INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

University of Alberta

Glycogen and Glucose Metabolism in Cardioprotection

By

Bernadine Heather Fraser 

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

Department of Pharmacology

Edmonton, Alberta

Fall, 1998

University of Alberta

Library Release Form

Name of Author: Bernadine Heather Fraser

Title of Thesis: Glycogen and Glucose Metabolism in Cardioprotection

Degree: Doctor of Philosophy

Year this Degree Granted: 1998

207 Douglas Avenue
Penticton, B.C. V2A 1N0

Date: Sept. 29/98

The education of man is never completed until he dies.

--Robert E. Lee

It is human nature to think wisely and act foolishly.

--Anatole France

The great pleasure in life is doing what people say you cannot do.

--Walter Bagehot

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Glycogen and Glucose Metabolism in Cardioprotection** submitted by **Bernadine Heather Fraser** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

Alexander S. Clanachan, PhD

Gary D. Lopaschuk, PhD

Koon K. Teo, MB, PhD

Sandra T. Davidge, PhD

Michael F. Allard, MD

Date: Sept 15, 1998

Abstract

Although suggested to play only a minor role in myocardial energy substrate metabolism, glycogen metabolism has been shown to contribute significantly to overall myocardial energy production. A new approach was developed to determine directly the contributions of glycogen turnover (simultaneous synthesis and degradation) to overall glucose metabolism in aerobic, ischemic and reperfused working rat hearts. Data obtained from studies described in this thesis indicate a substantial turnover of glycogen during aerobic, low-flow ischemic and reperfusion conditions. Evidence is also presented that suggests glycogenolysis does not proceed simply by either an ordered or random process. Studies described herein tested the role of glycogen metabolism in cardioprotection using three pharmacologically distinct classes of drugs. First, the cardioprotective agent, N^6-cyclohexyladenosine, (CHA, an adenosine A_1-receptor agonist) improved the recovery of mechanical function during reperfusion and decreased the rates of glycolysis and proton production from the hydrolysis of glycolytically derived ATP. CHA also altered glycogen turnover during reperfusion by stimulating glycogen synthesis, an effect that accelerated the recovery of post-ischemic glycogen content, possibly in response to improved functional and energetic state and/or inhibition of glycolysis. The time-course of glycogen turnover revealed a rapid rate of glycogenolysis during the first 5 min of low-flow ischemia that corresponded to a rapid rate of glycogen degradation. These changes in glycogen turnover were not reflective of the activities of the two enzymes controlling glycogen turnover, glycogen synthase and glycogen phosphorylase. The second class of drugs, the α-glucosidase inhibitors, did not

limit glycogenolysis as expected. Chronic 17β-estradiol, a member of a third class of drugs, estrogen receptor agonists, inhibited glycogen degradation and stimulated glucose oxidation in the heart during aerobic perfusion. Chronic, 17β-estradiol treatment was cardioprotective in association with increased glycogen content and glycogen synthase activity compared with untreated hearts. 17β-Estradiol-induced cardioprotection was not a result of improved metabolic coupling of glucose metabolism. Enhanced NO biosignaling was increased in post-ischemic hearts from chronically treated rats as both iNOS activity and cGMP content increased. These results show associations between glycogen turnover and post-ischemic mechanical function. Furthermore, 17β-estradiol-induced cardioprotection may arise from an action on glucose and glycogen metabolism and/or NO biosignaling.

Dedication

To Danial James Fraser, with love

Acknowledgements

I have many people to thank for their tremendous support and advice. Firstly and most importantly, I would like to give enormous credit to my supervisor, and mentor, Sandy Clanachan. His honesty, support and dedication were most appreciated.

In addition to my supervisor, I would also like to extend thanks to the other members of my committee, Drs. Lopaschuk, Teo and Davidge, who were instrumental in study design and encouragement of productivity.

The entire department was kind and helpful, but particularly Dr. Colmers for helping me through many difficult times and Drs. Schulz and Lopaschuk who were instrumental in my postdoctoral decision making process.

I owe a debt of gratitude to my closest and dearest friends whose friendship, love and support I cherish: Dr. Melissa Maybroda-Metcalfe, Julie-Ann Foster, Hillary Taylor, Richard Wambolt, Olga Jakiwczyk, Dr. Michelle Arnot, Laura Atkinson, Dr. Darrell Belke, Dr. Will Ford and Ray Kozak. I would also like to show my appreciation to Barbara Zeilnick-Drabik and Dr. Gandhi.

