University of Alberta

Cardioprotective Efficacy of Adenosine During and Following Mild Ischemia

by

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Dedication

To my beloved wife, Stella.

Abstract

Alterations in cardiac metabolism impair the recovery of function and efficiency following severe ischemia. However, whether modifications in substrate metabolism occur during the actual ischemia event is not well characterized. To address this, paced (5 Hz) working rat hearts perfused with Krebs-Henseleit solution containing (in mM) glucose (11), palmitate (1.2) and insulin (100μU/ml) were subjected to mild ischemia by reducing coronary flow by 31% (n=16), causing a 30% decrease in oxygen consumption accompanied by about 50% and 45% decline in work and efficiency, respectively. This was associated with a ~30% increase in proton production from glucose metabolism, secondary to a 20% increase in glycolysis. Adenosine (Ado, 100 μM), added pre-ischemia, did not affect work, efficiency or metabolism during mild ischemia. During reperfusion, Ado improved work and efficiency by 77% and 87%, respectively. These data show that inhibiting glycolysis during reperfusion may be efficacious in managing patients with coronary artery disease.

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Table of Contents

Lists of Tables

List of Figures

List of Abbreviations

ACC:	acetyl CoA carboxylase
AMP:	adenosine monophosphate
AMPK:	5`AMP-activated protein kinase
ATP:	adenosine triphosphate
BSA:	bovine serum albumin
CAD:	coronary artery disease
cAMP:	cyclic adenosine monophosphate
CHA:	N^6-cyclohexyladenosine
CO_2:	carbon dioxide
CoA:	coenzyme A
CPT 1:	carnitine palmitoyltransferase 1
Cr:	creatine
CrP:	creatine phosphate
DCA:	dichloroacetate
2-DG:	2-deoxy-D-glucose
FABP:	fatty acid binding protein
$FADH_2$:	flavin adenine dinucleotide
FDG:	[^{18}F]2-deoxy-2-fluoroglucose
F-6-P:	fructose-6-phosphate
F-1,6-BP:	fructose-1,6-bisphosphate
F-2,6-BP:	fructose-2,6-bisphosphate
GIK:	glucose-insulin-potassium
G-6-P:	glucose-6-phosphate
GLUTs:	glucose transporters
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
HK:	hexokinase
HPLC:	high pressure liquid chromatography
H_2O:	water
IHD:	ischemic heart disease
IPC:	ischemic preconditioning
3- KAT:	3-ketoacyl thiolase
K_{ATP}:	ATP-sensitive potassium channel
LDH:	lactate dehydrogenase
LV:	left ventricle
LVDP:	left ventricular developed pressure
MVO_2:	myocardial oxygen consumption
NADH:	nicotinamide adenine dinucleotide
NCE:	sodium-calcium exchanger
NHE:	sodium-hydrogen exchanger
O_2:	oxygen
PDH:	pyruvate dehydrogenase complex
PFK-1:	phosphofructokinase-1
PFK-2:	phosphofructokinase-2
PI3-K:	phosphoinositide 3-kinase
PKA:	protein kinase A
PKC:	protein kinase
SAH:	S-adenosylhomocysteine
TCA:	tricarboxylic acid cycle
TMZ:	trimetazidine

Chapter 1

Introduction

In order to maintain its constant pumping action, the heart requires a continuous supply of energy substrates, which in the presence of oxygen are metabolized to produce high-energy phosphates. During pathological conditions such as occur during ischemia, hypoxia or other stressful stimuli, the heart is unable to meet its energy requirement due to the limitation in O_2 supply. Thus, ischemia has been defined as an imbalance between myocardial O_2 requirement and supply resulting from the reduction in blood supply to the heart.

Clinically, myocardial ischemia is manifested in a group of diseases collectively called ischemic heart disease (IHD) or coronary artery disease (CAD). The first in line in the progression of disease is angina pectoris ("chest pain"), which occurs when the oxygen supplied by the coronary circulation is insufficient to meet the requirements of the myocardium.

Conventionally, the therapeutic objective for managing patients with coronary artery disease is to improve the balance between O_2 supply and demand that has been impaired by the ischemic condition. However, due to the metabolic derangements associated with CAD, a novel strategy of treatment is emerging, which optimizes myocardial energy metabolism. These agents, which are devoid of hemodynamic effects, promote glucose oxidation by reducing fatty acid oxidation or by enhancing glucose oxidation. Subsequently, glycolytic flux is reduced and the ensuing improvement of glucose metabolism reduces intracellular acidosis by enhancing the coupling of glycolysis to glucose oxidation.

An increased rate of proton production from glucose metabolism has been shown to impair post ischemic recovery of left ventricular (LV) function and efficiency.[8,9] However, although it is a significant source of proton production in the ischemic heart, whether glycolysis is beneficial or detrimental in the clinical situation still remains inconclusive.

Animal studies designed to study ischemia and reperfusion have employed both *in vitro* and *in vivo* techniques. However, due to lack of experimental models, the metabolic and functional alterations that occur in the heart during the critical ischemic period are poorly understood.

In the present study, we developed a new model of ischemia, which we termed "mild ischemia" to enable us better understand the relationship between cardiac function and metabolism during the critical period of ischemia. Moreover, the role of glycolysis and consequently of proton production and its relation to recovery of cardiac function and efficiency during mild ischemia and reperfusion was investigated.

1.1 Coronary Artery Disease

Although there have been improvements in developing agents to treat patients with different forms of cardiovascular disorders, lethality and morbidity associated with cardiovascular diseases still remain overwhelmingly high. In 2001, it was estimated that about 64,400,000 Americans suffer from various forms of cardiovascular diseases out of which about 23% result from coronary artery diseases (CAD).[1] Interestingly, CAD accounted for about 54% of all deaths arising from

cardiovascular diseases. The incidence of death due to cardiovascular dysfunction is now extending its boundaries to non-Western societies and catching up with other worldwide pandemics such as AIDS. A recent report revealed a stunning doubling of deaths in non-Western countries associated with various cardiovascular problems.[2] Governments worldwide therefore are spending huge sums of money to fund research aimed at finding appropriate targets to treat cardiovascular diseases.

In general, mortality due to cardiovascular diseases has declined over the past few years but CAD-related deaths remain a leading cause of death in the Western world.[1] Interestingly, about one-third of these patients have preceding angina. Thus, it is becoming increasingly important to understand the pathophysiology of disease to enable the design of better therapeutic modalities to manage these patients.

The etiology of disease for cardiovascular disorders is a subject of ongoing investigation but recent data have established a strong correlation between cardiovascular diseases and derangement of myocardial energy substrate metabolism.[2,3-5] This is due to narrowing of coronary vessels resulting in the limitation of coronary flow and hence, O_2 and substrate supply to the heart.[6]

One of the major consequences in the pathogenesis of myocardial ischemia is alteration in the utilization of energy substrates. The reduction in blood supply and O_2 delivery results in depletion of high-energy phosphate supply, thus, rendering the heart energetically compromised. Conventionally, the mainstay therapies for patients with CAD have aimed at either increasing blood flow, and hence O_2 supply to the heart, or reducing O_2 demand by the heart muscle.[6] Thus, common treatment modes include the use of vasodilators such as nitroglycerin, which relaxes the coronary

arteries and veins. By relaxing the veins, it reduces the amount of blood reaching the heart (preload) thus, reducing the workload and oxygen demand of the heart. ß-adrenoceptor antagonists and calcium channel blockers have also been used to reduce energy demand by reducing heart rate and cardiac output (see ref. 6 for review).

Recent evidence has revealed that a novel strategy of treating CAD is to enhance myocardial efficiency (cardiac work per oxygen consumed).[4, 7,8,9] This approach stems from the observation that CAD is closely associated with various metabolic derangements of the myocardium. Therefore, agents that alter myocardial energy substrate metabolism have been shown to enhance myocardial energy expenditure. These agents, now referred to as "metabolic modulators", have proven efficacious in animal and clinical studies.[3,4,5,10,11]

1.2 Cardiac Energy Metabolism

To maintain its high contractile activity, the heart requires a continuous supply of energy in the form of adenosine triphosphate (ATP). This is achieved by the aerobic metabolism of energy substrates, namely fatty acids, glucose, and to a lesser extent lactate and ketone bodies depending on the prevailing conditions and substrate availability.

During periods of O_2 depletion (ischemia and hypoxia), oxidative metabolism is slowed, and as a compensatory mechanism glycolysis is accelerated. Glycolysis, however, is by no means an efficient way of producing energy since it produces only 2 moles of ATP per mole of glucose metabolized compared to 30 and 105 moles of

ATP from glucose oxidation and palmitate oxidation, respectively (see References 4 and 12 for reviews). Moreover, it is accompanied by an increased rate of proton production, which is a trigger for a cascade of ionic exchanges ultimately resulting in intracellular Ca^{2+} overload, an event shown to induce several detrimental effects on the contractile and electrical activity of the heart.[9,13,14,15] Thus, during an ischemic episode, a greater proportion of the energy produced is used for non-contractile purposes to correct the ionic imbalance, making the heart inefficient in its energy utilization for contractile work.[8,16]

Upon reperfusion of a previously ischemic heart, oxidative metabolism is rapidly restored, but depending on the severity of ischemia both cardiac function and efficiency may remain impaired.[8,9,16,17,18] The extent of recovery has also been shown to be dependent on the energy substrate metabolized.[4,7,8,19,20] A myriad of evidence has shown that during reperfusion, fatty acids continue to dominate as the major source of ATP occurring at the expense of glucose oxidation.[8,9,10,19,21,22] This tends to exacerbate further the uncoupling of glucose metabolism, which has been initiated by the ischemic insult, resulting in a continued proton production (see below for details).[8,9,15,19,20,23,24]

In the clinical scenario of angina, pharmacological agents that optimize cardiac energy substrate utilization by shifting metabolism away from fatty acid oxidation to glucose utilization have proven very promising.[4,5,6,7,10,25,26]

1.2.1 Energy Metabolism under Normal Conditions

By its ability to utilize fuel substrates from different sources, the heart is described as being "omnivorous".[3] The high-energy requirement of the heart is met mainly by the aerobic metabolism of glucose and fatty acids. Under aerobic conditions, oxidation of fatty acids accounts for majority of energy production and provides about 60-80% of the total ATP for the heart while the rest comes from the oxidation of glucose and lactate.[4,6,11,12,]

1.2.1.1 Glucose Metabolism under Normal Conditions

Although it is not the major energy-producing substrate of the heart under physiological conditions, glucose metabolism becomes more important in the postprandial state as well as during periods of energetic stress.[12,27,28,29,30] The heart utilizes glucose from both endogenous sources (glycogen) and from exogenous glucose delivered from the bloodstream. The primary determinant of exogenous glucose utilization is its transport across the sarcolemma into the myocyte. This occurs along a concentration gradient and is mediated via a family of glucose transporters designated as GLUTs.[27,28,29] These are transmembrane proteins with 7 distinct isoforms, each coded by distinct genes (see References 27 and 28 for reviews). The mammalian heart expresses mainly the GLUT-1 and GLUT-4 transporters.[28,30,31] GLUT-1 is the most abundant isoform during fetal life with a gradual transition to GLUT-4 in adults.[28,30] GLUT-1 has a ubiquitous distribution in many cell types and is responsible for the basal uptake of glucose.[27,30,32,33,34] The GLUT-4 transporter is found mainly in intracellular storage vesicles where it is

translocated to the plasma membrane upon stimulation by the classical insulin pathway and other hormonal stimuli, such as adrenoceptor stimulation. It is therefore designated as the insulin-responsive transporter.[27,30,32,33,35,36]

Upon entry into the cell, glucose is rapidly phosphorylated by hexokinase, which commits glucose to further metabolism.[12,30] Glucose-6-phosphate (G-6-P), the reaction product, can enter either the glycolytic pathway or becomes a precursor for glycogen synthesis depending on the prevailing conditions and the presence of alternate substrates.[12,30,31] Glycolysis is the series of reactions that converts glucose into pyruvate, the substrate for glucose oxidation for the aerobically perfused myocardium (Fig 1.1). The rate-determining step in glycolysis is the phosphorylation of fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-BP), a reaction catalyzed by phosphofructokinase-1 (PFK-1).[12,30,31] PFK-1 is negatively regulated by ATP, citrate and protons, while adenosine monophosphate (AMP) and fructose-2,6-bisphosphate (F-2,6-BP) enhance its activity.[12,30,31]

Glycogen is the endogenous source of glucose for the heart and it is mobilized by glycogen phosphorylase to produce G-1-P, and consequently converted to G-6-P which can then be metabolized by the glycolytic pathway. Glycolysis becomes an important source of energy for the ischemic myocardium since it is capable of generating ATP anaerobically (see below). When one glucose molecule from exogenous sources is metabolized through glycolysis, it yields a net of two ATP molecules in contrast to 3 ATP molecules coming from glycogen metabolism.[12,30,31]

1.2.1.2 Fatty Acid Metabolism under Normal Conditions

Fatty acids are the predominant energy-producing substrate for the heart providing as much as 80% of the total ATP of the heart under normal conditions. Long chain fatty acids are transported in the plasma as triacylglycerols or bound to albumin as free fatty acids.[4,5,12,24,37,38,39,40] The mechanism of fatty acid uptake into the cytosol has been highly debated but it has been shown to occur via two major mechanisms; 1) a concentration difference of fatty acids between the plasma and the cytosol causing simple diffusion and 2) uptake mediated by sarcolemmal fatty acid transport proteins (see References 24 and 40 for reviews). In the cytosol, insoluble long chain fatty acids are bound by fatty acid binding proteins. Cytosolic long chain free fatty acids are activated by conversion to long chain fatty acyl-CoA esters, a reaction catalyzed by long chain acyl-CoA synthetases located on the cytosolic side of the outer mitochondrial membrane (Fig. 1.2).[41] The transport of acyl CoA moieties into the mitochondrial matrix is mediated by a system of three carnitine-dependent enzymes. Carnitine palmitoyltransferase-I (CPT-I), located in the inner mitochondrial membrane, is the first enzyme that converts the long-chain acyl CoA moieties into acylcarnitines. The transport of long-chain acylcarnitines into the mitochondrial matrix is facilitated by acylcarnitine-carnitine translocase. Finally, CPT-II catalyzes the regeneration of long chain fatty acyl CoA that then feeds into the β-oxidation spiral for subsequent metabolism in the mitochondrial matrix. The translocation of long chain fatty acyl CoAs across the mitochondrial membrane is limited by the activity of CPT-I.[12,24,42]

CPT-I is under potent inhibition by physiological concentrations of malonyl CoA, which is formed from acetyl CoA by the enzyme, acetyl CoA carboxylase.[4,42,43] It is imperative to note however, that short and medium chain fatty acyl CoAs bypass the acylcarnitine enzyme system since they readily transverse the mitochondrial membrane.[12,24,44,45,46] Studies utilizing different fatty acid chain lengths to determine fatty acid β-oxidation should therefore be interpreted with caution.

Fatty acid β-oxidation involves a system of four enzymes that cleaves fatty acyl CoAs into one acetyl CoA unit in each turn of the spiral producing one NADH and one $FADH_2$. The overall oxidation of fatty acids in the mitochondrial matrix is regulated by 3-ketoacyl thiolase (3-KAT), and thus is a very important enzyme in the regulation of myocardial energy metabolism.[4,12,41,47]

1.2.1.3 The Tricarboxylic Acid Cycle

During periods of oxygen availability, pyruvate, which is the major end product of glycolysis, is transported into the mitochondria where it is converted to acetyl CoA by the pyruvate dehydrogenase (PDH) complex. This rate-determining step of glucose oxidation forms a major regulatory point because it determines whether the chemical energy from glucose will be extracted efficiently.[4,11,12,13] Moreover, this reaction is regulated closely by end products of the metabolism of glucose and fatty acids.

The TCA cycle is the converging point for the oxidation of glucose and fatty acids. Acetyl CoA produced from pyruvate and β-oxidation enters the TCA cycle and combines with oxaloacetate for a subsequent catabolism to produce the reducing

equivalents, NADH and $FADH_2$, which feed into the electron transport chain.[12,24] Extensive studies by Randle in the 1960s showed that the oxidation of glucose and fatty acid is reciprocally regulated at this point.[48,49] These observations have been confirmed in recent studies.[7,8,9,19]

The electron transport chain is the series of redox reactions that transfers protons out of the mitochondria to build up a proton gradient across the mitochondrial membrane. This gradient then drives oxidative phosphorylation resulting ultimately in the production of ATP.[12] The final acceptor of protons is molecular O_2, hence, the indispensable role of O_2 in myocardial energy metabolism.[4,12,30] This suggests that in order to sustain a continual production of energy, there must be an uninterrupted supply of oxygen. Deprivation of O_2 as occurs during ischemia and hypoxia profoundly alters myocardial energy supply, which translates into impaired cardiac performance.[6,7,8,9,23]

When glucose is metabolized through glycolysis and further oxidized by the TCA cycle, there is a net yield of 30 ATP molecules per mole of glucose oxidized.[12] A complete oxidation of a typical long chain fatty acid (palmitate) yields a net of 105 ATP molecules under normal conditions.[4,12] Glucose is considered a more efficient generator of ATP since the ATP produced per mole of O_2 consumed is greater compared to that produced per mole of O_2 used in the oxidation of fatty acid.[4,12] Promoting glucose oxidation at the expense of fatty oxidation in the setting of myocardial ischemia forms the basis of employing metabolic modulators in treating CAD patients.[3-6,8,11,25,30,38,39]

Under normal physiological conditions, flux through the various energy-generating pathways of the heart is tightly balanced. Neely and Morgan[50] elegantly showed that the rate of ATP production by the heart is tightly coupled to the utilization of ATP. Other workers[23,51], who also revealed that myocardial ATP utilization perfectly matches the energy demand, have supported this finding. That cardiac function relies on the availability of energy substrates in their correct amounts suggests that alterations in the energy-generating pathways of the myocardium will result in abnormal function. Hence, during an ischemic attack, the ensuing deprivation of O_2 supply inhibits oxidative metabolism, triggering a sequelae of events consequently resulting in impairment of cardiac function.[4,5,20,17,23]

1.2.2 Energy Metabolism During Ischemia and Reperfusion

Myocardial ischemia is characterized by a reduction in the delivery of both energy substrates and O_2 to the heart due to reduction in coronary flow.[4-6] The severity and duration of ischemia determines the metabolic adaptation that occurs in the heart.[3,6,28] In both *in vivo* and *in vitro* animal studies, different models of ischemia have been defined depending on the clinical condition been investigated. During severe low-flow ischemia[18,52,53], coronary flow is reduced to an extent to support some metabolic activities but mechanical function ceases. In severe no-flow ischemia[8,9,23], heart perfusion is completely halted, thus, all measurable oxidative metabolism and mechanical function ceases. Glycolysis, which occurs under anaerobic conditions, can be assessed indirectly in this model by measuring tissue lactate content. Another model of ischemia employed mainly in *in vivo* animal

studies[7,54] is moderate ischemia where flow reduction is achieved through banding of the left anterior descending coronary artery. The problem with this model is the species variation in the presence of collateral blood vessels making interpretation of data difficult among different species.

Due to lack of appropriate experimental models to study myocardial energy substrate utilization during ischemia, the relationship between myocardial function and metabolism during ischemia is poorly understood. As noted above, myocardial energy utilization and performance are well matched under normal physiological conditions. A causal relationship between depletion of myocardial ATP content and depression of cardiac function has not been well characterized during the critical ischemia event.

1.2.2.1 Energy Metabolism During Ischemia

During severe ischemia, oxidative phosphorylation is markedly depressed resulting in a marked depletion of myocardial content of ATP and creatine phosphate.[4,6,12] Fatty acid oxidation is accelerated during moderate ischemia *in vivo*[4,5,7], due to an increase in the circulating levels of plasma fatty acids similar to levels seen in the myocardium of CAD patients.[4,5,55,56] This increase in fatty acid oxidation has been reported to be detrimental to the recovery of cardiac function post-ischemia.[4,7,8,9,10,17]

A large body of evidence supports the hypothesis that glycolysis is accelerated during ischemia[8,9,13,18,31,57,58] and the extent of increase is dependent on the availability of glycolytic substrates, i.e. insulin and glucose.[3,31,57] It has been

suggested that during no-flow ischemia, there is an initial increase in the rate of glycolysis followed by a decline as a result of impaired glucose delivery and feedback inhibition by accumulation of glycolytic end products, mainly lactate and protons.[3,6,31,57,58] During moderate ischemia *in vivo*, glycolytic rates persist because glucose is continually available and washout of glycolytic end products occurs.[3,6,7,31,59]

The mechanisms proposed to explain the increase in flux through glycolysis during ischemia are as follows: 1. Activation of the key regulatory enzyme of glycolysis, PFK-1[12,30,31], by the decreased AMP:ATP [29,30,31] ratio and 2. Accelerated glycogenolysis, which provides substrates to drive glycolysis.[28,30,31] Several lines of evidence suggest that the activation of AMP-activated protein kinase (AMPK) mediates the increase in glycolysis during ischemia.[177,178] The literature is however, divided on whether an increase in flux through glycolysis is detrimental or beneficial to the ischemic myocardium.[4,8,9,10,17,23,28,60-64]

Evidence that favors a protective effect of glycolysis during ischemia proposes that glycolytically-derived ATP is preferentially channeled to maintaining ion channel activity and preserving membrane integrity.[28,60-67] Further evidence for this hypothesis stems from the findings that glycolytic enzymes are associated with sarcolemmal ion channels.[65,68] Jeremy *et al*[69] demonstrated that in the isolated rabbit heart perfused with only glucose there is an improvement of mechanical function following global no-flow ischemia compared with hearts in which glycolysis was inhibited with iodoacetate. In the same study it was shown that depletion of glycogen stores prior to the ischemic insult resulted in a worsening of left ventricular

function. Addition of glucose or any other carbon source, pyruvate or acetate, during reperfusion enhanced functional recovery. The authors hypothesized from these findings that glycolysis during the early periods of reperfusion is necessary for recovery of function. This study did not, however, take into consideration the contribution of other important energy sources such as fatty acids. Moreover, the perfusate lacked insulin whose signaling mediates the uptake of exogenous glucose into the cells. The use of iodoacetate as an irreversible inhibitor of GAPDH indicates that glycolysis was completely attenuated, thus, indirectly inhibiting glucose oxidation because of depletion of the intermediate, pyruvate. Thus, the poor recovery of function seen in this study may not be due to absence of glycolysis, but could be a result of the heart being energetically compromised.

In a parallel and related work, Vanoverschelde and colleagues[67] also reported that in the isolated perfused rabbit hearts subjected to low-flow ischemia, enhancement of glycolysis by high glucose/insulin, dichloroacetate (DCA) or high glycogen during ischemia reduced the extent of ischemic injury and improved recovery of contractile function upon reperfusion. This was associated with reduction in the release of creatine kinase suggesting that glycolysis is important in preserving membrane integrity and viability of the cell. Other investigators in the field also show that glycolytically derived ATP is important for the activation of the Na^+-H^+ exchanger (NHE)[28,68], one of the mechanisms involved in the regulation of intracellular pH (pH_i) (see Refs. 71 and 72 for reviews).

