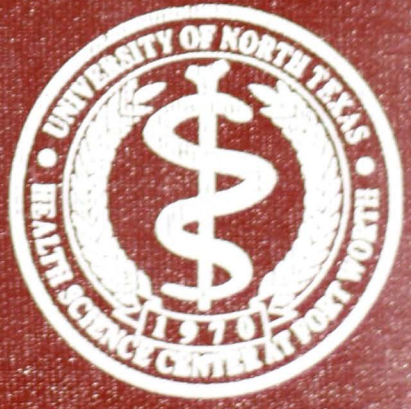
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Gao, Zhi-Ping, Role of Adenosine in Acute Hibernation of Guinea-Pig Myocardium Doctor of Philosophy (Biomedical Sciences), August, 1995; 111 pp; 3 tables; 15 figures, bibliography, 158 titles.

Myocardial hibernation is a state of depressed contractile function and energy demand during chronic ischemia. When coronary flow is restored, depressed contractile function can partially or completely recover to the pre-ischemic level, and ischemic injury of the myocardium is not evident. This project tested the hypothesis that endogenous adenosine mediates hibernation in guinea-pig myocardium. Isolated working guinea-pig hearts, perfused with glucose fortified Krebs-Henseleit buffer, were subjected to global low-flow ischemia. Left ventricular performance and cytosolic energy level were assessed. Lactate and purine nucleosides were measured in venous effluent. Hearts were perfused with [U-^{14}C]glucose to investigate the role of adenosine on glucose metabolism in myocardium. Left ventricular function in untreated hearts decreased by 80% and remained stable during ischemia, and completely recovered upon reperfusion. Neither adenosine receptor blockade with 8-*p*-sulfophenyl theophylline (8-SPT; 20 μM) nor *ecto* 5'-nucleotidase inhibitor α,β-methylene adenosine 5'-diphosphonate (AOPCP; 50 μM) affected left ventricular function either during ischemia or during reperfusion. Cytosolic energy level fell by 67% at 10 min ischemia in untreated hearts, but subsequently recovered to the pre-ischemic level despite continued ischemia. Adenosine receptor blockade increased

cytosolic energy level at 10 min ischemia relative to untreated hearts, but blunted the subsequent rebound of phosphorylation potential. Moreover, 8-SPT doubled ischemic lactate release. Adenosine receptor blockade also increased glucose uptake during pre-ischemia and hypoperfusion, but did not stimulate glucose oxidation. Crossover plots of glycolytic intermediates revealed that phosphofructokinase, a key rate-controlling step in glycolysis, was activated by adenosine receptor blockade in both pre-ischemic and hibernating myocardium. We conclude that 1) activation of adenosine receptors results in recovery of cytosolic energy level of moderately ischemic working myocardium, but this energetic recovery is not solely responsible for post-ischemic contractile recovery; 2) endogenous adenosine attenuates anaerobic glycolysis during myocardial hibernation by blunting phosphofructokinase activity.

ROLE OF ADENOSINE IN ACUTE HIBERNATION

OF GUINEA-PIG MYOCARDIUM

Zhi-Ping Gao, B.Med., M.Med.

APPROVED:

H. Fred Downey
Major Professor

Co-Major Professor

Committee Member

James L Caffrey
Committee Member

Patricia A. Gwirtz
Committee Member

Committee Member

Chair, Department of Physiology

Dean, Graduate School of Biomedical Sciences

ROLE OF ADENOSINE IN ACUTE HIBERNATION OF GUINEA-PIG MYOCARDIUM

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Zhi-Ping Gao, B.Med., M.Med.

Fort Worth, Texas

August, 1995

ACKNOWLEDGEMENT

This project was supported by grants to H. Fred Downey (R01-HL35056) and Robert T. Mallet (R29-HL50441) from the National Heart, Lung and Blood Institute, and by a grant to H. Fred Downey from the Texas Advanced Research Program.

I sincerely express my gratitude to my two supervisors, Drs. H. Fred Downey and Robert T. Mallet and to the chair of the Department of Physiology, Dr. Peter Raven, for their encouragement and leadership for the past three and half years. From them I learned the essence of science: creativity and honesty. In addition, I also very much appreciate the efforts of my committee members: Drs. James L. Caffrey, Eugene E. Quist, Patricia A. Gwirtz and Manus Donahue. Furthermore, I acknowledge the outstanding technical assistance of Wen-Lin Fan, Jie Sun, Blaine Payne, Chong-Hong He, and Arthur Williams, Jr.

I am also grateful for my husband, Sheng Wang, and my daughter, Han Wang, for their spiritual support and patience for helping me complete my doctoral degree. For my father and mother who cannot be with me at this moment, I miss you and would like you to share my happiness and glory.

My education and research training was supported by the Department of Physiology of University of North Texas Health Science Center at Fort Worth.

The following publications and abstracts are the results from this project.

PUBLICATIONS:

1. **Zhi-Ping Gao**, H. Fred Downey, Wen-Lin Fan, and Robert T. Mallet. Does interstitial adenosine mediate acute hibernation of guinea-pig myocardium? *Cardiovasc Res.* 1995;29:796-804.
2. **Zhi-Ping Gao**, H. Fred Downey, Jie Sun, and Robert T. Mallet. Adenosine receptor blockade enhances glycolysis in hypoperfused guinea-pig myocardium. (In preparation)

ABSTRACTS:

1. **Zhi-Ping Gao**, H. Fred Downey, Jie Sun, and Robert T. Mallet. Endogenous adenosine depresses glycolysis in acute myocardial hibernation. *Circulation* 1995 (submitted).
2. **Zhi-Ping Gao**, H. Fred Downey, Wen-Lin Fan, and Robert T. Mallet. An isolated working heart model of "hibernating" myocardium. *Proc Soc Exp Biol Med* In press.

3. **Zhi-Ping Gao**, H. Fred Downey, Wen-Lin Fan, and Robert T. Mallet. Interstitial adenosine does not mediate acute hibernation of guinea-pig myocardium. *FASEB J* 1995;9(4):A298.

4. **Zhi-Ping Gao**, H. Fred Downey, Wen-Lin Fan, and Robert T. Mallet. Decreased energy demand in ischemic guinea-pig myocardium. *FASEB J* 1994;8:A831.

5. **Zhi-Ping Gao**, Shang-Chiun Lee, and H. Fred Downey. Adenosine decreases oxygen consumption of control and isoproterenol stimulated minced canine myocardium. *FASEB J* 1993;7:A326.

TABLE OF CONTENTS

LIST OF TABLES

Chapter II

Table

LIST OF FIGURES

Chapter I

Figure

Chapter II

Figure

Chapter III

Figure

LIST OF ABBREVIATIONS

α_1R	α_1-adrenergic receptor
α_2R	α_2-adrenergic receptor
ADA	adenosine deaminase
ADO	adenosine
AF	aortic overflow
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ANOVA	analysis of variance
AOPCP	α,β-methylene adenosine 5'-diphosphate
ATP	adenosine triphosphate
[ATP]/ ([ADP][Pi])	cytosolic ATP phosphorylation potential
cAMP	cyclic adenosine monophosphate
CF	coronary flow
Cr	creatine
CrP	creatine phosphate
DAP	dihydroxyacetone phosphate
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenosine
F2P	fructose 1,6-bisphosphate

F6P	fructose 6-phosphate
LAC	lactate
G6P	glucose 6-phosphate
HR	heart rate
HPLC	high performance liquid chromatography
HYP	hypoxanthine
IMP	inosine 5'-monophosphate
INO	inosine
IP	inositol monophosphate
IP_2	inositol bisphosphate
IP_3	inositol 1,4,5,-triphosphate
Isch	ischemia
K_{ck}	creatine kinase equilibrium constant
LVP	left ventricular pressure
MVO_2	myocardial oxygen consumption
P_a	aortic pressure
PEP	phosphoenolpyruvate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
pH_i	intracellular pH
P_i	inorganic phosphate

PKC	protein kinase C
P_v	left atrial filling pressure
PYR	pyruvate
PRP	heart rate-pressure product
PVW	pressure-volume work
Rep	reperfusion
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SL	sarcolemma
8-SPT	8-*p*-sulfophenyl theophylline
$V_{ADO+INO}$	rate of adenosine + inosine release

CHAPTER I

INTRODUCTION

BACKGROUND

Myocardial `hibernation'

Myocardial ischemia is a major cause of morbidity and mortality in cardiac patients. The prognosis for post-ischemic recovery is, to a large extent, dependent on the severity of the ischemic insult. The metabolic characteristics of myocardial ischemia are increased lactate and adenosine release, and decreased cytosolic energy level. If the ischemic insult is sufficiently severe and prolonged, myocardial energy supply will be depleted, resulting in cell death due to loss of ionic homeostasis and membrane integrity. However, a different myocardial response can occur in moderate, rather than severe ischemic conditions. Ischemic myocardium can decrease, *i.e.* down-regulate, its energy demand by decreasing functional activity when energy supply is moderately limited. This myocardial `hibernation', as first recognized by Rahimtoola[1,2] in 1985, was described clinically in patients experiencing stable or unstable angina or coronary stenosis. In these patients, contractile depression was reversible by coronary bypass or angioplasty. Since then, it has been recognized that myocardial ischemia is potentially reversible and myocardial function can completely or partially recover following restoration of coronary flow. Myocardial `hibernation' is analogous to a bear hibernating in winter. When the

food supply decreases, the bear decreases its activity and metabolism. When spring comes and the food supply is restored, the bear resumes its activity and metabolism. Myocardium has the same capacity to reversibly decrease its functional and metabolic activity when energy supply decreases. The signal to trigger myocardial down-regulation (*i.e.* hibernation) is still unknown. But it is clear that an agent which can preserve myocytic energy stores during ischemia, will likely be protective to the heart.

Characteristics of hibernating myocardium

Several studies in experimental mammalian heart preparations have shown that hibernation is an adaptive process that protects the heart muscle from ischemic injury. Pantely *et al.*[3-5] induced hibernation in *in situ* porcine heart by reducing coronary flow by 25-50%. In these studies, subendocardial ATP and phosphocreatine contents had fallen appreciably after 5 min hypoperfusion. Despite persistent hypoperfusion, phosphocreatine content recovered to pre-ischemic level at 60 min hypoperfusion, while ATP content remained moderately decreased. Schultz *et al.*[6-9] reported a marked increase in lactate production at 15 min hypoperfusion followed by a modest decline toward control levels when coronary flow was lowered sufficient to depress systolic wall thickening by 50% in porcine heart *in situ*. Downing and Chen[10-12] developed an isolated, nonworking neonatal piglet heart model of hibernation in which coronary flow was lowered to 10% of control for 2 hr, and then restored to the pre-ischemic level. It was observed that the myocardial ATP and phosphocreatine concentrations were identical in both reperfused and

time control groups, although glycogen was lowered in the post-ischemic hearts. In studies of acutely hibernating isolated rat hearts, Keller *et al.*[13] and Schaefer *et al.*[14] both found that left ventricular performance fell in proportion to perfusion pressure, but completely recovered when perfusion pressure was restored, and the phosphocreatine level[14] also recovered despite the progressive loss of ATP. In summary, myocardial function decreases during ischemia, but can recover upon restoration of coronary flow, provided that the hypoperfusion is not too severe. Under these conditions, creatine phosphate is markedly decreased at the onset of ischemia, but can recover despite continued ischemia. This finding suggests that the hibernating myocardium lowered its energy requirements during hypoperfusion to restore the balance between energy demand and energy supply.

The protective effects of adenosine during myocardial ischemia

Adenosine metabolism

Figure 1 summarizes the pathways of adenosine production and degradation. The major sources of adenosine formation are dephosphorylation of 5'-AMP by 5'-nucleotidase and hydrolysis of S-adenosylhomocysteine (SAH) by SAH-hydrolase. In normoxic myocardium, adenosine is mainly formed from S-adenosylmethionine through the transfer of the methyl group to a variety of methyl acceptors. SAH is then hydrolyzed by SAH hydrolase to form adenosine and homocysteine. The overall adenosine production rate was reported as 0.8 nmol/min/g in isolated perfused guinea pig heart,

which is quite close to the hydrolysis rate of SAH (0.75 nmol/min/g).[15] However, during hypoxia or ischemia the primary source of adenosine formation shifted to the 5'-nucleotidase pathway.[16] The enzyme 5'-nucleotidase exists in two isoforms: an *ecto* 5'-nucleotidase which is bound to the extracellular surface of the sarcolemma, and a cytosolic 5'-nucleotidase in the myocyte cytoplasm.[17] Whether the primary source of adenosine formed during myocardial ischemia is derived from cytosolic 5'-nucleotidase or *ecto* 5'-nucleotidase remains unclear. Adenosine derived from AMP or SAH can be phosphorylated by adenosine kinase, forming AMP, or degraded to inosine by adenosine deaminase.

Adenosine receptors and signal transduction pathways

The protective effects of adenosine during myocardial ischemia and reperfusion could be mediated through receptor or through non-receptor mechanisms such as ATP replenishment. Purinergic receptors are classified as P_1 for adenosine selective receptors and P_2 for ATP/ADP receptors. P_1 receptors are further divided into adenosine A_1 and A_2 receptors. A_1 receptors are located in the membrane of cardiomyocytes and vascular smooth muscle.[18] The effects of adenosine binding to the A_1 receptor are mediated by an inhibitory guanine nucleotide binding protein (G_i).[19-21,67] A_1 receptors mediate negative chronotropic, dromotropic and inotropic responses.[18,22] A_2 receptors are located on the vascular smooth muscle epithelium,[18] and their activation increases cyclic AMP formation in epithelium.[19] Delayed onset of ischemic contracture by adenosine in isolated rat hearts also appears to be mediated by A_1 receptors.[23]

Adenosine and its analogs antagonize the increases in adenylate cyclase activity and the accumulation of cyclic AMP caused by catecholamine and forskolin.[24,25] However, adenosine does not attenuate the inotropic and electrophysiological effects of intracellularly applied cyclic AMP. Thus adenosine may exert its effect on any of the steps linking adrenergic receptor activation with cyclic AMP production. Recent biochemical and pharmacological studies in ventricular myocytes have provided evidence that adenosine A_1-receptors, which are linked to adenylate cyclase via G_i or G_o,[26,27] mediate the inhibitory effect of adenosine. Pertussis toxin, which induces ADP-ribosylation of a subunit of G_i and G_o, uncouples receptors from adenylate cyclase and greatly attenuates the contractile effects of adenosine and its analogs[28] and their inhibition of isoproterenol induced cyclic AMP accumulation.[29] In addition, Lasley *et al.*[20,30] recently examined the involvement of G_i in the protective effect of adenosine in isolated rat hearts. Inhibition of G_i by pertussis toxin blocked the protective effects of exogenous adenosine and adenosine A_1-receptor agonists. These preliminary findings suggest that adenosine cardioprotection is mediated by A_1-receptors coupled to G_i protein.

Adenosine A_1 receptors have recently been subclassified as A_1 and A_3 receptors[31,32] based on relative potencies of various adenosine agonists and antagonists. The findings of Armstrong *et al.*[33] and Liu *et al.*[34] implicated adenosine A_3 receptors in preconditioning of isolated rabbit cardiomyocytes and perfused rabbit hearts.

Adenosine and glucose metabolism

When the heart is subjected to severe ischemia, glycolysis initially increases to produce ATP which could delay the onset of ischemic contracture, maintain ischemic cellular ion homeostasis, and reduce post-ischemic dysfunction. As noted by Opie,[35] the overall rate of ATP turnover is crucial in maintenance of viability, especially the ATP derived from glycolytic flux. Thus, glycolysis plays a crucial role in supplying sufficient ATP to prevent derangement of ion homeostasis and cell death in ischemic myocardium. But when severe ischemia is prolonged, glycolysis will slow and eventually cease as a result of feedback inhibition of glycolytic enzymes due to accumulated hydrogen ion, NADH and lactate.

Recently, Mainwaring and coworkers[36] reported that adenosine, infused at 50 and 100 μg/min, increased myocardial glucose uptake in non-ischemic rat hearts by 80% and 140% respectively. During hypoxia, endogenous adenosine also appears to play an important role in glucose metabolism.[37] The hypoxia induced increase in glucose utilization could be attenuated by the adenosine receptor antagonist 8-(sulphophenyl)-theophylline. Law and Raymond[38] showed that adenosine could potentiate insulin-stimulated glucose uptake in canine myocardium in vivo. Additional support favoring adenosine-enhanced glycolysis,[39] was obtained in isolated perfused rat hearts, treated with adenosine plus the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenosine (EHNA) during low flow ischemia. These hearts exhibited increased lactate release and

a delayed onset of contracture. Adenosine receptor blockers, such as BWA 1433U, reduce lactate release and accelerate the onset of ischemic contracture. Omission of glucose from the perfusate eliminated the effects of the adenosine plus EHNA on lactate release or time to contracture. Owen *et al.*[40] reported that ATP derived from glucose metabolism, not total tissue ATP content, correlated best with the delay of onset of myocardial contracture.

Although several studies have provided evidence of adenosine increasing glycolysis during ischemia, there is still controversy in this field. Recently, Finegan *et al.*[41] reported that glycolysis, which was unaffected by ischemia *per se,* was inhibited by adenosine pre-treatment in isolated rat hearts. During reperfusion, glycolysis was also inhibited by adenosine treatment either concurrently or during the prior ischemia. Also, Dale *et al.*[42] reported inhibition of glycolysis by the adenosine A_1-receptor agonist N^6-(L-phenylisopropyl)adenosine (PIA). In addition, adenosine deaminase enhances the effect of insulin on glucose uptake. There is evidence that adenosine exerts a biphasic effect on glucose transport, *i.e.* glucose uptake was increased when adenosine was at low concentration, but decreased when adenosine concentration was increased.[38] Thus the effects of adenosine on glucose metabolism remain unresolved.

The effect of adenosine on myocardial energetic status

A number of studies have provided evidence that during ischemia and reperfusion, cardiac function is determined by cytosolic ATP phosphorylation potential, the main

determinant of Gibbs free energy of ATP hydrolysis, rather than total tissue ATP content or concentration *per se*. The reasons are (i) according to the results of various workers under different experimental conditions, the measured tissue ATP content varied considerably from 0.6 to 2.4 μmol/g wet wt, but cardiac function did not differ; (ii) if there is critical ATP level, the hearts could not survive below that level, yet several workers have demonstrated a dissociation between post-ischemic recovery of heart function and tissue ATP level;[44,61-63] (iii) cardiac contractility is only modestly decreased with greatly decreased ATP. In a normoxic heart model,[45] the turnover rate of ATP appeared sufficient to maintain membrane function, ion gradients and active force development, and to maintain near-normal ATP phosphorylation potential despite near total depletion of ATP and phosphocreatine stores.

The ATP phosphorylation potential ([ATP]/{[ADP][P_i]}) is the concentration term of the expression that describes the free energy of ATP hydrolysis (ΔG_{ATP}) in the cytosol, *i.e.* the change in chemical energy resulting from the hydrolysis of ATP to ADP and P_i:

$$\Delta G_{ATP} = \Delta G^{o}_{ATP} - RT\ln\{[ATP]/[ADP][P_i]\}$$

Over 95% of myocardial ADP is bound or compartmented,[43,68] so total ADP measured in myocardial extracts grossly overestimates free cytosolic, thermodynamically relevant ADP. Because direct measurements of cytosolic free [ADP] are not possible at present, the [ATP]/[ADP] ratio is estimated by means of the reversible reaction catalyzed by creatine kinase (CK):

$$Cr + ATP \rightleftarrows CrP + ADP + H^+$$

Thus,

$$[ATP]/[ADP] = \{[CrP]/[Cr][P_i]\} \cdot \{[H^+]/K_{CK}\}$$

Thus the creatine phosphate potential, $[CrP/[Cr][P_i]$, can serve as an indicator of energy state under many physiological conditions. Reperfusion dysfunction was characterized by a low cytosolic phosphorylation potential, due to accumulation of P_i and lactate.[46] A consistent correlation was found between ATP phosphorylation potential and reperfusion contractile function. Accordingly, interventions which increase the cardiac ATP phosphorylation potential, could prove beneficial to improve cardiac ischemic or reperfusion survival. Adenosine is one of the potential candidates. Bünger *et al.*[47] reported the effect of adenosine (100 μM) on cardiac adenylates and levels of creatine (Cr), creatine phosphate (CrP) and inorganic phosphate in paced Langendorff perfused hearts. In normoxic hearts, adenosine had no effect on cytosolic energy level, including CrP/Cr ratio and creatine phosphate potential. In the post-ischemic heart, on the other hand, adenosine significantly increased creatine phosphate potential and ATP level compared to the non-treated group.

What causes adenosine release during ischemia? There is a multifactorial control of purine nucleoside formation. According to the studies of Bünger *et al.*[48] and Headrick *et al.*,[49] total adenosine plus inosine release is the function of ATP phosphorylation potential or free [AMP]. Thus a decrease in ATP phosphorylation potential (an index of

cytosolic energy level) could trigger adenosine release. 5'-nucleotidase, an enzyme responsible for adenosine formation, is allosterically regulated by ATP, ADP, P_i, and magnesium.[64-66] Itoh *et al.*[64] reported that the 5'-nucleotidase was activated by ATP and ADP, and inhibited by P_i. However, the rate of AMP hydrolysis catalysed by the 5'-nucleotidase increased sharply with decreasing energy charge, *i.e.* ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]). Purine efflux also displayed a linear relation with cytosolic [AMP] during graded ischemia, consistent with mass action-dependent formation of adenosine in the ischemic heart.[65] Sullivan and Alpers[66] found magnesium completely relieved the nucleotide-inhibition of 5'-nucleotidase. The regulation of the two isoforms of 5'-nucleotidase, *i.e.* cytosolic 5'-nucleotidase and *ecto* 5'-nucleotidase, is somewhat different. High concentrations of ATP inhibit and AMP activates both isoforms of 5'-nucleotidase.[69,70] However, ADP activates cytosolic 5'-nucleotidase by increasing maximal velocity and affinity for AMP in canine myocytes.[69] Inhibition of *ecto* 5'-nucleotidase by ADP and/or ATP is a significant determinant of the rate of adenosine production in rat myocytes.[70] ATP phosphorylation potential of myocardium correlates significantly with purine nucleoside (adenosine + inosine) release (Figure 2).[48] Adenosine plus inosine release displayed a correlation with both the ATP phosphorylation potential (Figure 2A) and cytosolic free AMP concentration (Figure 2B). Purine nucleoside release increased exponentially as the phosphorylation potential fell and cytosolic AMP increased. Adenosine formation is regulated by availability of cytosolic AMP, and therefore is correlated with the phosphorylation state of ATP through the myokinase equilibrium.

Since adenosine is the precursor of adenosine triphosphate, it could bypass many preliminary steps in *de novo* purine synthesis pathway, accelerating AMP resynthesis by adenosine kinase. By this mechanism, adenosine could trigger a rapid increase in ATP levels following reperfusion.[50] In accordance with this proposal, exogenous adenosine has been found to retard myocardial ATP depletion, and this effect is associated with improved post-ischemic ventricular function.[51,52] Although myocardial ATP content is a poor index of cytosolic energy level, exogenous adenosine improved post-ischemic recovery possibly by contributing to the synthesis of ATP which increased cytosolic energy level.

Adenosine and oxygen consumption

Myocardial oxygen consumption is determined by myocardial contractility, heart rate and ventricular wall tension. Myocardial ischemia stimulates sympathetic outflow, causing norepinephrine release. Norepinephrine activates ß-receptors to increase contractility and heart rate which in turn increases myocardial oxygen consumption. Adenosine could inhibit norepinephrine release from adrenergic nerve endings.[53] Adenosine not only decreases oxygen consumption under basal conditions, but also partially antagonizes the effect of isoproterenol on oxygen consumption of minced myocardium.[54] The negative chronotropic and dromotropic effects of adenosine A_1 receptor activation also decrease myocardial oxygen consumption. Thus, adenosine

appears to reduce myocardial oxygen demand. This effect could conserve cellular ATP stores to maintain myocyte viability.

SPECIFIC AIMS

Down-regulation of myocardial energy demand or 'hibernation', is a state of depressed myocardial contractile function during coronary hypoperfusion which can be partially or completely reversed when coronary flow is restored. This metabolic down-regulation may protect the heart from ischemic injury during hypoperfusion. We have recently established an isolated working guinea-pig heart model of hibernation,[55,56] exhibiting many of the hallmarks of this phenomenon.[3-14] Our preliminary studies have shown that cytosolic energy level (ATP phosphorylation potential) falls sharply at the onset of ischemia, but later recovers appreciably despite continued ischemia. However, the mechanisms of this newly recognized, potentially beneficial phenomenon are still unknown. Adenosine has been found to be protective in ischemic myocardium.[57] We propose that adenosine, released during moderately severe ischemia, triggers down-regulation of myocardial energy demand. Thus the objective of this dissertation is to delineate the role of adenosine in the mechanism of myocardial hibernation.