Finally, I thank my family for their 30 years of unconditional support. Mom and dad you always told me I could do anything I put my mind towards. Thanks for believing in me. And last but not least, my little brother Danny, I am so proud of you. You three, are everything to me.

Table of Contents

Chapter

List of Tables

List of Figures

List of Abbreviations

ACC: acetyl-CoA carboxylase
ADP: adenosine diphosphate
AMP: adenosine monophosphate
AMPK: 5'AMP activated protein kinase
ATP: adenosine triphosphate
BSA: bovine serum albumin
BCA: bichinchonic acid
CAT: carnitine acetyltransferase
cAMP: cyclic adenosine monophosphate
CHA: N^6-cyclohexyladenosine
cGMP: cyclic guanosine monophosphate
CO: cardiac output
CO_2: carbon dioxide
CoA: coenzyme A
CK: creatine kinase
Cr: creatine
CrP: creatine phosphate
CPTI: carnitine palmitoyltransferase 1
CPTII: carnitine palmitoyltransferase II
DCA: dichloroacetate
DNA: deoxyribonucleic acid
DNJ: deoxynojirimycin
DTT: dithiothreitol
EDTA: ethylenediamine-tetraacetic acid
EGTA: ethylene glycol-bis(b-amino ethyl ether)N,N,N',N'-tetraacetic acid
eNOS: endothelial nitric oxide synthase
ER: estrogen receptor
ERE: estrogen response element
ERT: estrogen replacement therapy
$FADH_2$: flavin adenine dinucleotide
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
G_{in}: rate of glycogen synthesis
G'_{in}: apparent rate of glycogen synthesis
G_{out}: rate of glycogen degradation
G'_{out}: apparent rate of glycogen degradation
GTP: guanosine triphosphate
H_2O: water
HK: hexokinase
HPLC: high pressure liquid chromatography
HR: heart rate
HRT: hormone replacement therapy
iNOS: inducible nitric oxide synthase
IPC: ischemic preconditioning
LDH: lactate dehydrogenase
LFI: low-flow ischemia
LV: left ventricle
MI: myocardial infarction
mDNJ: N-methyl-deoxynojirimycin
MOPS: 3-[N-morpholino]propane sulfonic acid
NAD^+: nicotinamide adenine dinucleotide (oxidized)
NADH: nicotinamide adenine dinucleotide (reduced)
NO: nitric oxide
NOS: nitric oxide synthase
O_2: oxygen
PCA: perchloric acid
PDC: pyruvate dehydrogenase complex
PDH: pyruvate dehydrogenase
PFK: phosphofructokinase
PKA: protein kinase A
PKC: protein kinase C
SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis
S.E.: standard error of the mean
sGC: soluble guanylyl cyclase
SERM: selective estrogen receptor modulator
TCA cycle: tricarboxylic acid cycle
TBS: tris buffered saline
Tris: tris(hydroxymethyl)aminomethane

CHAPTER 1

Introduction

1.1) General Overview

Availability and usage of different metabolic substrates can profoundly influence the extent of "damage" to the heart during and following myocardial ischemia and thus optimization of energy substrate metabolism is an important mechanism to induce cardioprotection. Other groups have studied extensively the interactions between fatty acid and glucose metabolism during and following ischemia. However, the involvement of glycogen metabolism in cardioprotection is less well understood and this is the major focus of this thesis. Two experimental strategies have been used to study glycogen metabolism in heart. Firs[*] a novel approach was developed in order to measure glycogen metabolism during low-flow ischemia and reperfusion. Second, the relationships between alterations in glycogen metabolism were investigated. Using three distinct classes of drugs, 1) adenosine A_1-receptor agonists (N^6-cyclohexyladenosine) that have well documented cardioprotective effects, 2) α-glucosidase inhibitors (deoxynojirimycin and N-methyl-deoxynojirimycin) that have the potential to limit glycogen breakdown, and 3) estrogen receptor agonists (17β-estradiol) that alter glycogen metabolism and are cardioprotective. This introduction describes the background to the major components of this thesis, including myocardial ischemia, myocardial energetics, myocardial energy substrate metabolism and cardioprotective mechanisms.