Other compelling evidence to support the protective effect of increased rates of glycolysis is the use of glucose-insulin-potassium (GIK) in treating patients with

myocardial infarction. Several lines of evidence suggest that administration of GIK solution reduces size of infarct thus, enhancing myocardial function following infarction.[28,30,73-76] The beneficial effect of GIK treatment was attributed to enhancement of glucose utilization and hence, glycolysis.[28,77] It must, however, be emphasized that the presence of insulin inhibits the reliance on fatty acid oxidation as an energy source.[78,79] Therefore, the effectiveness of the GIK treatment could be due to a combination of enhanced glucose utilization and reduced fatty acid oxidation. Other workers however, failed to observe a protective effect of GIK treatment in reducing infarct size.[28,80] One clinical trial actually revealed that presence of glucose in cardioplegic solutions did not reveal any beneficial effect.[80] A recent randomized controlled trial in patients with ST-segment elevation myocardial infarction failed to show decrease in mortality.[81]

These observations were however, contrasted by Neely and Grotyohann[82] who demonstrated that depleting myocardial glycogen content before the onset of global ischemia resulted in improved function upon reperfusion. The authors speculated that the beneficial effect of reduced glycogen content prior to ischemia results from a decrease in deleterious end products secondary to reduced glycolytic rates. This hypothesis has since been supported by findings which showed that increased rate of glycolysis impairs recovery of function post-ischemia.[8,9,10,17,18,83]

1.2.2.2 Energy Metabolism During Reperfusion

The reinstitution of coronary flow following an ischemic insult has been associated with recovery of oxidative metabolism.[4,8,9,42,84] It has been shown that fatty

acid oxidation recovers to or above pre-ischemic levels.[8,9,17,84] This occurs at the expense of glucose oxidation. TCA cycle activity is rapidly restored with most of the acetyl CoA coming from fatty acids.[4,8,21,22,23,42,84] Glucose oxidation therefore becomes uncoupled from glycolysis and this becomes an important source of proton production.[4,8,9,12,13,42,84] Early reperfusion therapy following an ischemic insult has been shown to be imperative in salvaging the myocardium from further ischemic damage. Reperfusion is however, accompanied by cellular injury on its own referred to as "reperfusion injury".[85-88] This has been associated with impaired functional recovery of the heart and depression of cardiac efficiency.[4,8,9,10,18]

1.2.3 Mechanisms of Ischemia and Reperfusion Injury

The mechanisms proposed to account for the damage resulting from ischemia and reperfusion injury appear to be multifactorial. During myocardial ischemia, depletion of high-energy phosphates[89,90] has been suggested to depress mechanical function[4,8,9], since the contractile proteins require continuous energy supply to support their activity.[91]

During reperfusion of a previously ischemic myocardium a milieu of events causes myocardial functional recovery to be impaired. The nature of this injury depends on the extent of ischemic injury and the time to initiation of restoration of coronary flow.[89,92] Mechanisms proposed to account for this injury arise from factors that trigger intracellular signaling events ultimately leading to cellular damage. Generation of oxygen free radicals,[93,94] activation of neutrophils mediating myocardial and endothelial damage,[95] presence of vasoconstrictors (angiotensin and

endothelin) and intracellular calcium overload have all been shown to contribute to reperfusion injury.[88,92] Patients with myocardial ischemia and infarction on thrombolytic or fibrinolytic therapy have thus, benefited from a wide range of interventions including free radical scavengers, vasodilators, calcium channel antagonists and inhibitors of neutrophil activation.[85-88]

Emerging evidence suggests that alteration of myocardial energy substrate utilization is another factor contributing to ischemia and reperfusion injury.[3,4,8,9,11,17,18] The decreased pH_i arising from uncoupled glucose metabolism,[9,10,13,17] a condition which is exacerbated during reperfusion by increased fatty oxidation, has been shown to contribute to ischemia and reperfusion injury.[4,8,9,23,42]

1.2.3.1 Mechanism of Acidosis-Induced Myocardial Injury

Intracellular pH is regulated within a narrow range to maintain normal physiological function of intracellular proteins and other cellular components.[9,13,91] The source of intracellular protons and their fate during myocardial ischemia and reperfusion has therefore been a subject of intense investigation.

Myocardial ischemia accelerates proton production. It has been established that increased hydrolysis of glycolytically derived ATP during severe ischemia lowers intracellular pH.[12,13] When glucose is metabolized by glycolysis and the pyruvate subsequently oxidized by the TCA cycle, the net proton production is zero. However, during ischemia, because of the inhibition of oxidative metabolism, glycolysis becomes uncoupled from glucose oxidation. Consequently, there is a net

production of two protons for every molecule of glucose that is not subsequently oxidized.[12,13]

Intracellular acidosis has been shown to contribute to cellular injury in a variety of studies. In cardiac myocytes, intracellular acidosis causes a reduction in force development.[2,9,13,91] Protons have also been shown to reduce the sensitivity of the contractile proteins to Ca^{2+}, thus decreasing contractility.[91] Clinically, increased intracellular proton accumulation induces cardiac arrhythmias following myocardial ischemia.[91]

During reperfusion of the ischemic heart, extracellular pH quickly recovers creating a proton gradient across the membrane.[8,9,17] This ultimately activates the Na^+-H^+ exchanger (NHE), leading to increased intracellular Na^+.[71,72] The reverse mode of the Na^+-Ca^{2+} exchanger (NCX) is in turn stimulated hence, inducing Ca^{2+} overload (Fig. 1.3).[71,72,96] A large number of studies demonstrate that increased intracellular Ca^{2+} causes loss of membrane integrity and cell death.[97,98]

In the isolated working rat heart, it was demonstrated that during reperfusion of a previously ischemic myocardium, there is a reduction of efficiency of energy conversion as a large proportion of the energy produced is used to restore the acidosis-induced ionic imbalance.[8,9,17]

1.2.3.2 Alteration of Myocardial Energy Substrate Utilization: A Mechanism of Ischemia-Reperfusion Injury

It has been suggested that the energy substrate utilized during and following acute myocardial ischemia determines the extent of functional recovery of the

heart.[4,9,17,23] In the aerobically perfused rat hearts, Lopaschuk *et al*[15] demonstrated that fatty acid oxidation impairs the coupling of glycolysis and glucose oxidation with glucose oxidation been inhibited to a greater extent than glycolysis.[99] It was later shown in the isolated perfused rat heart that the presence of fatty acids in the perfusate impairs recovery of function following global no-flow ischemia compared with hearts perfused with glucose alone.[9,17,23,100] In a parallel study, it was shown that the presence of fatty acids in the perfusate delays the recovery of intracellular pH following severe ischemia.[8,9] These observations were supported by subsequent findings that increased β-oxidation during reperfusion uncouples glucose metabolism and increases proton production.[8,9,10,17] This was associated with depression of post-ischemic cardiac function and efficiency (LV work per MVO_2).[8,9,18] Finegan *et al* also showed in the isolated working rat heart that increased proton production during reperfusion secondary to increased glycolysis reduces recovery of myocardial function.[18,83]

To further support the hypothesis that substrate preference impairs functional recovery during reperfusion, it was shown that agents that modulate myocardial energy substrate utilization by shifting metabolism away from fatty acid oxidation enhance mechanical function and efficiency.[7,8,9,10] This strategy is achieved by either directly inhibiting fatty acid oxidation[4,8,11,30], increasing glucose oxidation via an increased PDH activity[9,14,17,101,102] or inhibiting glycolysis.[8,9,18,83] Lopaschuk and coworkers[47] showed that trimetazidine (TMZ), an inhibitor of fatty acid oxidation, improves mechanical function and cardiac efficiency during reperfusion in the isolated rat heart. This was accompanied by increased glucose oxidation. Recently,

clinical studies have reported beneficial effect of TMZ in patients with coronary artery diseases (See ref. 103 for review).

Finegan *et al* reported in the isolated rat hearts that addition of adenosine or the A_1-adenosine receptor agonist before or during reperfusion attenuates proton production that translated into improved recovery of mechanical function.[18,83] Accordingly, Liu *et al*[9] showed that addition of DCA, an activator of PDH, increases glucose oxidation and reduces proton production secondary to improved coupling of glycolysis to glucose oxidation. This also translated into enhanced LV work and efficiency during reperfusion. In that same study, when the NHE inhibitor, cariporide, was added prior to ischemia, myocardial energy metabolism was not affected but there was improved recovery of mechanical function and efficiency similar to the DCA group. The authors proposed that altering the source and fate of protons diverts energy from non-contractile activities to sustain heart function thus, enhancing cardiac efficiency.

Taken together, these findings suggest that an accelerated rate of proton production, which is exacerbated by increased fatty acid oxidation during reperfusion, is detrimental to post-ischemic myocardial function and efficiency. This evidence confirms the earlier report by Neely and Grotyohann[82] that decreasing the availability of glycolytic substrates by depleting glycogen stores before the onset of ischemia, enhances myocardial functional recovery. Whether this myocardial preference for energy substrate occurs during the actual period of ischemia has not been well characterized in the isolated perfused heart.

These observations have led to the development of metabolic therapy to treat patients with acute myocardial ischemia and myocardial infarction.[4,5,10,11,103] Indeed, clinically, trimetazidine, an inhibitor of fatty acid oxidation, has been approved in the management of patients with angina pectoris.[103]

Despite the enormous amount of data delineating the interplay between glucose and fatty acid metabolism under aerobic conditions and during reperfusion post-ischemia, the actual interaction between these two fuel substrates during the critical period of ischemia is not well defined. This is partly a consequence of lack of experimental approaches to investigate this interaction under conditions of physiological workload and substrate availability, hence, the present study will attempt to address this interaction. Moreover, the various interventions available to treat the damage resulting from ischemia and reperfusion aim to indirectly reduce intracellular acidosis by improving coupling of glycolysis to glucose oxidation.[8,9] Either increasing flux through PDH directly or reducing β-oxidation to relieve inhibition on glucose oxidation can achieve this improved coupling.[8,10,12,31] From the foregoing discussion, it is noted that increased rate of glycolysis, which is uncoupled from glucose oxidation during ischemia and reperfusion, is a significant source of intracellular proton accumulation.[8,9,17,18,83] It is therefore reasonable to hypothesize that directly decreasing glycolysis during and/or following ischemia will enhance myocardial mechanical function and efficiency by improving glucose coupling and reducing proton production. This stems from the observation that increased proton production impairs recovery of function and efficiency post-ischemia.[8,9,13,18,83] This will form a major objective of this study.

1.3 Glucose Uptake and Myocardial Ischemia

The importance of glucose to the ischemic myocardium has been highlighted in a number of studies.[28,74,75,104,105] The beneficial effects of glucose to treat patients with CAD has been known for a long time when administration of glucose improved symptoms in angina patients.[28,30] This suggested the basis for the addition of glucose and insulin to cardioplegic solutions prior to surgical procedures.[106,107,108] There is a divergent view on whether myocardial ischemia alters uptake of glucose. The disparity in the data available stems partly from the experimental model, the tracer used to measure the rate of glucose uptake, the extent of the ischemic event and on the degree of coronary flow reduction.[53] Thus, a better understanding of the mechanisms by which glucose is taken up during ischemia will help provide newer therapeutic strategies for ischemic heart patients.

1.3.1 Mechanisms of Glucose Uptake

The transmembrane transport of glucose in heart is mediated by the GLUT-1 and GLUT-4 glucose transporters.[28,34,35] Both isoforms are found in the mammalian myocardium and expressed by distinct genes.[28] The content and activity of the transporters in the membrane together with the transmembrane glucose concentration determine the rate of glucose transport.[28,29] GLUT-1 is the predominant isoform in the neonatal myocardium. The GLUT 4 transporter is located mainly in intracellular storage vesicles, but undergoes translocation to the plasma membrane upon hormonal stimulation (insulin), and hence, is referred to as the insulin-sensitive transporter.[28-33]

Glucose uptake is described as glucose transport and the subsequent phosphorylation to G-6-P by hexokinase.[29] Myocardial glucose uptake is determined by the metabolic status of the heart such that in the presence of other energy substrates such as fatty acids, lactate and glycogen, uptake is slowed.[28,29]

Insulin influences myocardial energy substrate utilization in a number of different ways. Upon stimulation of insulin receptors, there is an accelerated recruitment of mainly the GLUT-4 transporters via the phosphoinositide 3-kinase-dependent (PI 3-K) pathway.[29,109] Recently, it has been demonstrated in the rat myocardium that insulin also mediates the translocation of both GLUT-1 and GLUT-4[110] transporters to the plasma membrane.[111,112]

Myocardial glycogen is a storage form of glucose and its content also determines glucose uptake into the cytosol.[28,29] Intracellular glycogen content is regulated by stimuli that increase its synthesis or breakdown.[12,28] The rate limiting step in glycogen synthesis is catalyzed by glycogen synthase,[28,113] which is itself under phosphorylation and dephosphorylation control by a number of protein kinases including cyclic AMP-dependent protein kinase[28,113,114,115] and AMPK[52,117]. In response to increased energy demand such as exercise, glycogen mobilization is accelerated. Adrenergic stimulation which increases cytosolic levels of cAMP activates protein kinase A (PKA), which in turn phosphorylates and inhibits glycogen synthase.[12] Glycogen synthesis, is thus decreased with a concomitant increase in its breakdown. In fasted animals, acceleration of fatty acid β-oxidation has been shown to increase glycogen content possibly by inhibition of glycolysis and diverting G-6-P to glycogenesis.[12,28]

Glycogenolysis is regulated by glycogen phosphorylase and mechanisms such as adrenergic stimulation that deplete glycogen content increase the activity of this enzyme.[13,52] Depletion of glycogen has been shown to increase glucose uptake as a feedback mechanism to restore intracellular glycogen levels.[28,116] It is therefore reasonable to speculate that factors that deplete myocardial glycogen including ischemia, hypoxia and exercise (increased energy demand), should lead to increased glucose uptake. Indeed in skeletal muscle, glucose uptake is increased during exercise or increased contraction.[110] In working rat hearts, it has been demonstrated that there is a simultaneous regulation of the synthesis and breakdown of glycogen suggesting activation of both glycogen synthase and phosphorylase, a concept referred to as glycogen turnover.[52,114]

Recently, it has been reported in various studies that an important enzyme regulating myocardial glycogen content and glucose uptake is AMP-activated protein kinase (AMPK).[52,117,118] AMPK is a metabolic sensor that responds to acute changes in the AMP:ATP and Cr:CrP ratios of the cell.[117,118] AMPK is under both allosteric and phosphorylation regulation. An increase in the cytosolic levels of AMP increases AMPK activity.[118] One or more upstream kinases, termed AMPKKs, phosphorylate and increase AMPK activity up to a 100-fold.[118] Interestingly, AMP levels also regulate the activity of AMPKK.[117,118,119] A recent report however, shows that AMPK can be activated in the absence of any changes in the energy status of the cell and by AMPKK-independent mechanisms.[120] Moreover, it is now debated whether intracellular AMP levels influence the activity of AMPKK.[120]

When activated, AMPK has been shown to turn off energy-consuming pathways while simultaneously activating energy-generating pathways.[42,84,117,118] Thus, it was earlier suggested that AMPK increases fatty acid oxidation by releasing the inhibition of malonyl CoA on CPT-I secondary to inhibition of acetyl CoA carboxlyase (ACC).[42,84,100,117,119] In those studies glycogen synthesis was also demonstrated to be decreased by AMPK with exogenous glucose being diverted to glycolysis. This was associated with increased glucose uptake via increased translocation of GLUT-1 and GLUT-4 to the plasma membrane.[35,54,84,117]

The known influence of AMPK on myocardial glucose uptake has recently been challenged by studies which showed that activation of AMPK occurred without the accompanying increase in glucose uptake in the skeletal muscle.[121,122] The effect of AMPK on myocardial energy metabolism thus, remains controversial and research is underway to characterize clearly the mechanisms underlying the effect of AMPK on overall energy metabolism. The latter will be given a consideration in the present study.

1.3.2 Evaluation of Glucose Uptake During Myocardial Ischemia

Several experimental strategies have been employed to investigate the effect of ischemia on myocardial glucose uptake. Earlier studies by Morgan *et al*[123] demonstrated in the isolated working rat heart that anoxia stimulates glucose uptake, indexed as the disappearance of glucose from the perfusate. This effect was shown to be dependent on the rate of transmembrane glucose transport and phosphorylation and was augmented by the presence of insulin. Recently, other markers have been

used to assess glucose uptake in cardiomyocytes, isolated heart perfusions and under *in vivo* conditions.

The non-metabolizable glucose analogue, 2-deoxy-D-glucose (2-DG), has been used extensively to evaluate glucose uptake in animal studies, after it was first described by Sokoloff *et al*[124] in rat cerebral tissues. The rate of conversion of 2-DG to 2-DG-phosphate by hexokinase gives an indication of glucose uptake rate. Other workers have since employed this approach to estimate glucose uptake in the ischemic myocardium.[36,125,126] The problem with this approach is that 2-DG-phosphate is a "dead-end" of glucose metabolism and is not subject to further metabolism. Doenst and Taegtmeyer[126] reported that in isolated rat hearts perfused with both glucose and the analogue, 2-DG, underestimates glucose uptake in the presence of insulin. Moreover, the affinity of hexokinase for 2-DG is different compared to glucose.[127] Thus, accumulation of the tracer in the cytosol may inhibit further uptake under certain experimental conditions.[37,125,128] Since 2-DG is not metabolized, it may suggest that the heart becomes energetically compromised in these studies rendering interpretations from the studies complex.[29,128]

In vivo glucose uptake has been assessed using [^{18}F]2-deoxy-2-fluoroglucose (FDG), which has been employed to test the viability of the myocardium in patients diagnosed for coronary syndromes.[29,53] Like 2-DG, after transport into the cytosol, FDG is phosphorylated by hexokinase and becomes trapped since it is not amenable to further metabolism. The difference in the kinetic properties of 2-DG and FDG and the physiological substrate, glucose, is corrected for by a factor referred to as the lumped constant.[29] It has recently been shown that the lumped constant varied

depending on the metabolic status of the myocardium.[29,129] It was also demonstrated in the rat myocardium that FDG overestimates glucose uptake during ischemia while underestimating it upon reperfusion.[129]

The production of ^3H_2O from D[2-^{3}H]glucose has also been used by other workers to assess glucose uptake in the rat heart.[53,125,129,130,131] Detritiation of D[2-^{3}H]glucose occurs at the phosphoglucoisomerase step of glycolysis and it has been assumed to equal glucose uptake.[53] However, as noted earlier, upon transport, glucose is either metabolized via glycolysis or stored as glycogen. Therefore, the use of D[2-^{3}H]glucose may not reflect the actual rate of glucose uptake. Furthermore, some studies have used the isotope to estimate the rate of glycolysis.[132,133] In *in vivo* heart studies, the arteriovenous difference in the concentration of radiolabelled glucose is widely used to quantify glucose uptake.[27,134]

To further complicate the issue, few studies have actually assessed glucose uptake during the critical period of ischemia and have provided conflicting results. In the *in vivo* myocardium, transport of FDG measured by positron emission tomography was shown to increase in response to reduction in coronary flow.[135] During low-flow ischemia in the isolated rat heart, Neely *et al*[62] showed that the absolute rate of glucose uptake is reduced. This finding was supported in the Langendorff perfused rat heart subjected to low-flow ischemia that although glucose extraction increased with reduction in coronary flow, the absolute rate of glucose uptake was not affected.[53] Fraser *et al*[52] also showed in the isolated working rat heart subjected to low-flow ischemia that, glucose uptake rate is actually decreased by ischemia. Taken together, these observations suggest that glucose uptake rate is

either reduced or not affected by an ischemic insult. This could be a result of the severity in reduction of coronary flow and thus, glucose delivery.[53,135] It must be emphasized however, that in all of these studies because coronary flow is severely reduced, cardiac function is dramatically impaired. Thus, glucose uptake in these studies may not reflect the actual *in vivo* situation because of the reduced energy demand.

Other workers who are of the view that ischemia causes an increased recruitment of the GLUT transporters to the plasma, have suggested that ischemia may enhance glucose uptake. Sun *et al*[35] demonstrated in the Langendorff perfused rat heart that ischemia induces an increase recruitment of the GLUT-4 transporter to the plasma membrane, an effect enhanced by the presence of insulin. The authors suggested that this could partly account for the increase in glucose uptake induced by ischemia and insulin. Young *et al*[54] later showed in the canine myocardium that, low-flow ischemia leads to an increase in the uptake of glucose which was accompanied by increased translocation of both GLUT-1 and GLUT-4 proteins to the plasma membrane. In the *ex vivo* working rat heart, Bolukoglu *et al*[131] reported that coronary flow reduction achieved by lowering of the afterload, did not affect glucose uptake even in the presence of insulin. In a parallel study using the same model, it was demonstrated that low-flow ischemia enhanced glucose uptake only in the presence of a competing substrate such as lactate or a fatty acid.[130]

Glucose uptake during ischemia remains an elusive subject. Therefore, in the present study, we designed different approach to assess glucose uptake during mild ischemia under conditions of physiological substrate and workload to enable us

enlighten our understanding of the interplay between ischemia, workload and glucose uptake.

