate flux and glucose oxidation we observed in the reperfused heart.

Many studies have shown that strategies, which increase glucose oxidation directly (dichloroacetate)[31] or indirectly via inhibition of fatty acid oxidation (trimetazine)[143] are cardioprotective in the setting of ischemia due to the improved coupling of glycolysis to glucose oxidation and the reduction of myocardial acidosis. Therefore the effect of these PTMs on PDH activity and the resultant decrease in glucose oxidation has important implications on the functional recovery of hearts subjected to I/R. Further studies are required to assess the effects of these PTMs on PDH activity and glucose oxidation, in order to determine if they are potential targets for stimulating glucose oxidation in the ischemic heart.

Limitations

A limitation of the 2-D gel approach is that even gels with wide pH gradients can only separate at most 2000 proteins (in a crude heart homogenate), which gives

incomplete proteomic coverage.[350] In our case we isolated mitochondria to reduce the complexity of our samples and we utilized a narrower pH range to increase separation, but this limits identification of proteins with high or low isoelectric points. In addition, it is difficult to separate basic proteins with 2-D gels, as these proteins have the tendency to form streaks.[350]

Due to the denaturing conditions of the 2-D PAGE, we did not examine complex formation in the present study. However, recently Lopez-Campistrous *et al.* identified defects in assembly of respiratory complex I and V that are associated with suppressed respiration rates in the hypertensive brain stem.[351] Therefore additional experiments utilizing Blue-native PAGE and Western blotting are required in order to examine complex assembly and its downstream effects on enzymatic activity of these complexes. In addition it would be of interest to determine whether the identified PTMs of PDH affects complex assembly, which may account for the modification in myocardial energy substrate metabolism during I/R.

In silico sequence based predictions of PTMs have high rates of false positives due to the limited knowledge about the structural features required for these modifications.[352] In the present study, we utilized preliminary biochemical techniques in order to confirm the identity of predicted PTMs, however further experiments, some of which are discussed in chapter 9, are required in order to confirm that these PTMs occur *in vitro* and *in vivo,* as well as the effect of these modifications on enzymatic activity. In the case of the phosphorylation events, extensive experiments are required in order to confirm the identity of the predicted kinases.

In summary, we have identified novel changes in mitochondrial protein abundance and PTMs of metabolic proteins in response to I/R using a proteomics approach. Two molecular forms of $PDH_{\beta E1}$ were identified and partially characterized, which differ due to an O-palmitoylation PTM. Also, for the first time we show that I/R induces phosphorylation of both forms of $PDH_{\beta E1}$, and that this phosphorylation is associated with a decrease in glucose oxidation. As PDH is the rate-limiting enzyme for glucose oxidation, the regulation of beta subunit PTMs may be utilized in order to optimize myocardial metabolism during reperfusion.

CHAPTER 8.

Discussion and Conclusions

Chapter 8.

Discussion and Conclusions

Although the use of improved therapeutic strategies has decreased the overall mortality from cardiovascular disease, the number of Canadians living with chronic cardiovascular disease has increased, which has imposed a massive economic burden on society. A major component of ischemic heart disease involves modifications of myocardial energy metabolism, which correlate with post-ischemic recovery of myocardial function. A better understanding of the signaling cascades required for these I/R-induced modifications of metabolism and the direct downstream effects on the fluxes through these metabolic pathways would provide insight into the pathophysiology of cardiac I/R injury and could identify novel therapeutic targets for the treatment of ischemic heart disease.

This thesis addresses these issues and demonstrates that the regulation of myocardial energy substrate metabolism is an important determinant of post-ischemic function during reperfusion. In particular we demonstrate that the cardioprotective effect of insulin and its ability to inhibit AMPK is dependent on the concentration of fatty acid present in the perfusate, such that insulin may become detrimental to the recovery of function during reperfusion in the presence of a clinically relevant high concentration of fatty acids. Utilizing transgenic mouse models that either up- or down-regulate AMPK activity, we demonstrate that activation of AMPK shifts overall oxidative metabolism

away from glucose towards fatty acids and is not associated with cardioprotection, but may actually be detrimental to the recovery of function during reperfusion. A consequence of shifting mitochondrial oxidative metabolism away from glucose toward fatty acids is the uncoupling of glucose metabolism leading to myocardial acidosis. The role this acidosis plays in inducing post-ischemic contractile dysfunction was assessed in ssTnI transgenic mice. We demonstrate that expression of ssTnI impairs post-ischemic function potentially due to greater sensitivity of these hearts to pathologic Ca^{2+} concentrations outweighing the beneficial effect of reduced acidosis-induced desensitization of the contractile apparatus. Utilizing a proteomics approach we identify modifications to the mitochondrial proteome, including O-palmitoylation and phosphorylation of $PDH_{\beta E1}$, which may have important implications in the regulation of metabolism following ischemia. This chapter will justify the methodology utilized to make these observations and place these observations in the context of the current literature. The potential limitations and future directions associated with these studies will also be discussed.

Justification of Methodology

The Isolated Working Heart (Mouse/Rat)

Due to the small energy reserve and the need to meet the high demands of contraction and ionic homeostasis, the heart must produce an abundant supply of ATP, with this supply matching the workload and energy demand of the heart.[3] The isolated

working heart is a reproducible model, which has been used for over 40 years to assess a variety of physiological, biochemical, metabolic and pharmacological parameters.[353] This experimental model is ideal for the measurement and correlation of cardiac function and cardiac energy metabolism as the work performed by the heart is one of the major factors controlling the flux through oxidative metabolism. Therefore the ability to perfuse the isolated heart at a physiologic preload and afterload in the presence of energy substrates (glucose and fatty acids) allows the characterization of cardiac substrate metabolism at physiologically relevant workloads without the confounding effects of other organs and circulating hormones and adipokines. In addition, this model is well suited for the study of I/R due to the simultaneous measurement of contractile function and metabolism during the critical early period of reperfusion. This allows for the direct assessment of the effect of modifications in myocardial energy metabolism on the recovery of contractile function. Post-ischemic contractile function was utilized as a measure of ischemic damage as opposed to markers of apoptosis/necrosis because it has previously been demonstrated that apoptosis is only evident following 30 mins of ischemia and 60 mins of reperfusion in a Langendorff mouse heart.[354] In addition, apoptosis was only observed after 1 hr of reperfusion in rat hearts subjected to 45 mins of *in vivo* LAD ligation.[355] In our model of global no-flow ischemia, the hearts are unloaded and stop contracting early during ischemia potentially reducing the amount of irreversible damage. Taken together this would suggest that quantifiable infarcts may not be observed in these mouse hearts. However, as infarct size or other markers of apoptosis/necrosis have not been examined in the present studies, we cannot rule out that part of the observed contractile dysfunction during reperfusion may be due to irreversible

injury as opposed to reversible injury such as myocardial stunning. It has been previously reported that post-ischemic ventricular function and infarct size do not correlate as ischemic preconditioning can reduce infarct size but has no effect on ventricular function in an isolated perfused mouse heart.[356]