Numerous studies have shown adenosine to be a cardioprotective and therapeutic agent.[57] Adenosine mediates coronary vasodilation,[58] preservation of cardiac tissue and function in myocardial ischemia, antiadrenergic effects,[59] protection of endothelium and

prevention of microvascular obstruction, increased energy supply,[37,38] and development of new microvessels.[57] In addition, it can mimic the preconditioning phenomenon against myocardial ischemia.[60] Our preliminary studies have shown that adenosine release greatly increased at the onset of ischemia, but later returned to control level despite continuing ischemia. Moreover, cytosolic energy level, which fell markedly at the onset of ischemia, fully recovered as ischemia was prolonged due to the establishment of a new balance between energy supply and energy demand. We propose that adenosine, released during myocardial ischemia due to imbalance of energy supply/demand ratio, is involved in the protective mechanism of myocardial hibernation. The following hypotheses will be tested:

i) *Inhibition of adenosine formation by ecto 5'-nucleotidase attenuates functional and metabolic down-regulation during myocardial ischemia.*

ii) *Adenosine receptor antagonism blocks the protective effects of adenosine on functional and metabolic parameters.*

iii) *Interstitial adenosine depresses glycolytic flux and lactate release.*

SIGNIFICANCE

This study answers the following questions: i) Does interstitial adenosine mediate functional and metabolic down-regulation of myocardium during ischemia? ii) Does adenosine mediate down-regulation via adenosine receptors? iii) Does adenosine receptor blockade alter glycolytic flux and lactate release during myocardial ischemia? The significance of this project is as follows: i) If our first hypothesis is confirmed, then

adenosine must play a crucial role in initiating and/or maintaining ischemic myocardial down-regulation. Such a finding would provide impetus to the development of therapeutic approaches to increase adenosine formation or prevent its degradation during myocardial ischemia to prolong myocyte viability in clinical settings. ii) If adenosine mediates myocardial down-regulation via adenosine receptors, this finding would encourage the synthesis of new adenosine analogs with high potency, high efficacy and low toxicity to treat cardiac patients. iii) Since glycolysis is the only significant source of ATP during myocardial ischemia, it is important to delineate the effects of adenosine on glycolytic flux and lactate release. The results from this study will provide evidence to help physicians treat patients with myocardial ischemia.

REFERENCES

1. Rahimtoola SH. A perspective on the three large multicenter randomized clinical trials of coronary bypass surgery for chronic stable angina. Circulation 1985;72(suppl V): V123-135.

2. Rahimtoola SH. The hibernating myocardium. Am Heart J 1989;117: 211-221.

3. Pantely GA, Malone SA, Rhen WS, Anselone CG, Arai A, Bristow J,Bristow JD. Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. Circ Res 1990;67: 1481-1493.

4. Arai AE, Pantely GA, Anselone CG, Bristow J, and Bristow JD. Active downregulation of myocardial energy requirements during prolonged moderate ischemia in swine. Circ Res 1991;69: 1458-1469.

5. Bristow JD, Arai AE, Anselone CG, and Pantely GA. Response to myocardial ischemia as a regulated process. Circulation 1991;84: 2580-2587.

6. Schultz R, Guth BD, Pieper K, Martin C, and Heusch G. Recruitment of an inotropic reserve in moderately ischemic myocardium at the expense of metabolic recovery: a model of short-term hibernation. Circ Res 1992;70: 1282-1295.

7. Ehring T, Schultz R, and Heusch G. Characterization of "hibernating" and "stunned" myocardium with focus on the use of calcium antagonists in "stunned" myocardium. J Cardiovasc Pharmacol 1992;20(Suppl.5): S25-S33.

8. Schultz R, Rose J, Martin C, Brodde OE, Heusch G. Development of short-term myocardial hibernation: its limitation by the severity of ischemia and inotropic stimulation. Circulation 1993;88: 684-695

9. Rose J, Schultz R, Martin C, and Heusch G. Post-ejection wall thickening as a marker of successful short term hibernation. Cardiovasc Res 1993;27: 1306-1311.

10. Downing SE, and Chen V. Myocardial hibernation in the ischemic neonatal heart. Circ Res 1990;66: 763-772

11. Downing SE, and Chen V. Acute hibernation and reperfusion of the ischemic heart. Circulation 1992;85: 699-707

12. Downing SE, and Chen V. Effects of catecholamine stimulation on myocardial hibernation. Am Heart J 1992;124: 589-596

13. Keller AM, Cannon PJ. Effect of graded reductions of coronary pressure and flow on myocardial metabolism and performance: a model of "Hibernating" myocardium. J Am Coll Cardiol 1991;17: 1661-1670

14. Schaefer S, Carr LJ, Kreutzer U, and Jue T. Myocardial adaptation during acute hibernation: mechanisms of phosphocreatine recovery. Cardiovasc Res 1993;27: 2044-2051.

15. Llyod HGE, and Schrader J. The importance of the transmethylation pathway for adenosine metabolism in the heart. In: Gerlach E, Becker BF (eds): Topics and Perspectives in Adenosine Research. Berlin/Heidelberg, Springer-Verlag, 1987, pp. 199-207.

16. Darvish A, Chakraborty J, Britton SL, Metting PJ. Myocardial adenosine production via AMP-specific cytosolic 5'-nucleotidase. FASEB J 1990; 4:1071.

17. Newby AC, Worku Y, Meghji P. Critical evaluation of the role of ecto- and cytosolic 5'-nucleotidase in adenosine formation. In: Gerlach E, Becker BF (eds): Topics and Perspectives in Adenosine Research. Berlin/Heidelberg, Springer-Verlag, 1987, pp. 155-169.

18. Ely SW, and Berne RM. Protective effects of adenosine in myocardial ischemia. Circulation 1992;85: 893-904.

19. Olah ME, and Stiles GL. Adenosine receptors. Ann Rev Physiol 1992;54:211-225.

20. Lasley RD, and Mentzer RM. Pertussis toxin blocks adenosine A_1 receptor mediated protection of the ischemic rat heart. J Mol Cell Cardial 1993;25: 815-821.

21. Cooper DMF, Londos C, Rodbell M. Adenosine receptor-mediated inhibition of rat cerebral cortical adenylate cyclase by a GTP-dependent progess. Mol Pharmacol 1980;18: 598-601.

22. Belardinelli L, Linden J, and Berne RM. The cardiac effects of adenosine. Prog Cardiovasc Dis 1989;32: 73-97.

23. Lasley RD, Rhee JW, Van Wylen DGL, and Mentzer RM. Adenosine A_1 receptor mediated protection of the globally ischemic isolated rat heart. J Mol Cell Cardiol 1990;22: 39-47.

24. Dobson JG. Mechanism of adenosine inhibition of catecholamine-induced responses in heart. Circ Res 1983;52: 151-160.

25. West GA, Isenberg G, and Belardinelli L. Antagonism of forskolin effects by adenosine in isolated hearts and ventricular myocytes. Am J Physiol 1986;250: H769-H777.

26. Lohse MJ, Udena D, and Schwabe U. Demonstration of Ri-type adenosine receptors in bovine myocardium by radioligand binding. Naunyn Schmidedbergs Arch Pharmacol 1985;328: 310-316.

27. Linden J, Hollen CE, and Patel A. The mechanism by which adenosine and cholinergic agents reduce contractility in rat myocardium: correlation with cyclic adenosine monophosphate and receptor densities. Circ Res 1985;56: 728-735.

28. Wilson WW, West GA, and Hewlett EL. Attenuation of the inhibitory effects of adenosine by pertussis toxin in isolated guinea pig hearts. Fed Proc 1987;46: 6536 (abstr).

29. Hazeki O, Ui M. Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cell. J Biol Chem 1981;256: 2856-2862.

30. Lasley, RD, Barakat O, Van Wylen DGL, and Mentzer RM. Pertussis toxin inhibits adenosine-mediated preservation of postischemic myocardial function. Circulation 1991; 84(Suppl II): II-307 (abstr).

31. Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, Rivkees SA, Fink JS, and Reppert AM. Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. Mol Pharmacol 1993;44: 524-532.

32. Zhou Q-Y, Li C, Olar ME, Johnson RA, Stiles GL, and Civelli O. Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. Proc Natl Acad Sci USA 1992;89: 7432-7436.

33. Armstrong S and Ganote CE. Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A_3 receptor involvement. Cardiovasc Res 1994;28:1049-1056.

34. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, and Downey JM. Evidence that the adenosine A_3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. Cardiovasc Res 1994;28: 1057-1061.

35. Opie LH. Cardiac metabolism - emergence, decline, and resurgence. Part II. Cardiovasc Res 1992;26: 817-830.

36. Mainwaring R, Lasley R, Rubio R, Berne RM. Adenosine stimulates glucose uptake in the isolated rat heart. Surgery 1988;13: 445-449.

37. Wyatt DA, Edmunds MC, Rubio R, Berne RM, Lasley RD, and Mentzer RM. Adenosine stimulates glycolytic flux in isolated perfused rat hearts by A_1-adenosine receptors. Am J Physiol 1989;257: H1952-H1957.

38. Law WR, and Raymond RM. Adenosine potentiates insulin-stimulated myocardial glucose uptake in vivo. Am J Physiol 1988;254: H970-H975.

39. Lasley RD, and Mentzer RM. Adenosine increases lactate release and delays onset of contracture during global low flow ischaemia. Cardiovasc Res 1993;27: 96-101.

40. Owen P, Dennis S, and Opie LH. Glucose flux rate regulates onset of ischemic contracture in globally underperfused rat hearts. Circ Res 1990;66: 344-354.

41. Finegan BA, Lopaschuk GD, Coulson CS, and Clanachan AS. Adenosine alters glucose use during ischemia and reperfusion in isolated rat hearts. Circulation 1993;87: 900-908.

42. Dale WE, Hale CC, Kim HH, and Rovetto MJ. Myocardial glucose utilization: failure of adenosine to alter it and inhibition by the adenosine analogue N^6-(L-2-phenylisopropyl)adenosine. Circ Res 1991;69: 791-799.

43. Perry SV. The bound nucleotide of the isolated myofibril. Biochem J 1952;51: 495-499.

44. Neely JR, and Grotyohann LW. Role of glycolytic products in damage to ischemic myocardium: dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. Circ Res 1984;55: 816-824.

45. Hoerter JA, Lauer C, Vassort G, and Gueron M. Sustained function of normoxic hearts depleted in ATP and phosphocreatine: a ^{31}P-NMR study. Am J Physiol 1988;255: C192-C201.

46. Mallet RT, Hartman DA, and Bünger R. Glucose requirement for postischemic recovery of perfused working heart. Eur J Biochem 1990;188: 481-493.

47. Bünger R, Menzer RM, and Mukohara N. Effects of therapeutic adenosine on adenylates and creatine compounds in normal and reperfused guinea-pig heart. FASEB J 1992;6: A1250 (Abstr).

48. Bünger R, Mallet RT, and Kang YH. Guinea-pig cardiac free, bound and interstitial adenylates: energy-linked and energy-independent adenosine release. In: Imai S and Nakazawa M (eds). Role of Adenosine and Adenine Nucleotide in the Biological System. Elsevier, Amsterdam/New York/Oxford, 1991; pp. 337-353.

49. Headrick J, Clarke K, and Willis RJ. Adenosine production and energy metabolism in ischemic and metabolically stimulated rat heart. J Mol Cell Cardiol 1989;21: 1089-1100.

50. Forman MB, and Velasco CE. Role of adenosine in the treatment of myocardial stunning. Cardiovasc Drugs Ther 1991;5: 901-908.

51. Ely SW, Menzer RM, Lasley RD, Lee BK, and Berne RM. Functional and metabolic evidence of enhanced myocardial tolerance to ischemia and reperfusion with adenosine. J Thorac Cardiovasc Surg 1985;90: 549-556.

52. Reibel DK, and Rovetto MJ. Myocardial ATP synthesis and mechanical function following oxygen deficiency. Am J Physiol 1978;234: H620-H624.

53. Richardt G, Waas W, Kranzhöfer R, Mayer E, and Schöming A. Adenosine inhibits exocytotic release of endogenous noradrenaline in rat heart: a protective mechanism in early myocardial ischemia. Circ Res 1987; 61: 117-123.

54. Gao Z-P, Lee SC, and Downey HF. Adenosine decreases oxygen consumption of control and isoproterenol stimulated minced canine myocardium. FASEB J 1993;7: A326 (abstr).

55. Gao Z-P, Fan W-L, Downey HF and Mallet RT. An isolated working heart model of "hibernating" myocardium. Proc Soc Exp Biol Med (In press).

56. Gao Z-P, Fan W-L, Downey HF, and Mallet RT. Decreased energy demand in ischemic guinea-pig myocardium. FASEB J 1994;8: A831 (abstr).

57. Berne RM. Adenosine - a cardioprotective and therapeutic agent. Cardiovasc Res 1993;27: 2.

58. Hori M, and Kitakaze M. Adenosine, the heart, and coronary circulation. Hypertension 1991;18: 565-574.

59. Dobson JG Jr, Ordway RW, and Fenton RA. Endogenous adenosine inhibits catecholamine contractile responses in normoxic hearts. Am J Physiol 1986;251: H455-H462.

60. Mentzer RM, Bünger R, and Lasley RD. Adenosine enhanced preservation of myocardial function and energetics: possible involvement of the adenosine A_1 receptor system. Cardivasc Res 1993;27: 28-35.

61. Schaper J, Mulch J, Winkler B, Schaper W. Ultrastructural, functional, and biochemical criteria for estimation of reversibility of ischemic injury: a study of the effects of global ischemia on the isolated dog heart. J Mol Cell Cardiol 1979;11: 521-541.

62. Taegtmeyer H, Roberts AFC, Raine AEG. Energy metabolism in reperfused heart muscle: metabolic correlates to return of function. J Am Coll Cardiol 1985;6: 864-870.

63. Kupriyanov VV, Lakomkin VL, Steinschneider AY, Severina MY, Kapelko VI, Ruuge EK, Saks VA. Relationships between pre-ischemic ATP and glycogen content and post-ischemic recovery of rat heart. J Mol Cell Cardiol 1988;20: 1151-1162.

64. Itoh R, Jun OKA, Hisashi O. Regulation of rat heart cytosol 5'-nucleotidase by adenylate energy charge. Biochem J 1986;235: 847-851.

65. Headrick JP, and Willis RJ. 5'-Nucleotidase activity and adenosine formation in stimulated, hypoxic and underperfused rat heart. Biochem J 1989;261: 541-550.

66. Sullivan JM, Alpers JB. In vitro regulation of rat heart 5'-nucleotidase by adenine nucleotides and magnesium. J Biol Chem 1971;246: 3057-3063.

67. Fredholm BB. Adenosine receptors in the central nervous system. News in Physiological Sciences 1995;10: 122-128.

68. Hebisch S, Soboll S, Schwenen M, and Sies H. Compartmentation of high-energy phosphates in resting and working rat skeletal muscle. Biochim Biophy Acta 1984;764: 117-124.

69. Darvish A, Metting PJ. Purification and regulation of an AMP-specific cytosolic 5'-nucleotidase from dog heart. Am J Physiol 1993;264: H1528-H1534.

70. Meghji P, Pearson JD, Slakey LL. Regulation of extracellular adenosine production by ectonucleotidases of adult rat ventricular myocytes. Am J Physiol 1993;263: H40-H47.

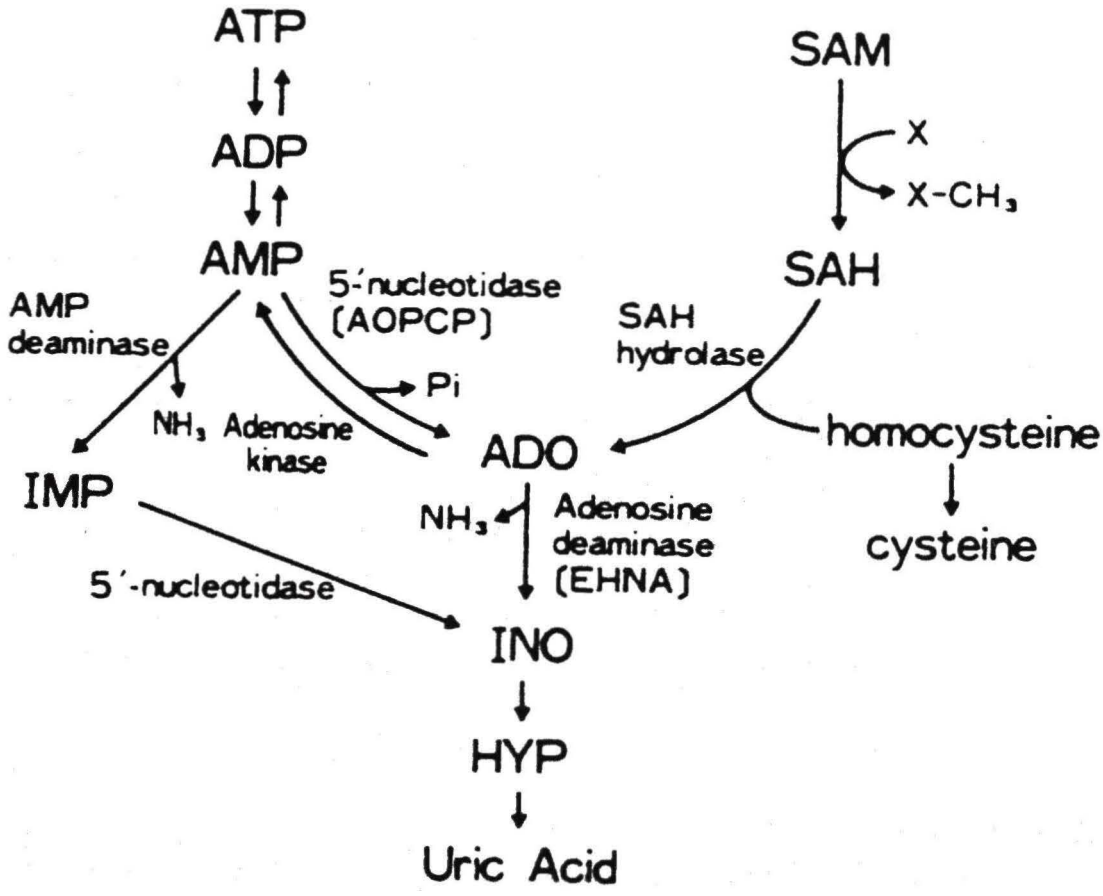

Figure 1. Schematic diagram of adenosine metabolism. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; ADO, adenosine; AMP, adenosine 5'-monophosphate; IMP, inosine 5'-monophosphate; INO, inosine; HYP, hypoxanthine; AOPCP, α,β-methylene adenosine 5'-diphosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine. (from Hori M and Kitakaze M, *Hypertension* 1991;18:(5):565-574).

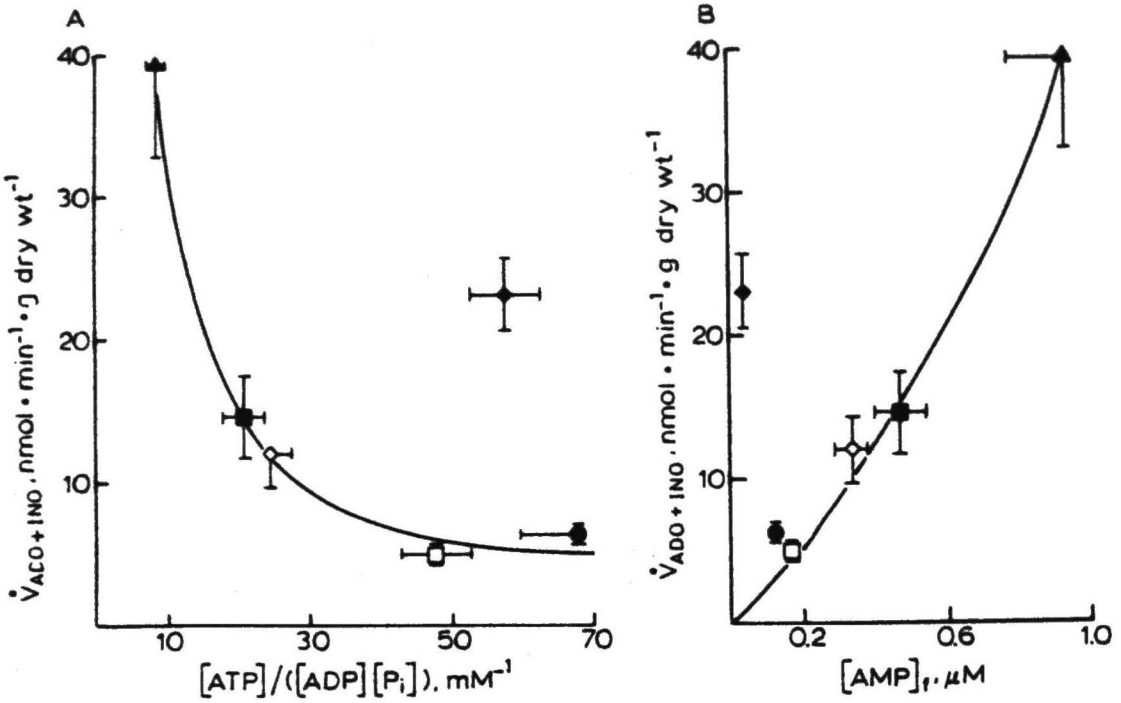

Figure 2. Coronary purine nucleoside release ($V_{ADO+INO}$) as functions of cytosolic [ATP]/([ADP][P_i]) (panel A) or [AMP]$_f$ (panel B) in isolated empty-beating, normoxic Langendorff guinea pig heart. Perfusion pressure was 87 cmH_2O. Energy-yielding substrates (all 5 mM; 5 U/l insulin) were: ▲, substrate-free; ◊, glucose; □, glucose + lactate; ●, glucose + pyruvate; ■, glucose + acetate; ✦, glucose (6 mM $MgSO_4$). Experiments were terminated by freeze-clamping the hearts at 30 min perfusion; nucleoside release was the mean of double determinations at 24 - 25 min perfusion. Data are means ± SE from 5 - 8 experiments. Cytosolic free Mg^{2+} concentration was taken as 0.42 mM in 0.6 mM $MgSO_4$ controls and 2.45 mM in 6 mM MgSO4 perfusions. Purine nucleosides (ADO = adenosine, INO = inosine) were measured using standard reverse-phase HPLC procedures. Intersection of solid line in panel B was statistically acceptable but intersection on the ordinate near 3 nmol/min · g dry wt was also statistically valid. (from Bünger R, Mallet RT, Kang YH. In: Imai S, Nakazawa M, eds. *Role of Adenosine and Adenine Nucleotide in the Biological System.* Elsevier: Amsterdam/New York/Oxford, 1991:337-353).

CHAPTER II

DOES INTERSTITIAL ADENOSINE MEDIATE ACUTE HIBERNATION OF GUINEA-PIG MYOCARDIUM?

Zhi-Ping Gao, H. Fred Downey, Wen-Lin Fan, and Robert T. Mallet

Cardiovascular Research 1995;29:796-804

ABSTRACT

Objective: This investigation tested the role of interstitial adenosine in protective down-regulation of myocardial energy demand during myocardial hibernation. ***Methods:*** Isolated, working guinea-pig hearts, perfused with glucose-fortified Krebs-Henseleit buffer, were subjected to 60 min global, low-flow ischaemia, followed by 30 min reperfusion. Left ventricular performance was assessed from heart rate-developed pressure product and pressure-volume work. Cytosolic energy level was indexed by creatine phosphate and ATP phosphorylation potentials measured in stop-frozen myocardium. Lactate and purine nucleosides (adenosine, inosine) were measured in venous effluent. ***Results:*** When coronary flow was lowered 80% for 60 min, heart rate-pressure product and pressure-volume work fell 87% and 75%, respectively, and stabilised at these low levels, but fully recovered when flow was restored. Myocardial ATP phosphorylation potential fell 67% during the first 10 min ischaemia, but subsequently recovered to pre-ischaemic levels despite continuing ischaemia, indicating down-regulation of myocardial energy demand. Lactate release increased about 10-fold during ischaemia and remained elevated until reperfusion. Purine nucleoside release varied reciprocally with phosphorylation potential, peaking at 10 min ischaemia, then gradually returning to the pre-ischaemic level during the subsequent 50 min ischaemia. The *ecto* 5'-nucleotidase inhibitor α,β-methylene adenosine 5'-diphosphonate (AOPCP, 50 μM) decreased ischaemic purine nucleoside release by 41%, but did not attenuate post-ischaemic contractile recovery. The unspecific adenosine receptor antagonist 8-*p*-sulfophenyl

theophylline (8-SPT, 20 μM) doubled ischaemic lactate release and lowered coronary venous purine nucleoside release by 21%. 8-SPT increased phosphorylation potential at 10 min ischaemia relative to untreated hearts, but blunted the subsequent rebound of phosphorylation potential. 8-SPT treatment during ischaemia resulted in a significantly higher cytosolic phosphorylation potential at 30 min reperfusion, but did not affect post-ischaemic contractile function. ***Conclusion:*** We conclude that activation of adenosine receptors results in recovery of cytosolic energy level of moderately ischaemic working myocardium, but this energetic recovery is not solely responsible for post-ischaemic contractile recovery.