1.4 Adenosine and Cardioprotection

Adenosine is an endogenous nucleoside, which exerts a variety of effects on the cardiovascular system. The physiological and pharmacological profiles of adenosine on the mammalian cardiovascular system have been well documented (see Refs. 136 and 137 for reviews). Adenosine is present in low concentrations in the cell under normal conditions but during periods of decreased energy production or high-energy demand, there is an increased generation of adenosine.[138,139]

Two major pathways have been identified to be involved in adenosine formation: 1. The dephosphorylation of 5'-AMP by 5'-nucleotidase which has been shown to occur during ischemia and hypoxia, and 2. The hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase which is responsible for adenosine formation under normal conditions.[138,139,140] The formation of adenosine is sensitive to the availability of oxygen in the cell suggesting that adenosine may play a critical role in the regulation of local flow in the heart.[136] Thus, when coronary flow is reduced during ischemia or energy demand increases, adenosine formation is increased.[136,147] Cytosolic adenosine is transported to the interstitial space by the membrane-bound nucleoside transporters.[140] Adenosine has a very short half-life (0.1-0.6 secs) and is catabolized either via deamination to inosine by adenosine deaminase or phosphorylated to AMP by adenosine kinase from where it can be salvaged by the nucleotide repletion pathway.[136,139,140]

Adenosine has been shown to have a ubiquitous distribution in almost all tissues of the body emphasizing the relevance of this molecule in maintaining the balance between metabolism and flow. Although the vascular effects of adenosine have been well established,[141,142] the cardioprotective efficacy have only recently been reported.[136,138,140,143]

The numerous effects of adenosine are exerted by acting on G-protein coupled surface receptors.[142,144,145] Currently four subtypes of the adenosine receptors; A_1, A_{2A}, A_{2B} and A_3 have been cloned and characterized and these surface receptors are expressed in various tissues of the mammalian body.[142,144] Available evidence suggests that the A_1 adenosine receptor subtype is coupled to the inhibitory G-protein (G_i-protein)[142,144] whose signaling results in the opening of sarcolemmal K^+_{ATP} channels[146] via activation of protein kinase C (PKC).[147] In the myocardium, this has been associated with the negative inotropic and negative chronotropic effect of adenosine.[140] Activation of the A_2-adenosine receptor stimulates a G_s-protein causing the well known vasodilation induced by adenosine on various vascular beds.[148] The coupling of the A_3-adenosine receptor subtype to G_i- and G_q-proteins[147] results in inhibition of adenyl cyclase.[142]

A large body of evidence suggests that the high concentration of adenosine produced during an ischemic event increases tolerance of the myocardium against damage.[136,139,140,145] The cardioprotective effect of adenosine against ischemia and reperfusion injury is thus, a subject of more intense investigation following the observation that brief episodes of ischemia renders the heart tolerant to a subsequent lethal ischemic insult.[149] This phenomenon referred to as ischemic preconditioning

(IPC), involves various triggers and mediators and the distinct pathways have been dissected although not fully understood (see Ref. 150 for review). Adenosine has been implicated as the prime candidate of IPC from observations that adenosine mimics the infarct-limiting effect of IPC.[145,150,151]

Indeed, various animal studies have shown that administration of adenosine or adenosine receptor agonists before global no-flow myocardial ischemia reduces myocardial infarction[140,145,152] and enhances recovery of function during reperfusion post-ischemia.[18,52,153,154] Additionally, it has been shown that the cardioprotective effectiveness of adenosine is afforded by the stimulation of the A_1 and A_3 adenosine receptors.[140,145] Addition of the A_1 adenosine receptor agonist, N^6-cyclohexyladenosine (CHA, half-life, 106 min)[182], prior to 30 min global ischemia in the isolated perfused rat heart, improves post-ischemic recovery of left ventricular function.[153] In that same study stimulation of the A_2 adenosine-receptors does not show any improvement in the recovery of function.[153] Transgenic mice overexpressing the cardiac A_1 adenosine receptors manifested an increased tolerance to ischemia and reperfusion-induced apoptosis, lending further support of the involvement of these receptors in adenosine mediated cardioprotection.[155] Recently, Jacobson *et al* reported that the anti-ischemic properties of adenosine is mediated via co-activation of both the A_1 and A_3 adenosine receptors by the novel binary A_1/A_3 agonists in mice hearts.[156]

These studies have been confirmed by the observation that increasing endogenous adenosine levels prior to ischemia attenuates ischemia-reperfusion damage and limits myocardial infarction.[145,157] This is achieved by reducing the

degradation of adenosine either via inhibiting adenosine deaminase,[157] adenosine kinase[145] or inhibiting the nucleoside transporters.[158] Clinically, adenosine is employed as an anti-arrhythmic agent[159] and also used as a supplement in cardioplegic solutions.[145,160]

The mechanism involved in the cardioprotection mediated by adenosine is not clearly understood. The proposed mechanisms of adenosine-induced cardioprotection against ischemia and reperfusion include: improvement in coronary flow regulating the local myocardial oxygen consumption,[136,137] preservation of myocardial ATP content via reduction in heart rate,[136,137,145] inhibition of platelet aggregation and inhibition of neutrophil activation.[145]

Although the cellular signaling pathway of adenosine receptor activation remains elusive, available evidence suggests that activation of ATP sensitive K^+ channels may be the end effector mediating most of these cytoprotective effects.[150,151] Emerging evidence now suggests that adenosine-induced cardioprotection could be via modulation of myocardial energy substrate metabolism.[18,52,83,161]

1.4.1 Adenosine and Myocardial Energy Metabolism

The role of adenosine on myocardial energy metabolism has stimulated interest, following the observation that myocardial substrate preference during ischemia is essential to recovery of function upon reperfusion. This effect was originally hypothesized to be due to salvage mechanisms that incorporate adenosine into the nucleotide pool to replenish intracellular ATP content.[152,162] Other workers, however, showed a dissociation between enhancement of myocardial ATP content

by adenosine with no effect on recovery of function post-ischemia.[163] This suggests that the protective effect of adenosine may be related directly to energy substrate use and it may be independent of its role as a precursor for ATP resynthesis. Adenosine has been shown to have various effects on glucose metabolism in different tissues. Wong *et al*[164] showed that adenosine activates PDH and increases glucose oxidation in adipocytes by potentiating insulin effects. Later, our laboratory demonstrated that when adenosine is given during normal aerobic perfusion in the isolated working rat heart perfused with palmitate and glucose as energy substrates, glycolysis is inhibited while glucose oxidation is increased.[161] Parallel to this observation, it was reported that in the isolated perfused rat heart, when added before low-flow ischemia or during reperfusion, adenosine inhibits glycolysis during reperfusion and increases glucose oxidation. This is accompanied by improved functional recovery post-ischemia.[18,83] The improvement in myocardial function was attributed to enhanced coupling of glycolysis and glucose oxidation, which results in reduced proton production during reperfusion. Fralix and coworkers[154] reported that following a 30 min global ischemia in the Langendorff perfused rat heart, adenosine inhibits the rate of glycolysis measured as lactate production and attenuates the decline in ATP content. Interestingly, this was accompanied by a decreased accumulation of intracellular Ca^{2+} and pH and an enhanced recovery of function compared to the untreated heart. These findings suggest that the cardioprotective effect of adenosine appear to involve modification of myocardial glucose metabolism resulting in reduced proton production from glycolytically derived ATP. The cellular signaling mechanism of the effect of adenosine on myocardial energy metabolism is unknown.

Moreover, whether adenosine alters myocardial energy substrate use during the critical ischemic insult remains to be established.

Other workers[153] have shown, however, that adenosine enhancement of functional recovery occurs via increase in glycolytic flux, contrary to results from our work and other laboratories.[18,52,83,154] From previous findings,[4,7,17,10] it has been shown that metabolic modulators such as TMZ and DCA, enhance the recovery of myocardial function and efficiency by enhancing the coupling of glucose metabolism secondary to inhibition of fatty acid oxidation.[4,17,10] An alternative strategy to enhance the coupling of glycolysis to glucose oxidation is to directly inhibit glycolytic flux.[18,83] It is therefore tempting to assume that adenosine by partially inhibiting glycolysis during the critical ischemia period may enhance mechanical functional recovery. Moreover, the observation that adenosine alters ion homeostasis by decreasing Ca^{2+} accumulation secondary to inhibition of intracellular proton production during reperfusion[154] suggests that adenosine enhances efficiency post-ischemia.

In the present study, we describe a novel *ex vivo* model of mild ischemia representative of angina, which will shed light on the metabolic status of the heart during the critical period of ischemia. In this model cardiac function and metabolism will be assessed simultaneously (see "Method" section for details of model). We believe this model will enrich our understanding of myocardial energy substrate metabolism during the actual ischemic event and help address certain aspects of metabolism, such as the effect of ischemia on glucose uptake. Previous studies have suggested that the cardioprotective effect of adenosine is partly accounted for by the

alteration of myocardial energy metabolism during reperfusion.[18,83,154] No study has evaluated the effect of adenosine on the relationship between, myocardial energy use, LV function and efficiency during the actual ischemic event. We therefore assessed the effect of adenosine on myocardial metabolism during and following 30 min period of mild ischemia in order to elucidate the mechanism by which adenosine enhances mechanical function and efficiency.

Hypothesis

Adenosine-induced alteration in cardiac energy metabolism enhances recovery of mechanical function and efficiency during reperfusion following mild ischemia by reducing myocardial proton production from exogenous glucose metabolism, secondary to inhibition of glycolysis.

Specific Objectives

1. To design a model of ischemia-reperfusion to enable the simultaneous assessment of myocardial energy metabolism and function during an actual mild ischemic period under conditions of physiological workload and substrate availability.
2. To evaluate the uptake of glucose during mild ischemia and reperfusion under the above perfusion conditions.
3. To assess the role of adenosine on myocardial energy metabolism, function and efficiency during and following mild ischemia.

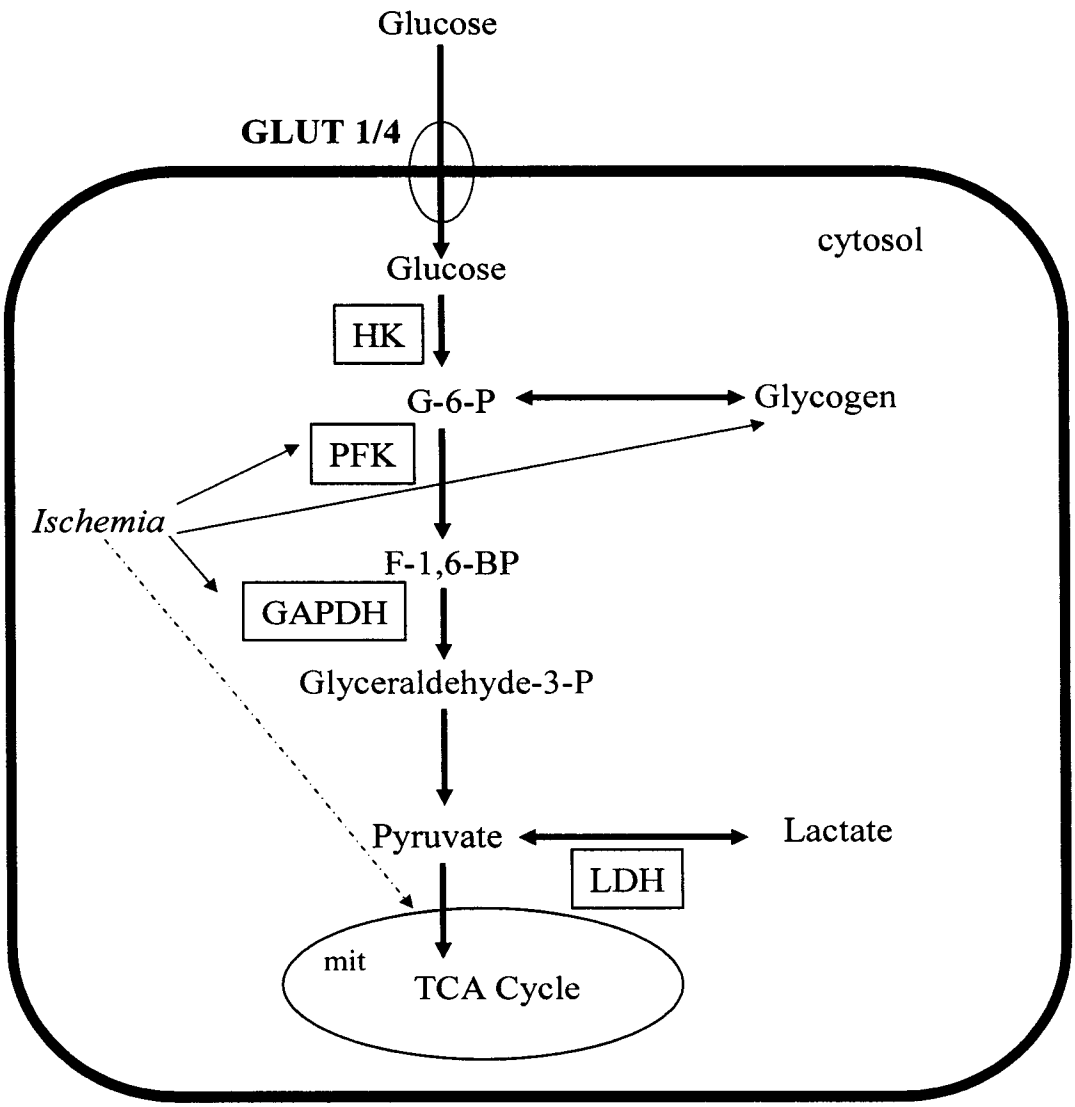

Fig.1.1. Regulation of glucose metabolism. Glucose entry occurs via the facilitative glucose transporters (GLUT 1/4). Hexokinase (HK) commits glucose to either glycolysis or glycogen synthesis. Rate of glucose metabolism is further regulated by phosphofructokinase (PFK) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). During ischemia, the activities of PFK and GAPDH are accelerated, while the tricarboxylic acid (TCA) cycle enzymes are inhibited. LDH, lactate dehydrogenase; mit, mitochondrial. Dashed lines indicate inhibition.

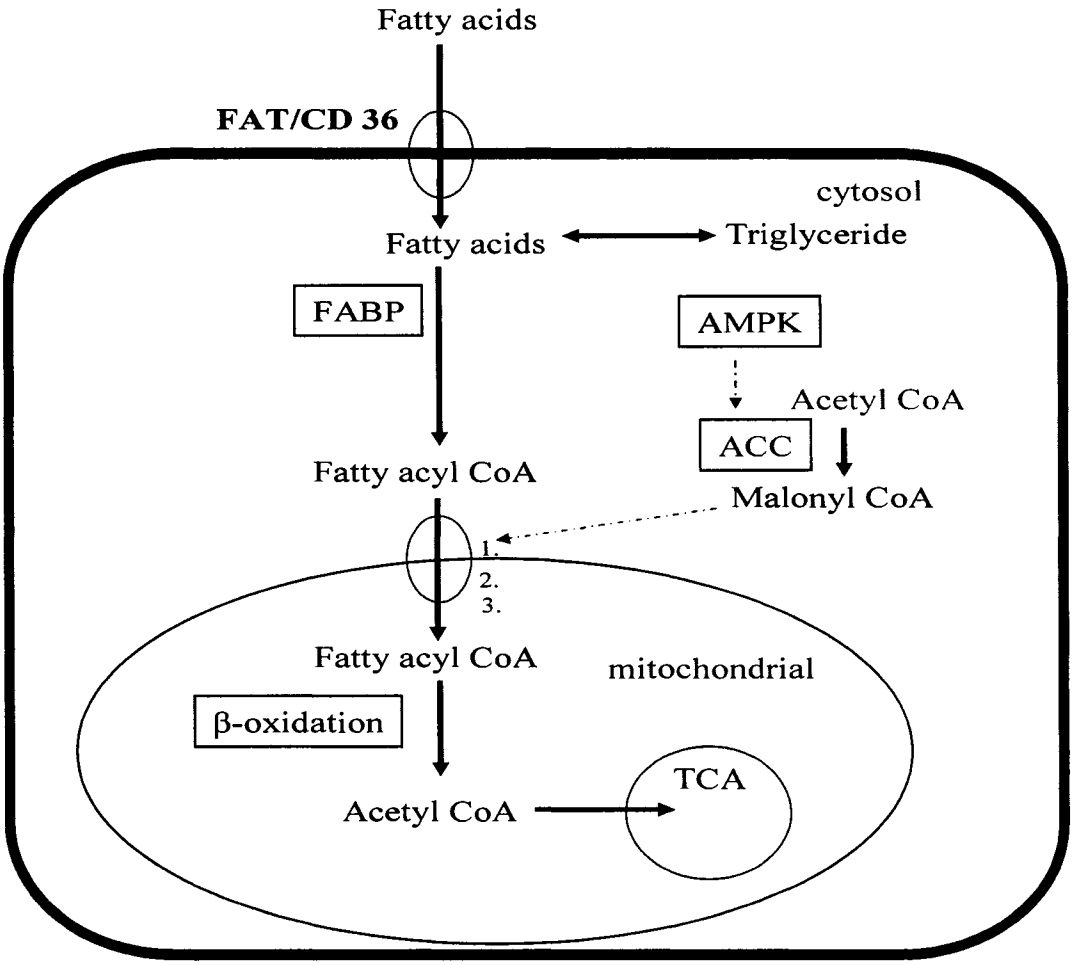

Fig.1.2. Regulation of fatty acid metabolism. This occurs via the transport of fatty acyl CoA into the mitochondrial by a system of carnitine-dependent enzymes (1, carnitine palmitoyltransferase-I; 2, acylcarnitine-carnitine translocase (CAT) and 3, carnitine palmitoyltransferase-II). Carnitine palmitoyltransferase-I, whose activity is regulated by malonyl CoA, is the rate determinant. Malonyl CoA is synthesized from acetyl CoA by acetyl CoA carboxylase (ACC), which is under phosphorylation control by AMP-activated protein kinase (AMPK). FABP, fatty acid binding protein; FAT/CD36, fatty acid transporter. Dashed lines indicate inhibition of activity.

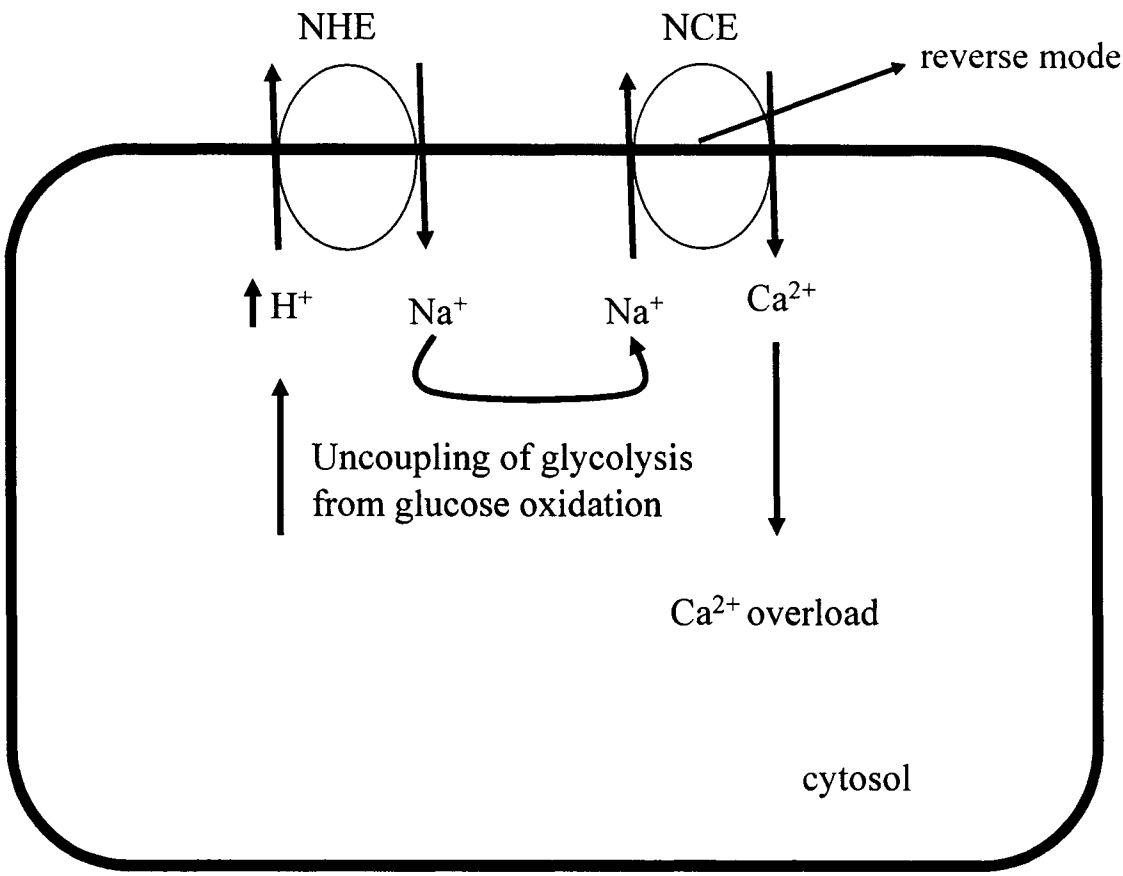

Fig.1.3. Clearance of intracellular protons (H^+) via the sodium-hydrogen exchanger (NHE). Uncoupling of glycolysis from glucose oxidation, which occurs during ischemia and reperfusion leads to intracellular acidosis. Increased activity of the NHE results in intracellular Na^+ overload. Consequently, this activates the reverse mode of the sodium-calcium exchanger (NCX) ultimately resulting in intracellular Ca^{2+} overload.

CHAPTER 2

Methods

2.1 Animal Use

All animal procedures, including housing, feeding, surgical protocols and euthanization received prior approval from the Health Sciences Animal Welfare and Policy Committee of the University of Alberta and were performed in accordance with the guidelines and standards set by the Canadian Council of Animal Care. All rats were allowed free access to food and water until the time of euthanization.

2.2 Materials

D-[^{3}H]glucose, D-[U-^{14}C]glucose and [9, 10-^{3}H]palmitate were purchased from PerkinElmer LIFE Sciences, Inc., Boston, MA. Bovine serum albumin was obtained from EQUITECH- BIO Inc. Kerville, TX. Hyamine hydroxide (methylbenzethonium hydroxide, 1 M in methanol solution) was obtained from CURTISS Laboratory Inc. Bensalem, PA. Ecolite scintillant was purchased from MP Biomedicals, Costa Mesa, CA. Dowex 1x4 anion exchanger resin (200-400 mesh chloride form) was obtained from Bio-Rad Laboratories Inc, Hercules, CA. Adenosine was obtained from Research Biochemicals International, Natick, MA. Phospho-AMPK-α (Thr 172), AMPK-α and goat anti-rabbit antibodies were purchased from Cell Signaling Technology.

2.3 Isolated Heart Perfusions

Male Sprague-Dawley rats (Biosciences Laboratories, 250-350g) were anesthetized with pentobarbital sodium (60 mg · kg^{-1}, intraperitoneally) and the hearts were rapidly excised (after opening of the thoracic region) and subjected to perfusion as described previously.[18,57,83] Briefly, the hearts were placed in ice-cold Krebs solution and the aorta cannulated immediately. A Langendorff perfusion, at a hydrostatic pressure of 60 mm Hg, was initiated using Krebs-Henseleit solution containing (in mM): KCl (4.7), NaCl (118), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25), and glucose (11), bubbled with carbogen (95% O_2-5% CO_2 mixture) for 12-15 min. During this period the heart was trimmed of excess non-cardiac tissues and the opening to the left atrium was cannulated. The pulmonary artery was also cannulated for the collection of perfusate samples for the measurement of myocardial oxygen consumption (MVO_2). Following this, the heart was switched to the working mode by clamping the Langendorff line and opening both the preload and afterload lines. The recirculating working perfusate (100 ml) was delivered from an oxygenator gassed with carbogen, and consisted of a modified Krebs-Henseleit solution containing palmitate (1.2 mM) pre-bound to 3% bovine serum albumin (BSA) and insulin (100 μU · ml^{-1}). The perfusate was maintained at a constant temperature of 37 °C by a water bath. Aortic developed pressure of 50-60 mm Hg was achieved by adjusting the air in the compliance chamber. Hearts were perfused at a constant preload and afterload of 11.5 and 80 mm Hg, respectively, and paced at 5 Hz using a Grass S88 stimulator. A diagrammatic representation of the perfusion apparatus is shown in Fig 2.1.

2.3.1 The Mild Ischemia Model

Following a 30 min baseline aerobic perfusion, hearts were subjected to 30 min mild ischemia using a device (Fig. 2.2) inserted between the aortic cannula and the compliance chamber, which is specifically designed to limit coronary perfusion (as described below). Basically, this device is composed of two main parts: 1) a one-way valve which allows a unidirectional flow of perfusate from the aortic cannula to the compliance chamber and 2) a diastolic backflow controller (a three-way tap), that allows the regulation of the amount of perfusate entering the coronary circulation during diastole. During aerobic perfusion, perfusate delivered to the left atrium through the preload line is pumped by the LV via the aortic cannula and through both the valve and the controller into the compliance chamber. The diastolic phase of the cardiac cycle causes the ventricles to relax enabling coronary perfusion through the three-way tap. Since perfusate can only pass through the diastolic backflow controller and not the one- way valve, adjusting the three-way tap can regulate the amount of solution perfusing the heart. Thus, mild ischemia can be induced by carefully and partially closing the three-way tap to obtain a coronary flow reduction that can sustain a new steady state of myocardial function and contractility. Initial experiments showed that a ~30% reduction in coronary flow (relative to preischemic values) at a heart rate of 300 beats $\cdot$ min^{-1} is sufficient to sustain a steady but impaired contractile function. Induction of mild ischemia was confirmed by a depression of diastolic pressure and an accompanying reduction in coronary flow.

2.3.2 Reperfusion After Mild Ischemia

Following a 30 min period of mild ischemia hearts were reperfused for a further 30 min by fully opening the diastolic backflow controller to the pre-ischemic state. Whenever present, adenosine (100 μM) was added 5 min prior to the induction of mild ischemia and was present throughout the entire periods of mild ischemia and reperfusion. Hearts were snap frozen at the end of the perfusion period with Wollenberger clamps cooled to the temperature of liquid N_2. The ventricular tissue was weighed and powdered in a mortar and pestle cooled to the temperature of liquid N_2 and the atria segment dried in an oven at 100°C for at least 24 hrs and weighed. About 60 mg of the powdered tissue was weighed to determine the dry weight-to-wet weight ratio and together with the dry atria weight and frozen ventricular tissue weight, the total dry weight of the heart was determined. The rest of the powdered tissue was stored at -80 °C for analysis of tissue metabolites and other enzymatic assays as described below.

2.4 Measurement of Mechanical function

Heart rate, peak systolic pressure (PSP) and diastolic pressure were measured using a Gould P21 pressure transducer connected to the aortic outflow line. Cardiac output (CO) and aortic flow were measured with Transonic T206 ultrasonic flow probes connected in the preload and afterload lines. The O_2 content of the perfusate entering and leaving the heart were assessed by YSI micro O_2 electrode (Microelectrodes, Inc, Bedford, NH) connected in the preload and pulmonary artery lines, respectively.