This experimental model has a number of advantages over other cell and muscle preparations for metabolic and functional studies. The isolated working heart has an advantage over the use of cell preparations, as isolated cardiomyocytes have oxidative rates that are orders of magnitude lower due to their quiescent nature and their non-physiological energy demand. The isolated working heart also has an advantage over isolated muscle preparations, such as papillary muscle, as these preparations rely on superfusion and the diffusion of substrates and O_2 across the muscle. In some cases superfusion may not be sufficient to supply the whole muscle, thereby producing an ischemic zone in the central core of the muscle, which would modify metabolism. Therefore, cell and muscle preparations are not as physiologically relevant as the isolated working heart for the study of energy metabolism.

Measurement of Myocardial Substrate Metabolism

Direct Measurement of Glycolysis

Metabolic flux through glycolysis can be directly measured in the isolated working heart using one of two stable radioactive tracers, [5-^{3}H]glucose or [2-^{3}H]glucose. All the studies described in this thesis utilize the [5-^{3}H]glucose tracer, as the use of [2-^{3}H]glucose as a tracer of metabolic flux through glycolysis has several limitations. Measurement of glycolysis using the [2-^{3}H]glucose tracer depends on the release of ^3H_2O from the carbon-2 of glucose at the phosphoglucose isomerase step of glycolysis. However the ^{3}H label at the carbon-2 position can undergo intramolecular exchange to the carbon-1 position, thus underestimating the metabolic flux through glycolysis. During extreme conditions such as total ischemia or abrupt anoxia, the regulation of glycolysis can be passed from PFK-1 to GAPDH.[3] Previous studies demonstrate that the fractional velocity of GAPDH activity is inhibited by I/R, thus restricting flux through glycolysis.[357] Consequently, as ^3H_2O is liberated from [2-^{3}H]glucose upstream of GAPDH this may lead to an overestimation of the actual rates of glycolysis. The use of [5-^{3}H]glucose as a metabolic tracer for glycolysis avoids this overestimation of metabolic flux through glycolysis as ^3H_2O is liberated from [5-^{3}H]glucose by the triose phosphate isomerase reaction and enolase reaction that is downstream of GAPDH.

A previous report suggests that the use of [5-^{3}H]glucose may overestimate the true rates of glycolysis based on the observations that rates of glycolytic flux from [5-^{3}H]glucose are nonlinear and that these rates are in excess of true rates of glycolysis calculated from the sum of the rate of lactate and pyruvate accumulation in the perfusate

and the rate of glucose oxidation.[358] The authors suggest that the discrepancy would be accounted for by the non-glycolytic detritiation by the transaldolase reaction of the nonoxidative portion of the pentose phosphate pathway.[358] However, the rates of true glycolysis may have been underestimated in the study due to very low rates of glucose oxidation, as subsequent studies show linear rates of glycolytic flux with good correlation between rates of glycolysis measured from the detritiation of [5-^{3}H]glucose and the calculated true rates of glycolytic flux.[359, 360]

Myocardial Acidosis from the Uncoupling of Glycolysis from Glucose Oxidation

In the studies described in this thesis, H^+ production was utilized as a measure of the uncoupling of glycolysis (measured directly using [5-^{3}H]glucose) from glucose oxidation (measured directly using [U-^{14}C]glucose). As H^+ production is dependent on the hydrolysis of glycolytically derived ATP in the absence of the mitochondrial oxidation of pyruvate, any increase in the rate of glycolysis above the rate of glucose oxidation results in an increase of H^+ production. The stoichiometry of H^+ production is dependent on the overall reaction of glycolysis:

$$\text{glucose} + 2\ \text{ADP} + 2\ P_i + 2\ \text{NAD}^+ \rightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+ + 2\ H_2O$$

Under aerobic conditions the resulting H^+s are co-transported with pyruvate into the mitochondria by the monocarboxylate transporter, where they are consumed by oxidative phosphorylation.[269] However when glucose oxidation is suppressed due to limited O_2 availability or by high rates of fatty acid oxidation, pyruvate is metabolized to lactate via LDH to maintain cytosolic levels of NAD^+:

$$2\ \text{pyruvate} + 2\ \text{NADH} + 2\ H^+ \rightarrow 2\ \text{lactate} + 2\ \text{NAD}^+$$

This gives an overall reaction for anaerobic glycolysis:

$$\text{glucose} + 2\ \text{ADP} + 2\ P_i \rightarrow 2\ \text{lactate} + 2\ \text{ATP} + 2\ H_2O$$

However if the glycolytically-derived ATP is hydrolyzed:

$$2\ \text{ATP} + 2\ H_2O \rightarrow 2\ \text{ADP} + 2\ P_i + 2\ H^+$$

The resultant reaction of anaerobic glycolysis with ATP hydrolysis is:

$$\text{glucose} \rightarrow 2\ \text{lactate} + 2\ H^+$$

Therefore measuring the flux through glycolysis and glucose oxidation provides a direct and accurate assessment of the H^+ production due to the degree of uncoupling between glycolysis and glucose oxidation. In addition, this calculation of H^+ production has been validated, as in a previous study in isolated working rat hearts utilizing a similar metabolic approach, the pH_i determined using ^{31}P NMR correlates very well with our calculated values.[32] Alternatively, the uncoupling of glycolysis and glucose oxidation can also be assessed indirectly by measuring the amount of lactate efflux into the perfusate. However this relationship is difficult to interpret, as the concentration of lactate in the perfusate is dependent on not only the efflux of lactate due to anaerobic glycolysis, but also its import back into the cardiomyocyte and subsequent mitochondrial oxidation.