INDEX TERMS: Adenosine, adenosine receptor blockade, inosine, myocardial ischaemia, ATP phosphorylation potential, myocardial hibernation, 5'-nucleotidase

ENZYMES: 5'-nucleotidase (EC 3.1.3.5), creatine kinase (EC 2.7.3.2)

ABBREVIATIONS: AOPCP: α,β-methylene adenosine 5'-diphosphonate; 8-SPT: 8-*p*-sulfophenyl theophylline; P_a: aortic pressure; P_v: left atrial filling pressure; MVO_2: myocardial oxygen consumption; pH_i: intracellular pH; HPLC: high performance liquid chromatography; CrP: creatine phosphate; Cr: creatine; P_i: inorganic phosphate; K_{CK}: creatine kinase equilibrium constant; ANOVA: analysis of variance; $V_{ADO+INO}$: rate of adenosine + inosine release

INTRODUCTION

Myocardial 'hibernation', first characterised in cardiac patients by Rahimtoola[1,2], is a state of depressed contractile function and energy demand in chronically ischaemic myocardium. Hibernating myocardium has been described clinically in patients experiencing stable or unstable angina or coronary stenosis. In these patients, contractile depression is reversed by coronary bypass or angioplasty. Several studies in experimental mammalian heart preparations have shown that hibernation is an adaptive process in the myocardium that protects the heart muscle from ischaemic injury. Pantely *et al.*[3, 4] induced hibernation in *in situ* porcine hearts by reducing coronary flow by 25-50%. In these studies, subendocardial ATP and creatine phosphate contents fell appreciably by 5 min hypoperfusion. Despite persistent hypoperfusion, creatine phosphate content recovered to the pre-ischaemic level at 60 min hypoperfusion, while ATP content remained moderately decreased. Schultz *et al.*[5] reported a marked increase in lactate production at 15 min hypoperfusion followed by a modest decline toward control levels when coronary flow was lowered sufficient to depress systolic wall thickening by 50% in porcine heart *in situ*. These workers also demonstrated that the ischaemic myocardium retained appreciable inotropic reserve which could be recruited by dobutamine.[5] In an earlier study, Fedele *et al.*[6] demonstrated in *in situ* porcine myocardium that an 80% reduction in coronary arterial diameter produced a similar biphasic response of lactate release and an apparent sustained reduction in myocardial oxygen consumption. Downing and Chen[7] developed an isolated, nonworking neonatal piglet heart model of hibernation

in which coronary flow was lowered to 10% of control for 2 hr, and then restored to pre-ischaemic level. They observed that myocardial ATP and creatine phosphate contents were identical in both reperfused and control groups, although glycogen was lowered in the post-ischaemic hearts. In studies of acutely hibernating isolated rat hearts, Schaefer *et al.*[8] found that left ventricular performance fell when coronary flow was decreased, but completely recovered when flow was restored; moreover, creatine phosphate content recovered during hypoperfusion after an initial decline despite progressive loss of ATP. These studies have raised the possibility that hibernation could be therapeutically induced in the clinical setting to protect the heart during surgery or other procedures causing a temporary reduction in coronary flow. However, this advance will require knowledge of the cellular mechanisms for hibernation.

Exogenous adenosine has been shown to protect the myocardium during ischaemic stress.[9] The potentially beneficial effects of adenosine include coronary vasodilation,[10] adrenergic antagonism,[11] attenuation of infarction,[12] and enhancement of glucose uptake[13] and glycolysis.[14] Adenosine treatment has also been shown to reduce post-ischaemic contractile dysfunction or 'stunning'.[15] During myocardial ischaemia, production and release of adenosine is sharply increased;[16, 17] thus, endogenous adenosine would be available to exert a protective effect, possibly by sustaining high energy phosphate reserves during ischaemia-induced reduction in contractile function. However, the role

of adenosine in mediating this down-regulation of myocardial energy demand during hibernation has not been examined.

The present study was undertaken to establish a model of metabolic down-regulation, *i.e.* myocardial hibernation, in the isolated perfused guinea-pig heart performing external work and to test the hypothesis that adenosine produced during moderate ischaemia is responsible for the metabolic down-regulation observed in this model. The proposed role of adenosine in modulating myocardial energy reserve and contractile function was studied with two different pharmacological approaches: inhibition of extracellular adenosine production by α,β-methylene adenosine 5'-diphosphonate, a potent inhibitor of the *ecto* 5'-nucleotidase,[18,19] and blockade of adenosine receptors during ischaemia with the non-selective adenosine antagonist 8-*p*-sulfophenyl theophylline. The present model provides an opportunity to delineate the metabolic effects of coronary hypoperfusion in the absence of confounding influences of autonomic stimulation and hormones, in a heart performing external work at physiological rates.

METHODS

Animal experimentation was approved by the institutional Animal Care and Use committee and conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1985). Hearts (n = 78) beating at intrinsic sinus rhythm, were isolated from methoxyflurane-anaesthetised Hartley guinea-pigs (400-600 g body mass),

and perfused as working hearts.[20] Perfusion medium was a modified Krebs-Henseleit bicarbonate buffer (38 °C, pH 7.35-7.40) aerated with 95% O_2:5% CO_2 and containing the following ionic composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, and $CaCl_2$ 1.0. 10 mM glucose (+ 5 U/L bovine insulin; Sigma, St Louis, MO, USA) was supplied as energy-yielding substrate. Water for perfusion media and analytical tests was freshly prepared (up to 24 h prior to use) by distillation (Corning low-output quartz still) and subsequent filtration (Barnstead Econo-pure water purification system). α,β-methylene adenosine 5'-diphosphonate (AOPCP) and 8-*p*-sulfophenyl theophylline (8-SPT) were purchased from Sigma and Research Biochemicals (Natick, MA, USA), respectively.

Left ventricular haemodynamics

Cardiac performance was assessed from spontaneous heart rate, aortic pressure (P_a), and left atrial filling pressure (P_v). These variables were continuously monitored with a multi-channel polygraph (Beckman Model R611). Coronary and aortic flows were measured by timed collections; cardiac output was taken as the sum of coronary and aortic flows. Myocardial energy expenditure was assessed from myocardial O_2 consumption, left ventricular pressure development per min (heart rate times (P_a - P_v)) and from left ventricular pressure-volume work (*i.e.* stroke work times heart rate), an index of power.

Blood gases and coronary effluent metabolites

PO_2 and PCO_2 of left atrial inflow and coronary venous effluent were measured polarographically in a Corning Model 178 pH/Blood Gas Analyzer. Myocardial oxygen consumption (MVO_2) was calculated as the product of arteriovenous oxygen content difference and coronary flow. Intracellular pH (pH_i) was calculated from measured coronary venous PCO_2 as previously described:[16]

$$pH_i = 7.524e^{-0.0008786 \cdot pCO2}$$

Lactate in coronary effluent was measured by enzymatic assay.[21] Coronary venous effluent purine nucleosides (adenosine, inosine) were analyzed by high performance liquid chromatography (HPLC; Shimadzu Instruments, Columbia, MD, USA). Coronary effluent was heated (100°C) for 8 min immediately following collection to inactivate adenosine deaminase and filtered (0.2 μm pore size) prior to HPLC analysis. 50 μl samples were injected onto an ODS-Hypersil reverse-phase 5 μm C-18 column (Keystone Scientific, Bellefonte, PA, USA). HPLC solvents were 100 mM $NH_4H_2PO_4$ (Fisher Scientific, Fair Lawn, NJ, USA) in 1% methanol at pH 5.3 (solvent A), and 25% methanol at pH 5.58 (solvent B). A nonlinear bisolvent gradient was employed at a flow rate of 1.3 ml/min: 0 - 4 min: 100% A; 4-9 min: 70% to 60% A; 9-15 min: 40% to 30% A; 15-19 min: 0% A. Absorbance of the eluate was monitored at 254 nm. Compounds were identified and quantified by comparing retention times and peak areas with known standards. Rates of purine and lactate release were taken as respective coronary effluent concentrations times coronary flow.

Experiments were terminated by freeze-stop with Wollenberger tongs pre-cooled to constant temperature in liquid N_2. Stop-frozen myocardium was powdered in a liquid N_2-cooled porcelain mortar. An aliquot of powder was desiccated to constant mass at 100°C for determination of total tissue water content. Myocardial metabolites were extracted with 0.3 N $HClO_4$ as described previously.[16] Metabolites (ATP, ADP, AMP, creatine phosphate (CrP), creatine (Cr), inorganic phosphate (P_i) and lactate) were assayed enzymatically[21] in a Perkin Elmer Lambda-2 dual wavelength uv/vis spectrophotometer (measuring wavelength: 337 nm; reference wavelength: 417 nm ; $\epsilon = 5.65\ M^{-1} \cdot cm^{-1}$).

Extracellular and intracellular solvent spaces

Extracellular solvent space was taken as the distribution volume of [^{14}C]inulin[18]. [^{14}C]inulin carboxylic acid (50 mg · liter^{-1}; specific activity = 0.33 μCi/mg; Amersham, Arlington Heights, IL, USA) was infused into the left atrium for 5 min prior to freeze-stop. Measurements in serial collections indicated that coronary effluent radioactivity plateaued within 3 min. Radioactivities in myocardial extracts and venous effluent were measured by standard liquid scintillation techniques.[20] Intracellular fluid space (ml · g^{-1} wet mass) was taken as the difference between total tissue water content and extracellular space and calculated as total tissue H_2O (ml · g^{-1} wet mass) - extracellular space (ml · g^{-1} wet mass). Intracellular concentration of P_i was calculated as [(total tissue content - extracellular content)/intracellular space], where extracellular P_i content equalled coronary effluent P_i concentration times [^{14}C]inulin space.

Cytosolic phosphorylation potential, [ATP]/([ADP][P_i]), was calculated from the mass action ratio of measured intracellular creatine kinase reactants:

$$[ATP]/([ADP][P_i]) = ([CrP][H^+])/([Cr][P_i]\cdot K_{CK})$$

where K_{CK} is the pH- and Mg^{2+}-dependent creatine kinase (CK) equilibrium constant. $[H^+]/K_{CK}$ was determined from the following equation:[22]

$$[H^+]/K_{CK} = 10^{(-0.87 \cdot pHi) + 8.31}$$

where cytosolic free Mg^{2+} is estimated at 0.6 mM.[22]

Experimental protocol

The experimental hypoperfusion/reperfusion protocol is summarised in Figure 1. Hypoperfusion was produced by decreasing P_a, *i.e.* coronary perfusion pressure, in two stages, from 90 cm H_2O to 45 cm H_2O for 15 min (mild hypoperfusion phase), then to 22.5 cm H_2O (ischaemia phase). P_a was held at this level for 60 min, then restored within 15 s to 90 cm H_2O (reperfusion) and held at this level for 30 min. P_v was maintained at 12 cm H_2O throughout the protocol. Hearts were stop-frozen at 30 min reperfusion. To measure myocardial energy metabolites at selected times during this protocol, hearts were stop-frozen at 15 min pre-ischaemia and at 10, 30, and 60 min ischaemia. In time control experiments, P_a and P_v were held at 90 cm H_2O and 12 cm H_2O, respectively, for 120 min, followed by freeze-stop.

In some experiments, hearts received 50 μM AOPCP from the beginning of the protocol until the end of the ischaemic phase (Figure 1). This concentration of AOPCP has been shown to maximally inhibit *ecto* 5'nucleotidase.[19] In other experiments, hearts received the unspecific adenosine receptor antagonist 8-SPT. Preliminary measurements were performed to determine the 8-SPT concentration which blocked the bradycardia resulting from adenosine administration. The heart rate response to a left atrial bolus injection of 150 μg adenosine was measured before and during 8-SPT infusion. A pronounced but transient bradycardia, including cessation of heart beats for several seconds, resulted from the adenosine bolus. 10 μM 8-SPT attenuated, and 20 μM 8-SPT completely blocked, the bradycardia. Accordingly, 20 μM 8-SPT was infused continuously from the beginning of the 15 min mild hypoperfusion period until reperfusion onset following 60 min severe ischaemia (Figure 1). To examine the concentration dependence of 8-SPT's effects, an additional 6 hearts were perfused with 200 μM 8-SPT and subjected to the ischaemia/reperfusion protocol. This higher concentration of the blocker prevented bradycardia following the adenosine bolus and also decreased adenosine-induced hyperaemia by 65 ± 13%.

Statistical analysis

Data are means ± SEM. Groups were compared by one way analysis of variance (ANOVA) in combination with Student-Newman-Keuls test for multiple comparisons.

Statistical analyses were performed with SigmaStat statistical software. P values < 0.05 were taken to indicate statistical significance.

RESULTS

Functional characteristics of ischaemic/reperfused working hearts

Isolated working guinea-pig hearts were subjected to the global, low-flow ischaemia/reperfusion protocol detailed in Figure 1. As expected, coronary flow fell in lockstep with aortic pressure, *i.e.* coronary perfusion pressure, and quickly stabilised during ischaemia at 20% of the pre-ischaemic level (Figure 2A). Left ventricular contractile function, assessed from heart rate-pressure product (Figure 2B) and pressure-volume work (Figure 2C) paralleled the changes in coronary flow: heart rate-pressure product and pressure-volume work fell to 42 and 68% of the respective pre-ischaemic levels when aortic pressure was decreased from 90 to 45 cm H_2O, and fell further to 13 and 25% of the pre-ischaemic levels when aortic pressure was lowered to 22.5 cm H_2O. Contractile function quickly stabilised and did not decline further as ischaemia was prolonged. During reperfusion, coronary flow and left ventricular function recovered within 15 min to the levels observed in the time control group; thus, post-ischaemic contractile dysfunction (*i.e.*, 'stunning') was not evident in the reperfused hearts compared to the time control level. In the time control group, heart rate-pressure product fell 11% during the 2 hr protocol and pressure-volume work fell 29%.

Metabolic features of ischaemic/reperfused hearts

Coronary venous PCO_2 increased sharply at onset of ischaemia from 41 ± 1 to 53 ± 2 mm Hg and remained at this level throughout ischaemia. The increased venous PCO_2 reflected a modest decline in intracellular pH from 7.26 to 7.19 (Figure 3A). Oxidative metabolism, assessed from myocardial oxygen consumption (MVO_2), fell from 2.5 ± 0.2 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$ initially to 1.7 ± 0.1 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$ during mild hypoperfusion and further to 0.92 ± 0.08 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$ at 5 min severe ischaemia. MVO_2, like coronary flow and left ventricular function, was essentially stable during the ischaemic period; at 60 min ischaemia, MVO_2 was 0.72 ± 0.09 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$. During reperfusion, recovery of oxidative metabolism paralleled contractile function; MVO_2 equalled 2.0 ± 0.2 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$ at 30 min. MVO_2 remained essentially constant during the 2 hr protocol of time control experiments, averaging 2.5 ± 0.1 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$ initially and 2.1 ± 0.2 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$ at 2 hr (P = n.s.).

Anaerobic glycolytic rate and the level of ischaemic stress during the protocol were assessed from myocardial lactate release. Lactate release rate during pre-ischaemia was 0.09 ± 0.03 μmol $\cdot min^{-1} \cdot g^{-1}$ wet mass. The initial reduction in P_a to 45 cm H_2O did not increase lactate release (Figure 3B). When P_a was further lowered to 22.5 cm H_2O, lactate release rose sharply, cresting at 0.76 ± 0.09 μmol $\cdot min^{-1} \cdot g^{-1}$ at 7 min. Lactate release remained elevated during the subsequent ischaemic period, and equalled 0.69 ± 0.09 μmol $\cdot min^{-1} \cdot g^{-1}$ at 60 min ischaemia. During reperfusion, lactate release fell

rapidly but tended to remain somewhat elevated relative to time controls. Lactate release in time control experiments remained below 0.1 μmol $\cdot$ min^{-1} $\cdot$ g^{-1} wet mass throughout the 2 hr protocol (Figure 3B).

In the untreated ischaemia group, purine nucleoside (adenosine + inosine) release did not increase during the initial 15 min mild hypoperfusion (Figure 4A). At the onset of severe ischaemia, coronary venous purine nucleoside release rose sharply by over tenfold ($P < 0.001$), peaking within 10 min at 3.6 ± 0.4 nmol $\cdot$ min^{-1} $\cdot$ g^{-1}. Ischaemic purine nucleoside release was markedly biphasic; after the initial peak, adenosine + inosine release fell progressively, and did not exceed the time control value at 60 min ischaemia. Purine nucleoside release remained low during 30 min reperfusion. In the time control group, purine nucleoside release was low and essentially constant at approximately 0.2 nmol $\cdot$ min^{-1} $\cdot$ g^{-1} wet mass.

Energy metabolites and phosphorylation potential

Table 1 presents myocardial contents of key energy metabolites and lactate as well as inorganic phosphate concentrations and cytosolic phosphorylation potentials at selected times during the experimental protocol. Creatine phosphate tended to fall in the first 10 min of ischaemia (P = n.s.), but by 60 min creatine phosphate content was 36% above the 10 min level ($P < 0.01$). The ratio of creatine phosphate/creatine fell 54% during the first 10 min ischaemia, then gradually to recovered to the pre-ischaemic level during the

following 50 min ischaemia (Table 1). ATP content did not change appreciably during the protocol. Intracellular P_i concentration increased 41% during the first 10 min ischaemia ($P < 0.05$), and subsequently fell to near the pre-ischaemic level (P = n.s.). Myocardial lactate content increased markedly at the onset of ischaemia, rising fivefold by 10 min. Myocardial lactate content fell significantly later in ischaemia (Table 1), while coronary effluent lactate release remained elevated (Figure 3B). At 30 min reperfusion, myocardial lactate content stabilised at the time control levels (Table 2).

Cytosolic energy level during ischaemia and reperfusion was indexed by cytosolic ATP phosphorylation potential (Table 1, Figure 5), which was determined from measured creatine kinase reactants (Table 1) according to the creatine kinase equilibrium (see Methods). ATP potential fell sharply at the onset of ischaemia. By 10 min of ischaemia, when purine nucleoside release was at its maximum rate, ATP phosphorylation potential had fallen by 67% relative to the pre-ischaemic baseline. Subsequently, cytosolic energy level progressively recovered despite continued ischaemia: by 60 min ischaemia, ATP phosphorylation potential was fully restored to the pre-ischaemic level. Thus, cytosolic energy level completely recovered in the face of ischaemic stress sufficient to markedly depress contractile function and trigger an appreciable, albeit transient, release of adenylate degradatives and significant, sustained lactate release. The relationship between coronary effluent purine nucleoside release and cytosolic ATP phosphorylation potential is shown in Figure 6. The data indicate that, as expected, purine nucleoside release is

strongly and inversely correlated with cytosolic energy level in both non-ischaemic and ischaemic myocardium. Thus, this moderately ischaemic working heart model exhibited several hallmarks of protective metabolic down-regulation during coronary hypoperfusion: full energetic recovery in the face of prolonged ischaemia, and normalization of contractile performance (*i.e.*, absence of stunning) during reperfusion.

Haemodynamic effects of adenosine receptor blockade and ecto 5'-nucleotidase inhibition

Next, experiments were undertaken to test the hypothesis that adenosine, present in the interstitial fluid and acting on sarcolemmal A_1 receptors, could mediate ischaemic down-regulation of metabolic demand. Two interventions were employed to examine two steps in the proposed pathway: interstitial adenosine formation, and interaction of adenosine with its sarcolemmal receptors. A series of hearts were treated with 8-*p*-sulfophenyl theophylline (8-SPT; Figure 1), a unspecific adenosine receptor antagonist, and a second series of hearts was treated with the selective *ecto* 5'-nucleotidase inhibitor α,β-methylene adenosine 5'-diphosphonate (AOPCP; Figure 1). Importantly, neither 8-SPT nor AOPCP altered coronary flow during mild hypoperfusion or ischaemia, relative to the untreated ischaemic hearts. Also, heart rate-pressure product (HR $\cdot$ (P_a - P_v): cm $H_2O \cdot min^{-1} \cdot 10^{-3}$) at 30 min reperfusion did not differ among the three groups (untreated ischaemia hearts: 15.1 ± 1.0; 8-SPT-treated ischaemia hearts: 15.6 ± 0.6; AOPCP-treated ischaemia hearts: 13.5 ± 1.1; P = n.s.), nor did left ventricular pressure-volume work differ ($mJ \cdot min^{-1} \cdot g^{-1}$: untreated ischaemia hearts: 76 ± 9; 8-SPT-treated ischaemia hearts:

89 ± 13; AOPCP-treated ischaemia hearts: 60 ± 19; P = n.s.; Figure 2). When 8-SPT concentration was increased to 200 μM, post-ischaemic recoveries of heart rate-pressure product (16.1 ± 1.3) and pressure-volume work (60 ± 20) did not significantly differ from that of the other groups. Thus, post-ischaemic contractile recovery was not attenuated by adenosine A_1 receptor blockade or by inhibition of interstitial adenosine production.

Metabolic consequences of adenosine receptor blockade and ecto 5'-nucleotidase inhibition

8-*p*-Sulfophenyl theophylline and, to a lesser extent, AOPCP intensified the intracellular acidification that occurred at the onset of ischaemia (Figure 3A). Intracellular pH remained depressed throughout the ischaemic period in the treated groups relative to untreated ischaemic hearts, especially in the 8-SPT group. Intracellular pH did not differ among the three ischaemia groups during reperfusion. Lactate release was markedly increased throughout 8-SPT treatment (Figure 3B), and remained significantly elevated during the first 15 min reperfusion. In contrast, lactate release was significantly increased only at 5 min ischaemia by AOPCP. Thus, A_1 receptor blockade, but not *ecto* 5'-nucleotidase inhibition, stimulated glycolytic flux during ischaemia and reperfusion.

Both 8-SPT and AOPCP attenuated but did not abolish the biphasic purine nucleoside release during ischaemia (Figure 4A). Thus, maximally effective inhibition[19] of *ecto* 5'-nucleotidase decreased adenosine + inosine release by only 41%, indicating that the *ecto*

enzyme is not the sole source of interstitial adenosine in ischaemic myocardium. Figure 4B reveals that 8-SPT treatment increased initial purine release relative to untreated hearts in the first three min of ischaemia. Later in ischaemia, however, purine release of 8-SPT treatment hearts fell below that of the untreated hearts.

The effects of ischaemia/reperfusion and adenosine antagonism on myocardial energy metabolites and phosphorylation potentials measured at 30 min reperfusion are summarised in Table 2. Total myocardial ATP and CrP contents, intracellular P_i concentration, and cytosolic phosphorylation potential were similar to the respective time control levels. Notably, AOPCP treatment during ischaemia did not diminish post-ischaemic recovery of ATP potential. Moreover, 8-SPT treatment during ischaemia was associated with appreciable increases in creatine phosphate content and cytosolic ATP phosphorylation potential and decreased intracellular P_i concentration during reperfusion. 8-SPT slowed the decline in cytosolic phosphorylation potential during the initial 10 min ischaemia ($P < 0.05$) but attenuated the subsequent recovery of phosphorylation potential as ischaemia was prolonged (Figure 5). Thus, pharmacological antagonism of interstitial adenosine during ischaemia blunted recovery of cytosolic energetics during low-flow ischaemia, but failed to attenuate post-ischaemic recovery of left ventricular mechanical function and myocardial energetics in this model of myocardial hibernation.

DISCUSSION

The overall goal of this investigation was to test the hypothesis that interstitial adenosine mediates down-regulation of myocardial energy demand, *i.e.* 'hibernation' during low-flow ischaemia in isolated working guinea-pig heart. The principal findings were as follows. Firstly, left ventricular contractile function fell quickly at the onset of ischaemia and remained depressed throughout the ischaemic period, but recovered completely following restoration of coronary flow. Thus, post-ischaemic cardiac dysfunction, *i.e.* 'stunning', was not evident in this model. Secondly, cytosolic energy level fell sharply at the onset of ischaemia, but significantly recovered later despite continued ischaemia, and was similar to time controls at 30 min reperfusion. Thirdly, inhibition of *ecto* 5'-nucleotidase partially attenuated coronary venous purine nucleoside release, but did not impair post-ischaemic contractile or energetic recovery. Lastly, blockade of adenosine receptors by 8-*p*-sulfophenyl theophylline (8-SPT) attenuated the recovery of cytosolic energy level during prolonged ischaemia, but failed to impair post-ischaemic recovery of contractile function and cytosolic energetics. Thus, this isolated working heart preparation exhibits many of the hallmarks of hibernating myocardium and therefore can be used as an experimental model of this newly recognised phenomenon. The present findings support a role for interstitial adenosine in mediating the protective metabolic down-regulation during myocardial hibernation.