All measurements were done at 10 min intervals throughout the perfusion protocol. Coronary flow ($ml \cdot min^{-1}$) was calculated as the difference between the CO and aortic flow. Hearts with coronary flow < 14 during aerobic perfusion were considered unhealthy and therefore excluded from analysis. Aortic developed pressure (mm Hg) was calculated as the difference between the aortic PSP and aortic diastolic pressure. LV work was assessed as CO (PSP – preload pressure). MVO_2 ($\mu mol \cdot min^{-1} \cdot g\ dry\ wt^{-1}$) was calculated by the Fick principle, which is the product of the arteriovenous difference in perfusate O_2 content and the coronary flow rate. Myocardial efficiency (%) is defined as the LV work done per O_2 consumed expressed as a percentage.

2.5 Measurement of Energy Substrate Metabolism

Assessment of the rates of myocardial energy substrate utilization was performed as previously described[9,18,52,83,165]. Briefly, perfusate samples (3 ml) were removed at 10 min intervals with a syringe from an injection port inserted between the oxygenator and preload line. Without exposure to air, the samples were slowly injected below 3 ml of mineral oil in sample containers. This prevented the release of gaseous CO_2. The steady-state rates of glycolysis and glucose oxidation were evaluated simultaneously by perfusing hearts with perfusate containing [5-^{3}H]glucose and [U-^{14}C]glucose. Additional groups of hearts were perfused with perfusate containing [9, 10- ^{3}H]palmitate for the assessment of fatty acid oxidation.

2.5.1 Measurement of Rate of Glycolysis

Flux through glycolysis was measured by the quantitative collection of ^3H_2O (liberated from the enolase stage of glycolysis).[18,53,83,154] ^3H_2O in 0.1 ml of perfusate samples (above) was separated from [^{3}H]glucose and [^{14}C]glucose using chromatographic columns containing Dowex 1-X4 anion exchanger resin (200-400 mesh) suspended in 0.4 M potassium tetraborate. The Dowex resin was stirred in 0.4 M potassium tetraborate overnight and 2 ml was aliqouted into the columns the following day. The column was washed adequately with distilled water to wash out the tetraborate and also to allow the tight packing of the resin. Perfusate samples (0.1 ml) were loaded onto the columns and eluted with dH_2O (0.9 ml) into vials, after which 4 ml of Ecolite scintillant was added. ^3H_2O and residual radiolabelled glucose were detected by a Scintillation counter programmed for counting dual-labeled isotopes. Corrections were made for the small amount of [^{3}H]glucose that passed through the column and also the spillover from the [^{14}C] into the [^{3}H] window were corrected by using standards containing only [^{14}C]glucose. The column was shown to retain about 98% of the total [^{3}H]glucose and [^{14}C]glucose. Average rates of glycolysis were expressed as μmol glucose metabolized · min^{-1} · g dry wt^{-1}.

2.5.2 Measurement of Glucose Oxidation

Rate of glucose oxidation was measured by the quantitative determination of $^{14}CO_2$ produced from [^{14}C]glucose at the pyruvate dehydrogenase step of glucose oxidation and also from the TCA cycle.[18,165,166] $^{14}CO_2$ released from the oxygenator

and trapped in hyamine hydroxide (40 ml) as well as the CO_2 dissolved in the perfusate as $H^{14}CO_3^-$ were used to assess myocardial glucose oxidation. Perfusate samples (1 ml), which had been stored under oil (see above) were removed and injected into test tubes containing 1 ml of 9 N H_2SO_4. The $^{14}CO_2$ liberated was trapped in vials containing filter papers saturated with hyamine hydroxide (0.4 ml), which has been tightly fixed onto rubber stoppers on top of the test tube. The samples were prepared in duplicates. The vials with the filter papers were removed after 1 hr and $^{14}CO_2$ counted using the standard counting procedures described above. Average rates of glucose oxidation were expressed as μmol glucose metabolized · min^{-1} · g dry wt^{-1}.

2.5.3 Calculation of Proton Production from Glucose Metabolism

If glycolysis is coupled to glucose oxidation the net proton production is zero.[9,12,18,165] However, when glucose metabolism is uncoupled (glycolysis exceeds glucose oxidation), the hydrolysis of the ATP results in the net proton production of 2 protons per molecule of glucose utilized. Thus, the overall average rate of proton production was calculated as: 2 · (rate of glycolysis – rate of glucose oxidation).

2.5.4 Measurement of Glycogen Content

Glycogen content of left ventricles was evaluated by a spectrophotometric assay following acid hydrolysis of tissue glycogen into glucosyl units[52]. Powdered frozen tissue (150-200 mg) was solubilized in 30% KOH (0.3 ml, 100 °C for 1 hr) to extract glycogen followed by precipitation with 2% Na_2SO_4 (0.2 ml) and 2.0 ml

absolute ethanol. Mixtures were stored at -20 °C overnight and centrifuged (3500 rpm, 5 min) the following day. The pellets were rinsed with 66% ethanol (2 ml) and then subjected to acid hydrolysis (1 ml 2 N H_2SO_4) to produce glucosyl units. Following neutralization (pH 6.8-7.0), the glucose content was determined using a spectrophotometric analysis at a wavelength of 475 nm against a standard glucose curve of 0-20 μg. Glycogen content was expressed as μmol glucosyl units · g dry wt^{-1}.

2.5.5 Assessment of Glycogen Synthesis

Since hearts were perfused with solution containing radiolabelled glucose ($^3H/^{14}C$), incorporation of radiolabelled exogenous glucose into the glycogen pool was used to determine the average rate of glycogen synthesized during the perfusion protocol[52]. This was measured by counting 0.1 ml of the above neutralized glycogen sample extract and 0.1 ml of the perfusate sample in Ecolite following the standard counting procedures described above. Glycogen synthesis was calculated as the difference between the total glycogen content and amount of incorporated radiolabelled glucose.

2.5.6 Determination of Glucose Uptake and Extraction

Upon entry into myocytes, glucose is either metabolized through the glycolytic pathway or converted into glycogen. Therefore, glucose uptake (μmol · min^{-1} · g dry wt^{-1}) was evaluated as the sum of the rate of glycolysis and amount of incorporation of radioactive glucose into glycogen (the rate of synthesis). Glucose

extraction was calculated from the rate of glucose uptake as a percentage of glucose delivery (glucose concentration · coronary flow)[52,53]. Thus,

% glucose extraction = (glucose uptake / glucose delivery) x 100

2.5.7 Measurement of Fatty Acid Oxidation

The liberation of ^3H_2O from [^{3}H]palmitate was used to measure quantitatively the rate of myocardial fatty acid oxidation as described previously[18,165]. To separate ^3H_2O from [^{3}H]palmitate, 1.88 ml of a chloroform:methanol mixture (1:2, v/v) was added to 0.5 ml perfusate samples and shaken gently. Further addition of 0.625 ml of chloroform followed by 0.625 ml of 2 M KCl:HCl (each time shaking the tube), resulted in the formation of two phases. After centrifugation, the top aqueous phase was removed using a Pasteur pipette into a new set of tubes and then treated with a mixture of chloroform (1 ml), methanol (1 ml), and KCl:HCl (0.9 ml). The mixture was centrifuged after which two 0.5 ml samples of the aqueous phase for each perfusate sample were aliquoted into scintillation vials containing 4 ml Ecolite scintillant. These were then counted for the determination of total ^3H_2O content taking into account the dilution factor and any residual [^{3}H]palmitate. This procedure resulted in ~99% extraction and separation of ^3H_2O from [^{3}H]palmitate. Average rates of palmitate oxidation were expressed as μmol [^{3}H]palmitate oxidized · min^{-1} · g dry wt^{-1}.

2.5.8 Calculation of Tricarboxylic Acid Cycle Acetyl CoA Production and Efficiencies of Energy Production and Utilization

For each molecule of glucose and palmitate that is oxidized, two and eight molecules of acetyl CoA, respectively, are produced for TCA cycle activity. Thus, the rate of acetyl CoA production was calculated as the sum of acetyl CoA produced from the measured rates of glucose and palmitate oxidation[12,165]. The efficiency of energy production was evaluated as the amount of acetyl CoA produced for each O_2 molecule consumed. Efficiency of energy utilization was calculated as the LV work done per acetyl CoA produced.

2.6 Measurement of High Energy Phosphate Levels and Cellular Metabolites*

Frozen, pulverized tissue (100 mg) was subjected to perchloric acid extraction (PCA) by homogenization in 6% PCA and 0.5 mM EGTA (1 ml) with pre-cooled mortar and pestle. The homogenates were centrifuged (2,500 rpm x 10 min) and 0.95 ml of freshly prepared 0.32 mM DTT was added to the resulting supernatant. These solutions were neutralized with 5 M K_2CO_3 and then centrifuged (2,500 rpm x 10 min). The high-energy phosphate content and their metabolites in the supernatant were measured using HPLC technique as outlined previously in detail.[52,167]

* HPLC analyses were performed by Mr. Ken Strynadka.

2.7 Western Immunoblotting for AMPK Phosphorylation

The level of AMPK phosphorylation was used as an indication for the activation of the enzyme. To determine the phosphorylated state of AMPK in the pulverized tissue, 50-60 mg of heart tissue was homogenized using a polytron apparatus at high speed for 30 sec. The lysis buffer contained 0.1 M Tris-HCl (pH 7.5, 4 °C), and (mM), NaF (50), sodium pyrophosphate (5), EDTA (1), EGTA (1), DTT (1), 10% w/v glycerol, 0.02% sodium azide, protease and phosphatase inhibitors, Triton-X. The protein content ($\mu mol \cdot mg\ protein^{-1}$) of the homogenates was determined by the Bradford protein assay as previously described (see below). A portion of the homogenate was used for immunoblotting. To 0.6 ml homogenate was added 0.3 ml 3X protein sample buffer containing 30% glycerol, 6% SDS 0.1% bromophenol blue, 0.13 M Tris (pH 6.8) and 3% ß-mercaptoethanol. The resulting mixture was boiled at 95 °C for 5 min and diluted appropriately in 2X sample buffer. SDS- polyacrylamide gels (10% separating and 3.5% stacking) were prepared for the electrophoresis of protein. Protein samples (40 µg) and prestained molecular weight markers (5 µl) were loaded into wells and run at 60 V until samples have entered the separating gel after which the voltage was increased to 120 V constant. The samples were then run until the blue dye was just before the bottom of the gel.

After carefully cutting off the stacking gel, the protein was transferred onto nitrocellulose membrane at 100 V constant (4 °C for 2 hrs). Following complete transfer, the gel was stained with 0.1% Coomasie blue dye for 5 min and destained to observe the loading of the gel. The membrane was incubated in blocking buffer containing 0.1% tween-20 and 5% milk solution in Tris-buffered saline (20 mM Tris

base; 137 mM NaCl; pH 7.6) overnight with constant shaking. The milk was rinsed off using 0.1% tween-TBS the following day and the membrane was incubated with primary antibody (rabbit polyclonal, 1:1000 dilution in primary antibody dilution buffer (0.1% tween-20 and 5% BSA in TBS), generated against threonine-172 of AMPK, for 2 hrs at room temperature with constant shaking. After washing in TBS-tween, the membrane was then incubated in secondary antibody (goat anti-rabbit antibody diluted 1:2000 in TBS-milk-tween) for 1 hr and then washed thoroughly. To detect the antibody, the membrane was incubated with enhanced chemiluminescence (ECL, Amersham LIFE Sciences) for 1 min, and then developed on X-ray film for 15 sec to 15 min depending on the degree of intensity of bands.

To determine total AMPK, the membrane was stripped thoroughly in 20 ml strip buffer (2% SDS; 100 mM β-mercaptoethanol; 62.5 mM Tris-HCl, pH 6.7) for 2 hrs at 50 °C. The membrane was then washed and blocked in milk as in above and incubated with primary antibody (rabbit polyclonal; 1:1000 dilution in primary antibody buffer) developed against the α-subunit of AMPK for 3 hrs at room temperature. After a series of three washes, the procedure for the phospho-AMPK was followed to detect the total AMPK. The ratio of phosphorylated to the total AMPK was quantified by densitometric analysis.

2.8 Bradford Protein Assay

The protein concentration of heart extracts was assessed by the

Bradford assay.[52] To 10 µl of homogenate was added 40 µl of dH_2O. Diluted protein solution (2 µl) was aliquoted into 1 ml Bradford reagent prepared in triplicates and the absorbance determined against a standard curve of BSA (0-20 µg) at 595 nm after mixing.

2.9 Experimental Protocols

The protocols for the heart perfusions are shown in Fig 2.4. Three series of hearts (A, B and C) were perfused. All hearts were perfused with Krebs-Henseleit solution containing [^{3}H]glucose and [^{14}C]glucose unless noted otherwise. Series A consisted of hearts subjected to Langendorff perfusion only and frozen at the end of 15 min. Another series of hearts was perfused aerobically and frozen after 30 min of baseline aerobic perfusion to determine baseline function and tissue metabolite content prior to mild ischemia.

Hearts in series B and C were assigned randomly to either a control group or an adenosine group. For series B hearts, hearts were subjected to 30 min baseline aerobic perfusion and frozen at the end of 30 min period of mild ischemia. Finally, a third series of hearts (C) were subjected to a 30 min aerobic perfusion, followed by 30 min mild ischemia, and reperfused for a further 30 min prior to freezing. An additional series of hearts were perfused with solution containing [^{3}H]palmitate for the measurement of palmitate oxidation rates. When present, adenosine (100 µM) was infused through the injection port 5 min prior to the induction of mild ischemia and was present throughout the period of perfusion. Perfusate samples (3 ml) and hyamine samples (0.25 ml) were collected at 10 min intervals for the assessment of

myocardial metabolic rates, as were the recordings of mechanical function. Tissue glycogen contents were measured at the different frozen times shown (Fig 2.4). Glucose uptake ($\mu mol \cdot min \cdot g\ dry\ wt^{-1}$) was measured as an average rate during aerobic baseline function (0-30 min), mild ischemia (40-60 min) and reperfusion (70-90 min). Myocardial tissue content of phospho- AMPK and total AMPK were determined at the frozen times indicated.

2.10 Statistical Analysis

Results are expressed as mean ± S.E.M. Comparisons between groups were done by two-way repeated measures analysis of variance followed by Bonferroni's test. Values were considered significant if $P<0.05$.

Standard Perfusion Apparatus

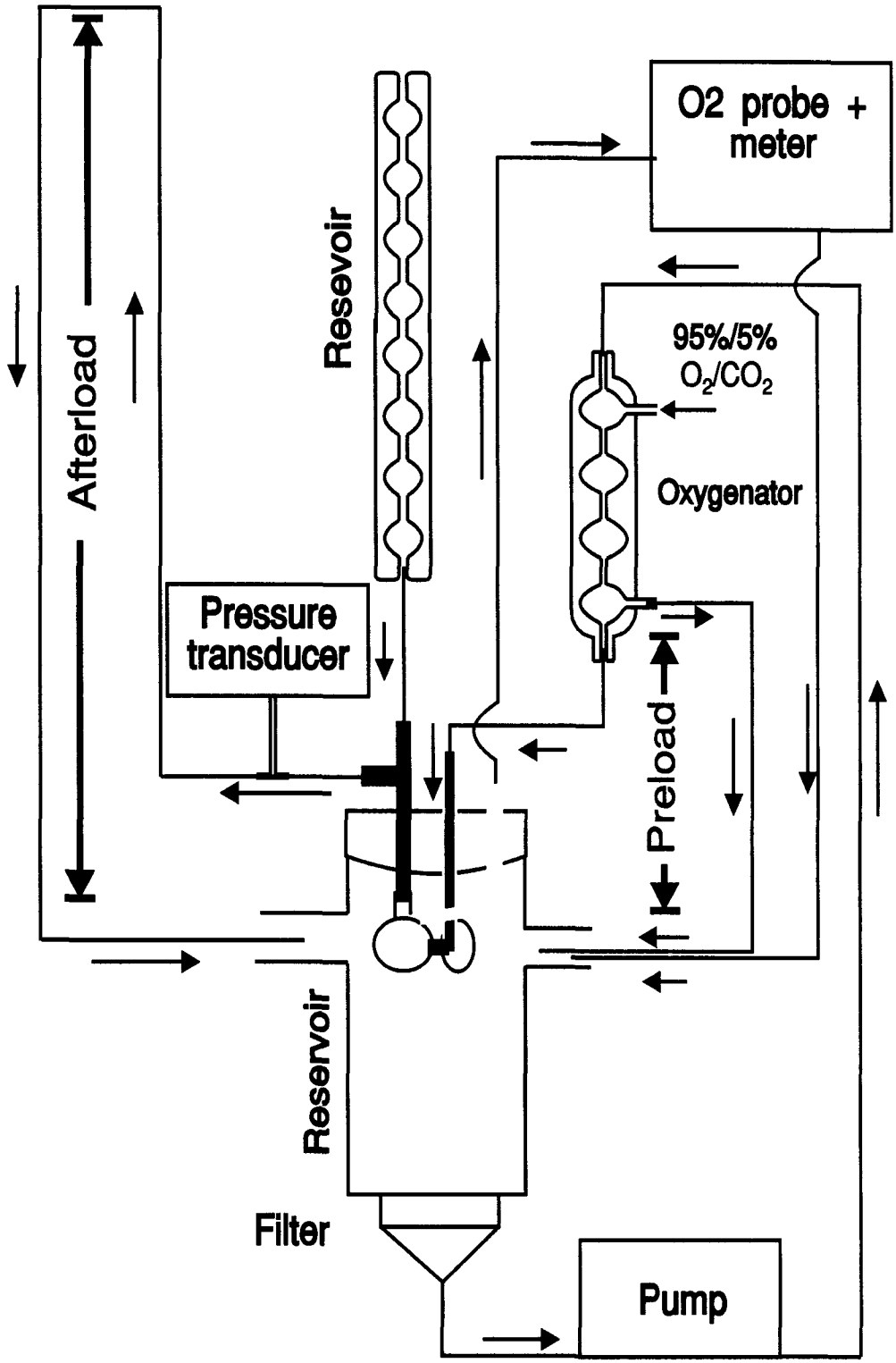

Fig 2-1. Schematic representation of the isolated heart perfusion apparatus. The diagram depicts a recirculation circuit whereby perfusate from the reservoir is pumped by the peristaltic pump into the thin-film oxygenator where it is gassed continuously. The perfusate via the preload line enters the left atrium and left ventricle, and is then ejected into the afterload line from where it returns to the perfusate reservoir. Following a 15 min of Langendorff (non-working) perfusion, the heart was switched into the working mode by clamping off the Langendorff line and opening the preload and afterload lines set at 11.5 mm Hg and 80 mm Hg, respectively. A pressure transducer connected to the aortic outflow line enabled the measurement of aortic systolic and diastolic pressures and heart rate. Cardiac output and aortic flow were measured by ultrasonic flow probes connected between the preload and afterload lines, respectively. Coronary flow was calculated difference between aortic flow and cardiac output.

Mild Ischemia Model *

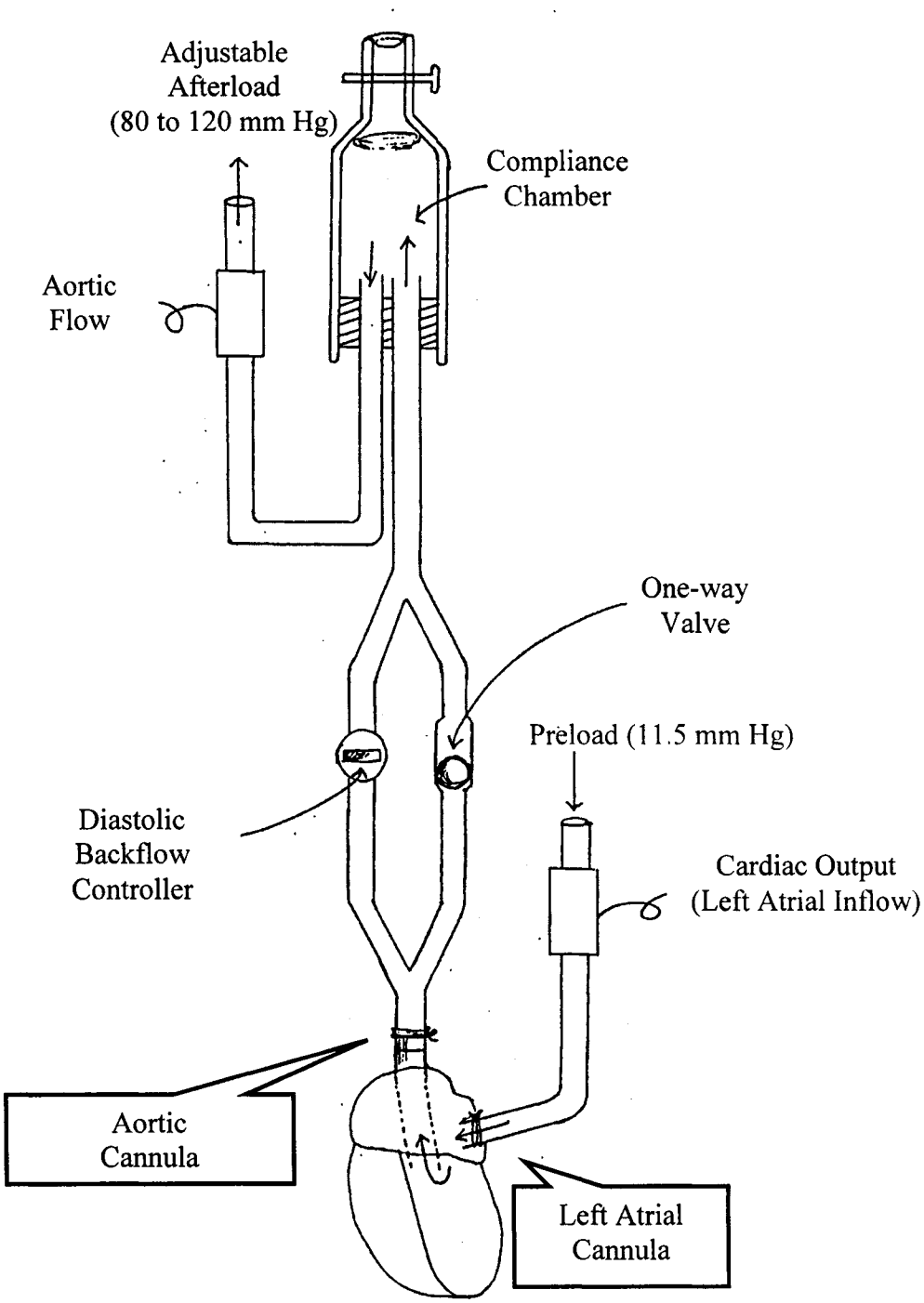

Fig 2-2. Diagrammatic representation of device used to induce mild ischemia. This device is inserted between the aortic cannula and the compliance chamber. The diagram illustrates how perfusate from the preload line enters the heart and via the aortic cannula is ejected into the compliance chamber, the volume of which has been adjusted to achieve an aortic developed pressure of 50-60 mm Hg. During the aerobic perfusion, the perfusate passes through both the diastolic backflow controller (3-way tap) and the one-way valve, which allows a unidirectional passage of perfusate. In the diastolic phase of cardiac function, the coronary circulation is perfused by perfusate returning only through the 3-way tap. Thus, in order to induce varying degrees of flow reduction (mild ischemia), the tap is partially closed until a coronary flow is attained which can still maintain contractile function. Induction of mild ischemia was confirmed by reduction of both coronary flow and aortic diastolic pressure. Reperfusion was achieved by returning the tap to its original state.