Regulation of Myocardial Energy Metabolism during Ischemia/Reperfusion and Consequences on Functional Recovery during Reperfusion

Insulin Modification of Myocardial Energy Metabolism and Cardioprotection

Insulin is shown to be cardioprotective in some animal models of I/R, with its beneficial effect being attributed to either a direct positive inotropic action or stimulation of anaerobic glycolysis, thus contributing to an increase in ATP production.[248-250] Despite encouraging laboratory observations, two randomized controlled trials, CREATE-ECLA and OASIS-6, show no benefit on any of the predefined endpoints of high-dose GIK therapy in patients with ST-elevated myocardial infarction and indicates that GIK may actually increase mortality during the three days following the ischemic event.[150, 151] These opposing results may be explained by our observations in Chapter 3. The majority of animal studies examining the role of insulin and GIK therapy in the regulation of glucose metabolism and cardioprotection have done so in isolated working hearts perfused either in the absence of fatty acids or in the presence of a low concentration of fatty acids.[248-250] However, levels of circulating fatty acids are elevated in most clinical forms of myocardial ischemia, thus the lack of benefit of insulin may be due to the high levels of circulating fatty acids.[33] In Chapter 3 we confirm previous observations that in the absence of fatty acids, insulin is cardioprotective and enhances recovery of contractile function during reperfusion.[248-250] Associated with the

cardioprotection is a stimulation of glycolysis and glucose oxidation during both the aerobic period and reperfusion. However, in the presence of a clinically relevant high concentration of fatty acids, the cardioprotective effect of insulin is lost and the presence of insulin becomes detrimental to the recovery of contractile function during reperfusion. Despite the dramatic change in functional recovery, insulin is still able to stimulate both glycolysis and glucose oxidation, although the presence of fatty acids blunts the insulin-induced increase in glucose oxidation during reperfusion.

In addition to the metabolic effects, insulin also inhibits AMPK.[152-154] We furthered the previous observation that fatty acids can attenuate the ability of insulin to inhibit AMPK by demonstrating that the inhibition occurs during ischemia and may play a role in insulin cardioprotection.[253] However, the mechanism by which insulin and fatty acids modify AMPK activity is unknown. Previous reports in isolated cells show that a supraphysiological concentration of insulin can activate Akt, which can in turn phosphorylate $AMPK_{\alpha 1/\alpha 2}$ on Ser485/491 leading to the inability of AMPK to be activated by phosphorylation at Thr172.[258, 361] We observe an activation of Akt, however this is not associated with an increase in the phosphorylation status of AMPK at Ser485/491. Thus, the insulin-induced inhibition of AMPK does not appear to involve Akt and may potentially be mediated by modifying the activity of the upstream AMPKKs. However, to date, an effect of insulin or fatty acids on the AMPKKs has not been demonstrated. Alternatively, insulin and palmitate are known to modify the activity of protein phosphatase 2A (PP2A), the phosphatase that dephosphoylates AMPK.[362-364] Therefore the regulatory effects of insulin and fatty acids on AMPK may be mediated by PP2A, however this possibility has not been examined in our model.

These differential effects of insulin on cardioprotection may be related to myocardial energetics, as hearts perfused with glucose as the sole substrate may be energetically compromised, such that addition of insulin would increase glucose uptake, glycolysis and glucose oxidation, thus increasing ATP production, which would be beneficial to the ischemic heart. In contrast, when hearts are perfused with a high concentration of fatty acids and glucose, the heart has a greater availability of substrates in order to produce ATP, therefore the competition of glucose and fatty acids for oxidative metabolism and the uncoupling of glucose metabolism and subsequent acidosis may play a more prominent role in post-ischemic dysfunction. This energetics issue may also account for the insulin-induced decrease in AMPK activity because if these hearts are energetically compromised in the absence of fatty acids, this stress would lead to AMPK activation. Addition of insulin could increase ATP production by stimulating glycolysis and glucose oxidation, therefore reducing stress and potentially reducing AMPK activation.

AMPK Modulation of Myocardial Energy Metabolism and Cardioprotection

Only a few studies have directly associated AMPK activity with functional recovery during reperfusion following ischemia, and they have reached differing conclusions.[192, 221, 222] Therefore the role that AMPK plays in the cardioprotection of the ischemic myocardium is still controversial.[220] We therefore examined the role of AMPK activation during I/R using two transgenic mouse models with opposite effects on AMPK activity; an AMPK Dn mouse which impairs ischemic-induced activation of AMPK

(Chapter 4) and a transgenic mouse overexpressing the $AMPK_{\gamma 2}$ R302Q mutation, which results in elevated I/R-induced AMPK activity (Chapter 5). We observe that AMPK Dn hearts have a greater reliance on glucose oxidation for oxidative metabolism and that functional recovery was either comparable to Wt hearts in the absence of insulin or slightly improved in the presence of insulin. In contrast, hearts overexpressing $AMPK_{\gamma 2}$ R302Q have a significant increase in palmitate oxidation at the expense of glucose oxidation such that nearly 90 % of the acetyl-CoA produced in these hearts is from palmitate. Associated with this is an elevated rate of glycolysis from endogenous substrates. Previous studies demonstrate that a higher reliance on fatty acid oxidation as a source of oxidative metabolism is associated with impaired recovery of function during reperfusion [31, 32], which we also observe in $AMPK_{\gamma 2}$ R302Q expressing hearts. These observations are consistent with the fact that activation of AMPK by I/R is not cardioprotective, however this is not a universally accepted theory.

Russell *et al.* demonstrate that $AMPK_{\alpha 2}$ KD mice have worse cardiac function during aerobic perfusion as well as during and following low-flow ischemia, associated with an inability to increase glucose uptake and glycolysis.[192] Surprisingly, rates of fatty acid oxidation and glucose oxidation did not change between the KD and Wt groups.[192] Interestingly those hearts were perfused with low levels of fatty acids (0.4 mM), which may maximize any benefit from glycolytic ATP production because of a decrease in detrimental effects of elevated rates of fatty acid oxidation on glucose oxidation and H^+ production. Associated with those metabolic and functional changes, KD hearts have increased cellular damage as observed by increased LDH and creatine kinase release, and an increase in apoptotic cells. These contradictory results may be due to the fact that the

KD mutation almost completely abolishes $AMPK_{\alpha 2}$ activity and also substantially blunts $AMPK_{\alpha 1}$ activity even under baseline conditions. Therefore during ischemia, total AMPK activity in the KD hearts is lower than AMPK activity in the basal state in Wt hearts. It is possible that there is some lower limit of AMPK activity that is required to maintain basal energetics, as ATP and PCr are suppressed in the KD hearts.[192] Therefore, activation of AMPK in this situation might be beneficial in order to improve cardiac ATP production. In contrast, our AMPK Dn hearts are not energetically compromised as they have similar levels of adenine nucleotides as Wt hearts, thus the shifting of oxidative metabolism away from palmitate toward glucose and decreasing H^+ production may play more important roles.