Isolated working heart model

The present isolated, working heart preparation offers several significant advantages over *in situ* heart models. It enables intrinsic cardiac function and energetics to be studied in the absence of potentially confounding humoral and neural effects, and facilitates rigorous experimental control of exogenous substrate supply, oxygen delivery, and left ventricular preload and afterload. Also, this isolated heart preparation performed physiological levels of external hydraulic work prior to ischaemia. A disadvantage of this preparation is that the perfusate does not contain erythrocytes and consequently has a lower O_2-carrying capacity than blood. Because of these differences with *in situ* hearts, it was essential to verify that the present isolated working heart model behaves in a manner comparable to *in situ* heart models, prior to studying the mechanism of acute metabolic downregulation.

The present isolated heart model exhibited several hallmarks of myocardial down-regulation which have been recently characterised in *in situ* myocardium[3-5, 23] and isolated perfused heart preparations.[7, 8, 24] When coronary flow was lowered 80% for 60 min, left ventricular contractile performance and myocardial oxygen consumption stabilised at decreased levels after an initial reduction, consistent with the recent findings of Downing and Chen.[24] Several metabolic features of the hypoperfused hearts indicated that they were indeed ischaemic: lactate release increased more than tenfold, cytosolic ATP phosphorylation potential fell sharply, and myocardial release of purine nucleosides was

markedly elevated. Creatine phosphate content tended to fall at ischaemia onset but increased significantly as ischaemia was prolonged, consistent with previous reports in *in situ*, regionally ischaemic, porcine myocardium[3] and isolated, nonworking, perfused rat heart.[8] Lactate release (Figure 3) was persistently elevated, and a moderate intracellular acidosis was present throughout the ischaemic period. This finding differed from that of Fedele *et al.*[6] and Arai *et al.*[4] who observed a decline in lactate release during prolonged ischaemia in *in situ* pig myocardium. However, the striking increase in myocardial lactate content (Table 1) during the first 10 min of ischaemia was largely reversed by 60 min, a finding consistent with the report of Arai *et al.*[4] Also in agreement with previous reports of other hibernation models,[8, 25] the working guinea-pig hearts achieved full recovery of ventricular function and cytosolic energy level after restoration of coronary flow. It therefore appears that the present low-flow ischaemic model is suitable for the study of metabolic down-regulation, *i.e.* acute myocardial 'hibernation'.

Down-regulation of myocardial energy demand

Gallagher *et al*[26] in 1983 proposed that myocardial energy requirements might be gradually decreased, *i.e.* down-regulated, during ischaemia until a new equilibrium is established between limited energy supply and reduced demand. In the present study, total myocardial ATP content did not decrease appreciably during the experimental ischaemia/reperfusion protocol (Tables 1, 2). However, ATP content alone does not provide quantitative information regarding cytosolic free energy level.[27] Accordingly, in

the present study cytosolic energy level was indexed by cytosolic ATP phosphorylation potential, the principal variable determinant of Gibbs free energy of ATP hydrolysis.[28] In the first 10 min ischaemia, cytosolic ATP phosphorylation potential fell 67%, indicating that myocardial energy consumption exceeded energy production during this period. Notably, the cytosolic energy status progressively improved as ischaemia was continued. In the absence of metabolic down-regulation, rates of ATP consumption would have exceeded the diminished ATP production, causing continuous depletion of myocardial high energy phosphates during ischaemia. Our findings therefore confirm that the myocardium has the capacity to down-regulate its energy demand in response to reduced energy supply, at least during relatively moderate ischaemic stress.

Arai *et al.*[4] proposed two models of myocardial metabolic down-regulation. In the passive down-regulation model, myocardial energy requirements gradually fall until a new balance is reached between residual ischaemic ATP production and utilization; according to this model, cytosolic energy level stabilises but does not rebound during protracted ischaemia. In the active down-regulation model, ATP utilization falls below flow-limited production, allowing recovery of ATP phosphorylation potential as ischaemia is prolonged. Our results suggest that the myocardial energy requirements are indeed actively down-regulated during ischaemia by decreasing ATP utilization, since ATP phosphorylation potential rebounded while oxidative phosphorylation, indexed by MVO_2, and anaerobic glycolysis, assessed from lactate release, remained stable during ischaemia.

While lactate release was persistently elevated during ischaemia in the present model, enhanced glycolytic energy production could be instrumental in adapting the myocardium to the ischaemic conditions.

Adenosine: a mediator of metabolic down-regulation?

Considerable experimental evidence has implicated adenosine as a mediator of two potentially cardioprotective mechanisms: antagonism of β-adrenergic stimulation,[11] and ischaemic preconditioning.[12] In ischaemic myocardium, adenosine is generated from AMP in the interstitium and cardiocyte cytosol by *ecto* 5'-nucleotidase and cytosolic 5'-nucleotidase, respectively.[29] Myocardial adenosine release is augmented during ischaemia, in parallel with a decline in cytosolic ATP phosphorylation potential and a corresponding increase in cytosolic free AMP.[30]

Binding of interstitial adenosine to A_1 receptors in the plasma membranes of cardiomyocytes has been proposed to initiate its antiadrenergic[11] and cardioprotective effects.[31] To test the possibility that down-regulation in the present model was induced by adenosine binding to the A_1 receptor, hearts were treated with the adenosine antagonist 8-SPT at a concentration sufficient to block the A_1 receptor-mediated negative chronotropic effects of intracoronary adenosine.[31] 8-SPT treatment did not impair post-ischaemic contractile recovery, and increased, rather than decreased, post-ischaemic cytosolic energy level. Interestingly, 8-SPT nearly prevented the rebound in cytosolic

energy level between 10 and 60 min ischaemia. This evidence suggests that activation of adenosine receptors by interstitial adenosine could be at least partially responsible for the observed recovery of cytosolic energy level during low-flow ischaemia.

We proposed that AOPCP, by inhibiting interstitial adenosine formation, would impede metabolic down-regulation during ischaemia if it were induced by interstitial adenosine, perhaps independent of adenosine receptors. In fact, AOPCP decreased purine nucleoside release by 41% during ischaemia, but had no discernable effect on post-ischaemic energetic or contractile recovery. This finding indicates that adenosine formed by *ecto* 5'-nucleotidase is not required for functional recovery following moderate hypoperfusion. The concentration of AOPCP applied in the present study, 50 μM, completely inhibited *ecto* 5'-nucleotidase in guinea-pig myocardium,[19] but only partially inhibited adenosine release during the present low-flow ischaemia (Figure 4). Thus, adenosine produced from other sources may have been sufficient to elicit down-regulation in the absence of adenosine receptor blockade. These findings also suggest that the cytosolic 5'-nucleotidase is an important source of adenosine in this moderately ischaemic working heart model.

Role of adenosine in myocardial glucose metabolism

A significant finding of the present study is that 8-SPT greatly increased glycolysis during ischaemia (Figure 3B) and elevated ATP phosphorylation potential in reperfused

myocardium (Table 2). Moreover, lactate release was stimulated by 8-SPT during the initial mild hypoperfusion phase, although purine nucleoside release remained at control levels indicating that ischaemia was absent. Thus, adenosine receptor blockade was associated with increased anaerobic glycolysis in both moderately hypoperfused and ischaemic myocardium. In non-ischaemic myocardium, glycolysis is a relatively minor contributor to overall myocardial ATP production. During ischaemia, oxidative metabolism is impaired, and anaerobic glycolysis assumes a crucial role in supplying sufficient ATP to maintain cellular integrity and to facilitate contractile recovery upon reperfusion.

The effects of adenosine A_1 receptor blockade on cytosolic energetics during ischaemia appeared contradictory. At the onset of ischaemia, A_1 blockade lessened the decline of ATP phosphorylation potential, while later in ischaemia the recovery of phosphorylation potential was clearly prevented (Figure 5). Furthermore, phosphorylation potential was appreciably higher in reperfusion in treated hearts compared with untreated controls, despite the lower phosphorylation potential at 60 min ischaemia. These data suggest a precarious balance may exist between adenosine receptor mediated enhancement of energy production from anaerobic glycolysis and a fall in energy level due to blockade of adenosine receptor-mediated down-regulation of energy demand. At 10 min ischaemia, glycolytic flux was appreciably higher in the 8-SPT treated group than in untreated controls, and this enhanced glycolysis was associated with a higher cytosolic energy level

(Figure 5). Thus, early in ischaemia glycolytic ATP production may overcome a competing adverse effect of adenosine receptor blockade. Later, glycolytic rate fell somewhat and at 60 min ischaemia did not differ between the untreated and 8-SPT treated hearts. At this time, the ability of glycolysis to sustain cytosolic energy level may have been overmatched by the impairment of down-regulation of energy demand due to adenosine receptor blockade. Following reperfusion, however, cytosolic phosphorylation potential was appreciably higher in the 8-SPT group than the untreated controls. This beneficial effect may have resulted from sustained stimulation of glycolysis during the first 15 min reperfusion (Figure 3B), when glycolysis is crucial for restoring cytosolic energy level.[32] Thus, overall the observed stimulation of anaerobic glycolysis by adenosine receptor antagonism appeared to promote cytosolic re-energization during reperfusion.

Activation of glycolysis by adenosine receptor blockade is consistent with recent reports that adenosine can inhibit glycolysis. Finegan *et al.*[33] found that glycolytic rate in ischaemic rat myocardium was lowered 45% by adenosine pretreatment. Glycolytic flux during reperfusion was inhibited by adenosine applied either concurrently or during the prior ischaemic period. Dale *et al.*[34] reported inhibition of glycolysis by the adenosine A_1-receptor agonist N^6-(L-phenylisopropyl)adenosine. In contrast to these reports, Mentzer and coworkers[31] found that adenosine infusions stimulated myocardial glucose uptake in non-ischaemic isolated rat hearts. Law and Raymond[35] also reported that

adenosine potentiates insulin-stimulated myocardial glucose uptake in canine myocardium *in vivo*. Isolated perfused rat hearts treated with adenosine plus the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenosine (EHNA) during low flow ischaemia exhibited increased lactate release and delayed onset of contracture,[36] while adenosine receptor blockers reduced lactate release and accelerated the onset of contracture. Omission of glucose from perfusate eliminated the effects of adenosine plus EHNA on lactate release or delay of contracture.[36] Thus, the effects of adenosine on glycolysis are controversial, but our findings support the view that interstitial adenosine inhibits anaerobic glycolysis in ischaemic myocardium.

In the present study, adenosine receptor blockade intensified intracellular acidosis during ischaemic stress. Evidence in the recent literature, as reviewed by Levine,[37] suggests that this moderate intracellular acidification could be protective in ischaemic and reperfused myocardium. Isolated ferret hearts reperfused at pH 7.4 were dysfunctional ('stunned'), whereas those initially reperfused at pH 6.6 functioned as well as controls not subjected to ischaemia.[38] Although 8-SPT lowered purine nucleoside release by 21% in our study (Figure 4A), this effect is probably not due to inhibition of 5'-nucleotidase by acidosis as may occur in severe zero-flow ischaemia,[39] because the present acidosis was moderate and intracellular pH did not fall below the optimum pH for *ecto* 5'-nucleotidase activity.[40] On the other hand, the modest reduction in purine nucleoside release could

have resulted from enhanced cytosolic energy level, secondary to increased glycolysis, during adenosine receptor blockade.

In summary, this study established an isolated working guinea-pig heart model exhibiting the hallmarks of acute myocardial hibernation when subjected to moderately severe ischaemic stress. In this model, hibernation was induced by interstitial adenosine released during myocardial ischaemia. The detailed mechanism of adenosine receptor-mediated metabolic down-regulation during ischaemia merits further investigation.

Acknowledgements

The technical assistance of Ms. Blaine Payne is gratefully acknowledged. This study was supported by grants to RTM (R29 HL50441) and HFD (R01 HL35056) from the National Heart, Lung and Blood Institute, and by a grant to HFD from the Texas Advanced Research Program.

REFERENCES

1. Rahimtoola SH. A perspective on the three large multicenter randomized clinical trials of coronary bypass surgery for chronic stable angina. *Circulation* 1985;**72**(suppl V):V123-V135.
2. Rahimtoola SH. The hibernating myocardium. *Am Heart J* 1989;**117**:211-221.
3. Pantely GA, Malone SA, Rhen WS, Anselone CG, Arai A, Bristow J, Bristow JD. Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. *Circ Res* 1990;**67**:1481-1493.
4. Arai AE, Pantely GA, Anselone CG, Bristow J, Bristow JD. Active downregulation of myocardial energy requirements during prolonged moderate ischemia in swine. *Circ Res* 1991;**69**:1458-1469.
5. Schultz R, Guth BD, Pieper K, Martin C, Heusch G. Recruitment of an inotropic reserve in moderately ischemic myocardium at the expense of metabolic recovery. A model of short-term hibernation. *Circ Res* 1992;**70**:1282-1295.
6. Fedele FA, Gewirtz H, Capone RJ, Sharaf B, Most AS. Metabolic response to prolonged reduction of myocardial blood flow distal to a severe coronary artery stenosis. *Circulation* 1988;**78**:729-735.
7. Downing SE, Chen V. Acute hibernation and reperfusion of the ischemic heart. *Circulation* 1992;**85**:699-707.

8. Schaefer S, Carr LJ, Kreutzer U, Jue T. Myocardial adaptation during acute hibernation: mechanisms of phosphocreatine recovery. *Cardiovasc Res* 1993;**27**:2044-2051.

9. Berne RM. Adenosine - a cardioprotective and therapeutic agent. *Cardiovasc Res* 1993;**27**:2.

10. Hori M, Kitakaze M. Adenosine, the heart, and coronary circulation. *Hypertension* 1991;**18**:565-574.

11. Romano FD, Dobson JG. Adenosine modulates β-adrenergic signal transduction in guinea-pig heart ventricular membranes. *J Mol Cell Cardial* 1990;**22**:1359-1370.

12. Liu GS, Thornton J, Van Winkle DM, Stanley AWH, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation* 1991;**84**:350-356.

13. Mainwaring R, Lasley R, Rubio R, Wyatt DA, Mentzer RM. Adenosine stimulates glucose uptake in the isolated rat heart. *Surgery* 1988;**103**:445-449.

14. Wyatt DA, Edmunds MC, Rubio R, Berne RM, Lasley RD, Mentzer RM. Adenosine stimulates glycolytic flux in isolated perfused rat hearts by A_1-adenosine receptors. *Am J Physiol* 1989;**257**:H1952-H1957.

15. Lasley RD, Mentzer RM. Adenosine improves recovery of postischemic myocardial function via an adenosine A1 receptor mechanism. *Am J Physiol* 1992;**262**:H1460-H1465.

16. Bünger R, Mallet RT, Hartman DA. Pyruvate-enhanced phosphorylation potential and inotropism in normoxic and postischemic isolated working heart. Near-complete prevention of reperfusion contractile failure. *Eur J Biochem* 1989;**180**:221-233.

17. Headrick JP, Matherne GP, Berr SS, Berne RM. Effects of graded perfusion and isovolumic work on epicardial and venous adenosine and cytosolic metabolism. *J Mol Cell Cardiol* 1991;**23**:309-324.

18. Headrick JP, Matherne GP, Berne RM. Myocardial adenosine formation during hypoxia: effects of ecto-5'-nucleotidase inhibition. *J Mol Cell Cardiol* 1992;**24**(3):295-303.

19. Dendorfer A, Lauk S, Schaff A, Nees S. New insights into the mechanism of myocardial adenosine formation. In: Gerlach E, Becker BF, eds. *Topics and Perspectives in Adenosine Research.* Berlin/Heidelberg: Springer-Verlag, 1987:170-187.

20. Bünger R, Sommer O, Walter G, Stiegler H, Gerlach E. Functional and metabolic features of an isolated perfused guinea pig heart performing pressure-volume work. *Pflügers Arch* 1979;**380**:259-266.

21. Bergmeyer HU. *Methods of Enzymatic Analysis*, 3rd English Ed. New York: Academic Press, 1983-7.

22. Mallet RT, Kang Y-H, Mukohara N, Bünger R. Use of cytosolic metabolite patterns to estimate free magnesium in normoxic myocardium. *Biochim Biophys Acta* 1992; **1139:** 239-247.

23. Schultz R, Rose J, Martin C, Brodde OE, Heusch G. Development of short-term myocardial hibernation. Its limitation by the severity of ischemia and inotropic stimulation. *Circulation* 1993;**88:**684-695.

24. Downing SE, Chen V. Myocardial hibernation in the ischemic neonatal heart. *Circ Res* 1990;**66:**763-772.

25. Keller AM, Cannon PJ. Effect of graded reductions of coronary pressure and flow on myocardial metabolism and performance: a model of "hibernating" myocardium. *J Am Coll Cardiol* 1991;**17:**1661-1670.

26. Gallagher KP, Matsuzaki M, Osakada G, Kemper WS, Ross J. Effect of exercise on the relationship between myocardial blood flow and systolic wall thicking in dogs with acute coronary stenosis. *Circ Res* 1983;**52:**716-729.

27. Kammermeier H. High energy phosphate of the myocardium: concentration versus free energy change. *Basic Res Cardiol* 1987;**82 (suppl 2):**31-36.

28. Veech RL, Lawson JWR, Cornell NW, Krebs HA. Cytosolic phosphorylation potential. *J Biol Chem* 1979;**254:**6538-6547.

29. Newby AC, Worku Y, Meghji P. Critical evaluation of the role of ecto- and cytosolic 5'-nucleotidase in adenosine formation. In: Gerlach E, Becker BF, eds.

Topics and Perspectives in Adenosine Research. Berlin/Heidelberg: Springer-Verlag, 1987: 155-169.

30. Bünger R, Soboll S. Cytosolic adenylates and adenosine release in perfused working heart. *Eur J Biochem* 1986;**159:**203-213.

31. Mentzer RM, Bünger R, Lasley RD. Adenosine enhanced preservation of myocardial function and energetics. Possible involvement of the adenosine A_1 receptor system. *Cardiovasc Res* 1993;**27:**28-35.

32. Mallet RT, Hartman DA, Bünger R. Glucose requirement for postischemic recovery of perfused working heart. *Eur J Biochem* 1990;**188:**481-493.

33. Finegan BA, Lopaschuk GD, Coulson CS, Clanachan AS. Adenosine alters glucose use during ischemia and reperfusion in isolated rat hearts. *Circulation* 1993;**87:**900-908.

34. Dale WE, Hale CC, Kim HD, Rovetto MJ. Myocardial glucose utilization: failure of adenosine to alter it and inhibition by the adenosine analogue N^6-(L-2-phenylisopropyl)adenosine. *Circ Res* 1991;**69:**791-799.

35. Law WR, Raymond RM. Adenosine potentiates insulin-stimulated myocardial glucose uptake in vivo. *Am J Physiol* 1988;**254:**H970-H975.

36. Lasley RD, Mentzer RM. Adenosine increases lactate release and delays onset of contracture during global low flow ischaemia. *Cardiovasc Res* 1993;**27:**96-101.

37. Levine RL. Ischemia: from acidosis to oxidation. *FASEB J* 1993;**7:**1242-1246.

38. Kitakaze M, Weisfeldt ML, Marban E. Acidosis during early reperfusion prevents myocardial stunning in perfused ferret hearts. *J Clin Invest* 1988;**82:**920-927.

39. Bak MI, Ingwall JS. Acidosis during ischemia promotes adenosine triphosphate resynthesis in postischemic rat heart. In vivo regulation of 5'-nucleotidase. *J Clin Invest* 1994;**93:**40-49.

40. Naito Y, Lowenstein JM. The 5'-nucleotidase from rat heart. *Biochemistry* 1981;**20:**5188-5194.

Table 1. Effects of myocardial ischaemia on myocardial energy metabolites and lactate.

			Ischaemia		
		Pre-ischaemia	10 min	30 min	60 min
	n	5	6	6	6
{ATP}	μmol/g dry	25.0 ± 0.8	24.3 ± 0.6	22.3 ± 0.8	23.6 ± 1.0
{ATP+ADP+AMP}	μmol/g dry	29.3 ± 0.9	27.1 ± 1.2	25.1 ± 1.3	28.3 ± 1.0
{CrP}	μmol/g dry	50.2 ± 3.2	42.6 ± 2.3	42.6 ± 3.6	58.0 ± 2.0†
{Lactate}	μmol/g dry	1.96 ± 0.67	10.26 ± 2.54*	6.90 ± 1.53*	3.51 ± 1.03†
[P_i]	mM	5.81 ± 0.45	8.19 ± 0.20*	6.77 ± 0.76	6.69 ± 0.47
{CrP}/{Cr}		1.77 ± 0.06	0.81 ± 0.10*	1.08 ± 0.18*	1.91 ± 0.22†
[CrP]/([Cr][P_i])	M^{-1}	324 ± 32	99 ± 14*	187 ± 53	229 ± 51†
[ATP]/([ADP][P_i])	mM^{-1}	24.3 ± 2.1	7.9 ± 1.1*	15.5 ± 4.0	25.4 ± 4.4†

Metabolites were measured in myocardium stop-frozen at selected times; data are means ± SE. { }: total myocardial metabolite content; [ ]: intracellular metabolite concentration; CrP: creatine phosphate; Cr: creatine; P_i: inorganic phosphate. Cytosolic ATP phosphorylation potential ([ATP]/([ADP][Pi])) was computed from creatine kinase equilibrium as detailed in METHODS. *: $p < 0.05$ *vs.* pre-ischaemia. †: $p < 0.05$ *vs.* 10 min ischaemia.

Table 2. Effects of ischaemia/reperfusion and adenosine antagonism on myocardial energy metabolites and lactate.

		Time Control	Isch/Rep	Isch/Rep + 8-SPT	Isch/Rep + AOPCP
	n	6	12	7	5
{ATP}	μmol/g dry	23.7 ± 0.6	22.6 ± 0.7	21.3 ± 0.4	21.9 ± 1.2
{ATP+ADP+AMP}	μmol/g dry	29.2 ± 0.8	27.5 ± 0.7	26.8 ± 0.4	26.3 ± 1.3
{CrP}	μmol/g dry	41.8 ± 1.8	43.8 ± 1.8	50.0 ± 1.8*†	40.0 ± 2.2
{Lactate}	μmol/g dry	1.64 ± 0.19	1.43 ± 0.29	1.49 ± 0.25	1.16 ± 0.15
$[P_i]$	mM	7.18 ± 0.77	7.45 ± 0.46	5.46 ± 0.46*†	8.44 ± 0.31
{CrP}/{Cr}		1.61 ± 0.14	1.53 ± 0.07	1.63 ± 0.08	1.73 ± 0.03
$[CrP]/([Cr][P_i])$	M^{-1}	237 ± 32	219 ± 22	319 ± 34†	206 ± 9
$[ATP]/([ADP][P_i])$	mM^{-1}	18.3 ± 2.3	17.8 ± 1.9	25.2 ± 2.6*†	15.3 ± 0.8

Metabolites were measured at 30 min reperfusion in the three ischaemia/reperfusion (Isch/Rep) groups, and at 120 min perfusion in the non-ischaemic time control group. Abbreviations and determination of ATP phosphorylation potential as in Table 1. Hearts were treated with α, β-methylene-adenosine diphosphate (AOPCP; 50 μM) or 8-sulfophenyl-theophylline (8-SPT; 20 μM) as described in Figure 1. *: $p < 0.05$ *vs.* time control, †: $p < 0.05$ *vs.* untreated Isch/Rep group.