* Drawing by Dr Manoj Gandhi

Assessment of Glucose Uptake

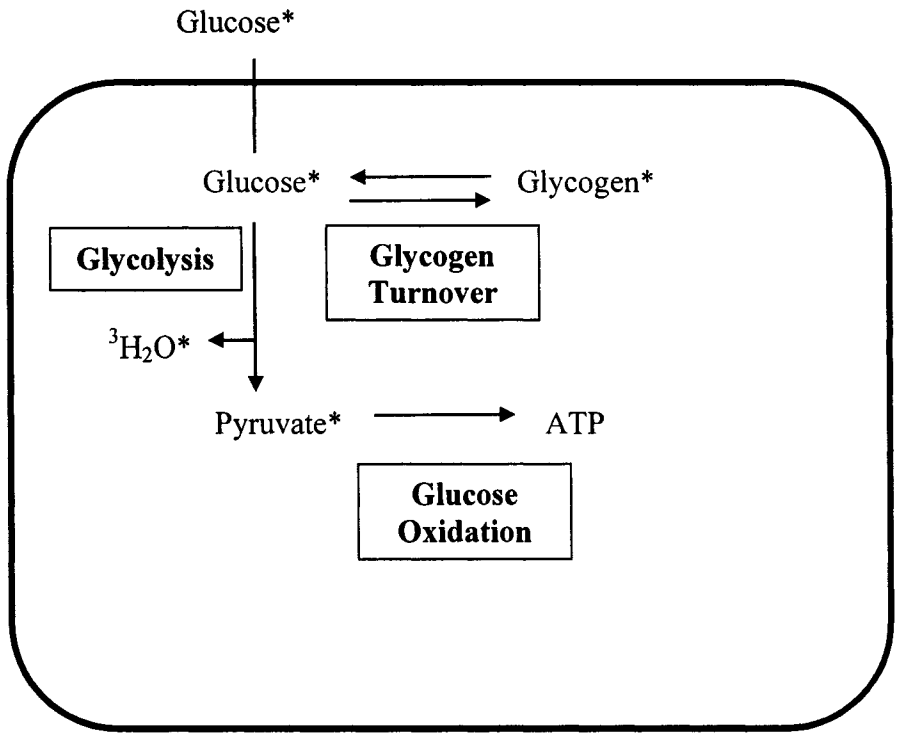

Fig 2-3. Schematic illustration of the assessment of glucose uptake. Glucose upon entry into cardiac cells is phosphorylated and metabolized to produce pyruvate (glycolysis) or stored in glycogen. The sum of the measured rate of incorporation of radiolabelled glucose into glycogen and the rate of glycolysis (quantitative measurement of ^3H_2O production) was used to assess the rate of glucose uptake.

* *Labelled glucose*

Experimental Protocols

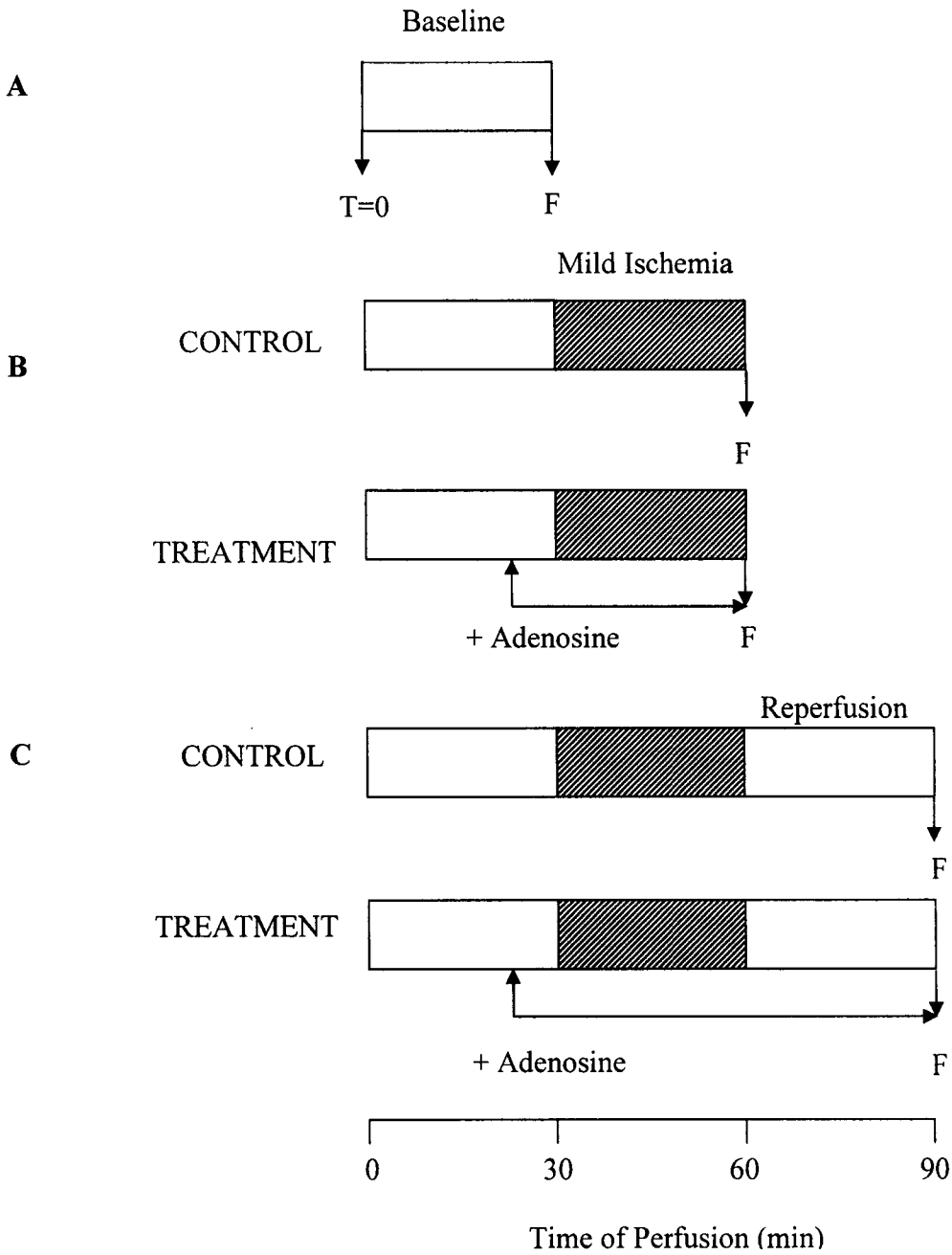

Fig 2-4. Protocol for series A, B and C heart perfusions. Series A shows hearts frozen at the end Langendorff perfusion (T=0) and after 30 min aerobic perfusion. Open bars represent aerobic baseline perfusion; Hatched bars, mild ischemia. Adenosine (Ado, 100 μM), when present, was added 5 min prior to mild ischemia. Downward pointing arrows (F), indicate times when hearts were frozen; upward pointing arrows, time of infusion of drug. See text for other details.

CHAPTER 3

Results

3.1 Effects of Adenosine on Mechanical Function

Fig. 3-1A, B and Fig. 3-2A and B show the effects of adenosine on CF, MVO_2, and the recovery of LV mechanical function and myocardial efficiency. During the initial 30 min period of normal aerobic perfusion, coronary flow, LV work, MVO_2 and myocardial efficiency were stable. Induction of mild ischemia resulted in about a 30% reduction in CF, which was accompanied, by a similar reduction in MVO_2. Under these conditions, LV work was impaired but remained steady and was decreased to approximately 50% of pre-ischemic baseline. Since the hearts were consuming more O_2 relative to work, myocardial efficiency was significantly impaired during mild ischemia (Fig 3-2B). During reperfusion, coronary flow recovered to about 80% of the pre-ischemic value (Fig 3-1A). Accordingly, MVO_2 recovered to a similar extent while LV work recovered slightly (Fig 3-1B and Fig. 3-2A). Hence, myocardial efficiency remained dramatically depressed during reperfusion (Fig 3-2A).

Addition of adenosine (100 μM) 5 min prior to the onset of mild ischemia had no effect on baseline aerobic function although it caused a transient increase in coronary flow mainly due to its vasodilatory properties (Fig 3-1A). During mild ischemia, the presence of adenosine did not alter any of the measurable mechanical function parameters. Upon reperfusion, adenosine caused a marked improvement in the recovery of both MVO_2 and LV work to approximately 96% and 77%, respectively, relative to aerobic control values (Fig 3-1B and Fig. 3-2A). The

enhancement of mechanical function by adenosine occurred in the absence of any significant effect on coronary flow. Thus, myocardial efficiency was improved during reperfusion by the presence of adenosine in the perfusate following 30 min mild ischemia compared to the control hearts (Fig 3-2B). This protective effect of adenosine was consistent with a previous study in the isolated rat heart that adenosine pre-treatment enhances post-ischemic LV function following severe ischemia.[83]

LV developed pressure (LVDP), coronary vascular resistance and heart rate peak systolic pressure product were not altered by mild ischemia and reperfusion in either group (Table 3-1). LVDP was however, significantly impaired during reperfusion, an effect which was significantly reversed by the presence of adenosine (Table 3-1). Since coronary flow was reduced to a similar extent during mild ischemia and recovered to almost similar levels during reperfusion in the two groups, it indicates that the adenosine-mediated improvement of LV work and myocardial efficiency post-ischemia is independent of the well known vasodilatory effect of adenosine. This observation was further supported by the lack of effect of adenosine on coronary vascular resistance (Table 3.1). This findings parallel previous report that adenosine does not affect hemodynamics of the working rat heart under aerobic conditions.[161]

3.2 Effects of Adenosine on Myocardial Metabolism

Fig. 3-3A and B show the effects of adenosine on the rates of glycolysis and glucose oxidation from the metabolism of exogenous glucose. The average rates of glycolysis and glucose oxidation during the 30 min period of baseline aerobic perfusion were similar to values reported previously for rat hearts perfused with 1.2 mM palmitate and paced at 5 Hz.[18,52,83,161] The average rate of glycolysis was similar in both groups during the initial 30 min baseline period of aerobic perfusion (Fig. 3.3A). As expected, the average rate of glycolysis increased during the 30 min period of mild ischemia (4.81 ± 0.26 vs. 3.88 ± 0.22 μmol · g dry wt^{-1} · min^{-1}, respectively, $P<0.05$) in the control group and this rate returned to pre-ischemic values during the subsequent 30 min period of reperfusion (Fig. 3.3A). Surprisingly, adenosine did not alter the steady state rate of glycolysis during mild ischemia. However, upon reperfusion, the presence of adenosine markedly inhibited the rate of glycolysis relative to the control value (2.49 ± 0.54 vs. 4.18 ± 0.33 μmol · g dry wt^{-1} · min^{-1}, $P<0.05$). The steady state rate of glucose oxidation was similar in both groups during baseline perfusion, mild ischemia and during reperfusion (Fig. 3-3B). Of interest is the observation that although MVO_2 decreased by about 30% of the baseline value during mild ischemia (Fig. 3-1A), oxidative metabolism of exogenous glucose remained unaltered (Fig. 3-3B). Since the rate of glycolysis increased during mild ischemia with no considerable effect on glucose oxidation, this resulted in a further imbalance of glucose metabolism. Thus, the calculated rate of proton production attributable to the metabolism of exogenous glucose (Fig. 3-4) significantly increased during mild ischemia in both groups (7.44 ± 0.58 vs. 5.41 ±

0.41 μmol · g dry wt^{-1} · min^{-1}, $P<0.05$). The increased rate of proton production was sustained throughout the period of reperfusion in the control hearts but adenosine dramatically reduced the rate of proton production during reperfusion (Fig. 3-4).

This observation strongly suggests that the impairment of mechanical function and myocardial efficiency during and following mild ischemia in the control hearts could be partly accounted for by the increased rate of proton production secondary to uncoupled glucose metabolism. Adenosine had no effect on glucose metabolism during mild ischemia, but dramatically improved glucose metabolism and lowered proton production during reperfusion, translating into enhanced recovery of mechanical function and efficiency.

As described previously,[166] fatty acid is the predominant energy substrate for the heart and contributes approximately 60-70% of the total rate of ATP generation. We subsequently assessed the effect of mild ischemia and reperfusion on the rate of palmitate oxidation in an additional group of hearts and addressed the role of adenosine on fatty acid oxidation. The rate of palmitate oxidation during baseline perfusion was similar in both groups (Fig. 3-5), and was identical to the value described for rat hearts perfused under similar conditions.[18] Palmitate oxidation rates were reduced modestly during mild ischemia and returned to pre-ischemic values during reperfusion. Adenosine did not modify palmitate oxidation during mild ischemia or reperfusion (Fig. 3-5). The 30% reduction of MVO_2 during mild ischemia could be accounted for by the modest reduction of palmitate oxidation. These data suggest that during periods of oxygen deprivation, oxidative metabolism of fatty acids is affected more compared with glucose metabolism.

3.3 Effects of Adenosine on TCA Cycle Acetyl CoA Production

Since hearts were perfused with radiolabelled glucose and palmitate in parallel perfusions, it is possible to determine the steady state rate of acetyl CoA production from both glucose and fatty acid oxidation in order to evaluate TCA cycle activity during baseline conditions, as well as during mild ischemia and reperfusion. As shown in Fig. 3-6, overall TCA acetyl CoA production was reduced during mild ischemia in control hearts and recovered to pre-ischemic values upon reperfusion. Adenosine did not affect TCA cycle activity during mild ischemia or reperfusion. An interesting observation seen in Fig. 3-6 is that even during mild ischemia residual oxidative metabolism of palmitate continues to be the predominant source of acetyl CoA. Moreover, the poor recovery of function and efficiency in control hearts is not attributable to depletion of energy production during reperfusion. Since total acetyl CoA production was not significantly different between the control and adenosine-treated hearts it indicates that the poor functional recovery of control hearts as well as efficiency is a consequence of either inefficiency in acetyl CoA production or utilization. To elucidate this mechanism further, we assessed both the efficiency of TCA acetyl CoA production and the efficiency of energy utilization. Fig. 3-7A shows that efficiency of energy production (acetyl CoA · MVO_2^{-1}) during mild ischemia and reperfusion was not different from pre-ischemic values. Furthermore, adenosine did not alter energy production efficiency during mild ischemia and reperfusion. Interestingly, efficiency of energy utilization (LV work · acetyl CoA^{-1}) was significantly decreased during mild ischemia and remained depressed during reperfusion (Fig. 3-7B) in control

hearts. The presence of adenosine prevented the inefficiency of energy utilization during mild ischemia and markedly improved this efficiency during reperfusion (0.83 ± 0.18 vs. 0.44 ± 0.09, $P<0.05$). This result was consistent with the impaired mechanical function and overall efficiency observed during and following mild ischemia in the control hearts.

3.4 High Energy Phosphate and Cellular Metabolite Levels

Myocardial contents of high-energy phosphates and other cellular metabolites are shown in Tables 3-2 and 3-3, respectively. Intracellular ATP content at the end of aerobic perfusion is similar to levels reported for rat hearts perfused with high fatty acid concentration (Table 3-2). As expected, mild ischemia resulted in a marked reduction of tissue ATP content in control hearts indicative of ischemic stress. This was further exacerbated throughout the period of reperfusion. Adenosine significantly preserved tissue ATP levels during mild ischemia and reperfusion. The level of CrP was not affected during mild ischemia but was significantly reduced upon reperfusion in control hearts. During reperfusion, adenosine maintained myocardial content of CrP. Tissue AMP content was not altered by mild ischemia and reperfusion in either group. During mild ischemia and reperfusion, Cr content was considerably reduced in control hearts and adenosine preserved the cellular content of this metabolite. Accordingly, there was no significant difference between the AMP to ATP ratio or the Cr to CrP ratio during mild ischemia and reperfusion. This further confirms the findings that adenosine does not alter the overall

myocardial high-energy phosphates, but rather enhances the efficiency of utilizing energy.

Adenosine and xanthine levels trended towards increase during mild ischemia and returned to preischemic levels after reperfusion in adenosine-treated hearts but were not altered in control hearts (Table 3-3). Interestingly, inosine, a breakdown product of adenosine, was elevated in adenosine-treated hearts during reperfusion relative to the control value. This indicated that a large portion of adenosine was metabolized during mild ischemia and reperfusion. ADP levels were high in adenosine-treated hearts during mild ischemia and after reperfusion compared to control hearts. GTP and hypoxanthine contents were not modified by mild ischemia and reperfusion and did not differ between control and adenosine-treated groups. IMP level increased time-dependently in control hearts but was not affected by the presence of adenosine (Table 3-3).

3.5 Glucose Uptake During and Following Mild Ischemia

Previous *in vivo*[54] and *in vitro*[35] animal studies have shown that severe no-flow ischemia stimulates intracellular glucose uptake, a view which forms the basis of intense investigation following other findings which failed to show any increase in glucose uptake during ischemia. These *in vitro*[35] animal studies were performed in quiescent cardiomyocytes with no energy demand or in whole heart experiments[131] during which myocardial work completely ceases due to the limitation of coronary flow. In this study we used a model of mild ischemia to assess glucose uptake under physiological workload and substrate availability. Myocardial glucose uptake was

measured as the sum of rate of incorporation of radiolabelled glucose into glycogen (index of glycogen synthesis) and the steady state rate of glycolysis, the two major pathways of exogenous glucose metabolism.

Fig. 3-9A shows the total glycogen content of hearts frozen at the end of Langendorff perfusion (time zero), 30 min aerobic, 30 min mild ischemia and after of 30 min reperfusion. Surprisingly, total glycogen content was not altered by mild ischemia and reperfusion in control hearts or by the presence of adenosine. The rate of incorporation of radiolabelled glucose into the intracellular glycogen pool was also determined to evaluate the net rate of glycogen synthesis. During mild ischemia, the amount of glucose incorporation into glycogen increased two-fold in the control hearts with a further increase during reperfusion indicative of increased glycogen synthesis (Fig. 3-9A). However, the actual extent of incorporation was not affected by mild ischemia. Adenosine did not modify glycogen synthesis during and following mild ischemia contrary to previous report[168] that showed an increase in glycogen content and synthesis by the presence of adenosine. Since total glycogen content was not altered by mild ischemia and reperfusion but mild ischemia resulted in an increase in the amount of glucose incorporation into glycogen synthesis, it may suggest a parallel degradation and synthesis of glycogen, (i.e glycogen turnover).

During aerobic perfusion, the average rate of glucose uptake is consistent with values reported for rat hearts perfused with a high concentration of fatty acid.[52] Although glucose uptake trended towards an increase during mild ischemia (6.45 ± 0.75 vs. 5.36 ± 0.59 $\mu mol \cdot g\ dry\ wt^{-1} \cdot min^{-1}$), it did not reach statistical significance in the control hearts (Fig. 3-9B). At the end of reperfusion, glucose

uptake returned towards pre-ischemic values. Adenosine had no effect on the average rate of glucose uptake during the periods of mild ischemia or reperfusion and values were similar to those of control hearts.

Glucose extraction during aerobic perfusion was 0.61 ± 0.08% and increased modestly during mild ischemia in both groups (Fig. 3-10B). Glucose extraction remained high in control and was decreased in adenosine-treated hearts.

Since the rate of glucose uptake may have been dependent upon myocardial energy demand, we normalized the rate of glucose uptake to LV minute work to assess uptake of glucose in relation to energy demand. Interestingly, glucose uptake per minute work increased dramatically (3-fold) during mild ischemia compared to aerobic values (Fig. 3-10). Normalized rate of glucose uptake returned towards pre-ischemic values during reperfusion. Adenosine had no effect on glucose uptake per LV minute work during mild ischemia compared to control hearts. During reperfusion, glucose uptake per LV minute work returned to baseline values and the presence adenosine decreased glucose uptake per LV minute work relative to control hearts (Fig 3-11).

3.6 Western Blot Analysis of AMPK

It has recently been reported that intracellular glycogen turnover and glucose metabolism is altered by AMPK activity,[27,84] a stress kinase shown to be activated during periods of energetic stress such as ischemia.[118] Upon stimulation by ischemia, AMPK is modified and modulates glucose uptake and fatty acid oxidation. AMPK

phosphorylation as an index of its activation was evaluated in the present study to determine its relationship with glucose uptake and energy substrate oxidation.

Fig. 3-11A is the western blot analysis of total and phosphorylated AMPK at the end of Langendorff perfusion, aerobic, mild ischemia and reperfusion. At the end of Langendorff perfusion, there was no phosphorylation of AMPK indicative of lack of stress during the initial surgical preparation of the hearts. AMPK phosphorylation increased significantly during the baseline working-mode aerobic perfusion relative to Langendorff hearts. During mild ischemia, AMPK phosphorylation increased significantly with a 2.5-fold increase in activation (Fig. 3-11B), consistent with increased glycolysis during mild ischemia (Fig 3-3A). This increase in AMPK phosphorylation occurred in the absence of any significant change in the AMP:ATP ratio of the myocardium. Upon reperfusion, AMPK phosphorylation returned towards pre-ischemic levels as indicated on the densitometric analysis of the western blots (Fig. 3-11B).

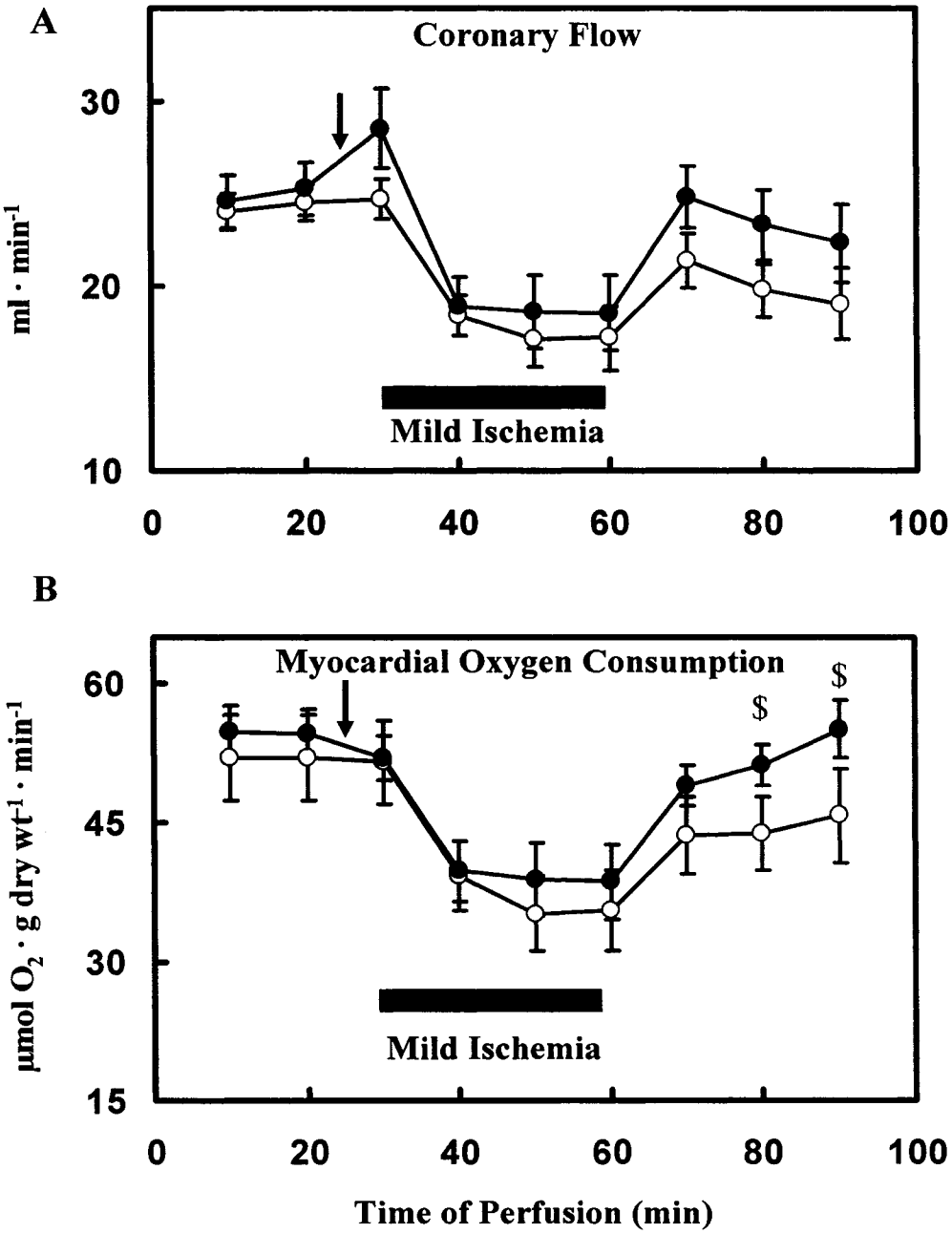

Fig. 3-1. Effects of adenosine on recovery of coronary flow (A) and MVO_2 (B) during reperfusion (70-90 min) following 30 min mild ischemia. Black bar represents period of mild ischemia; arrow, time of addition of adenosine. Values are mean ± S.E.M for hearts perfused in the absence (untreated, ○, n=15) or presence of adenosine (100 µM, ●, n=16). $ $P<0.05$ compared with untreated group.