Recent studies have examined the effect of I/R in an $AMPK_{\alpha 2}$ KO model. This model mimics to a greater extent the effects of the Dn model used in the present study, as $AMPK_{\alpha 2}$-associated activity is abolished but there is no effect on $AMPK_{\alpha 1}$-associated activity.[221, 222] In Langendorff perfused hearts in the absence of fatty acids and insulin, global ischemia results in an increase in lactate release in Wt mice, which is blunted in the $AMPK_{\alpha 2}$ KO.[222] Energetically, the KO hearts have similar AMP/ATP ratios during the aerobic period, however the AMP/ATP ratio is significantly elevated at the end of ischemia, but was not measured at the end of reperfusion. As previously observed in the Dn and KD mice, $AMPK_{\alpha 2}$ KO mice have a decreased time to start of ischemic contracture and greater maximal ischemic contracture during ischemia.[192, 221-223] Those observations are consistent with decreased rates of glycolysis.[133, 365, 366] Despite those metabolic and functional observations, the absence of $AMPK_{\alpha 2}$ does not exacerbate post-ischemic contractile dysfunction.[222] This is not surprising, as previous reports suggest

that ischemic contracture does not correlate with post-ischemic contractile function.[272, 273] This observation is further confirmed in a study showing that Wt hearts subjected to low-flow ischemia in the absence of fatty acids have better-preserved ATP levels during I/R, however this is associated with similar recovery of contractile function during reperfusion.[221] Yet, when hearts are subjected to low-flow I/R in the presence of fatty acids, the $AMPK_{\alpha 2}$ KO have a slight suppression of cardiac function during the immediate reperfusion period with similar contractility during late reperfusion.[221] Unfortunately it is difficult to determine why the addition of fatty acids to the perfusate is detrimental to the functional recovery only of the $AMPK_{\alpha 2}$ KO hearts as no metabolic parameters were assessed in this set of hearts.

This use of transgenic mice to assess the role of AMPK during I/R has raised a number of questions. As the transgene expression or KO of AMPK occurs for the entire lifetime of the mouse, there is potential for important compensatory changes in the AMPK signaling pathway, which may affect downstream signaling, including to myocardial metabolism and cardioprotection that are assessed in these studies, but also other indices that were not assessed in these studies. Using an inducible transgenic mouse could eliminate these long-term compensatory changes, such that AMPK activity could be modified shortly before the experiments are performed. Another important consideration is that AMPK is being modified and having its effects on AMPK activity during aerobic perfusion, ischemia and reperfusion. This makes the interpretation of the data difficult as during the aerobic period modification of AMPK activity may be changing indices such as adenine nucleotides and glycogen, that can affect post-ischemic recovery of function independent of the activation status of AMPK during ischemia. In

addition, utilizing these models also does not allow for separating out the downstream effects of AMPK activation during ischemia from the effects during reperfusion. As these studies have currently not been performed there is potential that AMPK could be beneficial during ischemia by providing an anaerobic source of ATP but may be detrimental during reperfusion by shifting oxidative metabolism away from glucose towards fatty acids, leading to an uncoupling of glucose metabolism and myocardial acidosis. When specific activators or inhibitors of AMPK are available, they may provide some insight into this question.

Additional evidence from this thesis demonstrates that reduced AMPK activation may be associated with cardioprotection, as modifications in AMPK activity may also play a role in the ability of insulin to confer cardioprotection (Chapter 3). In the absence of fatty acids, insulin inhibits AMPK and confers cardioprotection, however when fatty acids attenuate the ability of insulin to inhibit AMPK, insulin is no longer cardioprotective. Due to the direct metabolic effects of insulin, it is difficult to tease out the downstream effects of insulin versus AMPK, as they both can stimulate glucose uptake and glycolysis by acting on the same targets.[367] Interestingly, a previous study demonstrates that ischemia-induced acidosis can inhibit insulin signaling via Akt, suggesting that the insulin-induced modifications in AMPK activity, which we demonstrate occurs independent of Akt-induced phosphorylation of AMPK, may be more important than insulin-induced modifications in myocardial metabolism during ischemia.[153] However, as insulin signaling recovers quickly during reperfusion, insulin may play a more prominent role during reperfusion.[153] Therefore further studies are

required to further elucidate the roles of insulin signaling to AMPK and myocardial metabolism on the ability of insulin to confer cardioprotection during I/R.

The Role of Acidosis in Post-Ischemic Contractile Dysfunction

Many studies correlate post-ischemic contractile dysfunction with the ischemic-induced increase in acidosis associated with uncoupled glucose metabolism, however the mechanism by which acidosis impairs post-ischemic contractile function not well characterized.[31, 32, 121] Several mechanisms have been proposed including: 1) an inefficiency of energy utilization as ATP is taken away from contractile purposes in order to help reestablish ion homeostasis, or 2) a direct effect of H^+s on the contractile apparatus which causes a rightward shift in the Ca^{2+}-force relationship making contraction less efficient.[24, 126, 127, 130, 136-141] To investigate this second possibility, the role of I/R-induced acidosis on post-ischemic contractile function was examined in mice expressing ssTnI in place cTnI (Chapter 6). Expression of ssTnI does not modify metabolic fluxes in the heart, however contrary to previous reports ssTnI expression is detrimental to functional and energetic (lower AMP/ATP ratio in the ssTnI hearts) recovery in the presence of a high concentration of fatty acids in isolated working hearts.[286, 309] An increase in infarct size following I/R *in vivo* is also observed in the ssTnI hearts. Although it would be predicted that expression of ssTnI would be cardioprotective due to its resistance to acidosis-induced changes in Ca^{2+} sensitivity, ssTnI also plays a role in Ca^{2+} sensitivity of the contractile apparatus.[138, 279-285] Previous reports demonstrate that muscles expressing ssTnI have increased sensitivity to Ca^{2+} under physiological Ca^{2+} concentrations; therefore hearts expressing ssTnI may also be