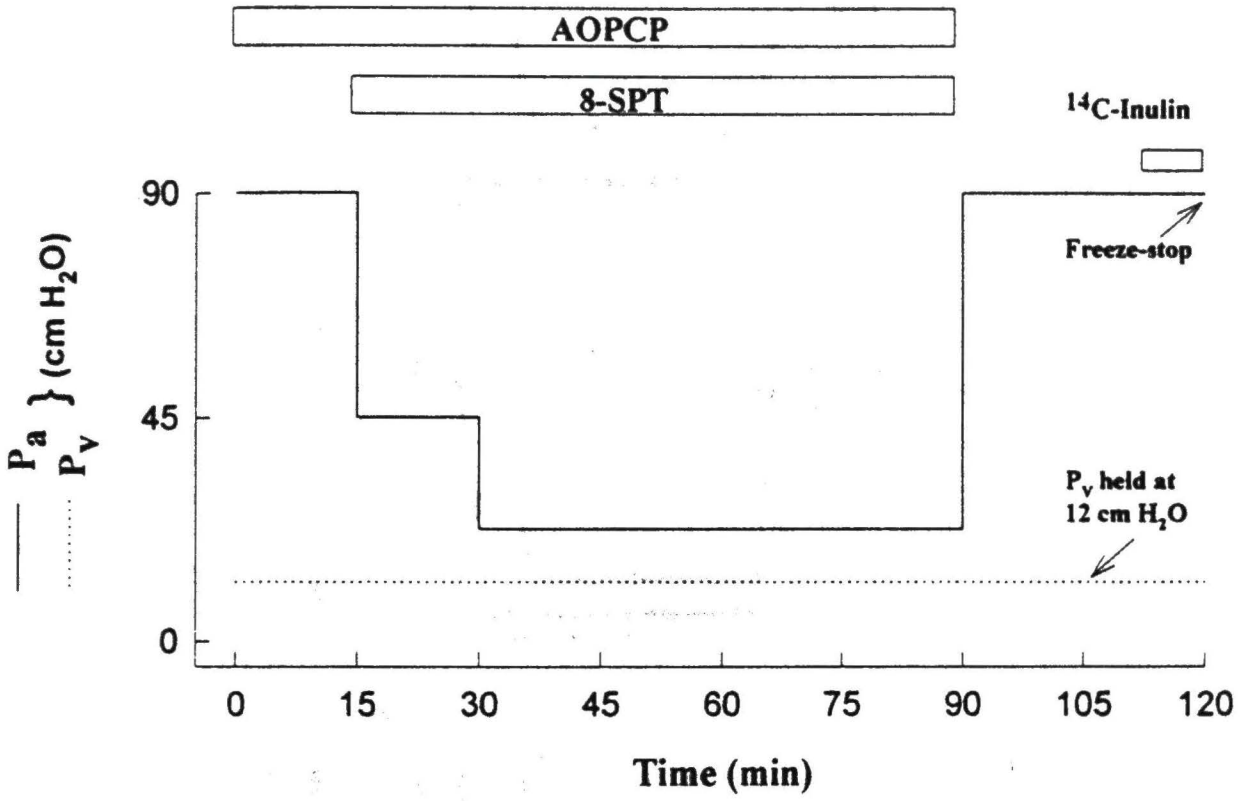

Figure 1. *Experimental ischaemia/reperfusion protocol.* Coronary hypoperfusion was produced by lowering aortic pressure (P_a) from 90 to 45 cm H_2O for 15 min, then to 22.5 cm H_2O for 60 min. Reperfusion was effected by restoring P_a to 90 cm H_2O. Left atrial filling pressure (P_v) was held at 12 cm H_2O throughout the protocol. [^{14}C]Inulin-carboxylic acid was infused for 5 min prior to freeze-stop. Duration of experimental treatment with α,β-methylene adenosine 5'-diphosphonate (AOPCP; 50 μM) or 8-*p*-sulfophenyl theophylline (8-SPT; 20 or 200 μM) is indicated by the open bars. In time controls, P_a was held at 90 cm H_2O for 120 min.

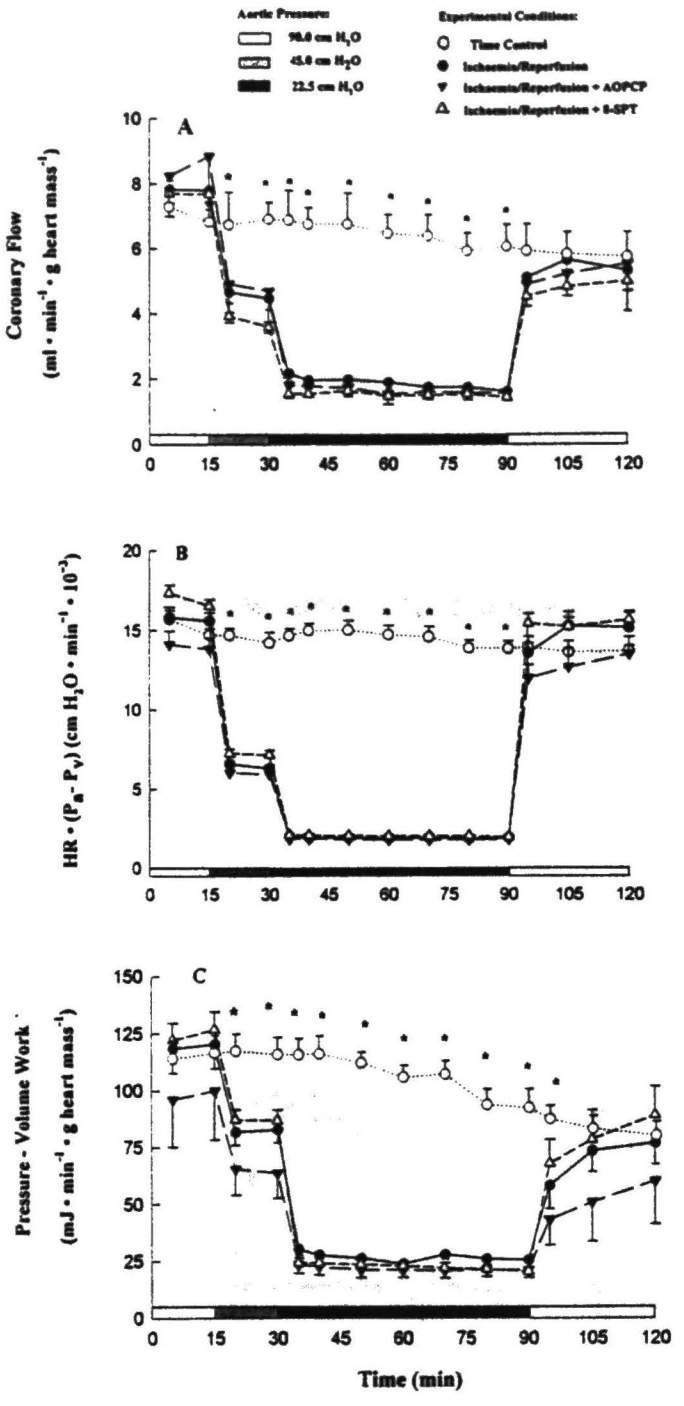

Figure 2. *Coronary flow (panel A), heart rate-pressure product (panel B) and left ventricular pressure-volume work (panel C).* Untreated ischaemia hearts were subjected to the ischaemia/reperfusion protocol illustrated in Figure 1. Nonischaemic time controls were perfused at P_a = 90 cm H_2O for 120 min. Means ± SE from 6-8 experiments. *: $P < 0.05$ *vs.* untreated ischaemia.

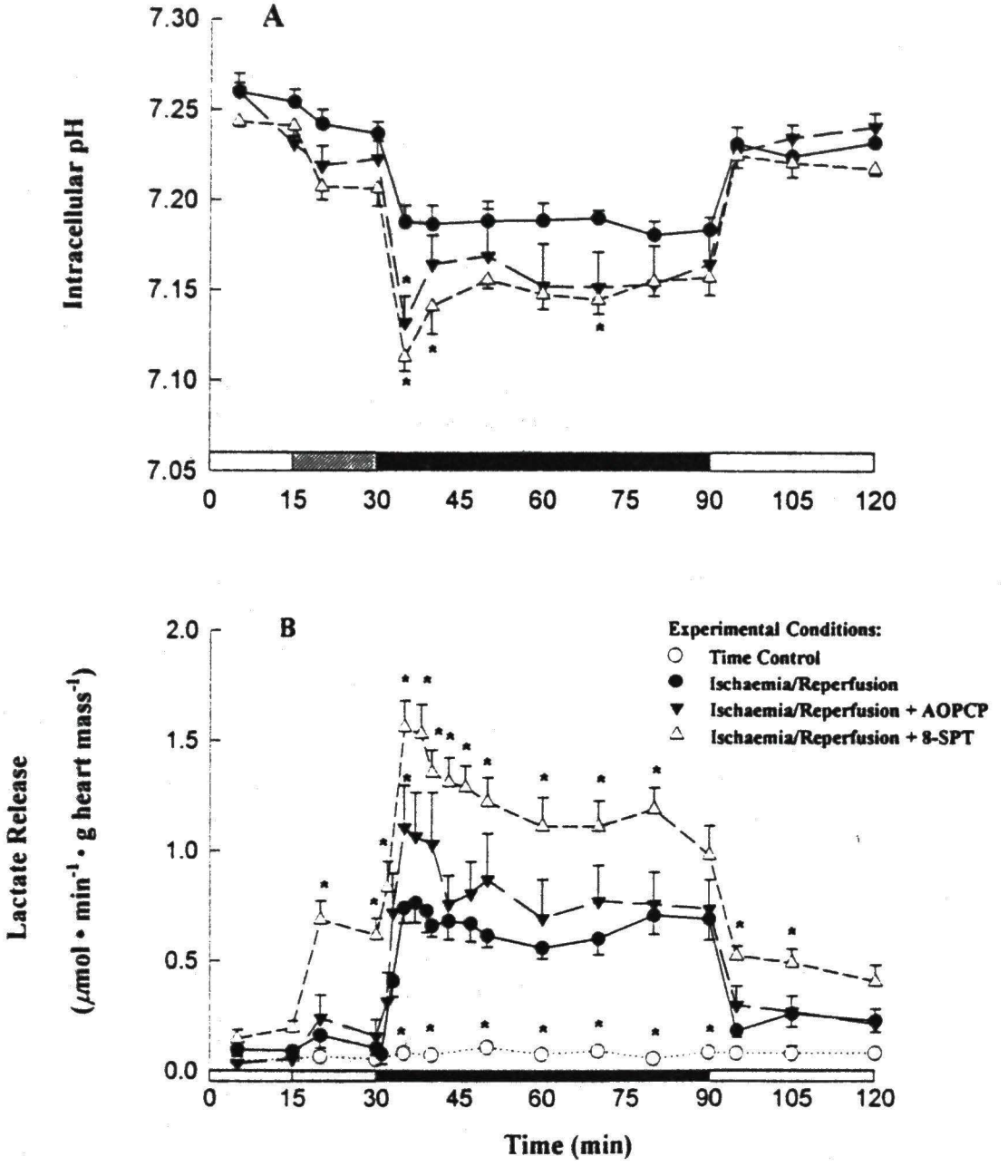

Figure 3. *Effects of ischaemia/reperfusion, adenosine receptor blockade, and 5'-nucleotidase inhibition on intracellular pH (panel A) and lactate release (panel B).* 8-*p*-sulfophenyl theophylline (8-SPT) was infused from 15 to 90 min; α,β-methylene adenosine 5'-diphosphonate (AOPCP) was infused from 0 to 90 min. *: $P < 0.05$ *vs.* untreated ischaemia.

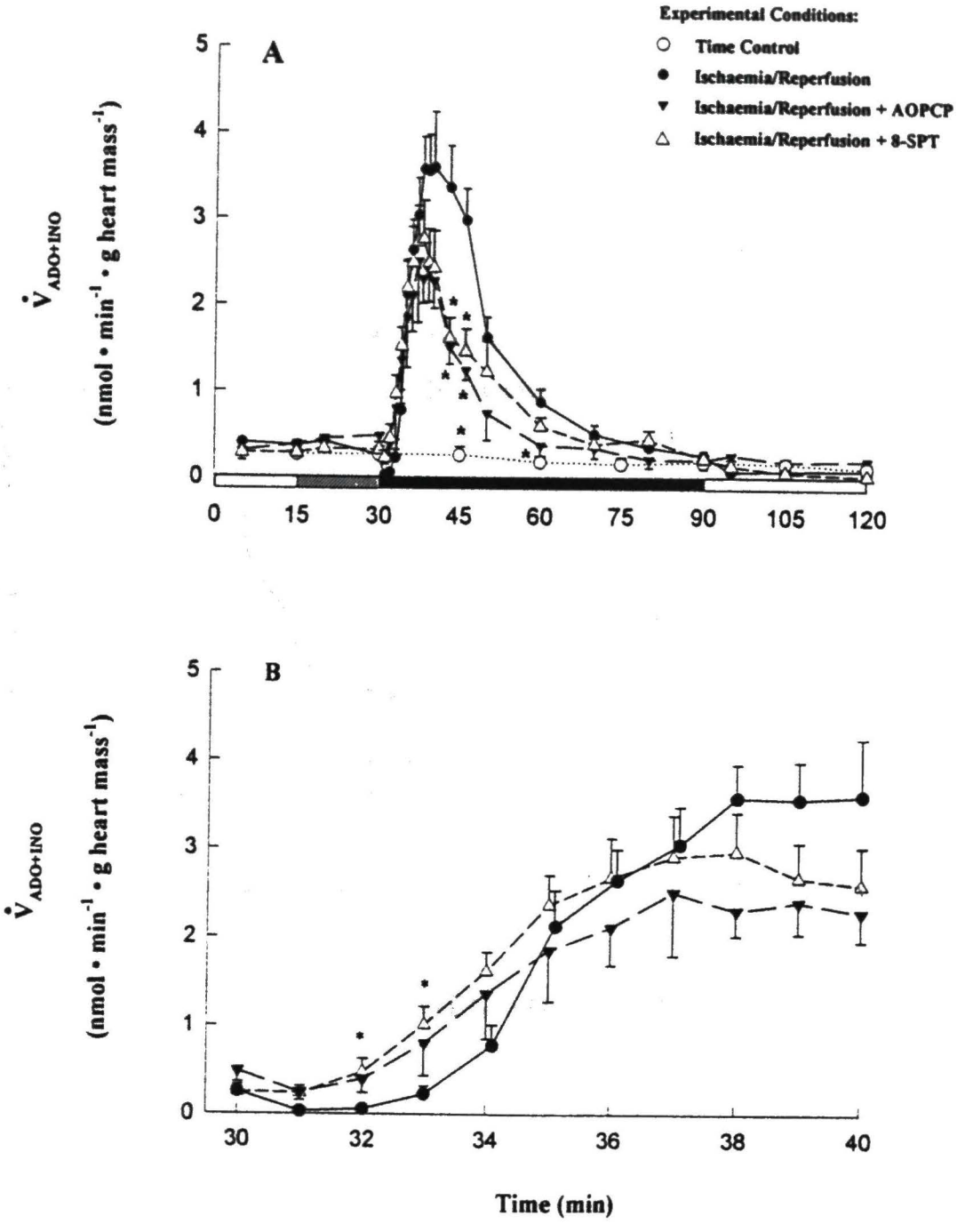

Figure 4. *Effects of ischaemia/reperfusion, adenosine receptor blockade, and 5'-nucleotidase inhibition on purine nucleoside (adenosine (ADO) + inosine (INO)) release.* Panel A presents nucleoside release during the full 120 min protocol; panel B depicts data from the three ischaemia groups (untreated, 8-SPT treated, and AOPCP treated) at 30-40 min, *i.e.* the first 10 min ischaemia. *: $P < 0.05$ *vs.* untreated ischaemia.

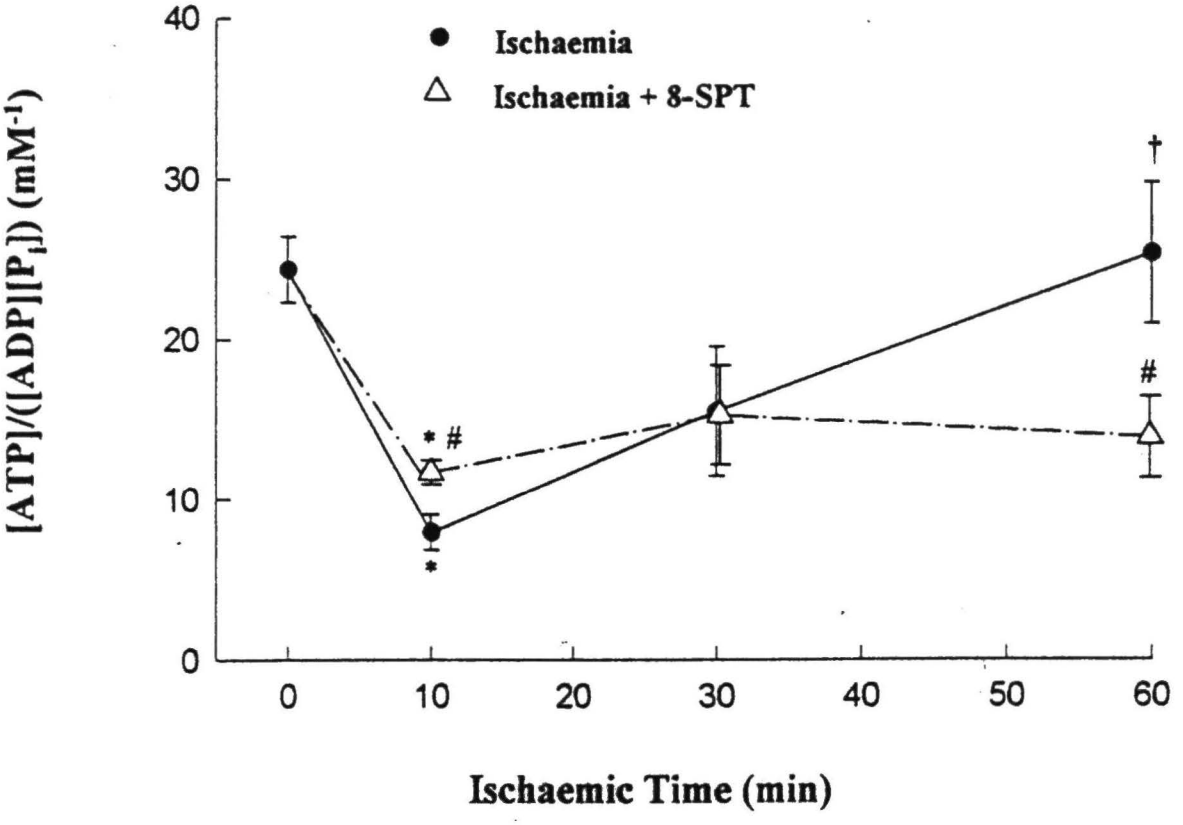

Figure 5. *Effects of adenosine receptor blockade on cytosolic ATP phosphorylation potential during global ischaemia.* Means ± SE for 6-8 measurements. Data at 0 min was obtained in pre-ischaemic myocardium at P_a = 90 cm H_2O. Ischaemic times refer to min perfusion at P_a = 22.5 cm H_2O, following 15 min perfusion at P_a = 45 cm H_2O. *: $P < 0.05$ *vs.* 0 min; †: $P < 0.05$ *vs.* 10 min; $^\#$: $P < 0.05$ *vs.* untreated group.

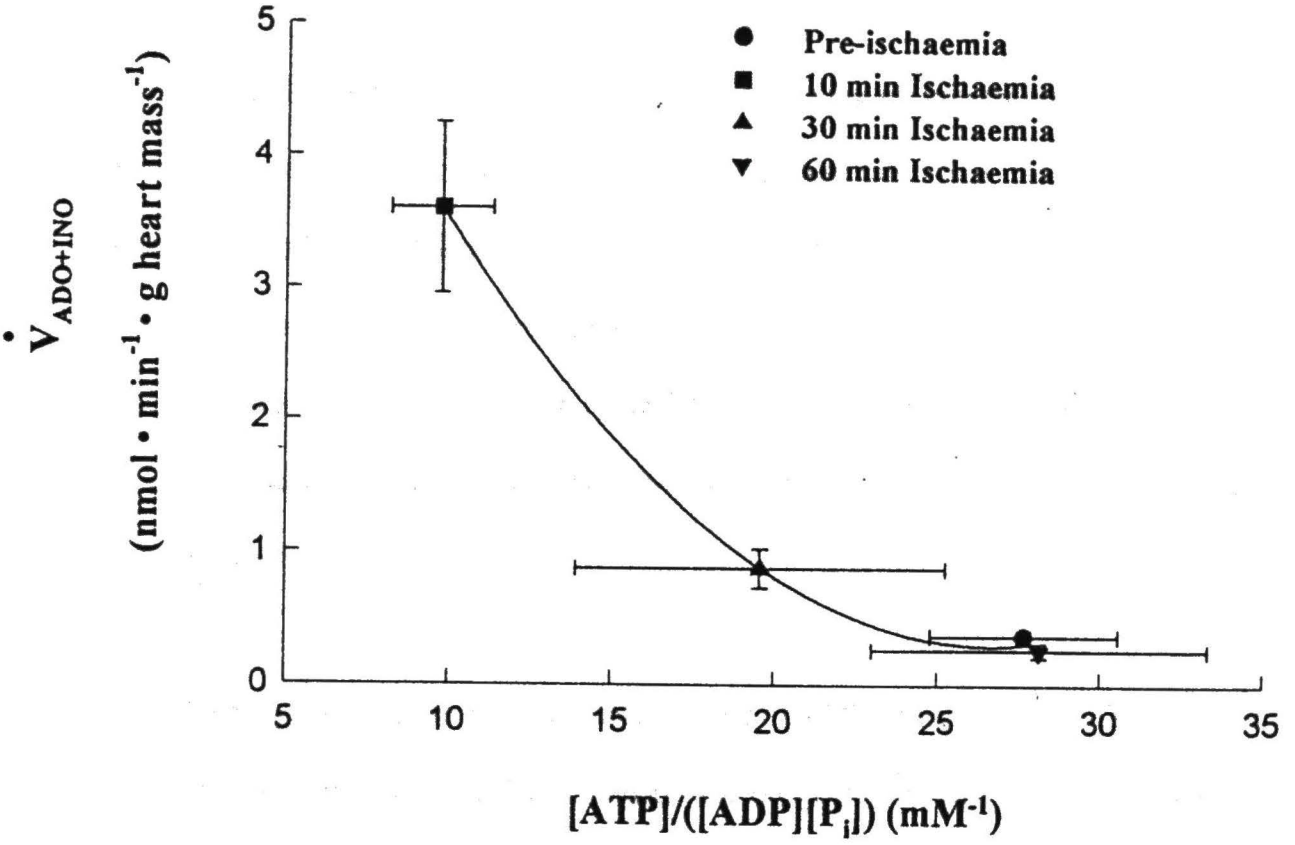

Figure 6. *Purine nucleoside release as a function of cytosolic ATP phosphorylation potential.* The curve, which depicts the least-squares nonlinear regression of the mean values, is defined by the equation: $V_{ADO + INO} = 8.5352 - 0.6175\{[ATP]/([ADP][P_i])\} + 0.0116\{[ATP]/([ADP][P_i])\}^2$; $r^2 = 0.999$.

TRANSITION

Our first investigation of the role of adenosine in mediating myocardial hibernation demonstrated: 1) Cardiac function fully recovers following 60 min ischemia, and neither adenosine A_1 receptor blockade nor *ecto* 5'-nucleotidase inhibition impairs recovery of contractile function; 2) Adenosine A_1 receptor blockade stimulates anaerobic glycolysis during ischemia, but does not increase purine nucleoside release; 3) Adenosine A_1 receptor blockade lessens the initial decline in cytosolic ATP phosphorylation potential during the first 10 min ischemia, but prevents the subsequent recovery of cytosolic energetics after 60 min ischemia.

Since glycolysis is the only significant source of ATP during myocardial ischemia, it is important to delineate the effects of adenosine on glycolytic flux and lactate release. Therefore, a second investigation was undertaken to evaluate the effects of adenosine on glucose metabolism in normoxic and ischemic myocardium. The results from this study will provide evidence to help solve the present discrepancy in the literature regarding the effect of adenosine on myocardial glucose metabolism. Thus, the following study was undertaken to investigate the effect of adenosine receptor blockade with 8-*p*-sulphophenyl theophylline on myocardial glucose metabolism in normoxic and ischemic guinea-pig hearts, and, therefore, to delineate the effect of endogenous adenosine on glucose

metabolism in hibernating myocardium. The results indicated adenosine receptor blockade increased glucose uptake, but did not alter glucose oxidation or glycogen degradation. Crossover plots of glycolytic intermediates revealed that phosphofructokinase, a key rate-controlling reaction in glycolysis, was activated by adenosine receptor blockade in both pre-ischemic and ischemic myocardium.