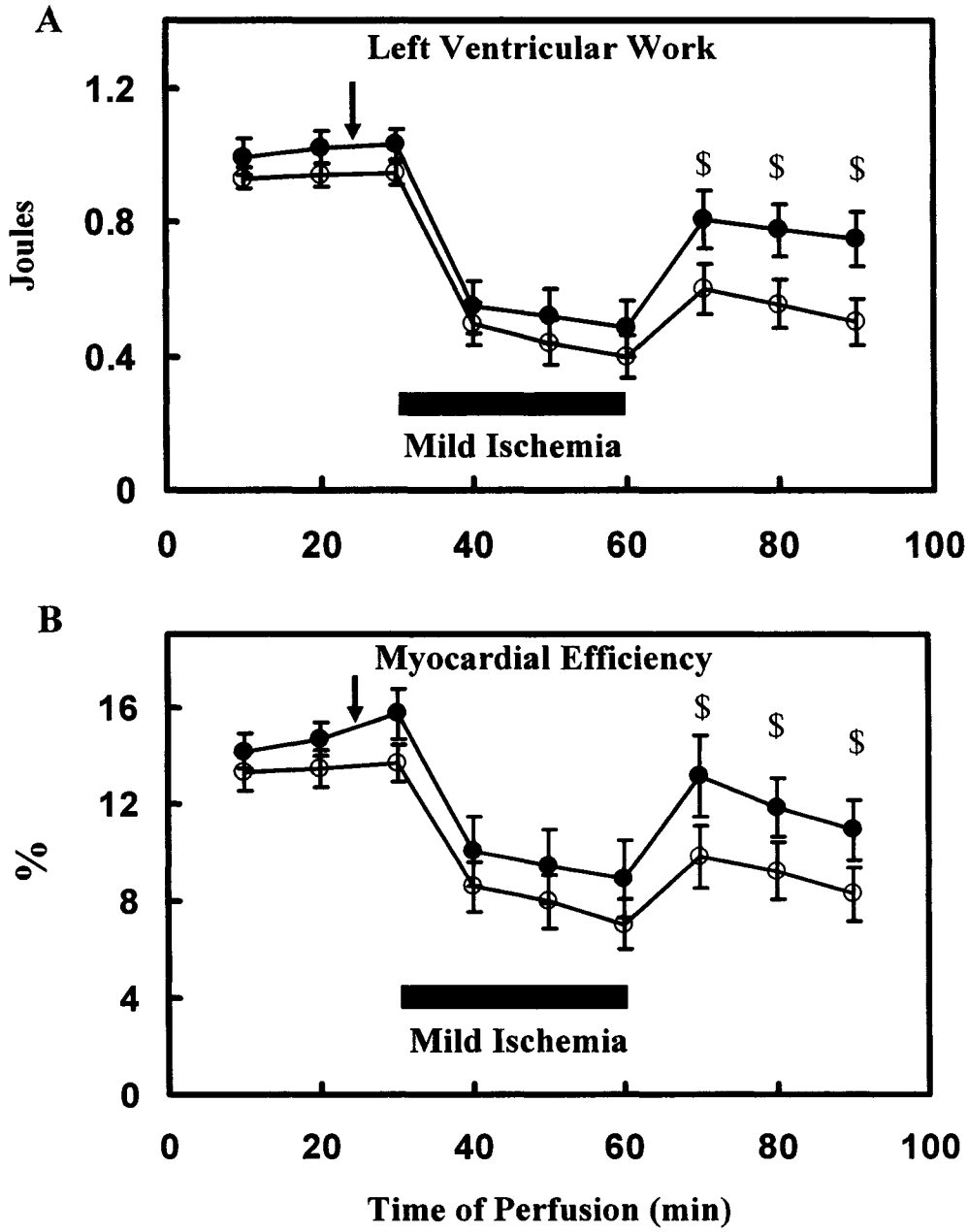

Fig. 3-2. Effects of adenosine on recovery of LV work (A) and cardiac efficiency (B) during reperfusion (70-90 min) following 30 min mild ischemia. Black bar represents period of mild ischemia; arrow, time of addition of adenosine. Values are mean ± S.E.M for hearts perfused in the absence (untreated, ○, n=15) or presence of adenosine (100 μM, ●, n=16). $ $P<0.05$ compared with untreated group.

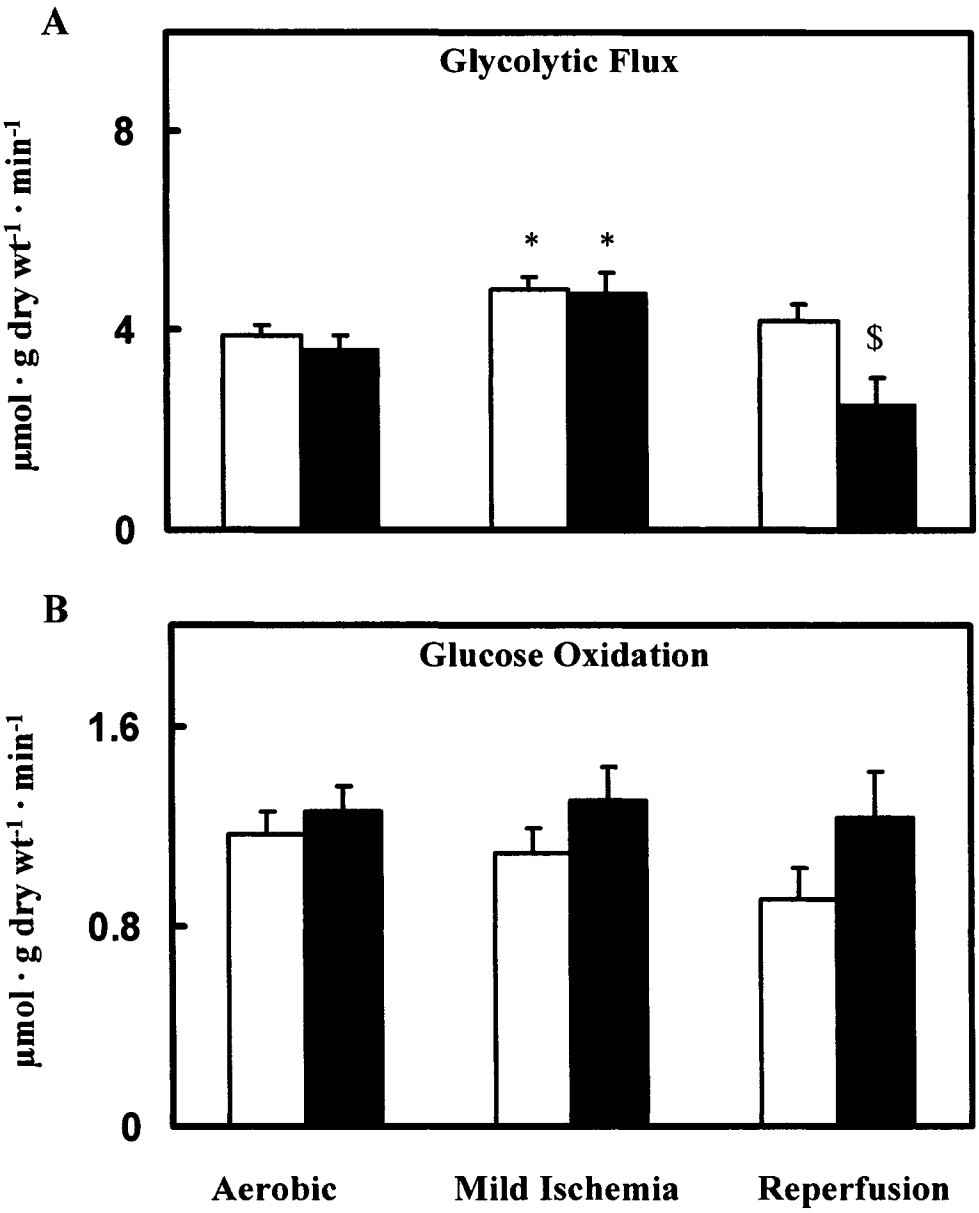

Fig. 3-3. Effects of adenosine on average rates of glycolysis (A) and glucose oxidation (B) during reperfusion (70-90 min) following 30 min mild ischemia. Values are means of averaged rate for each perfusion phase ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 μM, ■, n=16). * $P<0.05$ compared with aerobic group; $ $P<0.05$ compared with untreated group.

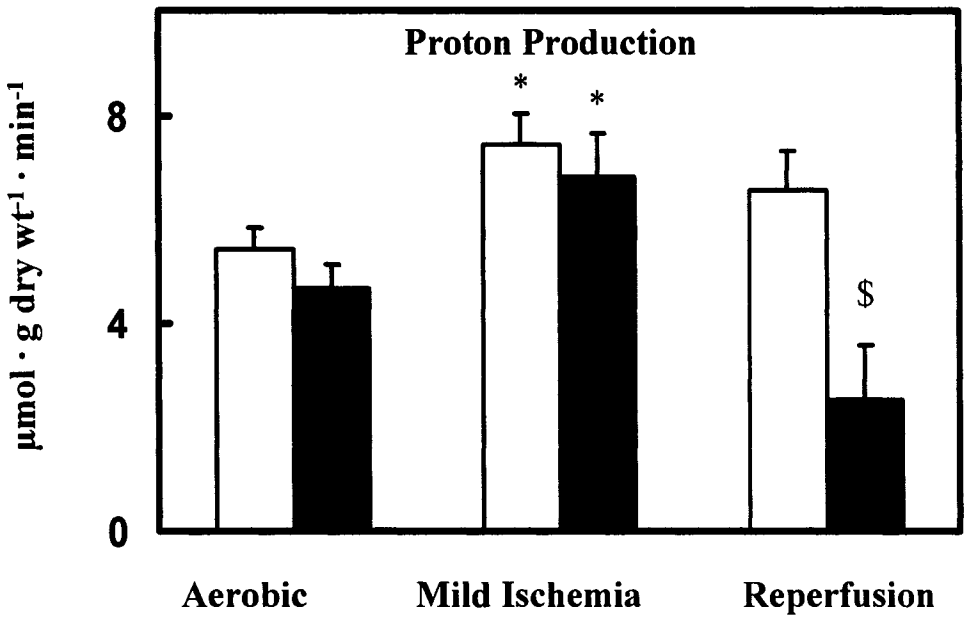

Fig. 3-4. Effects of adenosine on calculated rate of proton production during reperfusion (70-90 min) following 30 min mild ischemia. Values are means of averaged calculated rate for each perfusion phase ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 μM, ■, n=16). * $P<0.05$ compared with aerobic group; $ $P<0.05$ compared with untreated group.

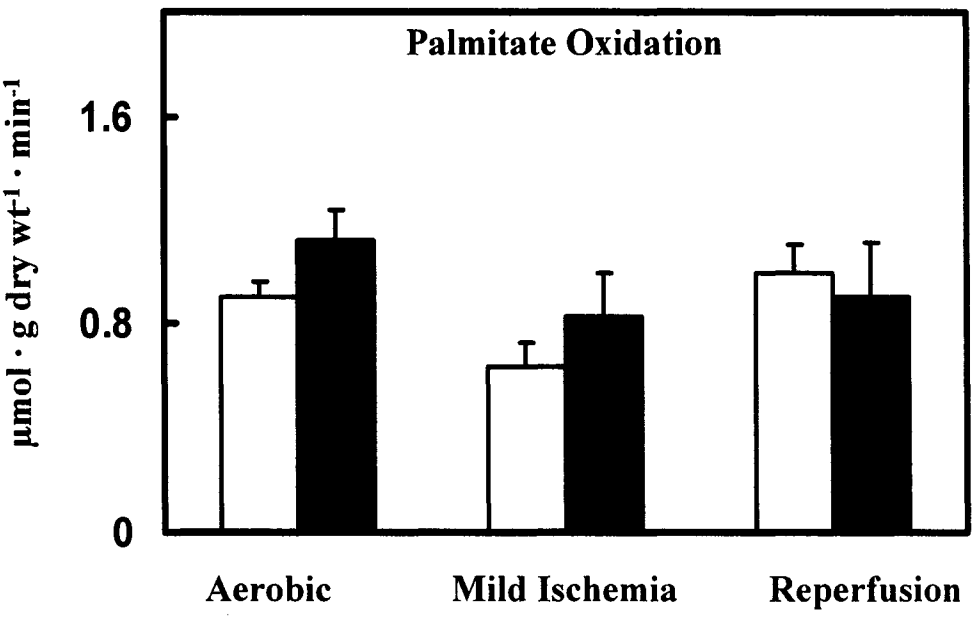

Fig. 3-5. Effects of adenosine on average rate of palmitate oxidation during reperfusion (70-90 min) following 30 min mild ischemia. Values are means of averaged rate for each perfusion phase ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 µM, ■, n=16).

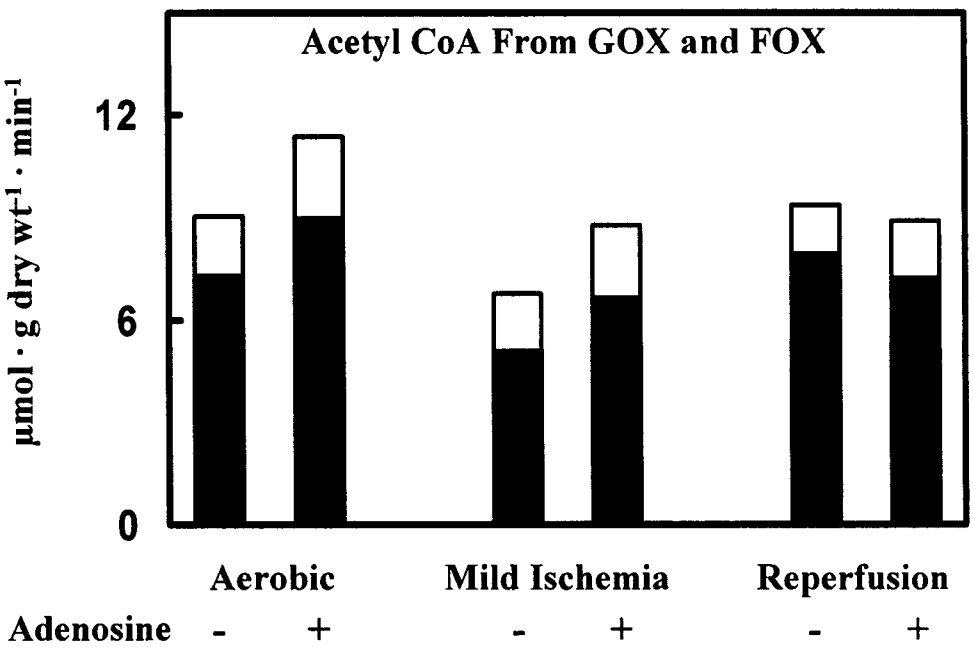

Fig. 3-6. Effect of adenosine on total acetyl CoA production from palmitate oxidation (■) and glucose oxidation (□) during reperfusion (70-90 min) following 30 min mild ischemia. Values are sums of the means of acetyl CoA produced from glucose oxidation and fatty acid oxidation of hearts perfused with 1.2 mM palmitate and 11 mM glucose in the absence (untreated, -, n=6) or presence of adenosine (100 µM, +, n=7).

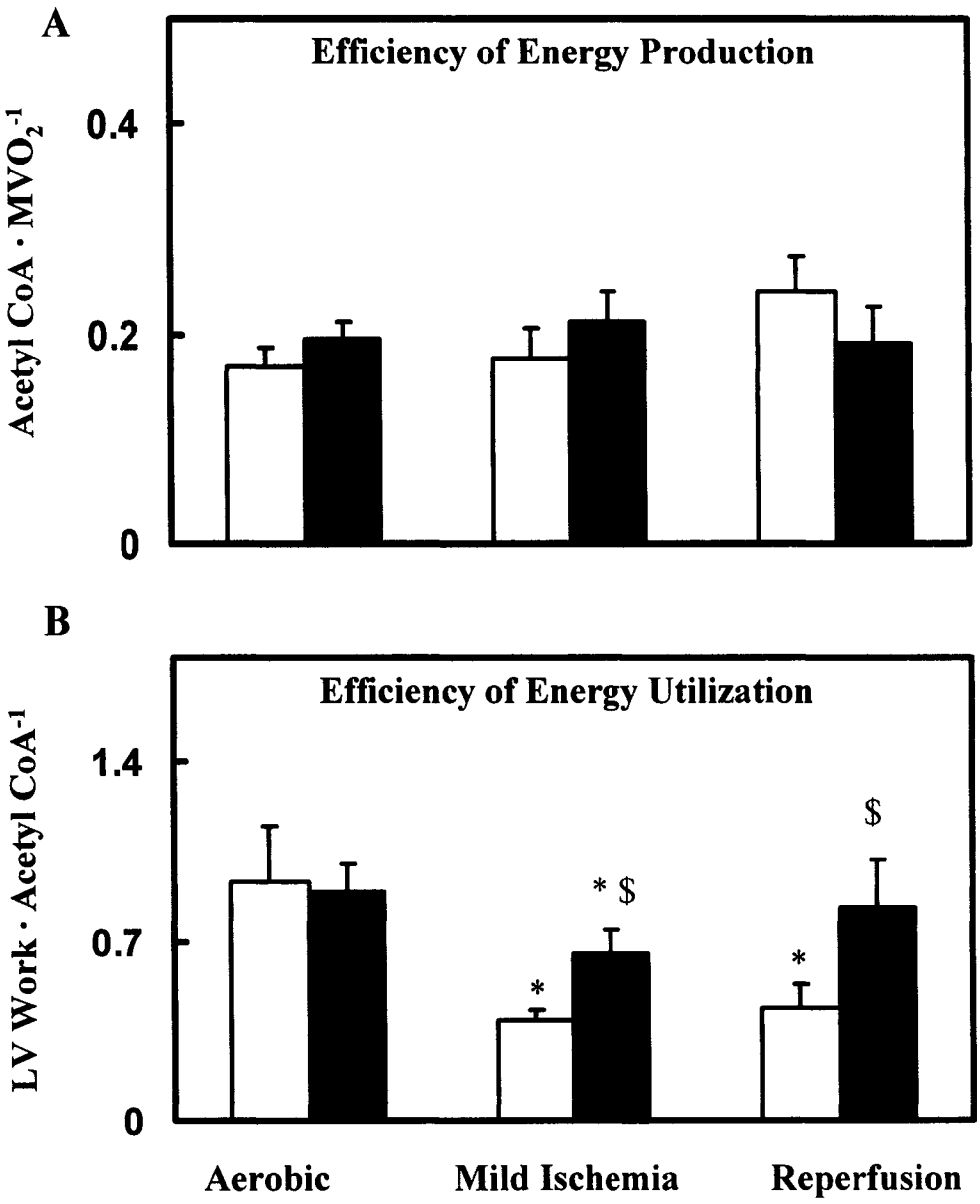

Fig. 3-7. Effects of adenosine on efficiency of energy production (A) and efficiency of energy utilization (B) during reperfusion (70-90 min) following 30 min mild ischemia. Values are means ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 μM, ■, n=16). * $P<0.05$ compared with aerobic group; $ $P<0.05$ compared with untreated group.

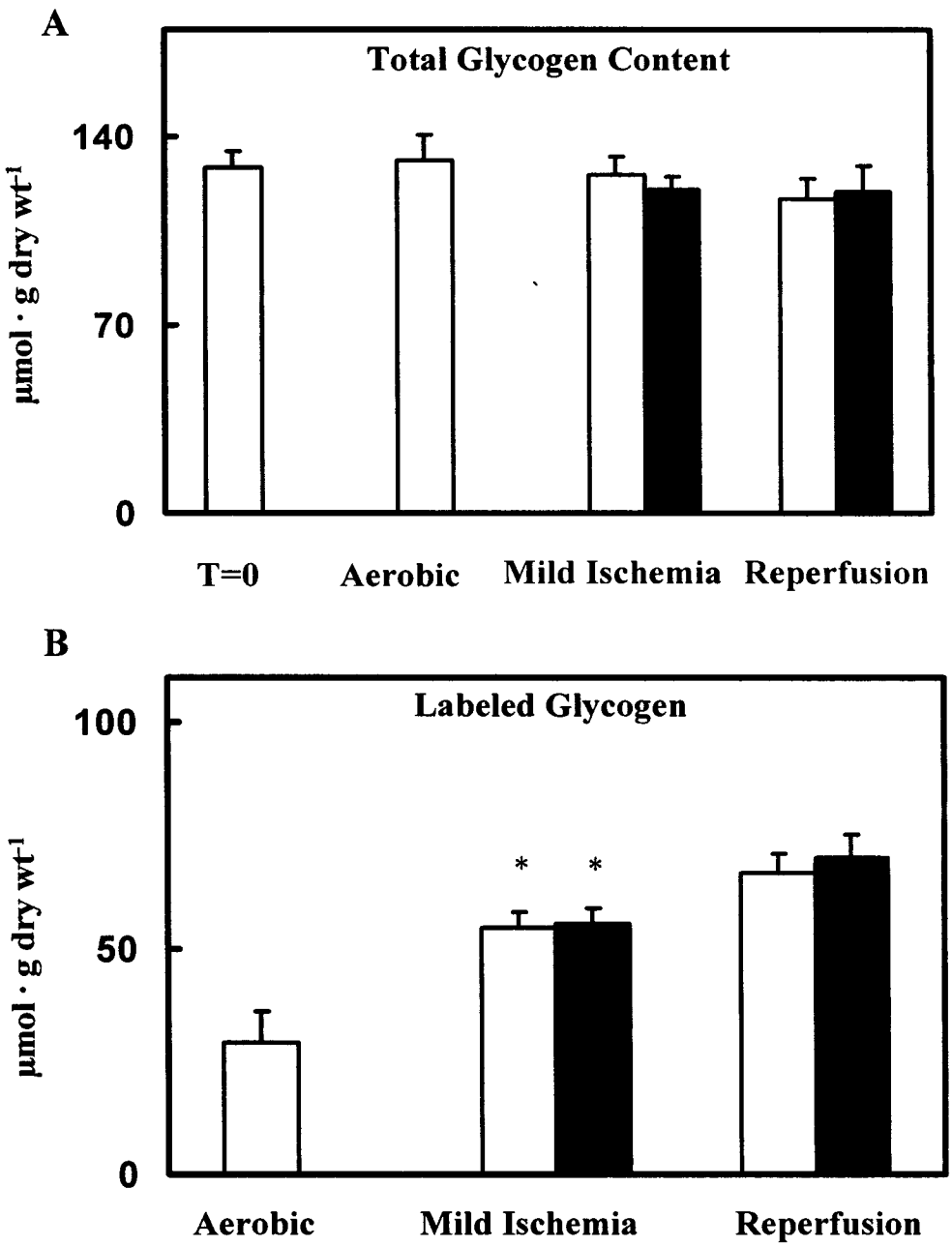

Fig. 3-8. Effects of adenosine on total glycogen content (A) and radiolabeled glycogen (B) during reperfusion (70-90 min) following 30 min mild ischemia. Values are means ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 μM, ■, n=16). * $P<0.05$ compared with aerobic group.