more sensitive to pathological Ca^{2+} concentrations leading to an increase in Ca^{2+}-induced dysfunction.[138, 280-282] It is possible that the greater sensitivity of the ssTnI hearts to Ca^{2+} overload may outweigh the beneficial effects of the resistance to acidosis-induced Ca^{2+} desensitization. In support of this concept, the cTnI and ssTnI isoforms are 70 % homologous, with the cTnI having a unique N-terminal extension of 32 amino acids that contains two serines (position 23 and 24), which are phosphorylation sites for PKA. Phosphorylation of these sites results in a decrease in myofilament Ca^{2+} sensitivity, and thus induces cardiomyocyte relaxation.[368, 369] Therefore during I/R, phosphorylation of these sites by PKA may decrease myofilament Ca^{2+} sensitivity and potentially protect the Wt hearts from pathological Ca^{2+}-induced post-ischemic contractile dysfunction. In addition, we confirmed previous results that ssTnI expressing hearts have diastolic dysfunction *in vivo*.[279, 296] As I/R induces diastolic dysfunction as well, the pre-existing diastolic dysfunction in the ssTnI mice may be exacerbated when subjected to I/R, resulting in impaired ventricular filling and cardiac output that may partially account for their poor functional recovery during reperfusion and increase in infarct size.[4] In addition, TnI cleavage by Ca^{2+} activated calpain is thought to play a role in myocardial stunning.[370, 371] Indeed, when the major degradation product of TnI (TnI_{1-193}) is expressed in a transgenic mouse it results in ventricular dilation, systolic dysfunction and reduced myofilament Ca^{2+} sensitivity.[372] Due to these observations, an attractive hypothesis would be that ssTnI is more susceptible to proteolytic cleavage by Ca^{2+} activated proteases, however no difference in I/R-induced troponin cleavage was found in a previous study with the ssTnI mice.[286]

Few studies have addressed the effects of modifications of myofilament function on the pathophysiology of I/R injury as the myofilaments have largely been considered to be bystanders in I/R injury, simply subjected to the effects of Ca^{2+} overload and energy depletion.[368] However, a number of studies demonstrate a role of the myofilaments in heart failure. For example, myofilament isoforms shifts in TNT, titin and myosin heavy chain may contribute to impaired systolic and diastolic function in failing hearts.[373-376] In addition, mutations that result in Ca^{2+} sensitization of the sarcomere are associated with hypertrophic cardiomyopathy.[377-379] Therefore further studies are warranted to examine the effect of the replacement of cTnI with ssTnI on the mechanisms of I/R-induced cardiac dysfunction including structural and functional changes in the myofilaments and the interplay of among them, effects on Ca^{2+} cycling and sensitivity to pathological Ca^{2+} overload, the energetics of contractility during I/R and intracellular signaling pathways that modify them.[368] In addition, transgenic expression of the cTnI A164H mutation results in a reduction in NCX expression and a decrease in baseline and peak Ca^{2+} concentrations, therefore the effect of ssTnI on Ca^{2+} homeostasis as well as excitation and contractile coupling should also be examined.[305]

I/R Induced Changes in the Mitochondrial Proteome

We utilized a proteomics approach in order to provide a more global picture of the I/R-induced changes in mitochondrial proteins. Using this approached we observe that I/R induces changes in the mitochondrial levels of 32 proteins in ischemic hearts. The five proteins with greatest changes in total abundance are long chain acyl-coenzyme A dehydrogenase, ATP synthase (α subunit), NADH-ubiquinone oxidoreductase (24 kDa

subunit), ATP synthase (D subunit) and 2 isoforms of PDH (βE1 subunit) (Chapter 7). As PDH plays a critical role in the regulation of myocardial metabolism, and is the rate-limiting enzyme for glucose oxidation, we endeavored to better characterize the two molecular forms of the βE1 subunit. The major difference between the two forms of $PDH_{\beta E1}$ is a novel O-palmitoylation PTM to the more acidic form of $PDH_{\beta E1}$. PDH is localized to the inner mitochondrial membrane in cardiac tissue [349], therefore we hypothesize that palmitoylation of PDH results in its recruitment to the inner mitochondrial membrane, whereby it could interact with the monocarboxylate transporter. If this is the case, then a reduction in the O-palmitoylated form of PDH during I/R would result in a reduction of the inner mitochondrial membrane localization of PDH, which may account for the reduction in pyruvate flux and glucose oxidation we observe in the reperfused heart. In addition, we demonstrate for the first time that I/R triggers the phosphorylation of both forms of the $PDH_{\beta E1}$. Using *in silico* methods, we show that the insulin receptor kinase is the putative kinase for the more basic form, while PKC_{ζ} or PKA is the putative kinase for the more acidic form. Although a previous study shows that I/R can induce the translocation of PKC_{δ} to the mitochondria to modify PDH activity, there is no evidence of the insulin receptor kinase or PKA translocating to the mitochondria.[341] Therefore further experiments are required in order to delineate the role of these phosphorylation and palmitoylation events on PDH activity and the regulation of glucose oxidation during I/R.

Interestingly, a recent proteomic study examining chronic hibernating myocardium corroborates some of our observation as they also demonstrate that protein

abundance of $PDH_{\beta E1}$ is suppressed and this is associated with a decrease in total PDH activity.[380]

Limitations

The studies in this thesis utilize the isolated working heart method, which has many benefits as described in the previous sections, but also has some limitations. These hearts are perfused with crystalloid perfusate, which requires relatively high rates of coronary flow due to the low O_2 carrying capacity of the perfusate. Despite this drawback, the crystalloid perfusate is capable of delivering the required amounts of O_2 due to a high partial pressure of O_2 by gassing the perfusate with carbogen (95 % O_2 and 5 % CO_2). Even gassing with 70 % O_2 is sufficient to sustain basal function and an increase in function in response to a positive inotrope without sustaining injury.[261]