CHAPTER III

ADENOSINE RECEPTOR BLOCKADE ENHANCES GLYCOLYSIS IN GUINEA-PIG MYOCARDIUM

Zhi-Ping Gao, H. Fred Downey, Jie Sun, and Robert T. Mallet

In preparation: Cardiovascular Research

ABSTRACT

We previously characterized a model of acute hibernation in guinea-pig heart and found adenosine receptor blockade blunted rebound of cytosolic energy level and stimulated anaerobic glycolysis during prolonged ischemia. ***Objectives:*** This study investigated the hypothesis that interstitial adenosine mediates cardioprotective mechanisms in hibernating myocardium by depressing glycolysis. ***Methods:*** Isolated, working guinea-pig hearts, perfused with glucose-fortified Krebs-Henseleit buffer, were subjected to 10 min ischemia (coronary flow 20% of baseline). Adenosine receptor blockade was produced by 8-*p*-sulfophenyl theophylline (20 μM). Glucose oxidation and lactate production from exogenous glucose were assessed from [U-^{14}C]glucose derived $^{14}CO_2$ production and ^{14}C-lactate release, respectively. Glycolytic intermediates and glycogen were measured in extracts of stop-frozen pre-ischemic and hibernating myocardium. ***Results:*** Adenosine receptor blockade did not affect left ventricular function assessed from heart rate-pressure product and pressure-volume work although coronary flow was slightly reduced. Adenosine receptor blockade increased glucose uptake ($p < 0.05$) during normoxia by 63% and during hypoperfusion by 51%. Adenosine receptor blockade increased lactate production from exogenous glucose ($p < 0.05$) by 139% during normoxia and 50% during hypoperfusion, but it did not stimulate glucose oxidation. Glycogen degradation during hypoperfusion was not increased by adenosine receptor blockade, so glycogen was not the sole source of enhanced lactate production. Crossover plots of glycolytic intermediates revealed that phosphofructokinase, a key rate-controlling

step in glycolysis, was activated by adenosine receptor blockade in both pre-ischemic and hibernating myocardium. ***Conclusion:*** Endogenous adenosine attenuates anaerobic glycolysis during myocardial hibernation by blunting phosphofructokinase activity. Decreased glycolysis may enhance myocardial hibernation by reducing intracellular acidosis and preventing Ca^{2+} overload.

INDEX TERMS: adenosine, adenosine receptor blockade, glycolysis, glycogen, myocardial ischemia, myocardial hibernation.

ABBREVIATIONS: ANOVA: analysis of variance; DAP: dihydroxyacetone phosphate; F6P: fructose 6-phosphate; F2P: fructose 1,6-bisphosphate; G6P: glucose 6-phosphate; LAC: lactate; PEP: phosphoenolpyruvate; PYR: pyruvate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; P_a: aortic pressure; P_v: left atrial filling pressure; 8-SPT: 8-*p*-sulfophenyl theophylline.

ENZYMES: phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.9); lactate dehydrogenase (EC 1.1.1.28); aldolase (EC 4.1.2.13).

INTRODUCTION

The phenomenon of 'hibernating myocardium' as first described by Rahimtoola[1,2] has forced a reassessment of the dogma that prolonged myocardial ischemia always produces irreversible injury. In hibernating myocardium, contractile function is depressed during prolonged ischemia, but partially or completely recovers to the pre-ischemic level when coronary flow is restored. Metabolically, hibernating myocardium is characterized by an initial decrease but subsequent rebound of creatine phosphate as ischemia is prolonged. Several studies have established models of myocardial hibernation in different mammalian species.[3-8] However, the mechanism of this adaptive and cardioprotective process is still unknown.

Our previous investigation of the role of adenosine in mediating myocardial hibernation[9] has shown: 1) cardiac function fully recovered following 60 min ischemia, and neither adenosine A_1 receptor blockade nor *ecto* 5'-nucleotidase inhibition impaired post-ischemic recovery of contractile function; 2) adenosine A_1 receptor blockade stimulated anaerobic glycolysis during ischemia, but did not increase purine nucleoside release; 3) adenosine A_1 receptor blockade lessened the initial decline in cytosolic ATP phosphorylation potential during the first 10 min ischemia, but prevented the subsequent recovery of cytosolic energetics as ischemia was prolonged. A number of studies have reported that exogenous adenosine inhibits glycolysis during myocardial ischemia,[14-17, 31] whereas other studies found that exogenous adenosine stimulates glycolysis in ischemic

and normoxic conditions.[18-20] The present study was undertaken to investigate the effect of adenosine receptor blockade with 8-*p*-sulphophenyl theophylline (8-SPT) on myocardial glucose metabolism in normoxic and ischemic guinea-pig hearts, and, thus, to delineate the role of adenosine in hibernating myocardium. Since glycolysis is the only significant source of ATP during myocardial ischemia, it is important to delineate the effects of adenosine on glycolytic flux and lactate release. To accomplish this objective, adenosine receptors were blocked with 8-SPT before and during cardiac ischemia. Glycolysis and glucose oxidation were monitored from ^{14}C-lactate and $^{14}CO_2$ production, respectively, from [U-^{14}C]glucose.

METHODS

Heart perfusion

Animal experimentation was approved by the institutional Animal Care and Use committee and conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1985). Hearts (n = 22), beating at intrinsic sinus rhythm, were isolated from methoxyflurane-anaesthetised Hartley guinea-pigs (400-600 g body mass), and perfused as working hearts.[10] Perfusion medium was a modified Krebs-Henseleit bicarbonate buffer (38 °C, pH 7.37-7.47) fortified with 10 mM glucose and 5 U/L bovine insulin (Sigma Company, St. Louis, MO, USA) and aerated with 95% O_2:5% CO_2.[9] [U-^{14}C]glucose and 8-SPT were purchased from DuPont (Boston, MA, USA) and Research Biochemicals (Natick, MA, USA), respectively.

Left ventricular hemodynamics

Cardiac performance was assessed from spontaneous heart rate, aortic pressure (P_a), and left atrial filling pressure (P_v). These variables were continuously monitored with a multi-channel polygraph (Beckman Model R611). Coronary and aortic flows were measured by timed collections; cardiac output was taken as the sum of coronary and aortic flows. Myocardial energy expenditure was assessed from left ventricular pressure development per min (heart rate times (P_a - P_v)) and from left ventricular pressure-volume work (i.e. stroke work times heart rate), an index of power.

Measurement of ^{14}C-glucose oxidation

The hearts received [U-^{14}C]glucose (specific activity: 2 mCi/mol glucose) throughout the experimental protocol. The rates of $^{14}CO_2$ release stabilized after about 20 min perfusion (Figure 5B), indicating that isotopic steady state had been attained. The release of $^{14}CO_2$ was corrected to the specific activity of ^{14}C-glucose in the arterial perfusion medium. Aliquots of coronary venous effluent and arterial perfusion medium were collected in 25 ml Warburg flasks. The venous effluent and arterial samples (1-8 ml) were acidified (pH = 1.25-1.50) with 1 N HCl 0.5 - 1 ml. $^{14}CO_2$ was trapped in 0.4 ml benzethonium hydroxide (Sigma) in a center well (Kontes Glassware, Vineland, NJ) without exposure to air; samples were incubated overnight in a Dubnoff metabolic oscillating incubator (GCA Corporation). Nonmetabolic $^{14}CO_2$ and other volatile impurities of the radioactive tracers were measured in acidified samples of arterial

perfusion media.[11] The specific radioactivity of [U-^{14}C]glucose in arterial and venous effluent samples was calculated by dividing DPM by glucose concentration.

^{14}C-lactate release from exogenous glucose

^{14}C-labeled compounds were separated by anion-exchange chromatography.[32] A freshly prepared 2 ml Dowex column (Dowex 1, 1 × 8, formate form, 200-400 mesh) was used to separate ^{14}C-glucose,^{14}C-lactate and ^{14}C-pyruvate.[11] Elution profile was determined by putting a solution containing known amounts of unlabeled glucose, lactate and pyruvate on column and analyzing the eluent. 20 ml H_2O separated all the glucose from other materials; 20 ml 0.15 N formic acid eluted lactate completely; 20 ml 2.0 N formic acid eluted pyruvate. The eluted fractions were measured by enzymatic methods in spectrophotometer. 5 ml of venous effluent samples were loaded on the column and ^{14}C-glucose,^{14}C-lactate and ^{14}C-pyruvate were separated by using the above eluting procedure. ^{14}C-labelled compounds were measured by liquid scintillation counting. [U-^{14}C]glucose in the perfusion medium contained small amounts of ^{14}C-lactate and ^{14}C-pyruvate. These contaminants were measured by chromatographing arterial perfusate samples collected at 10, 30, and 50 min perfusion. ^{14}C-lactate and ^{14}C-pyruvate measured in venous effluent were corrected by subtracting the arterial contamination. Rates of coronary venous ^{14}C-lactate and ^{14}C-pyruvate release samples were measured as disintegrations per minute (DPM), which was subtracted from DPM in arterial fractions. DPM were determined from counts per minute by a standard quench curve. Lactate and pyruvate concentrations

in coronary effluent were measured by enzymatic assay.[13] The specific activities of ^{14}C-lactate and ^{14}C-pyruvate were expressed as DPM per micromole.

Measurement of glycolytic intermediates and glycogen

Experiments were terminated by freeze-clamp with Wollenberger tongs pre-cooled to constant temperature in liquid N_2. The frozen ventricular myocardium was pulverized and extracted with 0.3 N perchloric acid as described previously.[9] For glycogen measurement, an aliquot of powder was extracted with 0.6 N $HClO_4$ and neutralized with 1 N $KHNO_3$.[13] Glycolytic intermediates (glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (F2P), dihydroxyacetone phosphate (DAP), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), phosphoenolpyruvate (PEP), pyruvate (PYR) and lactate (LAC)) and glycogen were assayed in a Perkin Elmer Lambda-2 dual wavelength uv/vis spectrophotometer (measuring wavelength: 337 nm; reference wavelength: 417 nm ; $\epsilon = 5.65\ M^{-1} \cdot cm^{-1}$) by standard enzymatic tests.[12,13] Glucose concentrations in arterial and venous effluent samples were also assayed enzymatically.[13] Glucose uptake was taken as the product of the arteriovenous glucose concentration difference times coronary flow.

Experimental protocol

The experimental protocols are summarized in Figure 1. The study was divided into two phases. Phase 1 examined the effect of adenosine receptor blockade on glucose

metabolism in normoxic working guinea-pig hearts. In these experiments, hearts were perfused with 10 mM [U-^{14}C]glucose (specific activity: 2 mCi/mol) for 70 min at 90 cm H_2O aortic pressure (P_a) and 12 cm H_2O left atrial filling pressure (P_v). After 40 min baseline perfusion, hearts were perfused 30 min with 20 μM 8-SPT. Experiments were terminated by freeze-clamp. Phase 2 examined the effect of adenosine receptor blockade on glucose metabolism in ischemic working guinea-pig heart. After 30 min baseline perfusion, ischemia was produced by decreasing P_a, *i.e.* coronary perfusion pressure, in two steps, from 90 cm H_2O to 45 cm H_2O for 15 min (mild hypoperfusion), then to 22.5 cm H_2O (ischaemia) for 10 min. P_v was maintained at 12 cm H_2O throughout the protocol. Hearts metabolized 10 mM [U-^{14}C]glucose (specific activity: 2 mCi/mol). 8-SPT (20 μM) was continuously infused beginning at 20 min baseline perfusion. Experiments were terminated at 10 min ischemia and hearts were stop-frozen.

Statistics

Data are expressed as mean ± SEM. Single comparisons of means were performed using two-tailed student's *t*-test. Repeated measurements were compared by one way analysis of variance (ANOVA). These analyses were performed with SigmaStat statistical software (Jandel; San Rafael, CA, USA). P values < 0.05 were taken to indicate statistical significance.

RESULTS

Functional characteristics of adenosine receptor blockade in normoxic working hearts

Isolated working hearts were subjected to 40 min baseline perfusion (P_a = 90 cm H_2O), followed by 30 min perfusion with 8-SPT (20 μM) (Figure 2). Adenosine receptor blockade with 8-SPT did not affect left ventricular function, assessed from heart-rate pressure product (Figure 2A), but tended to decrease pressure-volume work (Figure 2B), albeit not significantly. Thus, adenosine receptor blockade with 8-SPT in normoxic hearts did not appreciably affect left ventricular function except to slightly lower coronary flow (Figure 2C).

Effects of adenosine receptor blockade on glucose metabolism in normoxic hearts

Figure 3 illustrates the effects of adenosine receptor blockade with 8-SPT on glucose uptake, glucose oxidation, and glycolysis. 8-SPT tended to increase glucose uptake, albeit not significantly (Figure 3A). $^{14}CO_2$ formation plateaued after 20 min perfusion with [U-^{14}C]glucose, indicating that isotopic steady state had been achieved. 8-SPT did not alter glucose oxidation compared to baseline perfusion (Figure 3B). ^{14}C-Lactate release remained essentially constant in the first 40 min normal perfusion, then adenosine receptor blockade with 8-SPT increased ^{14}C-lactate release by 110% ($P < 0.05$) (Figure 3C).

Functional characteristics of adenosine receptor blockade in ischemic working hearts

When P_a was lowered to 45 cm H_2O (mild hypoperfusion) and then to 22.5 cm H_2O (ischemia), left ventricular function and coronary flow paralleled the decreases in aortic pressure. Heart-rate pressure product (Figure 4A), pressure-volume work (Figure 4B), and coronary flow (Figure 4C), fell to 40, 55 and 64% of the respective baseline levels when aortic pressure was decreased from 90 cm H_2O to 45 cm H_2O; these values fell further to 12, 16 and 33% of baseline when aortic pressure was lowered to 22.5 cm H_2O. Importantly, 8-SPT neither altered heart rate-pressure product nor pressure-volume work. 8-SPT caused modest decreases of coronary flow at baseline perfusion and mild hypoperfusion of 17 and 19% respectively, compared with the untreated group ($P < 0.05$), but it did not affect coronary flow during ischemia. Thus, adenosine receptor blockade did not alter left ventricular function, but did moderately increase coronary resistance prior to ischemia.

Effects of adenosine receptor blockade on glucose metabolism in ischemic myocardium

Glucose uptake in the untreated group did not change significantly (Figure 5A) either in hypoperfusion or ischemia, averaging ($\mu mol \cdot min^{-1} \cdot g^{-1}$) 0.99 ± 0.25 initially and 1.15 ± 0.21 at 55 min, respectively. 8-SPT increased glucose uptake by 63% ($P < 0.05$), and by 51% ($P < 0.05$) at normal perfusion and mild hypoperfusion. Figure 5B illustrates the effects of adenosine receptor blockade on glucose oxidation. Glucose oxidation assessed from production of $^{14}CO_2$ stabilized after 20 min [U-^{14}C]glucose

perfusion, as in Phase 1. Glucose oxidation fell in lockstep with aortic pressure, stabilizing at 0.179 ± 0.014 μmol · min^{-1} · g^{-1} during ischemia. Interestingly, adenosine receptor blockade with 8-SPT neither altered glucose oxidation in normal perfusion, nor in ischemia. ^{14}C-lactate release did not change markedly until aortic pressure was lowered to 22.5 cm H_2O in the untreated group (Figure 5C). ^{14}C-lactate release rose sharply from (DPM · min^{-1} · g heart $mass^{-1}$) 269 ± 23 at 15 min mild hypoperfusion to 1680 ± 230 at 10 min ischemia ($P < 0.05$). In normoxic, mild hypoperfusion and ischemic conditions, 8-SPT (20 μM) enhanced ^{14}C-lactate release by 2.4, 6, and 1.5 fold respectively, *v.s.* untreated hearts ($P < 0.05$). These results indicate adenosine receptor blockade increases glycolysis, at least in part by enhancing metabolism of exogenous glucose.

Effects of adenosine receptor blockade and ischemia on glycolytic intermediates

Crossover plots of glycolytic intermediates are shown in Figure 6. Crossover plots are constructed by expressing the myocardial contents of all intermediates of the glycolytic pathway in the experimental condition as a percentage of the respective metabolite contents in the control condition. In normoxic myocardium, adenosine receptor blockade with 8-SPT increased myocardial contents of fructose 1,6-bisphosphate, phosphoenolpyruvate, and lactate, to 139, 133, and 262% of the respective control levels ($P < 0.05$; Figure 6A). In addition, it decreased dihydroxyacetone phosphate and pyruvate to 46 and 49% of the untreated levels ($P < 0.05$). Thus, 8-SPT increased lactate/pyruvate

ratio, an index of cytosolic NADH/NAD, nearly four fold, indicating pronounced cytosolic reduction due to adenosine receptor blockade.

To further define the effects of ischemia and adenosine receptor blockade on glycolytic enzymes, the ratios of glucose 6-phosphate/fructose 6-phosphate, fructose 1,6-bisphosphate/fructose 6-phosphate, fructose 1,6-bisphosphate/glucose 6-phosphate and 3-phosphoglycerate/dihydroxyacetone phosphate were determined in untreated and 8-SPT treated hearts in pre-ischemic and ischemic conditions (Table). In pre-ischemic hearts, 8-SPT increased the ratio of glucose 6-phosphate/fructose 6-phosphate ($P < 0.05$), indicating that glucose 6-phosphate production was increased relative to its conversion to fructose 6-phosphate by phosphoglucoisomerase. Because fructose 6-phosphate level was not decreased by 8-SPT, the change in this ratio could have occurred as a result of increased glucose uptake and/or hexokinase activation. 8-SPT also increased the ratios of fructose 1,6-bisphosphate/fructose 6-phosphate, and 3-phosphoglycerate/ dihydroxyacetone phosphate ($P < 0.05$), indicating that phosphofructokinase and the glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase couple were activated by adenosine receptor blockade during pre-ischemia. Ischemia itself tended to activate phosphofructokinase, as suggested by the ratio of fructose 1,6-bisphosphate/ fructose 6-phosphate ($P = 0.09$). During ischemia, 8-SPT increased ratios of fructose 1,6-bisphosphate/fructose 6-phosphate and fructose 1,6-bisphosphate/glucose 6-phosphate

relative to both pre-ischemic 8-SPT treated hearts and untreated ischemic hearts ($P < 0.05$), indicating that ischemia and adenosine receptor blockade synergistically activated phosphofructokinase.

Myocardial ischemia increased fructose 1,6-bisphosphate and phosphoenolpyruvate to 160 and 136% of the non-ischemic levels (Figure 6B). As expected, ischemia increased myocardial lactate content and lactate/pyruvate ratio more than eight fold, revealing a pronounced cytosolic reduction. Increased fructose 1,6-bisphosphate, and lactate content also indicated phosphofructokinase was stimulated during ischemia. Thus, both 8-SPT and ischemia can stimulate glycolysis in guinea-pig myocardium. Next, we studied the effect of adenosine receptor blockade on glycolysis in ischemic myocardium. Fructose 1,6-bisphosphate, lactate and 2-phosphoglycerate in 8-SPT treated hearts were 160, 239 and 57% of the respective control levels ($P < 0.05$; Figure 6C), indicating that adenosine receptor blockade increased glycolysis during myocardial ischemia. In addition, although fructose 1,6-bisphosphate content increased (Figure 6C), the substrates of phosphofructokinase, glucose-6-phosphate and fructose-6-phosphate, did not decrease. This suggests that production of hexose monophosphate from exogenous glucose and endogenous glycogen stores must have increased to keep up with the pace of increased glycolysis. As noted above, glucose uptake was increased during adenosine receptor blockade (Figure 3A, 5A).

As expected, myocardial glycogen content fell appreciably during 10 min ischemia (Figure 7). However, 8-SPT did not alter the rate of glycogen degradation. Interestingly, glycogen content rebounded to the pre-ischemic level after 60 min ischemia and 30 min reperfusion in untreated, 8-SPT treated and AOPCP treated groups from our previous study.[9] Thus, adenosine receptor blockade increased glucose uptake and glycolysis in both normoxic and ischemic myocardium, but did not appreciably alter glycogenolysis.

DISCUSSION

The objective of this study was to investigate the mechanism by which adenosine receptor blockade enhances glycolysis in pre-ischemic and ischemic working guinea-pig myocardium. The major findings of the study are: 1) Adenosine receptor blockade markedly increased lactate production from exogenous glucose, but did not stimulate glucose oxidation. 2) Adenosine receptor blockade did not increase glycogen degradation during the first 10 min ischemia; thus enhanced lactate production during this period resulted from increased uptake and metabolism of exogenous glucose. 3) Crossover plots and ratios of glycolytic intermediates revealed that phosphofructokinase, a key rate-controlling step in glycolysis, was activated by adenosine receptor blockade in both pre-ischemic and ischemic myocardium.

We recently reported[9] that adenosine receptor blockade with 8-SPT increased lactate release during ischemia in isolated working guinea-pig myocardium. The increased

lactate release in these hearts persisted during the initial 15 min reperfusion, although 8-SPT infusion was discontinued following ischemia. These findings are consistent with the results of several recent studies. Finegan *et al.*[14] reported that 100 μM adenosine pretreatment inhibited glycolysis during ischemia and reperfusion in isolated nonworking rat hearts. These workers also found that glucose oxidation was inhibited during myocardial ischemia, but this was not altered by adenosine. Moreover, 25 μM adenosine inhibited glycolytic flux by 15% at constant flow in unpaced nonworking rat hearts.[16] Dale *et al.*[15] also reported that the adenosine A_1 receptor agonist N^6-(L-2-phenylisopropyl)adenosine inhibited glycolysis by 50% in perfused rat hearts. Fralix *et al.*[17] showed that 20 μM adenosine attenuated glycolysis during ischemia and decreased lactate accumulation in nonworking rat hearts. Furthermore, Vander Heide *et al.*[31] found that adenosine significantly slowed rates of ATP depletion, glycogen utilization and lactate accumulation during the first 20 min of coronary occlusion in *in situ* canine myocardium, suggesting that glycolysis was inhibited during ischemia. These results and those of the present study indicate that endogenous adenosine inhibits glycolysis in normoxic and ischemic myocardium. Accumulation of protons produced by glycolysis could contribute to cardiac injury during ischemia and lead to contractile dysfunction by promoting Ca^{2+} overload secondary to Na^+/Ca^{2+} exchange during reperfusion.[42,43] Pre-ischemic glycogen reduction or glycolytic inhibition was found to improve post-ischemic recovery of hypertrophied rat hearts.[44] Thus adenosine, by inhibiting glycolysis and attenuating intracellular acidification during ischemia, could exert a protective effect.

However, several recent studies reported that adenosine increased, not decreased, glycolytic flux in normoxic, ischemic, and hypoxic myocardium.[18-20] Wyatt *et al.*[18] found that 100 μM adenosine increased glycolysis in normoxic and hypoxic nonworking rat hearts. 10 μM adenosine also increased lactate production in isolated nonworking guinea-pig hearts.[19] Furthermore, Janier *et al.*[20] reported that adenosine stimulated glycolysis and delayed the onset of contracture in ischemic rabbit hearts. These findings are inconsistent with our present study and our previous report,[9] and other evidence summarized above[14-17, 31] that adenosine inhibited glycolysis in myocardium. The discrepancies are difficult to explain, although they may be due to species, nutritional state, or methodological differences in estimating glycolysis in perfused hearts. The different results may be due in part to the presence of insulin in our studies[9] and those of Fralix *et al.*[17] and Finegan *et al.*,[14] whereas Wyatt *et al.*[18] did not add insulin to their perfusion medium. The differences could also be due to the different experimental conditions. Fralix *et al.*,[17] Finegan *et al.*,[14] and we studied low-flow ischemia, while Wyatt *et al.*[18] studied hypoxic and normoxic conditions. Although Janier *et al.*[20] also used low-flow ischemia, their perfusion medium contained 0.07 U/L insulin and 0.4 mM bovine serum albumin. Buxton *et al.*[16] reported that 25 μM adenosine, in the presence of 10 U/L insulin, produced a modest decrease of glycolytic flux at constant flow, but caused a modest increase in glycolytic flux at constant pressure, in nonworking rat hearts. In the present study, we measured glucose metabolism and glycolytic intermediates in the same hearts, while none of the previous studies had quantified these indices simultaneously. Thus, the results from

this investigation provide more direct evidence in support of the view that adenosine inhibits glycolysis in normoxic and ischemic myocardium.

Glycolysis is accelerated in myocardium under two conditions: ischemia[24] and increased workload.[25] As shown in Figure 6B, the levels of all the glycolytic intermediates in ischemic hearts were above the corresponding normoxic levels. Rovetto *et al.*[26] and Williamson *et al.*[27] reported that acceleration of phosphofructokinase in anoxic and ischemic myocardium resulted in increased tissue levels of fructose 1,6-bisphosphate and dihydroxyacetone phosphate. In the present study, acceleration of phosphofructokinase by ischemia also resulted in increased levels of these glycolytic intermediates. On the other hand, adenosine receptor blockade, which also stimulated glycolysis, did not uniformly increase all glycolytic intermediates.