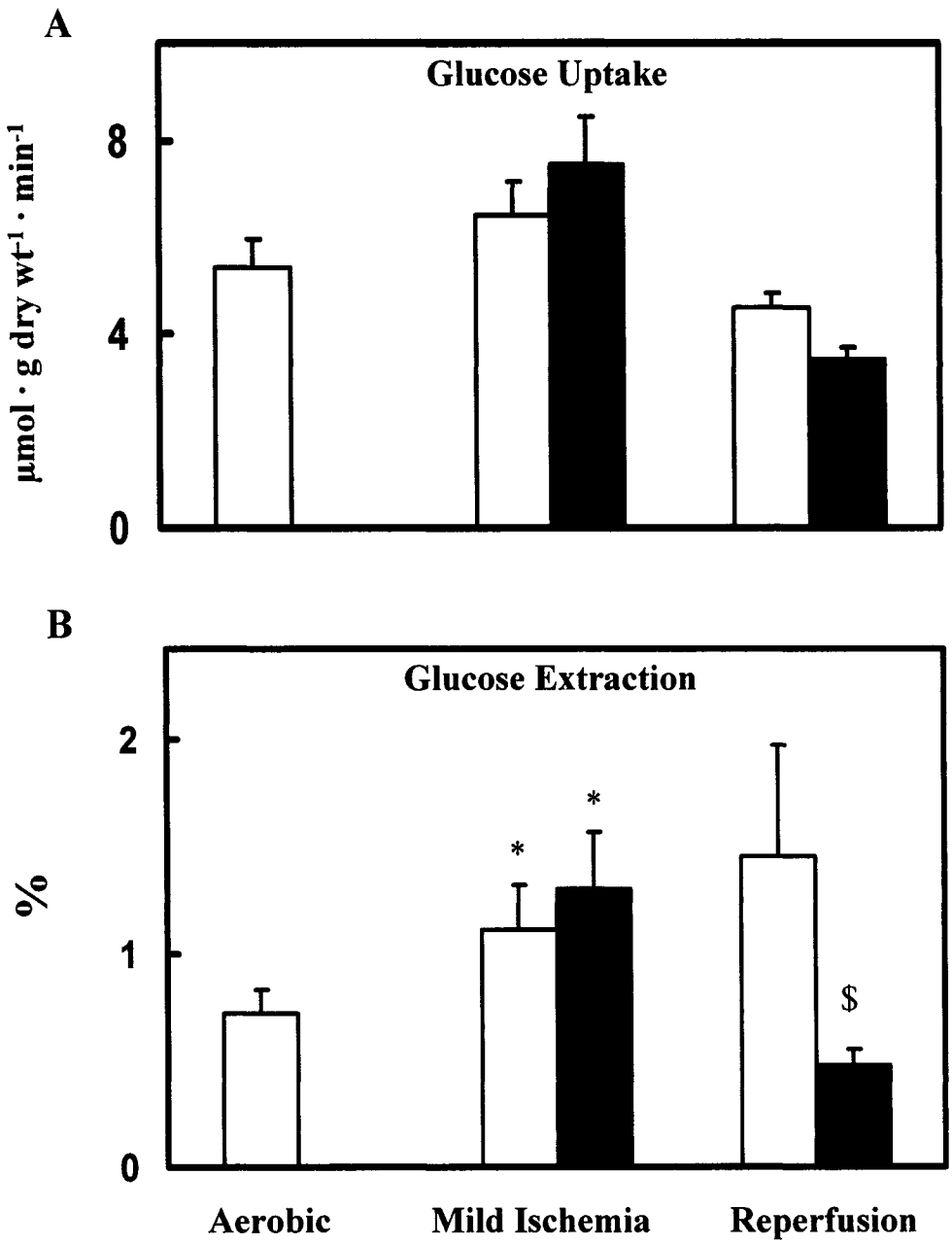

Fig. 3-9. Effects of adenosine on glucose uptake (A) and glucose extraction (B) during reperfusion (70-90 min) following 30 min mild ischemia. Values are means ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 µM, ■, n=16). * $P<0.05$ compared with aerobic group; $ $P<0.05$ compared with untreated group.

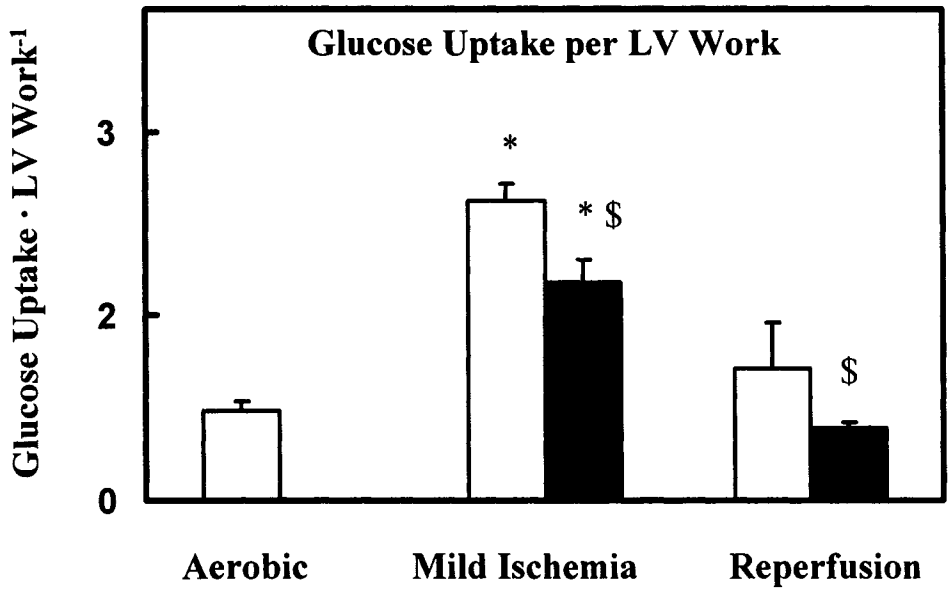

Fig. 3-10. Effects of adenosine on glucose uptake per unit work during reperfusion (70-90 min) following 30 min mild ischemia. Values are means ± S.E.M of hearts perfused in the absence (untreated, □, n=15) or presence of adenosine (100 μM, ■, n=16). * $P<0.05$ compared with aerobic group; $ $P<0.05$ compared with untreated group.

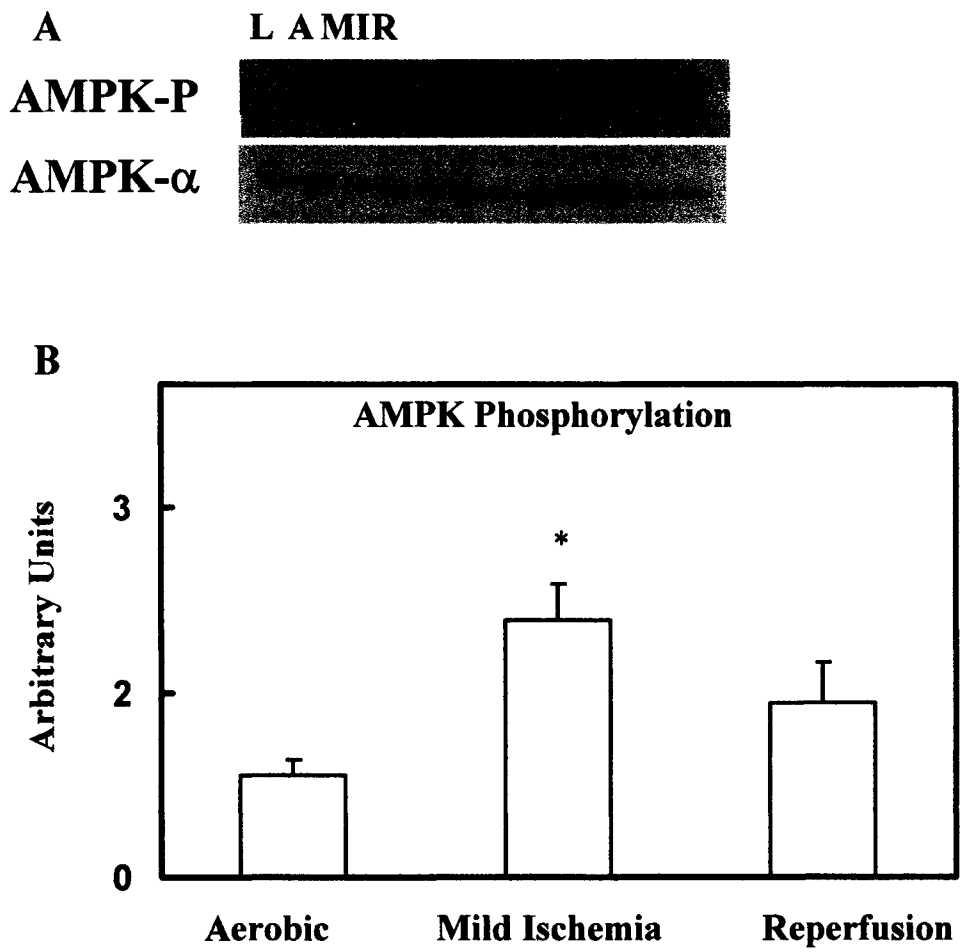

Fig. 3-11. Western blots (A) of total AMPK (AMPK-α), phosphorylated AMPK (AMPK-P) and densitometric analysis (B) of phospho-AMPK normalized for total AMPK at time zero (L, Langendorff), during aerobic baseline (A, 0-30 min) and reperfusion (R, 70-90 min) following 30 min mild ischemia (MI). Values are means ± S.E.M. * $P<0.05$ compared with aerobic baseline (n=4).

Table 3-1. Effects of adenosine on mechanical function during reperfusion (70-90 min) following 30 min mild ischemia.

Parameter Measured	Aerobic	Mild Ischemia	Reperfusion
LVDP (mm Hg)			
Control (16)	52.8 ± 1.9	61.0 ± 5.8	34.8 ± 3.6 *
Adenosine (15)	52.5 ± 1.7	62.1 ± 3.9	40.1 ± 3.3 * $
Coronary Vascular Resistance (ml/min/mm Hg)			
Control (16)	0.26 ± 0.01	0.24 ± 0.02	0.24 ± 0.02
Adenosine (16)	0.27 ± 0.02	0.26 ± 0.02	0.28 ± 0.02
Work (HR*PSP) (mm Hg/min*10^4)			
Control (16)	3.9 ± 0.04	3.4 ± 0.20	3.4 ± 0.11
Adenosine (16)	3.9 ± 0.05	3.3 ± 0.19	3.6 ± 0.10

Mechanical function was determined at 10 min intervals and averaged for each perfusion phase. Adenosine (100 μM) was added 5 min prior to the onset of mild ischemia and was present throughout the perfusion period. Data are mean ± S.E.M; values in closed bracket represent n observations; *$P<0.05$ compared with aerobic group, $ $P<0.05$ compared with untreated group.

Table 3-2. Effects of adenosine on myocardial high-energy phosphate content of hearts frozen at the end of reperfusion (70-90 min) following 30 min mild ischemia.

	Langendorff	Aerobic	Mild Ischemia	Reperfusion
ATP (μmol/g dry wt)				
Control (6)	28.7±2.3	28.1±2.7	18.6±1.3*	15.2±.0.7
Adenosine (6)			23.6±1.2*	19.3±1.7
AMP (μmol/ g dry wt)				
Control (6)	1.64±0.21	2.05±0.16	1.64±0.16	1.78±0.19
Adenosine (6)			2.23±0.17	2.38±0.23
AMP/ATP				
Control (6)	0.06±0.0	0.08±0.01	0.09±0.01	0.12±0.01
Adenosine (6)			0.01±0.01	0.12±0.01
CrP (μmol/ g dry wt)				
Control (6)	86.3±5.3	49.8±5.9	50.1±9.6	36.6±3.9
Adenosine (6)			62.6±6.7	49.5±6.6
Cr (μmol/ g dry wt)				
Control (6)	73.6±5.9	88.4±7.2	75.5±4.8	68.1±4.3
Adenosine (6)			82.0±4.4	73.9±4.0
Cr/CrP				
Control (6)	0.86±0.05	1.84±0.13	1.72±0.23	2.02±0.33
Adenosine (6)			1.39±0.17	1.60±0.35

Data are mean ± S.E.M; values in closed bracket represent n observations; * $P<0.05$ compared with aerobic group.

Table 3-3. Effects of adenosine on myocardial cellular metabolite content of hearts frozen at the end of reperfusion (70-90 min) following 30 min mild ischemia.

	Langendorff	Aerobic	Mild Ischemia	Reperfusion
ADP (μmol/g dry wt)				
Control (n=6)	8.6±1.1	11.21±1.1	7.5±0.4	7.0±.0.2
Adenosine (n=6)			9.2±0.6	9.2±1.1
Adenosine (μmol/ g dry wt)				
Control (n=6)	0.14±0.01	0.2±0.02	0.26±0.09	0.11±0.04
Adenosine (n=6)			0.45±0.06	0.18±0.03
IMP (μmol/g dry wt)				
Control (n=6)	0.04±0.01	0.12±0.01	0.20±0.09	0.45±0.31
Adenosine (n=6)			0.45±0.07	0.40±0.13
Inosine (μmol/g dry wt)				
Control (n=6)	0.07±0.05	0.03±0.01	0.06±0.02	0.04±0.02
Adenosine (n=6)			0.13±0.02	0.16±0.04
Xanthine (μmol/g dry wt)				
Control (n=6)	0.57±0.2	0.41±0.09	0.68±0.19	1.48±0.43
Adenosine (n=6)			1.29±0.18	1.94±0.43
Hypoxanthine (μmol/g dry wt)				
Control (n=6)	0 (6)	0.14±0.14	0	0.08±0.03
Adenosine (n=6)			0	0.14±0.05
GTP (μmol/g dry wt)				
Control (n=6)	1.19±0.08	1.28±0.11	1.19±0.1	1.05±0.05
Adenosine (n=6)			1.31±0.06	1.00±0.11

Data are mean ± S.E.M; values in closed bracket represent n observations; * $P<0.05$ compared with aerobic group.

CHAPTER 4

Discussion

4.1 Major Findings

In this study, we describe a novel heart model of mild ischemia and reperfusion whereby mild ischemia is induced by reducing coronary flow to such an extent that LV mechanical function will remain stable but at an impaired level. The uniqueness of this model lies in the ability to evaluate simultaneously the relationship between alterations of cardiac energy substrate metabolism, LV mechanical function and efficiencies of O_2 and energy utilization during the critical period of ischemia and under conditions of physiological workload and substrate availability. The cardioprotective efficacy of adenosine during and following mild ischemia and its effect on energy substrate utilization was also assessed. This model afforded the ability to determine directly glucose uptake during mild ischemia and reperfusion. In general, this model of ischemia was developed to simulate the clinical situation of angina in a controlled *ex vivo* environment in order to enhance our understanding of myocardial substrate modification during the actual ischemia insult and upon reperfusion.

The period of mild ischemia in the isolated working rat hearts was characterized by a marked depression of LV work and the efficiency of oxygen utilization. During reperfusion following mild ischemia, coronary flow and MVO_2 returned to 80% of aerobic baseline, but LV mechanical function and hence, efficiency, remained impaired. This implies that the depression of LV work and efficiency may a consequence of flow-independent mechanisms.

To assess directly the influence of mild ischemia and reperfusion on energy substrate metabolism, hearts were perfused with solution containing radiolabelled glucose and palmitate. We demonstrated that mild ischemia caused a modest reduction of palmitate oxidation which returned to baseline values during reperfusion. The rate of glycolysis increased during mild ischemia and recovered to pre-ischemic values during the subsequent reperfusion phase. Glucose oxidation was not altered during mild ischemia but trended towards a decrease upon reperfusion. Hence, the rate of proton production attributable to the metabolism of exogenous glucose was accelerated during mild ischemia and reperfusion as a result of uncoupled glucose metabolism. This may account for the depression of LV work and efficiency as previously demonstrated by Liu *et al*[9] in the isolated rat heart following severe ischemia.

In addition, we showed that mild ischemia resulted in a reduction of overall TCA cycle acetyl CoA production which recovered to baseline values during reperfusion. Interestingly, just as seen during the initial aerobic perfusion, palmitate oxidation was the predominant energy substrate during mild ischemia. Surprisingly, it was revealed that during mild ischemia and reperfusion, the efficiency of energy production as assessed by acetyl CoA per O_2 consumed, was not affected. However, during mild ischemia, efficiency of energy utilization was dramatically depressed and remained impaired during reperfusion post-ischemia. Myocardial tissue ATP content was markedly reduced during mild ischemia with a further decrease during reperfusion, an indication of ischemia stress. The level of CrP was not altered by mild ischemia but was reduced during reperfusion.

To address the issue of potential changes in glucose uptake during the critical ischemic insult, we examined the rate of incorporation of radiolabeled glucose into myocardial glycogen. Surprisingly, neither mild ischemia nor reperfusion altered total myocardial glycogen content. Although the amount of incorporated glucose into glycogen increased during mild ischemia and reperfusion, mild ischemia did not affect the average rate of glucose incorporation into glycogen. This suggests that the net rate of glycogen synthesis was not influenced by mild ischemia, but that glycogen turnover continued during mild ischemia. A major limitation of our approach of assessing glycogen content is the fact that it was measured at the end of an entire 30 min period, instead of on a minute to minute basis. This may partly account for the lack of effect of mild ischemia because glycogen turnover varies widely depending on varying stress conditions.

In contrast to previous data suggesting that ischemia enhances glucose uptake,[27,35] under the conditions used in the present study, it was demonstrated that during mild ischemia and reperfusion post-ischemia the average rate of glucose uptake was not altered. Interestingly, we showed that during mild ischemia, there was a marked increase in normalized rate of glucose uptake which returned towards pre-ischemic values during reperfusion. In order to elucidate the regulatory role of AMPK on energy substrate metabolism, we measured AMPK phosphorylation as an index of its activity in hearts extracts frozen at the end of each perfusion phase. We demonstrated that AMPK phosphorylation was elevated during mild ischemia independent of changes in the AMP:ATP ratio, which has been shown previously to

provoke AMPK activation.[118] During reperfusion, AMPK phosphorylation returned to baseline aerobic levels.

From the observation that increased rates of proton production during mild ischemia and reperfusion may be responsible for the impairment of LV function and efficiency, we further assessed the cardioprotective potential of adenosine during and following mild ischemia. Adenosine given prior to mild ischemia did not affect baseline aerobic function, although it did cause a transient increase in coronary flow, an indication of its vasodilatory property. During mild ischemia, adenosine did not alter LV work, MVO_2 or myocardial efficiency. During reperfusion, adenosine enhanced recovery of LV work and efficiency, irrespective of any effect on coronary flow. In accordance with previous observations in models of severe ischemia by Finegan *et al*[18,83] and Fraser *et al*,[52] this protective effect of adenosine was associated with a reduction in proton production from hydrolysis of glycolytically-derived ATP during reperfusion. Adenosine did not modify glucose oxidation during mild ischemia but it did improve the coupling of glycolysis to glucose oxidation during reperfusion, an effect that translated into improved LV work and efficiency.

Adenosine-mediated cardioprotection during reperfusion involved an improvement in the efficiency of energy utilization, but not the efficiency of energy production during mild ischemia itself. This improvement in efficiency during reperfusion may account for the enhanced recovery of LV work and efficiency of O_2 utilization seen at reperfusion. These observations suggest that loss of coupling between glycolysis and glucose oxidation during mild ischemia, with the resultant accumulation of intracellular protons, could account for the impairment of LV work

observed during mild ischemia and reperfusion in control hearts. Thus, an increased proportion of the energy generated during mild ischemia and reperfusion may be diverted to correct the acidosis-induced ionic imbalances rather than to support contractile activity.

Our data suggest that uncoupling glycolysis from glucose oxidation leading to increased rate of proton production during mild ischemia and reperfusion contributes to myocardial injury and impairs recovery of work and efficiency. Thus, inhibition of flux through glycolysis could be an attractive target of pharmacotherapy in treating patients presenting with clinical ischemia such angina pectoris.

The effect of adenosine on myocardial glucose uptake was assessed during mild ischemia and reperfusion. It was demonstrated that during mild ischemia and reperfusion, adenosine had no effect on total glycogen content during mild ischemia and reperfusion. The average rate of glucose uptake was not altered by adenosine during mild ischemia and reperfusion. This implies that the inhibitory effect of adenosine on glycolysis does not involve alteration of the transport process, but may likely involve its effect on a glycolytic enzyme, possibly PFK-1.

4.2 Experimental Approach

In the present study, we describe a model of ischemia which we termed "mild ischemia" in an attempt to simulate the clinical situation of angina. Although it may not be the direct representation of the clinical scenario, this model enables the direct evaluation of the relationship between myocardial energy substrate metabolism in concert with mechanical function during the actual period of ischemia. The rats were

allowed unlimited assess to food and water until the time of sacrifice in order to maintain endogenous contents of glycogen and triglyceride. This was important because alteration in the content of these storage molecules markedly influences LV work and energetic status during *ex vivo* perfusion.[52,131] A high concentration of fatty acid was also present in the perfusate to simulate the levels of fatty acid seen in the plasma of patients with clinical ischemia.[4,5,6,84]

The value of baseline LV work was consistent with values obtained in previous studies using rat hearts perfused under similar conditions.[18,52,83,161] This indicates that the apparatus for inducing mild ischemia does not affect baseline function, hence, during mild ischemia, any changes observed are a consequence of the experimental design and not due to alteration in baseline function. To overcome the negative chronotropic effect of adenosine, pacing was maintained throughout the period of perfusion in both of the experimental groups. The mode of inducing mild ischemia is unique because unlike previous models of mild ischemia *in vitro*,[130,131] hearts were working against an afterload comparable to that seen physiologically. Moreover, observations from this model of mild ischemia may not be complicated by the interference of endogenous metabolites, which is a major setback in interpreting results from *in vivo* models of mild ischemia. Thus, the model of mild ischemia used in the present study enabled elucidation of cardiac energy metabolism during the critical period of ischemia which has hitherto remained largely uncharacterized.

The relationship between mechanical function, MVO_2 and energy production has not been well established during the critical ischemic episode. This mild

ischemia model provides the ability to assess the link between these parameters and how they are altered during mild ischemia. This study may thus, represent the *ex vivo* equivalent of angina, whereby ischemia is indicated by a moderate reduction in blood supply to the cardiac muscle and which underlies the symptoms experienced by angina patients. It must be emphasized, however, that clinically, angina manifests as a regional ischemia (heterogeneous ischemia) as compared to the present model which may represent a global homogeneous ischemia.

A major advantage of this model is the ability to measure directly glucose uptake during mild ischemia. In the present study, assessing directly glucose uptake as the sum of the average rates glycolysis and glycogen synthesis, which are the two major pathways involved in exogenous glucose metabolism, may be a truer representation of the physiological situation.

Our model of ischemia had some limitations. First, it failed to show significant changes in oxidative phosphorylation as evidenced in lack of effect on glucose oxidation and only a modest reduction in palmitate oxidation, which have been shown in the classical severe low- and no-flow ischemia models to be halted completely. During severe ischemia, all measurable mechanical function ceases with complete halting of coronary flow and MVO_2. In this mild ischemia model, since oxidative metabolism was not altered considerably, it may be argued whether ischemia was actually present in the myocardium. During the subsequent reperfusion phase, however, the poor recovery of function and efficiency may suggest an injury resulting from the preceding ischemic insult. Prolonging the extent of the ischemia

period and the severity of reduction in coronary flow could be one strategy to overcome this limitation.

Another indicator of ischemic stress is increased glycogenolysis during severe global ischemia, an effect which was not shown in the present study. From the present data, it was shown that during mild ischemia, glucose delivery was not limiting. Hence, the myocardium relied on the provision of exogenous glucose to support glycolysis instead of conversion into intracellular glycogen. This could account for the lack of effect on glycogen breakdown seen in the present study. Our data demonstrating glycogen turnover and reduced tissue ATP content during mild ischemia do indicate the presence of ischemic and/or metabolic stress.