Although our perfusion conditions attempt to match plasma levels of fatty acids, glucose and insulin, our perfusate composition is not completely physiological due to the lack of other hormones, adipokines, lipoproteins and carbohydrates (lactate and pyruvate). In addition we do not assess the contribution of endogenous substrates (glycogen and triglycerides) to the overall metabolic rates in these studies. In a number of the chapters in this thesis we identify modification in the metabolic flux through glycolysis. While glycolysis is one of the major pathways of glucose utilization, the conversion of glucose to glycogen and the shunting of glucose to the pentose phosphate pathway also contribute to glucose utilization, however these pathways are not examined in this thesis. Several of the studies in this thesis are performed in the presence or

absence of 100 μU/mL of insulin in order to reproduce previous experimental conditions in the literature.[248-250] However, it is worth noting that the condition of no insulin would never be observed physiologically. In addition, the combination of high fatty acids and high insulin could promote glycolytic uncoupling and has been suggested to not be a physiologic condition *in vivo*. However, 1.2 mM palmitate is not an extremely high *in vivo* concentration of circulating fatty acids in a mouse, which has basal fatty acid levels between 0.7 and 0.8 mM during the fed state, which can rise to over 1.1 mM in the fasted state.[381] Normal serum insulin levels in the mouse range between 10 and 50 μU/mL and can rise much higher in diabetic models.[382] Previous work has suggested that although 100 μU/mL of insulin is added to the perfusate, much of this insulin can bind to glass resulting in a circulating perfusate insulin concentration of approximately 50 μU/mL (personal communication, A.S. Clanachan). This substrate milieu of high fatty acids and insulin may also be observed in the early insulin-resistant state.[383] Therefore, we acknowledge that modification of the perfusate composition and measurement of the turnover of endogenous substrates may alter the observed changes in myocardial metabolism and post-ischemic cardiac function and modify our interpretation of the data.

Although many cardioprotective agents have been described in the basic laboratory, few of these have been shown to have clinical efficacy. This lack of translation may be due to a number of reasons, one of which may be the use of an isolated heart/global ischemia model of I/R, as this is an overly simplistic form of I/R that is applied to otherwise healthy hearts from normal animals.[384] This is in sharp contrast to the clinical situation where the appearance of the symptoms of ischemia heart disease

develop over time and are associated with the physiological adaptation to the underlying pathophysiology.

In these studies, $AMPK_{\alpha 1}$- versus $AMPK_{\alpha 2}$-associated activity is not separated out, thus total activity and phosphorylation status of AMPK was reported. This limits our ability to delineate isoform specific effects of modifying AMPK activity during I/R. In addition, all the studies described in this thesis determine enzyme activities under V_{max} conditions; therefore any changes in enzyme activity related to modifications in K_m would not be detected.

A limitation of the 2-D gel approach is that even gels with wide pH gradients can only separate at most 2000 proteins (in a crude heart homogenate), which gives incomplete proteomic coverage.[350] In our case we isolated mitochondria to reduce the complexity of our samples and we utilized a narrower pH range to increase separation, but this limits identification of proteins with high or low isoelectric points. In addition, it is difficult to separate basic proteins with 2-D gels, as these proteins have the tendency to form streaks.[350]

Future Directions

Insulin Modification of Myocardial Energy Metabolism and Cardioprotection

The studies described in Chapter 3 identify differential effects of insulin on myocardial metabolism, AMPK activity and cardioprotection, dependent on the level of fatty acids in the perfusate. However the mechanisms underlying these observations require further characterization. Our data suggest there are two insulin signaling pathways in the heart, one that is fatty acid sensitive and modifies AMPK activity and one that is fatty acid insensitive and signals to myocardial metabolism. In addition, insulin and AMPK can both stimulate glucose uptake and glycolysis by acting on the same targets.[367] Therefore the identification of these pathways and separation of the metabolic effects of insulin from its effects on AMPK activity is important therapeutically. Thus, it would be interesting to repeat our I/R experiments in transgenic mice where AMPK cannot be activated ($AMPK_{\alpha 2}$ Dn or KO) and where AMPK is constitutively active and cannot be inhibited by insulin. Alternatively, in order to separate the effects of insulin during ischemia from its effects during reperfusion, insulin may be added to the perfusate at the end of the aerobic perfusion period and just prior to reperfusion. Insulin significantly increased Akt activity in our hearts, however this was not associated with increased phosphorylation of $AMPK_{\alpha}$ Ser485/491 as previous described using supraphysiological insulin concentrations.[258, 361] However, insulin may still be mediating its metabolic effects via Akt signaling. In addition, Akt is also an

essential component of the Reperfusion Injury Salvage Kinase (RISK) pathway, which has been shown to protect the heart from reperfusion injury.[385] Therefore the assessment of the role of insulin and fatty acids in the regulation of the RISK pathway may provide some insight into the cardioprotective ability of insulin. Alternative pathways by which insulin may modify AMPK activity should also be examined. These include: 1) insulin- and fatty acid-induced modification of the activity, localization or association of the known AMPKKs with AMPK, and in the case of MIF, whether insulin and fatty acids can modify either its release from the heart or its downstream signaling pathway [178], and 2) insulin and palmitate modifies PP2A activity, the major phosphatase isoform that dephosphorylates and inactivates AMPK, therefore the role of PP2A in insulin cardioprotection should also be examined.[362-364]

Perfusion of hearts in the absence of insulin is not physiological therefore further studies should compare insulin regulation of myocardial metabolism and cardioprotection under more physiological conditions, using basal and supraphysiological concentrations of insulin to examine its cardioprotective effect.[248-250, 386] In addition, insulin and GIK therapy should also be examined in an *in vivo* LAD ligation model of I/R, with particular emphasis placed on modification of infarct size and circulating insulin and fatty acids levels, in order to determine whether insulin's major action is direct on the heart, or indirect via decreasing circulating fatty acid levels.