1.2) Myocardial Ischemia

Cardiovascular disease is the leading killer of men and women in Canada and the United States. According to statistics from the Heart and Stroke Foundation of Alberta and N.W.T. (www.hsfacal.org/hrt_stats.html), approximately 40% of all deaths in Canada are a result of heart disease or stroke, with similar overall mortality rates in both men and women. The American Heart Association® states that the overall risk of developing heart disease occurs approximately 10 years earlier in males than females. However, after menopause the rate of heart disease is the same and even worsens as women age (www.americanheart.org/Scientific/Hsstats98/02about.html). Several modifiable risk factors that influence the incidence of heart disease include: smoking, hypertension, obesity and stress (Kirk-Gardner *et al.* 1992; Anding *et al.* 1996). Uncontrollable risk factors may also exist and these include age, gender and heredity. Although prevention of myocardial ischemia, by controlling risk factors, is the primary objective, the presence of uncontrollable risk factors stress the importance of developing additional treatments for ischemia.

Under normal physiological conditions, the oxygen demand of the heart is proportional to the level of work being performed by the heart. The pathological condition of myocardial ischemia occurs when either blood flow (oxygen supply) is limited, or workload (oxygen demand) is increased beyond the capacity for supply. The most common disease is coronary artery disease due to atherosclerosis causing partial or complete obstruction in the arterial lumen. Coronary artery disease and the less frequent coronary vasospasm limit oxygen supply, whereas increased oxygen demand can occur

with exercise, emotional stress, hypertension and tachycardia. Severe ischemia, if left untreated, can result in myocardial infarction. Ischemia can also occur during several types of cardiac surgery; however, modern surgical techniques utilize interventions that limit the extent of ischemic injury.

In experimental investigations of myocardial ischemia, two models of ischemia are typically described. The first is no-flow ischemia where coronary flow to the heart is completely stopped. Experimental no-flow ischemia can be investigated *in vivo* and *ex vivo*. A second form of experimental ischemia is termed low-flow ischemia, where coronary flow to the heart is severely reduced but not completely eliminated. Experimental low-flow ischemia can also be performed either *in vivo* or *ex vivo*. The relevance of either type of experimental ischemia to pathological conditions *in vivo* is subject to debate. During transplantation procedures, the organ is devoid of blood flow, whereas during myocardial infarction (MI) and coronary artery bypass surgery there may be residual collateral blood flow. Limitations in blood flow may be transient as during angina, more long-term as during coronary artery bypass surgery or cardiac transplantation or permanent as a consequence of untreated myocardial infarction. Therefore, it is important to consider the type of experimental ischemia that most appropriately mimics the clinical situation of interest. In this thesis work, a low-flow ischemia model was used. This has the advantage for the present investigations in that it is possible to collect coronary effluent and thereby measure directly myocardial metabolism throughout the ischemic period.

1.3) Myocardial Energy Production

The major source of energy for the cell is adenosine triphosphate (ATP). In the heart, oxidation of fatty acids provides the majority of the ATP required for myocyte function. The heart, however, is omnivorous and will generate its energy from fatty acids, glucose, lactate as well as other energy substrates depending upon substrate availability. As a result of oxidation of each of these energy substrates, $NADH + H^+$ and $FADH_2$ are generated by dehydrogenases in the mitochondria. These reduced equivalents, and those produced during glycolysis, then enter the electron transport chain resulting in the conversion of ADP to ATP by oxidative phosphorylation. ATP is then transported into the cytosol where it can be used for muscle (myocyte) contraction, as well as other energy-dependent processes (reviewed in Opie, 1991).

The generation of ATP by the mitochondria is controlled by the TCA cycle and the flow of reduced equivalents (NADH and $FADH_2$) into the electron transport chain. Therefore, when oxygen is limited as in hypoxia or ischemia, the TCA cycle slows and ATP generation subsequently decreases. The major regulator of the TCA cycle is the ratio between NAD^+ and NADH (reviewed in Salway, 1994). During ischemia the ratio of $NADH/NAD^+$ shifts so that NADH accumulates in the mitochondria and inhibits β-oxidation of fatty acids (Liedtke, 1981) as well as inhibiting pyruvate dehydrogenase (PDH) thereby inhibiting acetyl-CoA production for the TCA cycle (Fig. 1-1). In addition, in response to increased mitochondrial NADH, the activities of α-ketoglutarate dehydrogenase and isocitrate dehydrogenase decrease to slow the TCA cycle.