4.3 Effect of Adenosine on Mechanical Function and Efficiency During Mild Ischemia

In this study, LV work was used as an index of cardiac mechanical function as previously described.[8,9,52,165] The values of coronary flow and MVO_2 seen during baseline aerobic perfusion were in agreement with numerous previous studies which used this model of isolated rat heart perfusion.[54,186]

Following stable LV work during aerobic perfusion, function was markedly impaired during mild ischemia during the reduction in coronary flow. Important to this observation is the finding that work was steady but impaired throughout the period of 30 min mild ischemia, suggesting that the reduction in coronary flow was sufficient to sustain a new steady state of LV mechanical function.

Our data revealed that adenosine failed to alter LV work during mild ischemia. There have not been any previous studies to investigate the effect of adenosine on mechanical function during the actual period of ischemia. In a low-flow model of severe ischemia in the isolated rat heart, during which coronary flow was reduced to 0.1 ml/min, adenosine given prior to ischemia did not reverse the depressed heart LV function during the ischemic period.[18,83]

The efficiency of oxygen utilization was severely impaired during mild ischemia corresponding to the reduction in coronary flow. Adenosine had no effect on MVO_2 during mild ischemia, and thus did not alter myocardial efficiency.

4.4 Effect of Adenosine on Mechanical Function and Efficiency During Reperfusion

During reperfusion post-ischemia, untreated hearts showed poor recovery of mechanical function relative to adenosine-treated hearts. The beneficial effect of adenosine on mechanical function was independent of coronary flow since the level of flow returned to similar levels in both groups. This suggests that the poor recovery of function in control hearts could be a result of injury that was initiated during the period of mild ischemia or early reperfusion. Our data parallel findings from previous studies which showed that administration of adenosine or an A_1-adenosine receptor agonist before the onset of low-flow or global no-flow ischemia improves functional recovery post-ischemia.[18,52,83,153,154] Peart *et al* (2001)[169] demonstrated in the isolated mouse heart that increasing endogenous adenosine levels with adenosine metabolism inhibitors enhanced recovery of left ventricular

developed pressure (LVDP) following a 20-min period of severe ischemia. Recently, the same group demonstrated that adenosine given during ischemia improved recovery of LVDP in the Langendorff perfused mouse heart although the extent of recovery was less compared with the group in which adenosine was given during ischemia and reperfusion.[170] Fralix *et al* (1993)[154] also showed that adenosine administration before a period of severe ischemia delayed the onset of contracture and improved LVDP during reperfusion of Langendorff perfused rat hearts. The cardioprotective effect of adenosine has also been demonstrated when adenosine was given during reperfusion alone.[18] The present study and previous data appear to demonstrate that the benefits of adenosine are manifested during reperfusion and not during the period of ischemia.

The present data also reveal that adenosine enhances the recovery of myocardial efficiency during reperfusion consistent with previous studies following severe ischemia in the isolated working rat hearts. This improvement in efficiency is a consequence of improved myocardial LV work and MVO_2 relative to the untreated hearts. The mechanism for the cardioprotective effects of adenosine did not appear to be related to modulation of the coronary vasculature since coronary flow recovered to similar levels in each group.

4.5 Adenosine and Cardiac Energy Substrate Metabolism During Mild Ischemia

A growing body of evidence now supports the proposition that myocardial mechanical performance is a function of the energetic status of the heart.[4,5,11,13] The

system of perfusion developed by Neely *et al*[186] has been used extensively to study myocardial energy metabolism and its relation to function under controlled conditions. There is however, little information on the relation between myocardial function and energy metabolism during the actual period of ischemia in the isolated working heart. The present study offered the opportunity to assess simultaneously LV function and metabolism during ischemia and how alteration in energy metabolism affects recovery of function and efficiency post-ischemia.

Our data show that during mild ischemia, glycolytic flux is accelerated relative to baseline aerobic values. That glycolysis is elevated during ischemia has been shown in some previous studies.[18,83,154] However, other studies have provided conflicting results. In the globally ischemic myocardium, it has been argued that accumulation of glycolytic metabolites and reduction in glucose delivery inhibits the rate of glycolysis.[65,67,68] In our study, since glycolytic substrates (insulin and glucose) were available and metabolites were washed out during mild ischemia, glycolytic rates were sustained. Numerous previous studies have provided conflicting evidence on whether glycolysis is beneficial or detrimental to the ischemic heart. Evidence to support a beneficial role of glycolysis suggests that glycolytically derived ATP may be utilized preferentially to support membrane pump activity.[28,65,67,68] It must be noted however, that there has not been any conclusive data to demonstrate compartmentation of ATP. Moreover, increased glycolytic flux is associated with accumulation of deleterious intermediates which impair recovery of function during reperfusion. Our result showing that glycolysis is increased during mild and reperfusion supports the hypothesis that increased glycolytic rates is

detrimental.[18,83,154] This may possibly result from uncoupling of glycolysis from glucose oxidation leading to increased proton production from glycolytically derived ATP.

It has been known for sometime that fatty acid oxidation and glucose metabolism are reciprocally regulated.[4,5,12,18,48] Our data showing that palmitate oxidation was reduced during mild ischemia may have led to an increase in glucose oxidation. This was not observed in our study which may suggest that factors regulating these two pathways may become more complex during ischemia. In the isolated working rat heart, it was shown that the presence of fatty acids in the perfusate suppresses glucose oxidation to a larger extent than glycolysis.[165] The uncoupling of glucose metabolism caused by increased fatty acid oxidation during ischemia and reperfusion has previously been shown to contribute to ischemia and reperfusion-induced injury.[4,7,8,9,17] In our model of ischemia, increase flux through glycolysis may be the major contributing factor to ischemic injury. That adenosine did not affect glucose oxidation or fatty acid oxidation supports this hypothesis. In aerobically perfused rat hearts, Finegan *et al*[161] did not show any effect of adenosine on fatty acid oxidation. Subsequently, it was demonstrated that adenosine-mediated enhancement of function during reperfusion is accompanied by improvement of coupling between glycolysis and glucose oxidation.[18,83] Taken together, those findings and our data suggest that lack of adenosine cardioprotection during mild ischemia is a consequence of derangement of cardiac energy metabolism.

4.6 Effect of Adenosine on Cardiac Energy Metabolism During Reperfusion

Myocardial energy substrate utilization has been well documented during reperfusion both in the isolated heart [4,9,10,12,17,18] and under *in vivo* conditions.[5,7] However, the metabolic status of the heart following mild ischemic conditions in the isolated heart has not been addressed. Our study revealed that during reperfusion post-ischemia, glycolytic flux was sustained with glucose oxidation trending to decrease. The suppression of glucose oxidation may have resulted from either a reduced demand due to a lower workload or the complete recovery of palmitate oxidation to pre-ischemic values. Previous studies have reported that the rapid recovery of palmitate oxidation during reperfusion uncouples glucose metabolism and is a potential mechanism for depression of LV function and efficiency.[4,7,8,9,17,84] Our data support this hypothesis. The finding that adenosine dramatically decreased the rate of proton production resulting from inhibition of glycolysis upon reperfusion is consistent with previous data which showed inhibition of glycolysis by adenosine during reperfusion following severe ischemia.[18,52,83] This was associated with improved recovery of LV work and cardiac efficiency

Our data support the evidence that adenosine primarily modifies glucose use by decreasing flux through glycolysis leading to improved coupling of glycolysis and glucose oxidation during reperfusion. The resulting decrease in proton production may contribute to the improvement of LV work and cardiac efficiency observed during reperfusion. The failure of adenosine to modulate glycolysis during mild ischemia may have accounted for the depression of LV work and efficiency observed in the adenosine-treated hearts.

4.7 Effect of Adenosine on Cardiac Energy Production and Utilization During Mild Ischemia and Reperfusion

Cardiac energy production was indexed in this study as the TCA cycle acetyl CoA produced from palmitate oxidation and glucose oxidation. The finding that palmitate oxidation contributed the majority of the total acetyl CoA production during mild ischemia is in agreement with previous studies in aerobic hearts.[21,165] The reduction in TCA cycle activity coupled to the marked reduction of tissue ATP content in both groups likely accounts for the impairment of LV work during mild ischemia. Other studies[171,172] failed to show any correlation between myocardial ATP content and LV function suggesting that reduction of myocardial ATP content may not necessarily account for the depression of function. This is in keeping with our data that although tissue ATP was well preserved in adenosine-treated hearts during mild ischemia, LV work was not enhanced by adenosine.

Our data showing that palmitate is the predominant energy substrate during mild ischemia supports the hypothesis that energy substrate preference during ischemia determines cardiac function.[4,12,17,42,84] Cardiac energy can be influenced by altering the efficiency of energy production or the efficiency of energy utilization. An interesting observation in our study is that during mild ischemia and reperfusion, efficiency of energy utilization is markedly depressed in untreated hearts although energy production efficiency was unaltered. Our finding that adenosine prevented the inefficiency of energy utilization during reperfusion supports the hypothesis that impairment of mechanical function in ischemic heart patients is a consequence of

poor utilization of energy. This is consistent with the failure of adenosine to influence the efficiency of energy production during mild ischemia or reperfusion.

It has been postulated that one of the mechanisms of enhancing post-ischemic functional recovery in ischemic patients is to enhance the efficiency of energy utilization.[4,7,83] We provide here evidence to support this hypothesis. Furthermore, we suggest that reducing proton production by directly inhibiting the rate of glycolysis could play a major role in improving efficiency of energy utilization in the post-ischemic heart.

4.8 Glucose Uptake During Mild Ischemia

Various studies have suggested that promoting glucose utilization during an ischemic insult is beneficial to functional recovery (e.g. insulin treatment).[4,17,28,106] However, whether glucose uptake is altered during ischemia is speculative. Glucose transport is the influx of glucose into the cytosol mediated by facilitative diffusion. Glucose uptake on the other hand is the subsequent phosphorylation of the transported glucose, which commits glucose to further metabolism.[28,29] The major determinants of myocardial glucose uptake have been shown to be the density and activity of glucose transporters in the plasma membrane [28,29] and also the concentration gradient of glucose between the extracellular space and the cytosol.[3,28,29]

Phosphorylated glucose can further be broken down to pyruvate (glycolysis)[12] or be incorporated into cytosolic glycogen depending on prevailing conditions. Our data demonstrating that total glycogen content was not altered although labeled

glucose incorporation into intracellular glycogen increased during mild ischemia suggests the turnover of glycogen. During mild ischemia in the isolated working rat heart, Bolukoglu *et al*[131] showed no significant difference in the glycogen content relative to baseline aerobic values. Taken together, these results suggest that during mild ischemia, there is no net rate of glycogen synthesis. Furthermore, our findings support previous data that glycogen degradation and synthesis, which is controlled by activities of glycogen phosphorylase and glycogen synthase respectively, are simultaneously activated.[52,114,173]

The effect of adenosine on glycogen turnover has been examined in a number of studies and the results have been conflicting. Our data show that adenosine does not influence myocardial glycogen content and glycogen synthesis rate during mild ischemia. This confirms previous finding by Fraser *et al*[52] that selective A_1-adenosine receptor activation does not influence glycogen content and glycogen turnover during severe ischemia.

We further demonstrate that the rate of glucose uptake (sum of the rates of glycolysis and glycogen synthesis) was not significantly altered during mild ischemia but extraction of glucose increased. The increase in glucose extraction was modest when compared to the high values obtained for rat hearts during severe low-ischemia perfused under similar conditions.[52,57] This implies that with a coronary flow reduction of 30%, glucose availability is not rate-limiting. Our data also suggest that upon entry into the cytosol, exogenous glucose is preferentially metabolized to pyruvate with no significant change in glycogen content.

It has been suggested previously that glucose uptake is determined by the concentration of glucose in the perfusate such that at higher glucose concentrations, rates of uptake saturate.[174] The present data supports this observation, since a relatively high concentration of glucose was used. Another factor shown to inhibit glucose uptake is the presence of fatty acid [3,28] in the perfusate and this could contribute to the lack of effect of mild ischemia on glucose uptake. Since fatty acids and insulin are both present *in vivo*, our data suggest that under the appropriate physiological conditions, glucose uptake is not markedly altered by ischemia. Bolukoglu *et al*[131] showed that glucose uptake remained unchanged when glucose was the sole substrate during mild ischemia in the isolated rat heart. In agreement with our data, Fralix *et al*[154] failed to see alteration of glucose uptake during severe ischemia.

Our finding contrasts that of King and Opie[53] who demonstrated in the rat heart that restriction of coronary flow results in reduction of absolute glucose uptake measured as rate of detritiation of D[2-^{3}H]glucose. Neely *et al*[58] also demonstrated that absolute rate of glucose uptake was reduced in the anoxic rat hearts. Other studies however, showed that mild ischemia stimulated glucose uptake when the perfusate contained both glucose and a fatty acid source.[130] In the latter study, during ischemia, energy demand was reduced since afterload pressure was dramatically decreased as part of the system to reduce coronary flow. In the *in vivo* dog heart, Young *et al*[54] reported that glucose uptake is stimulated during mild ischemia. The disparity between our data and the above observations could be accounted for partly by the extent of coronary flow reduction and the method used to assess glucose

uptake in each study. Since energy demand was maintained during mild ischemia in our study in the presence of physiological substrates, our data may imply that glucose uptake is not altered during mild ischemia.

The failure of adenosine to influence glucose uptake during mild ischemia is supported by previous reports in the isolated rat heart that adenosine does not stimulate glucose uptake in the presence of insulin. [154,175] The implication of this finding is that the effect of adenosine on glucose utilization during mild ischemia does not appear to involve modulation of glucose uptake.

Interestingly, we observed that the rate of glucose uptake normalized for LV work markedly increased during mild ischemia relative to aerobic values. This observation supports the hypothesis that cardiac energy substrate metabolism is the primary determinant of mechanical function.

4.9 Glucose Uptake During Reperfusion

It has been suggested that, during reperfusion when coronary flow is restored following an ischemic event to normal aerobic conditions, glycogen stores are resynthesized.[52,176]. Whether this occurs following mild ischemia in the isolated working heart is not clear. Our data show that, glycogen content was not modulated by reperfusion despite an increase in the amount of labeled glucose into glycogen. This implies that transmembrane transport of glucose still occurred but at a rate similar to the pre-ischemia rate. This may also suggest that glycogen turnover continued throughout reperfusion reflecting the lack of reperfusion to alter glucose

uptake. This observation is consistent with our finding that mild ischemia does not alter glycogen content.

4.10 AMP-Activated Protein Kinase (AMPK) Regulation of Cardiac Energy Substrate Metabolism During Ischemia and Reperfusion

Recently, the regulation of myocardial energy substrate metabolism by the cellular energy sensor, AMPK,[42,84,118] has been a subject of intense investigation. In response to energetic stress, AMPK exerts a number of regulatory effects on myocardial metabolism.[42,84,118] When high energy phosphate levels fall, it has been shown that AMPK activity is enhanced, which is associated with an increase in fatty acid oxidation and a phosphorylation and inhibition of ACC.[42,84,100] AMPK activation has also been shown to regulate glucose metabolism by causing translocation of GLUT-4 to the surface membrane.[35,54] Conflicting data do not correlate the increase in GLUT-4 translocation to an increase in absolute glucose uptake in the myocardium.[121,122] We therefore evaluated the association between AMPK activity, glucose uptake and overall fuel substrate use in our model of mild ischemia. Our data revealed that AMPK phosphorylation increased dramatically during mild ischemia relative to baseline level, indicative of ischemic or metabolic stress. AMPK activation did not parallel the rate of glycogen synthesis and hence, glucose uptake, that occurred during mild ischemia. The increase activation of AMPK was also independent of changes in the AMP:ATP ratio of the cell. This suggests alternative mechanisms of AMPK activation, possibly involving an upstream kinase as suggested by other workers.[121,122] Interestingly, this is consistent with the increase in

flux through glycolysis, an effect which has also been shown to be mediated by AMPK.[177,178] Surprisingly, AMPK activation did not modulate fatty acid oxidation during mild ischemia as would be expected based on previous reports.[84,118]

AMPK activation has been shown to influence intracellular glucose metabolism by various mechanisms. By mediating the translocation of GLUT-1 and GLUT-4 from intracellular pools to the plasma membrane, AMPK enhances glucose transport.[54,118] Young *et al*[173] showed in rat skeletal muscle that AMPK activates glycogen phosphorylase via phosphorylase kinase, resulting in glycogen breakdown. Recently, it has been shown in rabbit skeletal muscle that AICAR activation of AMPK leads to phosphorylation and inactivation of glycogen synthase.[179] Taken together, the above findings support a critical role of AMPK on glycogen metabolism. Furthermore, it confirms the proposition that AMPK activity increases energy production and/or turns off energy consumption pathways.

Our data demonstrate that AMPK activity during mild ischemia does not correspond to alteration of glycogen metabolism consistent with recent findings in the aerobic rat heart upon AMPK activation by AICAR.[179] However, since glycolysis increased during mild ischemia, it implies that in this model, activation of AMPK during mild ischemia predominantly affects flux through glycolysis. This effect of AMPK on glycolysis could possibly be due to its activation of PFK-2 and a consequent increase in F-2,6-BP concentration.[177,178] Further experiments are however, required to test this hypothesis. These data also indicate that activation of AMPK is dissociated from glucose uptake since we did not observe any significant change in the rate of glucose uptake. Our data contrast findings by Young *et al*[54] that

low-flow ischemia is accompanied by activation of AMPK which causes an increase translocation of GLUT-1 and GLUT-4 culminating to an increase in glucose uptake. The disparity between these observations and ours could be due to differences in experimental models and/or the severity of the ischemia.

Interestingly, the impairment of LV mechanical function during mild ischemia attributable to increased glycolysis may be a result of AMPK activation. This suggests that AMPK activation during mild ischemia is detrimental to functional recovery as shown by other workers.[42,83] This sharply contrasts recent findings by Russell *et al*[180] in the transgenic mouse heart expressing a kinase dead (KD) form of AMPK. The authors demonstrated that compared to wild type hearts, KD hearts showed poor functional recovery associated with impaired glucose uptake and reduced fatty acid oxidation during reperfusion post-ischemia.

During reperfusion, AMPK phosphorylation returned to baseline levels, in agreement with return of glycolysis and fatty acid oxidation to aerobic levels. Based on our observation that adenosine inhibits glycolysis during reperfusion, it is tempting to speculate that adenosine may inhibit AMPK activity during reperfusion. By so doing, PFK-2 activity will be reduced which might have resulted in the marked reduction of glycolysis during reperfusion. Measurement of the effect of adenosine on the extent of AMPK phosphorylation during mild ischemia and reperfusion is the subject of further studies. Fraser *et al*[52] actually demonstrated that the A_1- adenosine receptor agonist, CHA, inhibits AMPK activity during low-flow severe ischemia and reperfusion. Another reasonable suggestion from our data is

that AMPK activation may be involved in uncoupling which leads to increased acidosis and thus, impairment of functional recovery as well as efficiency.

4.11 Adenosine and Cardioprotection: Mechanism of Action

Various mechanisms have been proposed to account for the cardioprotection afforded by adenosine. The improvement of functional recovery and limitation of infarct size by adenosine has been associated with its vasodilatory properties.[136,145] In the present study, this mechanism is unlikely because coronary flow recovered to similar extent in control and adenosine-treated hearts. Another mechanism shown to be involved in adenosine-induced cardioprotection is the inhibition of neutrophil activation[154] which does not play a role in this study since the perfusate lacked the plasma cellular constituents. More recently, the ability of adenosine to reduce reperfusion injury has been associated with modulation of myocardial energy substrate use.[18,83,161,154] The latter mechanism is supported by the findings from the present study.

The observations that the calculated proton production increases during mild ischemia due to uncoupling of glycolysis and glucose oxidation, may explain the reduction in LV mechanical function. Thus, a greater proportion of intracellular ATP was diverted to correct acidosis-induced ionic imbalances. This accounts for the impairment of efficiency of energy utilization shown during mild ischemia. Adenosine did not affect LV function and myocardial efficiency during mild ischemia because it had no considerable effect on glucose metabolism and proton production. This parallels previous studies that adenosine or the A_1-adenosine

receptor agonist administered prior to low-flow ischemia had no effect on function during the period of ischemia.[18,52,83]

During reperfusion, the presence of adenosine inhibited proton production secondary to inhibition of glycolysis. This translated into improved recovery of function and efficiency of O_2 and energy utilization. Thus, by preventing the acidosis-induced Na^+ and Ca^{2+} overload, energy was utilized for contractile function rather than to correct ionic homeostasis in the adenosine-treated heart. A likely explanation for adenosine cardioprotection during reperfusion is the enhancement of the efficiency of energy utilization. These observations are consistent with previous data in which improvement of coupling with DCA, an activator of PDH, or inhibition of Na^+/H^+ exchanger activity improved post-ischemic functional recovery and efficiency.[9]

Alternatively, A_1 adenosine receptor activation via stimulation of PKC, has been demonstrated to be protective following a period of severe ischemia. This was associated with activation of the mitochondrial K_{ATP}-channel.[144,150] The resulting decrease in intracellular Ca^{2+} overload has been shown to account for the protection afforded by A_1 receptor activation by preserving myocardial high-energy phosphate level. The fact that high-energy phosphate levels recovered to similar extent in both experimental groups in our study, implies that the beneficial effect of adenosine may be independent of K_{ATP}-channel activation. Ford *et al* also reported that the improvement LV work following severe ischemia was independent of the activation of K_{ATP}-channel.[181]

The inhibitory effect of adenosine on glycolysis has been shown in previous studies, and it is paralleled by an increase in functional recovery.[18,83,161,154] The actual signaling mechanism is not known. It can be speculated from our study that adenosine-induced inhibition of glycolysis occurs at the PFK-1 step. Whether adenosine directly interacts with the kinase allosterically remains to be established. A more likely explanation is that adenosine by inhibiting AMPK activity reduces the activity of PFK-2.[192] Subsequently, the reduction of F-2,6-BP concentration, which is a very potent activator of PFK-1, leads to decreased flux through glycolysis.[192,193] Another plausible reason is that adenosine has been shown to preserve intracellular ATP by incorporation into the high energy pool via the purine salvage pathways.[145] The latter is unlikely in our study because there was no significant changes in the ATP:AMP ratios during mild ischemia and reperfusion.

4.12 Conclusions

We have designed a novel model of mild ischemia which enables the investigation of the relationship between function and myocardial energy substrate metabolism under conditions of physiological workload and appropriate energy substrates.

Our data indicate that mild ischemia increases proton production from hydrolysis of glycolytically derived ATP, leading to an impairment of LV mechanical function and efficiency. The depression of efficiency could be a consequence of diverting energy to correct acidosis-induced Na^+ and Ca^{2+} overload. The increased AMPK activation did not correspond with increased glycogen

synthesis and glucose uptake. The uncoupling of glycolysis and glucose oxidation during mild ischemia resulting in increased production of protons contributed to reperfusion injury culminating in poor recovery of LV function and cardiac efficiency. The presence of adenosine during mild ischemia, did not affect glycolysis, glucose oxidation, fatty acid oxidation or glucose uptake. Thus, adenosine had no effect on mechanical function and efficiency during mild ischemia.

Upon reperfusion, adenosine inhibited glycolysis, improving the coupling of glucose metabolism, and subsequently reducing proton production from exogenous glucose. This translated into a marked improvement in LV work and efficiency, possibly due to reduction in acidosis-induced intracellular Na^+ and Ca^{2+} accumulation. We further demonstrated that adenosine does not affect the efficiency of energy production but rather enhances the efficiency of energy utilization.

Our results suggest that increase flux through glycolysis is a major contributing factor to ischemia and reperfusion injury. Pharmacological interventions that target glycolysis during the ischemic event may be efficacious in the management of patients with CAD.

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