AMPK Modification of Myocardial Energy Metabolism and Cardioprotection

The studies described in Chapter 4 and 5 using transgenic mice to modify AMPK activity demonstrate that activation of AMPK during ischemia is not cardioprotective but may actually be detrimental to the recovery of mechanical function. The mechanisms underlying this lack of cardioprotection require further characterization. Additional hearts freeze clamped at the end of the aerobic period and ischemic period are required to examine the energetic status and level of glycogen of the hearts prior to and following ischemia, and to determine if either the AMPK Dn or the $AMPK_{\gamma 2}$ R302Q mutation modifies either of these factors, thus contributing to overall cardioprotection. This is of particular importance in the case of the $AMPK_{\gamma 2}$ R302Q mice as it is still controversial as to the effect of these mutations on AMPK activity: we observe a greater AMPK activation in the mutant mice at the end of reperfusion, yet previous work demonstrates that basal AMPK is suppressed in these mutant mice, therefore we are observing a greater ischemic-induced activation of AMPK.[211, 277] However, this must be confirmed in our working heart model. In addition, data from transgenic mouse models must be interpreted with caution, as there may be significant compensatory changes due to the expression of the transgene for the lifetime of the animal. In particular, glycogen overload in the $AMPK_{\gamma 2}$ R302Q mice may have significant effects on post-ischemic contractile function, therefore studies that glycogen deplete these hearts prior to ischemia would eliminate this confounding factor. Alternatively the contribution of glycogen to myocardial glycolysis and glucose oxidation could be assessed in a modified pulse-chase protocol using [5-^{3}H]glucose and [U-^{14}C]glucose.[387] In addition, the development of a

conditional/inducible transgenic mouse, where the mutation could be acutely regulated, could also eliminate alterations due to compensatory changes occurring in these mice.

Until recently there has been a relative lack of specific pharmacological agents to modify AMPK activity. We have tried to modify AMPK activity with AICAR and compound C, however at concentrations required to modify AMPK they were toxic to the isolated working hearts (unpublished data). However, with the availability of A-769662, an AMPK activator, there is potential to pharmacologically manipulate AMPK, which would also eliminate the confounding compensatory effect of chronic transgene expression.[388] The availability of specific non-toxic pharmacological agents that modify AMPK activity would be essential in order to determine the downstream effects of AMPK activation during ischemia versus the effects during reperfusion and correlate these with post-ischemic contractile function. Independent of whether AMPK activity is modified pharmacologically or genetically these studies should be followed up *in vivo* using the LAD ligation model of I/R, in order to determine the effect of modifying AMPK activity in a more physiological and clinically relevant animal model.

AMPK plays important regulatory roles in signaling pathways other than metabolism, such as protein synthesis and cell growth (elongation factor-2 [389, 390] and mammalian target of rapamycin [391]), NO production (endothelial NOS [392]), gene expression (p300 [393], peroxisome proliferators activated receptor γ co-activator-1α [394], hepatocyte nuclear factor-4α [395], carbohydrate response element binding proteins [396], and transducer of regulated CREB-2 [397, 398]), mitochondrial biogenesis (nuclear respiratory factor-1 [399]), and ion transport (cystic fibrosis transmembrane conductance regulator [400])

and it would be important to define the role of these mutations in these pathways and how they may relate with cardioprotection.

In particular, the role that AMPK plays in apoptotic cell death is still controversial, as AMPK activation appears to be pro-apoptotic in some cases but anti-apoptotic in others.[192, 225, 401-410] AMPK activation is associated with the induction of apoptosis in a number of cell models, including hepatocytes, Min6 cells, pancreatic β cells and neuroblastoma cells.[405-408, 410] Potential mechanisms of the pro-apototic effect of AMPK include upregulation of p53, a pro-apoptotic protein, activation of caspase-3, inhibition of cell cycle progression and activation of the JNK pathway.[405, 407, 410] In addition, it has been demonstrated that AMPK mediates the pro-apoptotic translocation of Bax to the mitochondria during simulated ischemia in the neonatal cardiomyocyte.[411] However, many of the investigations in the heart suggest that AMPK plays an anti-apoptotic role. Russell *et al.* demonstrated that their AMPK KD mouse was more susceptible to I/R induced apoptosis and necrosis, thus concluding that AMPK is anti-apoptotic in the ischemic heart.[192] AMPK activation has also been implicated in the anti-apoptotic effects of adiponectin as adiponectin KO mice have higher levels of I/R induced apoptosis and supplementation with adiponectin activates AMPK and inhibits apoptosis.[225] These authors also demonstrated that adiponectin inhibited hypoxia-induced apoptosis in ventricular myocytes and this protection was lost when cells were pretreated with AMPK Dn.[225] AMPK has also been demonstrated to impair palmitate induced apoptosis in cardiac myocytes.[409] The anti-apoptotic effect of AMPK is also observed in tumor cells, thymocytes, astrocytes and umbilical vein endothelia cells.[401-404] These conflicting results on the role of AMPK in apoptosis may be due to the fact that

apoptosis requires ATP, therefore in cases where cells can no longer be salvaged, AMPK activation would be pro-apoptotic by stimulating catabolic pathways and producing ATP, while in other cases this stimulation of ATP production may allow the cells to recover from an energetic crisis.[412] Therefore further investigation is required to assess the role of AMPK in apoptosis and post-ischemic contractile dysfunction in the heart.

The Role of Acidosis in Post-Ischemic Contractile Dysfunction

The studies described in Chapter 6 demonstrate that cardiac specific expression of ssTnI is not cardioprotective in the setting of I/R. However, the mechanisms underlying this lack of cardioprotection require further characterization. In particular, a comprehensive study of the perturbation of ionic homeostasis during I/R in the ssTnI mouse is required in order to separate the pathological effect of acidosis versus Na^+ and Ca^{2+} overload.[6, 127, 130] Two methods could be potentially useful to correlate intracellular ionic concentrations and cardiac function in the isolated working heart, namely fluorescent probes and nuclear magnetic resonance (NMR). Fluorescent probes have been developed to measure intracellular pH (2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and carboxy-seminaphthorhodafluor (SNARF-1)), Na^+ (sodium benzofuran isophthalate (SBFI)) and Ca^{2+} (1-[2-amino-5-(6-carboxy-2-indolyl)phenoxy]-2-(2-amino-5-methylphenoxy) ethane- N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester) (indo-1AM) and 5-oxazolecarboxylic acid, 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acid (Fura-2)) in isolated cells. Therefore, it is theoretically possible to assess the rates of glycolysis, glucose

oxidation and H^+ production and correlate these not only to contractile function, but also to intracellular concentrations of H^+, Na^+ and Ca^{2+}. Alternatively, these parameters can be measured using nuclear magnetic resonance (NMR), using ^{23}Na (dysprosium triethylene teraminehexaacetic acid (DyTTHA)), ^{19}F (acetoxymethyl ester of 5F-1,2-bis(2-aminophenoxy)ethane-N,N,N`,N`-tetra-acetic acid (BAPTA)) and ^{31}P NMR for measurement of Na^+, Ca^{2+} and pH and high-energy phosphates, respectively.[413, 414] Overall the measurement of intracellular pH, Na^+ and Ca^{2+} would be important in order to determine the mechanism by which ssTnI expression impairs post-ischemic contractile function, and would also provide important data for interpreting the role of AMPK activation during I/R.