Generally, the production of ATP increases with increased energy demand.

However, the increased activity of the TCA cycle is limited by the rate at which the rate-limiting enzymes function (reviewed in Salway, 1994). During periods of increased work, as energy demand outstrips supply, NADH levels decrease thereby stimulating the activity of the dehydrogenases. Accordingly, the TCA cycle responds by increasing generation of reduced equivalents for cellular respiration and oxidative phosphorylation. Further, Ca^{2-} levels also control the activities of TCA cycle enzymes. As cytosolic Ca^{2-} levels rise, mitochondrial Ca^{2+} levels also increase, causing activation of the PDH and TCA cycle dehydrogenases (isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) (reviewed in Opie, 1991).

Creatine phosphate (CrP) is also an important energy source in the heart. CrP is in equilibrium with cytosolic ATP levels through the action of creatine kinase. CrP levels decrease to a greater extent than ATP during ischemia. CrP is not utilized in myocyte contraction, but this reduction occurs because it is used to replenish the pool of ATP (reviewed in Salway, 1994) that is ultimately used as the energy source. Both the Cr/CrP ratio and the AMP/ATP ratio have been used as indices of metabolic stress.

Under normal physiological conditions, approximately 60-70% of the ATP generated by the cardiomyocyte is used in contraction (including Ca^{2+} uptake by the sarcoplasmic reticulum), another 10-15% for active transport by the Na^{+}-K^{-} pump, less than 5% for the generation of action potentials, and a minute amount to phosphorylate proteins (reviewed in Opie, 1991). Consequently, the heart is highly efficient at converting free energy into mechanical energy.

During ischemia, ATP is catabolized to produce inorganic phosphate and ADP that can be further metabolized to AMP and subsequently adenosine, inosine and

hypoxanthine. Indirectly, the production of these metabolites indicates that ATP content is decreasing and that anaerobic glycolysis may be occurring in an attempt to maintain ATP synthesis. However, in contrast to work performed in this thesis, direct measures of glycolysis are rarely undertaken. During ischemia, the AMP/ATP ratio is increased due to an increase in AMP associated with a decrease in ATP. Thus, the AMP/ATP ratio may be used as an indicator of the energy status of the cell (i.e. an index of "stress") (Hardie & Carling, 1997). This ratio is of particular relevance to this thesis as the relative energy status of the cell regulates the activities of various cellular metabolic enzymes, which ultimately may influence post-ischemic mechanical function.

1.4) Energy Substrate Metabolism in the Heart

1.4.1) Metabolism of Exogenous Substrates

Exogenous fatty acids are the primary contributor to energy production within the cell. Fatty acid oxidation normally contributes 60-70% of the overall ATP requirement (Neely & Morgan, 1974) and can contribute as much as 90% of the ATP in diabetes or fasting (Neely & Morgan, 1974; Saddik & Lopaschuk, 1994). Free fatty acids in the plasma are bound to albumin and enter cells by either facilitated transport (protein-dependent) or passive diffusion through the cell membrane. In the cytosol, fatty acids (palmitate is the most abundant) are activated by the addition of CoA and delivered into the mitochondria by the carnitine palmitoyltransferase system (CPT). In the mitochondria, fatty acids are broken down progressively into 2-carbon units of acetyl-

CoA by the β-oxidation pathway (reviewed in Opie, 1991). Acetyl-CoA then proceeds to the TCA cycle and is oxidized producing reduced equivalents that enter oxidative phosphorylation for ATP generation (Fig. 1-3).

Once glucose enters the cell, it is converted to glucose-6-phosphate by the enzyme hexokinase. Glucose-6-phosphate then can be stored as glycogen, enter the pentose phosphate pathway (this pathway does not function to any significant extent in adult heart) or enter glycolysis (reviewed in Salway, 1994). In this thesis, glycolysis refers to the enzymatic conversion of glucose-6-phosphate to yield pyruvate. Glycolysis of exogenous glucose is a process that does not require oxygen and results in the net production of 2 molecules of ATP per molecule of glucose (reviewed in Opie, 1991). In reality, glycolysis produces 4 molecules of ATP, but 2 are consumed as part of the catabolism of glucose. When oxygen is absent (anaerobic conditions), the 2 pyruvate molecules produced from glycolysis are converted into 2 lactate molecules. The fate of pyruvate is dependent upon a number of factors, one of which is oxygen availability. The regulation of this pathway will be discussed below (Section 1.4.3.2, Fig. 1-2).