Adenosine receptor blockade with 8-SPT increased myocardial lactate content in pre-ischemic and ischemic myocardium, demonstrating that glycolysis was accelerated in both conditions. Thus, the results from our study suggest that endogenous adenosine inhibits glycolysis in these two conditions. 8-SPT treatment increased fructose 1,6-bisphosphate and decreased dihydroxyacetone phosphate (Figure 6A). Two explanations for these findings are: 1) phosphofructokinase was activated; 2) aldolase was inhibited. The results support the first explanation. If aldolase were inhibited, lactate production could not have concurrently increased. However, aldolase was not activated to the same

extent as phosphofructokinase, thus, myocardial fructose 1,6-bisphosphate content increased, while dihydroxyacetone phosphate content decreased in 8-SPT treated hearts. Moreover, increased ratios of fructose 1,6-bisphosphate/fructose 6-phosphate and 3-phosphoglycerate/dihydroxyacetone phosphate by adenosine receptor blockade indicate that phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase/phosphoglycerate kinase were activated during pre-ischemia. As expected, phosphofructokinase was activated by the present moderate ischemia; adenosine receptor blockade activated this key rate-controlling enzyme even further in the ischemic setting (Table). Phosphoenolpyruvate, the substrate of pyruvate kinase, accumulated and pyruvate, the product of the enzyme fell during 8-SPT treatment of normoxic myocardium (Figure 6A). These findings indicate that pyruvate kinase, the third rate-controlling step in glycolysis, might not be activated to the same extent as the other rate-controlling glycolytic enzymes during adenosine receptor blockade. Furthermore, 8-SPT seems to accelerate pyruvate kinase during ischemia, but the activity of glyceraldehyde-3-phosphate dehydrogenase seems not to have increased compared with other glycolytic enzymes as indicated by a 43% decrease in 2-phosphoglycerate content (Figure 6C).

Regulation of phosphofructokinase is extremely complex. The general properties of the regulation of phosphofructokinase are as follows:[28] 1) inhibition by a high concentration of ATP; 2) inhibition by citrate at an inhibitory concentration of ATP; 3) relief of the inhibition caused by ATP, P_i, AMP, cAMP, ADP, fructose 6-phosphate, and

fructose 1,6-bisphosphate; 4) cooperativity of fructose 6-phosphate binding. Negative effectors decrease the enzyme's affinity for fructose 6-phosphate and the positive effectors increase its affinity. From our previous investigation,[9] 8-SPT treatment increased ADP content but decreased CrP content at 10 min ischemia. Thus, increased ADP production may activate phosphofructokinase. In addition, a recent study[29] reported that phosphofructokinase from sheep heart had been shown to be phosphorylated by Ca^{2+}/calmodulin protein kinase (CaM-Kinase) as well as cAMP-dependent protein kinase (PKA). Phosphorylation by either CaM-Kinase or PKA resulted in an increase in sensitivity to ATP inhibition and a small but consistent decrease in K_i for ATP. Activation of adenosine A_1 receptor can inhibit cAMP production and cAMP induced PKA activity.[48] On the other hand, 8-SPT, by blocking A_1 receptors, could increase cAMP and promote phosphofructokinase phosphorylation by protein kinase. However, it is unlikely that 8-SPT stimulated glycolysis by this mechanism since phosphofructo-kinase phosphorylation would inhibit, not activate, glycolysis.[29] Recently, adrenergic receptors were shown to mediate the activation of heart phosphofructokinase and phosphorylase. The relative potencies of catecholamines to activate phosphofructokinase was norepinephrine = epinephrine > isoproterenol, confirming a predominantly α-adrenergic mechanism for activation of these enzymes.[45] α-Adrenergic-receptor-mediated activation occurred independent of changes in cAMP.[46] The extensive study from Clark and Patten's laboratory was conducted to assess the role of Ca^{2+} in the α-adrenergic control of glucose uptake and phosphofructokinase.[47] Omission of Ca^{2+} from perfusion

medium resulted in: (i) cessation of contractions, (ii) loss of protein and of catalytic units of phosphofructokinase, (iii) failure of epinephrine to activate phosphofructokinase. Adenosine A_1 receptor couples to membrane pertussis toxin-sensitive G protein family (G_i). These G proteins can mediate the inhibition of adenylyl cyclase, activation of several types of K^+ channels, and inactivation of some types of voltage-dependent Ca^{2+} channels. Our results suggest that endogenous adenosine decreases glycolysis in guinea-pig hearts by inhibiting the activity of phosphofructokinase. Adenosine, by enhancing K^+ outflow and inactivating voltage-dependent Ca^{2+} channels, decreases action potential duration, reduces intracellular Ca^{2+} concentration during depolarization and increases membrane polarity during repolarization. Thus, adenosine may inhibit phosphofructo-kinase activity and glycolysis by decreasing intracellular Ca^{2+} concentration. Hazen *et al.*[30] reported the rapid and reversible association of phosphofructokinase with myocardial membranes during myocardial ischemia. They demonstrated that the majority of both phosphofructokinase mass and activity is translocated from the cytosol to a membrane-associated compartment prior to the onset of irreversible myocytic injury and that translocated phosphofructokinase is catalytically inactive. Adenosine may influence reversible association of phosphofructokinase with myocardial membranes by promoting its translocation from cytosol to membrane. Direct experimental evidence to support this hypothesis requires further investigation.

During myocardial ischemia, glucose and fatty acid oxidation are inhibited.[25] Thus glycolysis is essential to produce ATP to prevent ischemic damage, and facilitate functional recovery upon reperfusion. However, high glycolytic rates associated with low coronary flow could also cause accumulation of lactate and protons, and thus prove detrimental. Since intracellular pH decreases during ischemia, both H^+-Na^+ exchange and Na^+-Ca^{2+} exchange rates will increase, resulting in intracellular Ca^{2+} accumulation. Ca^{2+} accumulation can cause mitochondria eruption, cell swelling and finally cell death. We previously found that adenosine receptor blockade with 8-SPT caused more acidosis during myocardial ischemia.[9] These previous results and the present finding suggest that endogenous adenosine could lessen myocardial acidosis and Ca^{2+} accumulation during ischemia.

The effect of adenosine on glucose transport is more complex than its effect on glycolysis according to recent literature. Adenosine has been shown to have both inhibitory and facilitatory effects upon glucose transport. Fuller *et al.*[33] reported that in isolated rat hearts, removal of endogenous adenosine with adenosine deaminase increased insulin-stimulated glucose uptake and lactate release. In skeletal muscle, adenosine deaminase had been shown to stimulate glucose uptake,[40] and adenosine receptor blockade by either 8-cyclopentyl-1,3-dipropylxanthine (CPDPX) or 8-phenyltheophylline has been found to exert similar effects.[41] These reports are consistent with the results of our present study, indicating that interstitial adenosine has an inhibitory effect upon glucose

transport. The adenosine A_1 receptor agonist PIA inhibited glucose uptake in cardiomyocytes and sarcolemmal vesicles by binding to the glucose transporter.[15] Another study showed adenosine antagonist PD-115,199[23] can block glucose uptake, but adenosine A_1 blocker CPDPX does not block glucose transport. This suggests adenosine A_1 receptor stimulation is not a requirement for altering glucose uptake. These studies further suggest that adenosine may affect glucose uptake by an additional non-receptor mediated mechanism.

In contrast to the evidence summarized above, several studies reported that adenosine facilitates glucose transport and metabolism. Adenosine has been demonstrated to increase insulin-stimulated glucose uptake and transport in myocardium.[21, 34] Angello *et al.*[22] proposed that adenosine and insulin mediate glucose uptake in normoxic rat hearts by different mechanisms. Previous studies demonstrated that insulin primarily promotes translocation of glucose transporters from an intracellular site, increasing their density on the surface of the cell membrane,[36,37] and by increasing the intrinsic activity of glucose transporters.[38] Adenosine, on the other hand, was found to affect glucose transporter function rather than density, in adipocytes.[39] How adenosine affects glucose transport in myocardium is still unknown. Furthermore, Vergauwen *et al.*[35] reported that adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle. They proposed that interstitial adenosine, generated from ATP during muscle contraction, activated adenosine receptors to stimulate insulin-

dependent glucose transport. Adenosine receptor antagonism inhibited the increase in muscle glucose uptake at the onset of muscle contractions. Interestingly, neither adenosine nor its antagonists affected glucose uptake in resting skeletal muscle.[35]

In summary, this study demonstrates that adenosine receptor blockade by 8-SPT increases lactate production from exogenous glucose by accelerating phosphofructokinase activity, and by increasing glucose uptake. However, delineation of the specific mechanism by which 8-SPT activates phosphofructokinase will require further investigation.

Acknowledgements

This study was supported by grants to RTM (R29 HL50441) and HFD (R01 HL35056) from the National Heart, Lung and Blood Institute, and by a grant to HFD from the Texas Advanced Research Program.

REFERENCES

1. Rahimtoola SH. A perspective on the three large multicenter randomized clinical trials of coronary bypass surgery for chronic stable angina. *Circulation* 1985;72(suppl V):V123-V135.

2. Rahimtoola SH. The hibernating myocardium. *Am Heart J* 1989;117:211-221.

3. Pantely GA, Malone SA, Rhen WS, Anselone CG, Arai A, Bristow J, Bristow JD. Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. *Circ Res* 1990;67:1481-1493.

4. Arai AE, Pantely GA, Anselone CG, Bristow J, Bristow JD. Active downregulation of myocardial energy requirements during prolonged moderate ischemia in swine. *Circ Res* 1991;69:1458-1469.

5. Schultz R, Guth BD, Pieper K, Martin C, Heusch G. Recruitment of an inotropic reserve in moderately ischemic myocardium at the expense of metabolic recovery: a model of short-term hibernation. *Circ Res* 1992;70:1282-1295.

6. Fedele FA, Gewirtz H, Capone RJ, Sharaf B, Most AS. Metabolic response to prolonged reduction of myocardial blood flow distal to a severe coronary artery stenosis. *Circulation* 1988;78:729-735.

7. Downing SE, Chen V. Acute hibernation and reperfusion of the ischemic heart. *Circulation* 1992;85:699-707.

8. Schaefer S, Carr LJ, Kreutzer U, Jue T. Myocardial adaptation during acute hibernation: mechanisms of phosphocreatine recovery. *Cardiovasc Res* 1993;27:2044-2051.

9. Gao Z-P, Downey HF, Fan W-L, Mallet RT. Does interstitial adenosine mediate acute hibernation of guinea-pig myocardium? *Cardiovas Res* 1995;29:796-804.

10. Bünger R, Sommer O, Walter G, Stiegler H, Gerlach E. Functional and metabolic features of an isolated perfused guinea pig heart performing pressure-volume work. *Pflügers Arch* 1979;380:259-266.

11. Bünger R. Compartmented pyruvate in perfused working heart. *Am J Physiol* 1985;249:H439-H449.

12. Mallet RT, Hartman DA, Bünger R. Glucose requirement for postischemic recovery of perfused working heart. *Eur J Biochem* 1990;188:481-493.

13. Bergmeyer HU. *Methods of Enzymatic Analysis*, 3rd English Ed. New York: Academic Press, 1983-7.

14. Finegan BA, Lopaschuk GD, Coulson CS, Clanachan AS. Adenosine alters glucose use during ischemia and reperfusion in isolated rat hearts. *Circulation* 1993;87:900-908.

15. Dale WE, Hale CC, Kim HD, Rovetto MJ. Myocardial glucose utilization: failure of adenosine to alter it and inhibition by the adenosine analogue N^6-(L-2-phenylisopropyl)adenosine. *Circ Res* 1991;69:791-799.

16. Buxton DB, Kjaer-Pedersen K, Nguyen A. Metabolic effects of adenosine in the isolated perfused rat heart. *J Mol Cell Cardial* 1992;24:173-181.

17. Fralix TA, Murphy E, London RE, Steenbergen. Protective effects of adenosine in the perfused rat heart: changes in metabolism and intracellular ion homeostasis. *Am J Physiol* 1993;264:C986-C994.

18. Wyatt DA, Edmunds MC, Rubio R, Berne RM, Lasley RD, Mentzer RM. Adenosine stimulates glycolytic flux in isolated perfused rat hearts by A_1-adenosine receptors. *Am J Physiol* 1989;257:H1952-H1957.

19. Gutierrez-Juárez R, Madrid-Marina V, PiÑa E. Adenosine effect on lactate and pyruvate production in hypoxic guinea pig heart. *Int J Biochem* 1993;25:725-729.

20. Janier MF, Vanoverschelde JJ, Bergmann SR. Adenosine protects ischemic and reperfused myocardium by receptor-mediated mechanisms. *Am J Physiol* 1993;264:H163-H170.

21. Law WR, Mclane MP, Raymond RM. Adenosine is required for myocardial insulin responsiveness in vivo. *Diabetes* 1988;37:842-845.

22. Angello DA, Berne RM, and Coddington NM. Adenosine and insulin mediate glucose uptake in normoxic rat hearts by different mechanisms. *Am J Physiol* 1993;265:H880-H885.

23. Murphy E, Fralix TA, London RE, Steenbergen C. Effects of adenosine antagonists on hexose uptake and preconditioning in perfused rat heart. *Am J Physiol* 1993;265:C1146-C1155.

24. Kobayashi K, Neely JR. Control of maximum rates of glycolysis in rat cardiac muscle. *Circ Res* 1979;44:166-175.

25. Opie LH. Myocardial ischemia-metabolic pathways and implications of increased glycolysis. *Cardiovascular Drugs and Therapy* 1990;4:777-790.

26. Rovetto MJ, Whitmer JT, Neely JR. Comparison of the effects of anoxia and whole heart ischemia on carbohydrate utilization in isolated working rat heart. *Circ Res* 1973;32:669-711.

27. Williamson JR. Glycolytic control mechanisms. II. Kinetics of intermediate changes during the aerobic-anoxic transition in perfused rat heart. *J Biol Chem* 1966;214:5026-5036.

28. Uyeda K. Phosphofructokinase. *Advances in Enzymology & Related Areas of Molecular Biology* 1979;48:193-244.

29. Mahrenholz AM, Lan L, and Mansour TE. Phosphorylation of heart phosphofructokinase by Ca^{2+}/Calmodulin protein kinase. *Biochemical Biophysical Research Communications* 1991;174:1225-1259.

30. Hazen SL, Wolf MJ, Ford DA, Gross RW. The rapid and reversible association of phosphofructokinase with myocardial membranes during myocardial ischemia. *FEBS Letters* 1994;339:213-216.

31. Vander Heide RS, Reimer KA, Jennings RB. Adenosine slows ischemic metabolism in canine myocardium in vitro: relationship to ischemic preconditioning. *Cardiovasc Res* 1993;27:669-673.

32. Cooper TG. *The tools of biochemistry.* New York: John Wilkey & Sons, Inc. 1977;p136-168.

33. Fuller SJ, Sugden PH. Stimulation of protein synthesis, glucose uptake and lactate output by insulin and adenosine deaminase in the rat heart. *FEBS Lett* 1986;201:246-250.

34. Law WR, Raymond RM. Adenosine potentiates insulin-stimulated myocardial glucose uptake in vivo. *Am J Physiol* 1988;254:H970-H975.

35. Vergauwen L, Hespel P, Richter EA. Adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle. *J Clin Invest* 1994;93:974-981.

36. Devaskar SU, Mueckler MM. The mammalian glucose transporters. *Pediatr Res* 1992;31:1-13.

37. Watanabe T, Smith MM, Robinson FW, Kono T. Insulin action on glucose transport in cardiac muscle. *J Biol Chem* 1984;259:13117-13122.

38. Zaninetti D, Greco-Perotto R, Assimacopoulos-Jeannet P, Jeanrenaud B. Effects of insulin on glucose transport and glucose transporters in rat heart. *Biochem J* 1988;250:277-283.

39. Kuroda M, Honner RC, Cushman SW, Londos C, Simpson IA. Regulation of insulin-stimulated glucose transport in the isolated rat adipocyte: cAMP-independent effects of lipolytic and antilipolytic agents. *J Biol Chem* 1987;262:245-253.

40. Leighton B, Lozeman FJ, Vlachonikolis IG, Challiss RJA, Pitcher JA, Newsholme EA. Effects of adenosine deaminase on the sensitivity of glucose transport, glycolysis and glycogen synthesis to insulin in muscles of the rat. *Int J Biochem* 1988;20:23-27.

41. Challiss RAJ, Richards SJ, Budohoski L. Characterization of the adenosine receptor modulating insulin action in rat skeletal muscle. *Eur J Pharmacol* 1992;226:121-128.

42. Terracciano CMN, Macleod KT. Effects of acidosis on Na^{+}/Ca^{++}exchange and consequences for relaxation in guinea pig cardiac myocytes. *Am J Physiol* 1994;267:H477-H487.

43. Tani M, Neely JR. Role of intracellular Na^{+} in Ca^{++} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. *Circ Res* 1989;65:1045-1056.

44. Allard MF, Emanuel PG, Russell JA, Bishop SP, Digerness SB, Anderson PG. Pre-ischemic glycogen reduction or glycolytic inhibition improves post-ischemic recovery of hypertrophied rat hearts. *Am J Physiol* 1994;267:H66-H74.

45. Patten GS, Filsell OH, Clark MG. Epinephrine regulation of phosphofructokinase in perfused rat heart. *J Biol Chem* 1982;257:9480-9486.

46. Clark MG, Patten GS. Adrenaline activation of phosphofructokinase in rat heart mediated by α-receptor mechanism independent of cyclic AMP. *Nature* 1981;292:461-463.

47. Clark MG, Patten GS. Adrenergic control of phosphofructokinase and glycolysis in rat heart. *Current Topics in Cellular Regulation* 1984;23:127-176.

48. Olah ME, and Stiles GL. Adenosine receptors. *Ann Rev Physiol* 1992;54:211-225.

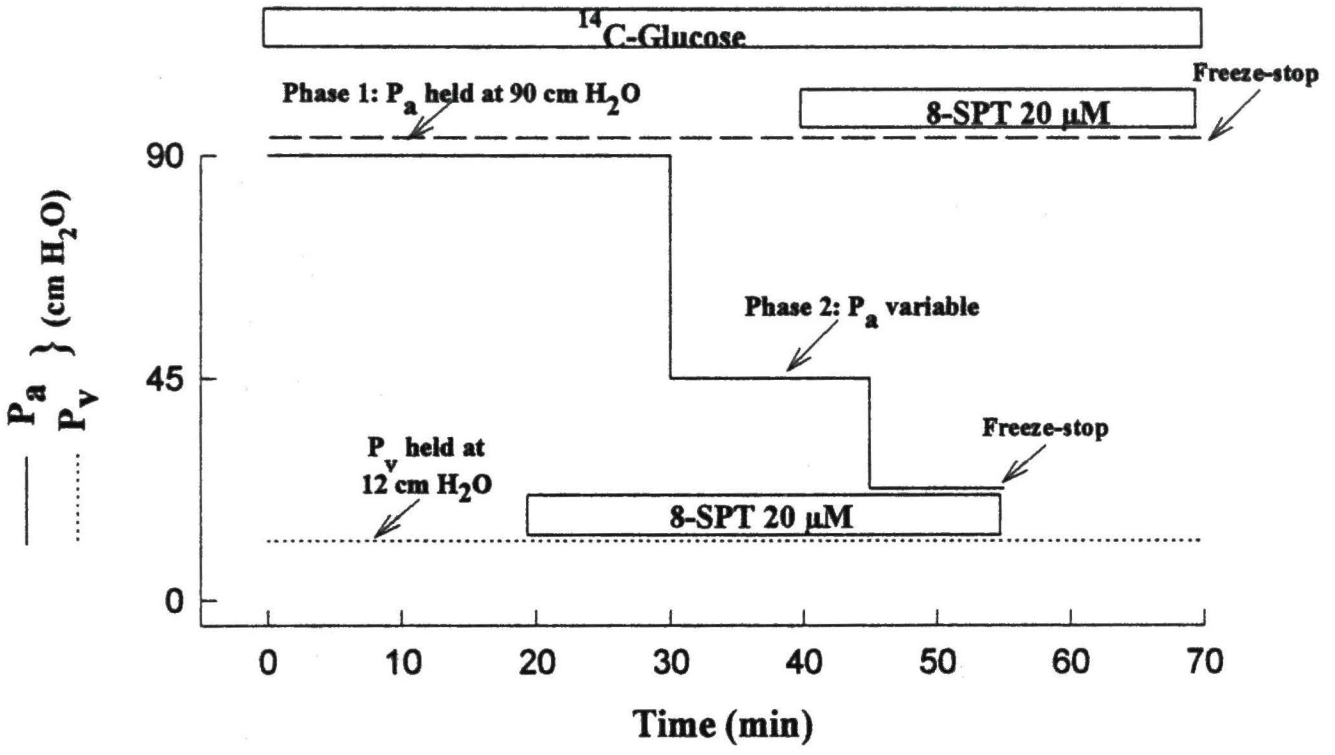

Figure 1. *Experimental protocol.* Phase 1: Hearts were first subjected to 40 min normal perfusion, followed by 30 min perfusion with 20 μM 8-SPT. Phase 2: Coronary hypoperfusion was produced by lowering aortic pressure (P_a) from 90 (30 min normal perfusion) to 45 cm H_2O for 15 min (mild hypoperfusion), then to 22.5 cm H_2O for 10 min (ischemia). Left atrial filling pressure (P_v) was held at 12 cm H_2O throughout each protocol. [U-^{14}C] glucose was perfused throughout the whole experimental protocol for both phase 1 and phase 2 experiments. 8-*p*-sulfophenyl theophylline (8-SPT; 20 μM) is indicated by the open bars.

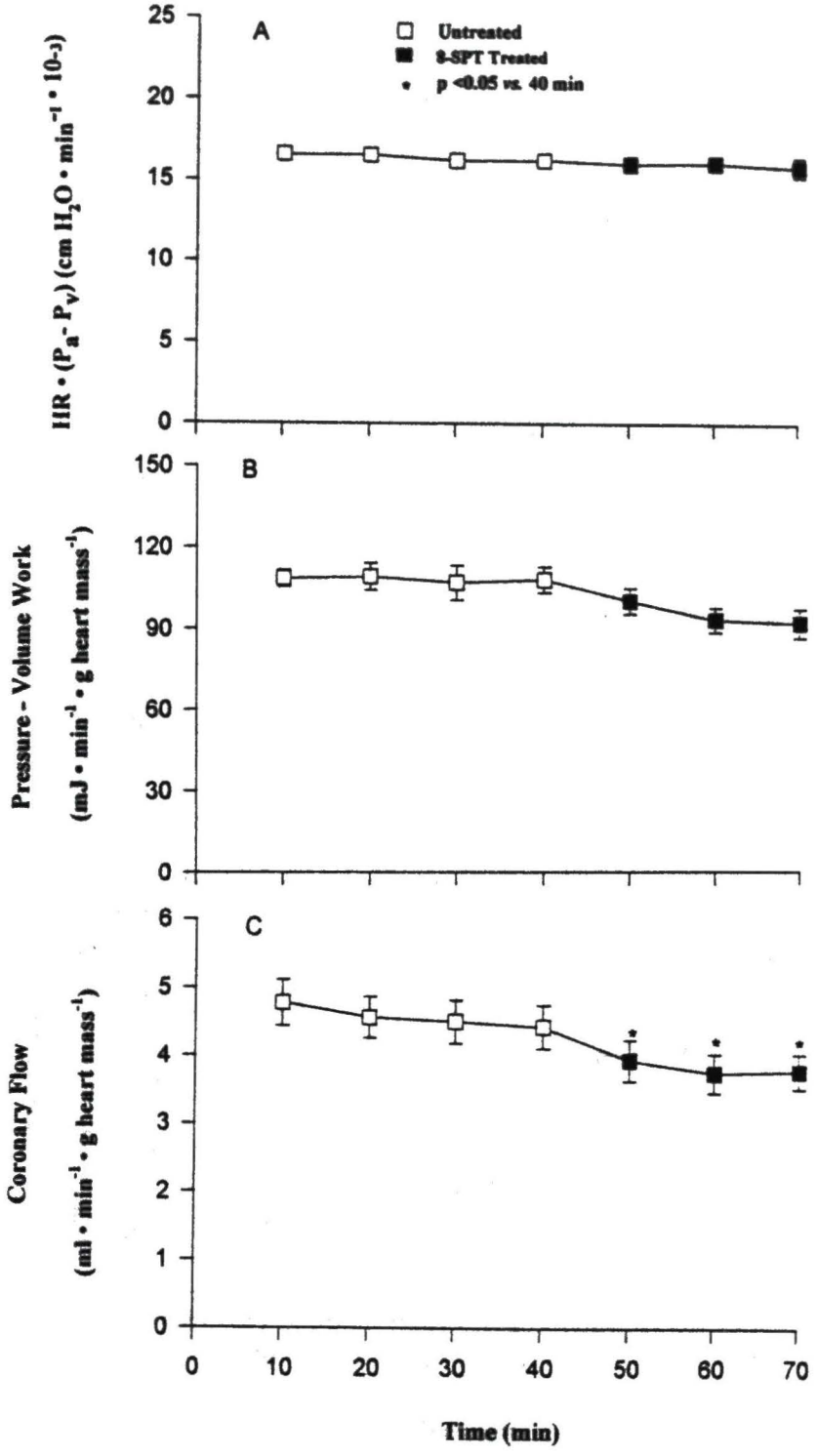

Figure 2. *Heart rate-pressure product (panel A), left ventricular pressure-volume work (panel B), and coronary flow (panel C) in normoxic myocardium.* Hearts were subjected to the experimental protocol illustrated in Figure 1. Means ± SE from 6 experiments.