I/R Induced Changes in the Mitochondrial Proteome

The studies described in Chapter 7 have identified a number of mitochondrial proteins whose abundances are modified by I/R, one of which is PDH, which we demonstrate to have both phosphorylation and O-palmitolyation PTMs. These observations have important implications on the role of myocardial metabolism during I/R, however their physiological effects require further characterization. We found an additional 27 mitochondrial proteins that were modified by I/R, which require MS identification. For a more robust mitochondrial proteomic analysis of I/R, all the spots on the 2-D gels should be subjected to MS identification to give an overall global picture of the protein modification induced by I/R.[321, 322] Proteomics analysis using 2-D gels can only reliably separate approximately 2000 proteins (in a crude whole heart homogenate) on a wide range pH gradient, therefore to get a better coverage of the whole

mitochondrial proteome complementary techniques are required.[350] One approach is a shotgun approach that initially digests the protein sample with trypsin, then separates the peptide fragments using liquid chromatography and then introduces them to a tandem MS for identification. This technique can identify an additional 1500 proteins, some of which overlap with the 2-D gel analysis, leading to a better coverage of the mitochondrial proteome.[350] In addition, using ingenuity pathways analysis to assess the protein interactions, we could identify a network of proteins which are modified by I/R.[415]

Although novel PTMs of PDH have been identified in Chapter 7, further biochemical and physiological studies are required to determine the actual effects on PDH activity and the regulation of glucose metabolism. Further studies are also required to determine if O-palmitoylation occurs in isolated working hearts perfused in the presence of alternative fatty acids such as oleate, to ensure that the palmitoylation does not occur simply due to excess available palmitate. Identification of the PTMs were determined in silico with supporting biochemical evidence, however further biochemical evidence is required to support these observations. Phosphorylation of the $PDH_{\beta E1}$ could be further demonstrated by immunoprecipitating PDH and developing western blots using anti-phosphotyrosine and anti-phosphoserine/threonine antibodies. Extensive experiments are required to prove the ability of the putative kinases to phosphorylate PDH, including the use recombinant proteins to determine if these kinases can phosphorylate PDH *in vitro*, the use of site direct mutagenesis to determine the specific sites of phosphorylation and to determine the effect of these phosphorylation events directly on PDH activity. Based on the *in vitro* experiments, phospho-specific antibodies directed against $PDH_{\beta E1}$ could be used to elucidate the effect of I/R on phosphorylation at

these sites. Palmitoylation of PDH could be further characterized using chemical labeling of putative sites or by using palmitoylation inhibitors (2-fluoropalmitate, cerulenin and tunicamycin).[416-418] Further *in vitro* experiments are required to determine the effect of palmitoylation on PDH activity and what effect this has on PDH localization and activity in the cardiomyocyte.

Further experiments could also examine the effect of ischemia alone on the mitochondrial proteome to better define the role of reperfusion in post-ischemic cardiac dysfunction. In addition, interesting experiments would include using a cardioprotective method (such as use of preconditioning, or metabolic modulator) to determine if some of mitochondrial proteome changes are reversed with treatment.[323]

Therapeutic Potential of Modulation of AMPK Activity for Ischemia/Reperfusion Injury

The studies described in this thesis demonstrate that activation of AMPK during I/R is not associated with cardioprotection, but may actually be detrimental to the recovery of function during reperfusion. Based on these observations the use of AMPK activators for the treatment of ischemic heart disease would not be recommended. In addition, modification of AMPK activity may have unforeseen, potentially damaging effects due to the numerous downstream targets of AMPK, which are involved in many cellular signaling pathways including among others, regulation of myocardial energy substrate metabolism, protein synthesis, NO production, mitochondrial biogenesis, ion transport and gene expression.[412] In general, modifying AMPK *in vivo* in a heart specific

manner would be extremely challenging as the AMPK isoforms found in the heart are also widely expressed in other tissues. However, global modification of AMPK activity is not an attractive approach either, given that AMPK is involved many processes, including appetite regulation in the hypothalamus, insulin secretion in the pancreatic islet β-cells, and modulation of skeletal muscle energy substrate metabolism as well as in pathological conditions such as cancer and the metabolic syndrome.[412, 419-421] Thus, unless modulation of AMPK activity can be induced in a tissue-specific manner, the potential of using AMPK as a therapeutic target is limited.

Conclusions

In conclusion, the studies described in this thesis demonstrate that the regulation of myocardial energy metabolism is an important determinant in the recovery of post-ischemic contractile function during reperfusion, which has clinical utility in the treatment of ischemic heart disease. These studies demonstrate that:

1) The cardioprotective effect of insulin and its ability to modify AMPK activity is highly dependent on the concentration of fatty acid present in the perfusate, however the regulation of glucose metabolism occurs independently of fatty acid availability. These observations have important implications in the use of insulin therapy for the treatment of ischemic heart disease.
2) Activation of AMPK during ischemia is not cardioprotective in the presence of a clinically relevant concentration of fatty acids, but may actually be detrimental to the recovery of cardiac function during reperfusion. The lack of cardioprotection

is associated with a shift of overall oxidative metabolism away from glucose towards fatty acids, thereby uncoupling glucose metabolism and accelerating the rate of H^+ production.

3) Expression of ssTnI impairs recovery of post-ischemic contractile function potentially due to a greater sensitivity of these hearts to post-ischemic pathological Ca^{2+} concentrations.

4) A proteomics approach has identified I/R-induced changes in the mitochondrial proteome that may play an important role in I/R-induced changes in myocardial metabolism. O-palmitoylation and phosphorylation post-translational modifications were demonstrated to occur in one of the identified proteins, $PDH_{\beta E1}$. As PDH is the rate-limiting step of glucose oxidation, these post-translational modifications may have important implications in the regulation of glucose metabolism during I/R.

Taken together these studies have elucidated an important role of myocardial energy metabolism in the pathophysiology of I/R-induced cardiac dysfunction and have implicated a number of pharmacological targets that may be exploited in order to manipulate myocardial energy metabolism and limit I/R injury.

CHAPTER 9.

References

Chapter 9.

References

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