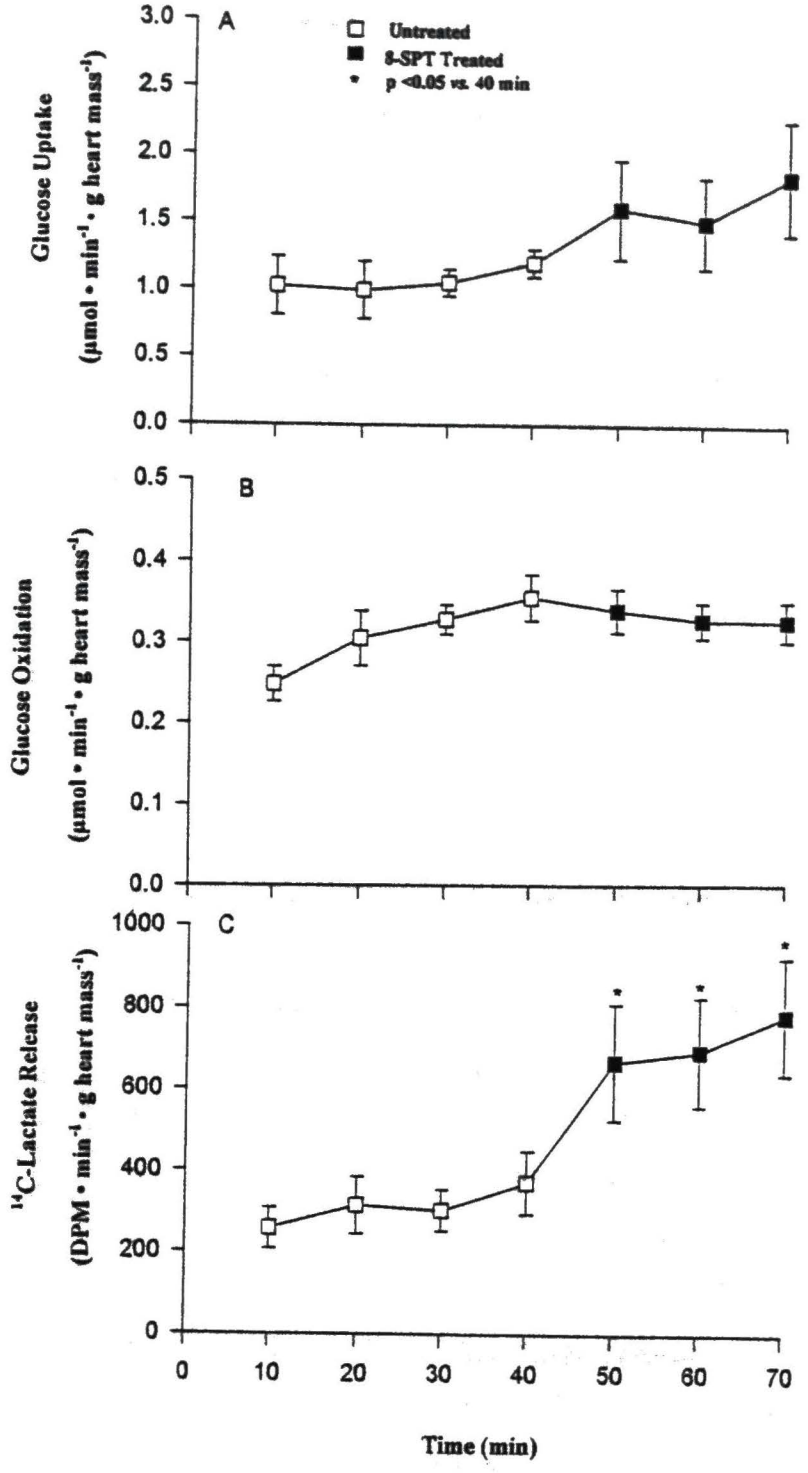

Figure 3. *Effects of adenosine receptor blockade on glucose uptake (panel A), glucose oxidation (panel B), and lactate release (panel C) in normoxic myocardium.* 8-*p*-sulfophenyl theophylline (8-SPT) was infused from 40 to 70 min. Means ± SE from 6 experiments.

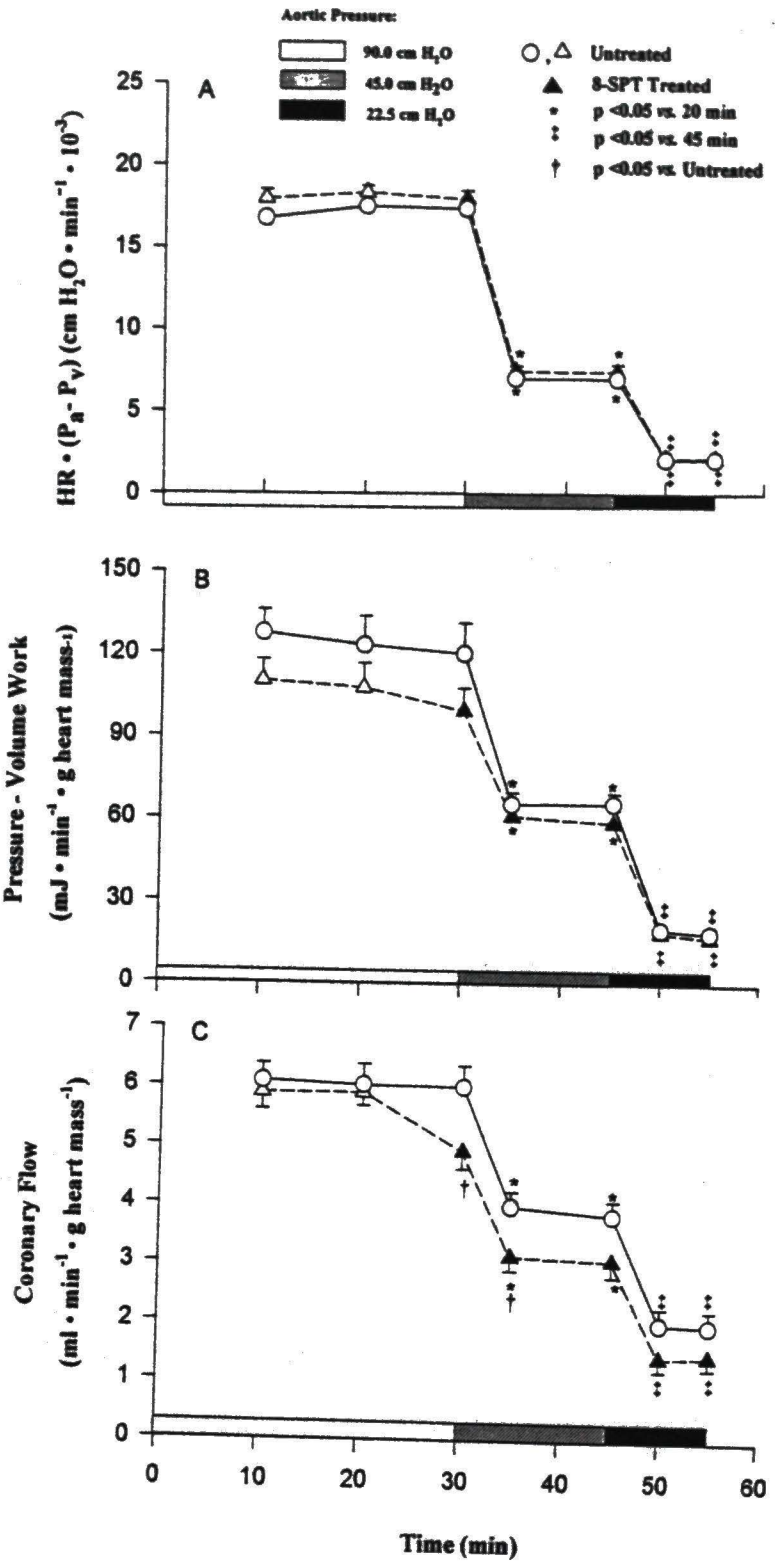

Figure 4. *Heart rate-pressure product (panel A), left ventricular pressure-volume work (panel B), and coronary flow (panel C) in ischemic myocardium.* Hearts were subjected to the experimental protocol illustrated in Figure 1. Means ± SE from 8 experiments.

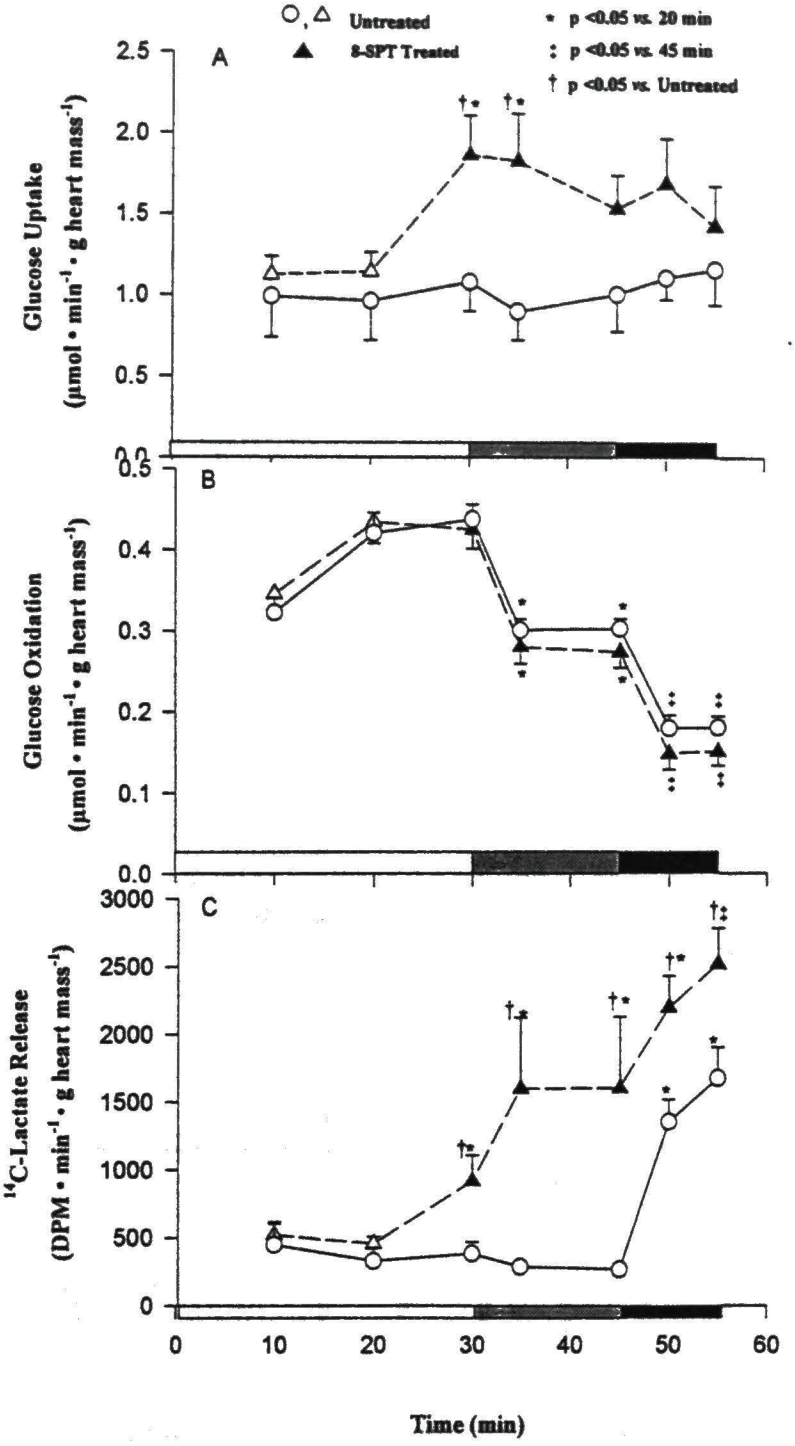

Figure 5. *Effects of adenosine receptor blockade on glucose uptake (panel A), glucose oxidation (panel B), and lactate release (panel C) in ischemic myocardium.* 8-*p*-sulfophenyl theophylline (8-SPT, 20 μM) was infused from 20 to 55 min. Means ± SE from 5-8 experiments.

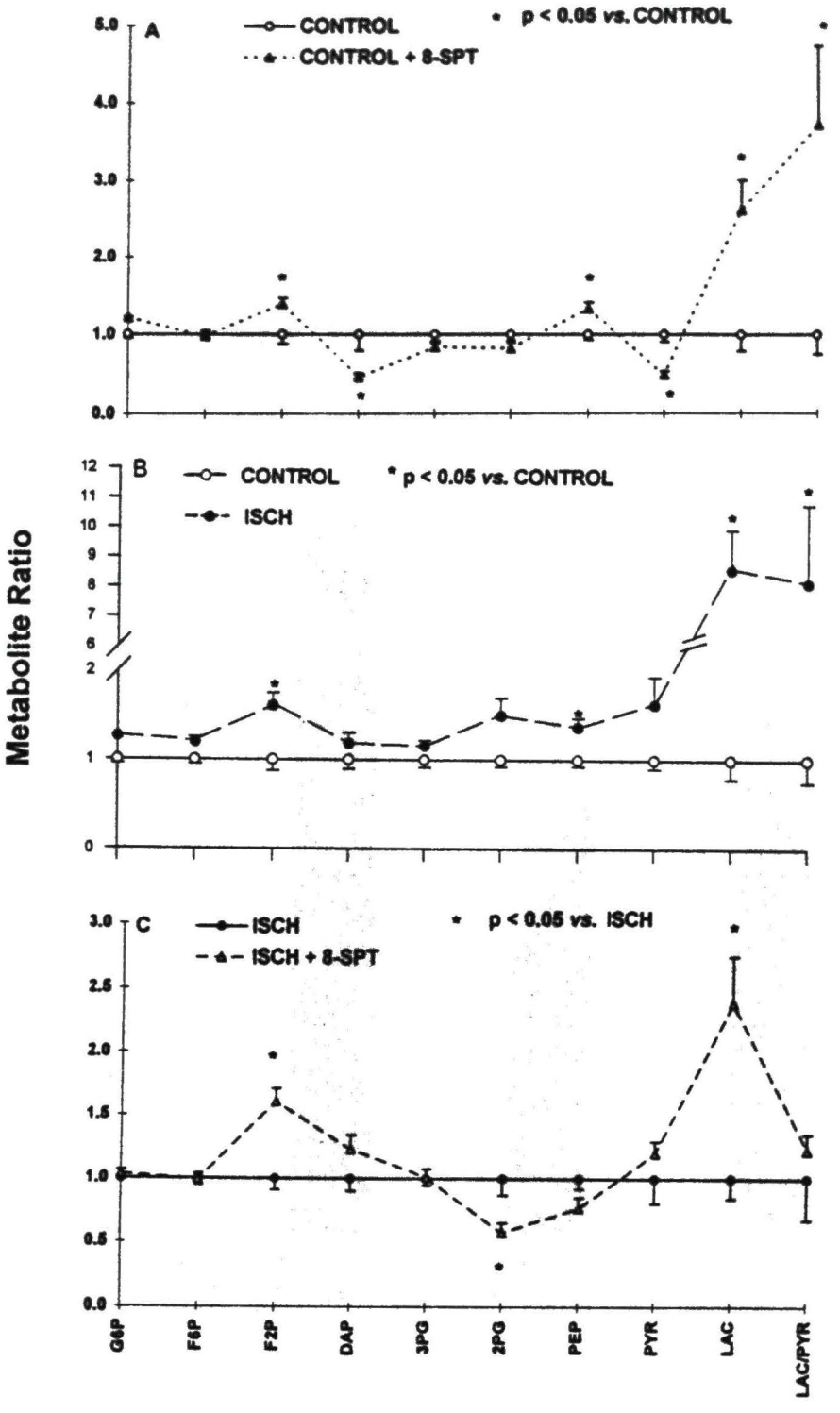

Figure 6. *Crossover plots of glycolytic intermediates and cytosolic redox metabolites in phase 1 (panel A), and phase 2 studies (panels B, C).* G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; F2P: fructose 1,6-bisphosphate; DAP: dihydroxyacetone phosphate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; LAC: lactate. Means ± SE from 6-8 experiments.

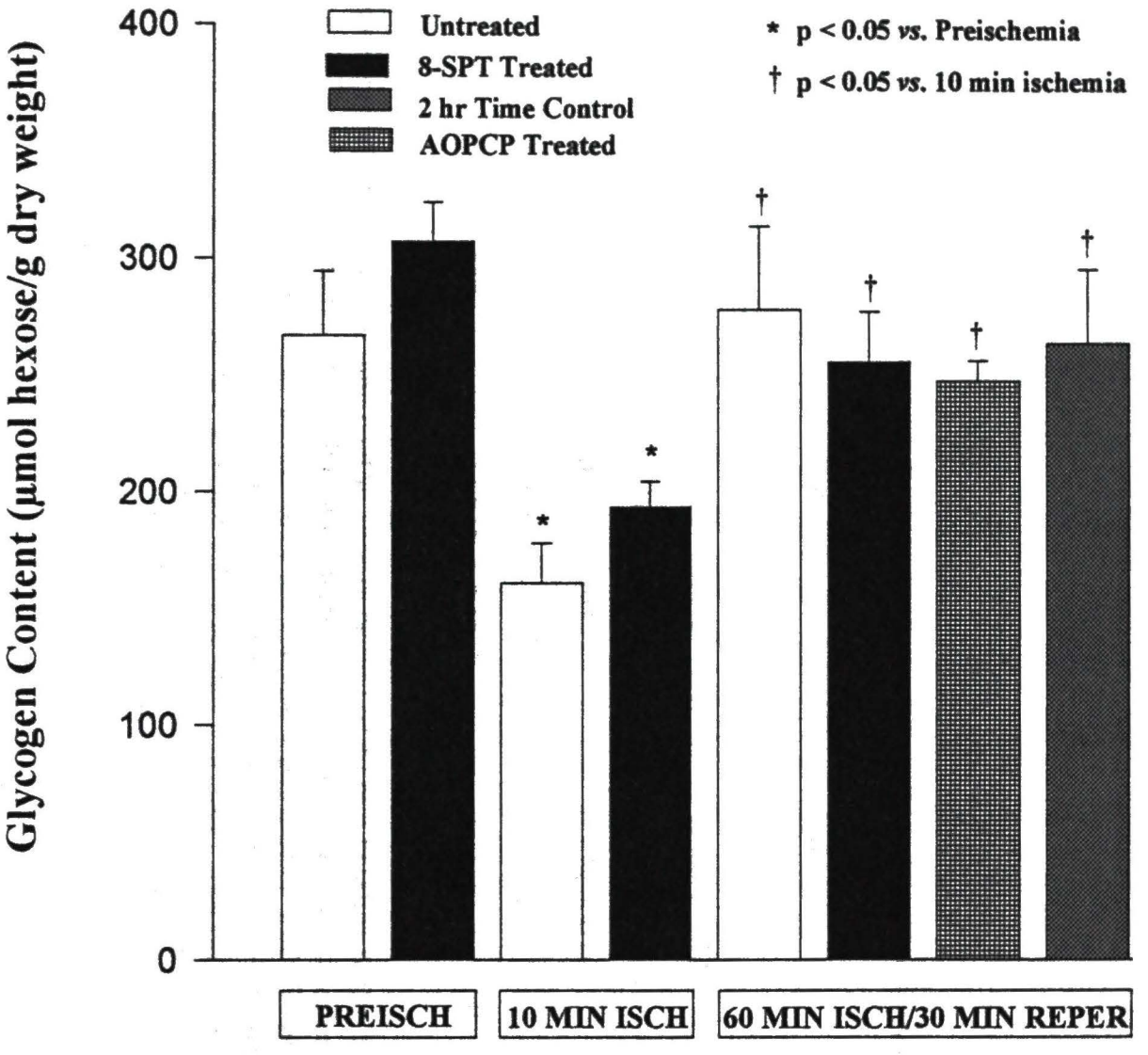

Figure 7. *Effects of ischaemia/reperfusion and adenosine receptor blockade on myocardial glycogen contents.* 8-SPT, 20 μM was infused in treated hearts. Means ± SE from 6-8 experiments. PREISCH: Phase 1 study with or without 8-SPT; 10 MIN ISCH: Phase 2 study; 60 MIN ISCH/30 MIN REPER: Results from our previous study (Chapter II).

Table. Effects of adenosine receptor blockade on glycolytic intermediate ratios during pre-ischemia and ischemia.

	Pre-ischemia	Pre-ischemia + 8-SPT	Ischemia	Ischemia + 8-SPT
n	6	6	8	8
G6P/F6P	4.611 ± 0.216	5.620 ± 0.362*	4.880 ± 0.256	5.091 ± 0.234
F2P/F6P	1.272 ± 0.108	1.948 ± 0.156*	1.746 ± 0.221	2.859 ± 0.265*‡†
F2P/G6P	0.290 ± 0.034	0.351 ± 0.032	0.358 ± 0.041	0.560 ± 0.038*‡†
3PG/DAP	1.875 ± 0.192	2.826 ± 0.183*	1.834 ± 0.142	1.524 ± 0.118†
LAC/PYR	5.386 ± 1.347	26.947 ± 8.134*	44.053 ±14.367*	53.850 ± 5.666*†

Metabolite ratios were measured at 70 min time control (Pre-ischemia) or 40 min baseline perfusion + 30 min 8-SPT (20 μM) (Pre-Ischemia + 8-SPT) in Phase 1 study. Metabolite ratios were measured in Ischemia and Ischemia + 8-SPT groups at time indicated in Figure 1. DAP: dihydroxyacetone phosphate; F6P: fructose 6-phosphate; F2P: fructose 1,6-bisphosphate; G6P: glucose 6-phosphate; LAC: lactate; PYR: pyruvate; 3PG: 3-phosphoglycerate; 8-SPT: 8-*p*-sulfophenyl theophylline (20 μM). G6P/F6P: an index of phosphoglucoisomerase activity; F2P/F6P and F2P/G6P: indexes of phosphofructokinase activity; 3PG/DAP: an index of glyceraldehyde 3-phosphate dehydrogenase/phosphoglycerate kinase activity; LAC/PYR: an index of cytosolic redox state *i.e.* NADH/NAD ratio. *: $p < 0.05$ *vs.* Pre-ischemia. †: $p < 0.05$ *vs.* Pre-ischaemia + 8-SPT. ‡: $p < 0.05$ *vs.* Ischemia.

CONCLUSIONS

This study partially delineates the role of adenosine in hibernating myocardium. The results demonstrate that in this model 1) cardiac function fully recovers following 60 min ischemia, and neither adenosine A_1 receptor blockade nor *ecto* 5'-nucleotidase inhibition impairs recovery of contractile function; 2) adenosine A_1 receptor blockade stimulates anaerobic glycolysis during ischemia, but does not increase purine nucleoside release; 3) adenosine A_1 receptor blockade lessens the initial decline in cytosolic ATP phosphorylation potential during the first 10 min ischemia, but prevents the subsequent recovery of cytosolic energetics after 60 min ischemia; 4) adenosine receptor blockade markedly increases lactate production from exogenous glucose, but does not stimulate glucose oxidation; 5) adenosine receptor blockade does not increase glycogen degradation during the first 10 min ischemia; thus enhanced lactate production during this period results from increased uptake and metabolism of exogenous glucose; 6) crossover plots of glycolytic intermediates reveal that phosphofructokinase, a key rate-controlling step in glycolysis, is activated by adenosine receptor blockade in both pre-ischemic and ischemic myocardium. The conclusions from this investigation are:

1. The isolated guinea-pig working heart preparation developed and studied in this investigation exhibits many of the hallmarks of hibernating myocardium and

therefore can be used as an experimental model of this newly recognized phenomenon.

2. In this model, an ischemia induced increase in interstitial adenosine mediates the rebound of cytosolic energy level by stimulating adenosine receptors. This improvement in energetics is not essential for complete recovery of contractile function in the post-ischemic period.

3. This ischemia induced increase in interstitial adenosine attenuates anaerobic glycolysis by blunting phosphofructokinase activity.

The following experiments are proposed to further delineate the role of adenosine in hibernating myocardium: In the same model of myocardial hibernation, 1) the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenosine (EHNA), should decrease glycolysis during ischemia. Adenosine deaminase should increase glycolysis and blunt rebound of the phosphorylation potential at the end of 60 min ischemia as did the adenosine receptor blocker, 8-SPT; 2) Ca^{2+} antagonists should decrease the activity of phosphofructokinase at 10 min ischemia. This experiment might prove that the inhibiting effect of adenosine on glycolysis is mediated by Ca^{2+} antagonism.