Under aerobic conditions, pyruvate enters the mitochondria, where it is converted to acetyl-CoA via the PDH complex, and is subsequently oxidized by the TCA cycle (Fig. 1-1). The TCA cycle is found in all cell types with the exception of red blood cells (these cells do not possess mitochondria). Oxidation of glucose involves a complex series of enzymatic conversions that ultimately produce CO_2, H_2O, GTP and reduced equivalents (NADH and $FADH_2$) for ATP synthesis in the electron transport chain.

1.4.2) Metabolism of Endogenous Substrates

Triglycerides are a storage form of long chain acyl groups that are esterified to glycerol. The contribution of triglycerides to ATP production can be substantial (10-50%), but it is dependent upon competition from exogenous fatty acids (Saddik & Lopaschuk, 1991). Although exogenous energy sources provide the majority of substrates for ATP generation, under certain conditions, particularly fatty acid-free perfusion, the contribution from the endogenous triglycerides can become significant (Saddik & Lopaschuk, 1992).

Another endogenous myocardial energy source is glycogen. The contribution of glycogen to aerobic ATP production from glucose metabolism can be as much as 40% (Goodwin *et al.* 1995; Henning *et al.* 1996). Much like the turnover of triglycerides in the heart, (Saddik & Lopaschuk, 1991; Saddik & Lopaschuk, 1992; Saddik & Lopaschuk, 1994) glycogen also turns over (Goodwin *et al.* 1995; Henning *et al.* 1996). The central component of the glycogen molecule is the protein glycogenin. Glycogenin is an autocatalytic component of glycogen, which when stimulated by Mn^{2-} and auto-glucosylation, primes glycogen synthesis (Pitcher *et al.* 1988). Glucosyl units attach to glycogenin and accumulate to form one of two forms of glycogen, an acid-insoluble (proglycogen) or an acid-soluble (macroglycogen) form. The distribution of proglycogen relative to macroglycogen is tissue-dependent with limited distribution of proglycogen in the liver, but with as much as 50% in the heart. This suggests an enormous potential for rapid synthesis of macroglycogen in the heart where it could be readily mobilized under different nutritional states or stress. It further suggests that different glycogen synthase

isozymes may exist which are specific for the conversion of glycogenin to proglycogen and for the conversion of proglycogen to macroglycogen. It has also been suggested, because there are two forms of glycogen, that turnover may be an oscillatory process between a stable pool of proglycogen and macroglycogen. This turnover process may not involve glycogenin (Alonso *et al.* 1995).

Glycogen stores increase when glycogen synthase is activated and a glucosyl unit from UDP-glucose is added to the glycogen molecule by an α(1-4) linkage. Once the chain becomes 11 residues long, a branching enzyme is activated and branches of glycogen form by breaking a string of 7 residues from the growing chain and rejoining it by an α(1-6) linkage to an interior point, at least, 4 residues from an existing branch (reviewed in Salway, 1994). Conversely, when glycogen is degraded (glycogenolysis) the enzyme glycogen phosphorylase removes a glucosyl residue from the end of the strand at the α(1-4) bond. At branch points, a debranching enzyme, α-glucosidase, removes a glucosyl unit at the α(1-6) bond, thereby exposing further α(1-4) bonds for phosphorylase to continue degrading. The release of a glucosyl unit from glycogen uses only 1 molecule of ATP in contrast to 2 used from exogenous glucose. Therefore, more ATP per mole of glucose is produced from glycogenolysis than from the glycolysis of exogenous glucose. However, overall there is no net advantage from glycogenolysis relative to glycolysis as ATP is consumed in glycogen synthesis.

1.4.3) Regulation of Energy Substrate Metabolism

1.4.3.1) Regulation of Fatty Acid Oxidation

Carnitine palmitoyltransferase 1 (CPT1) is located on the outer mitochondrial membrane and catalyzes the conversion of long-chain acylcarnitine to long-chain acyl-CoA, and is the rate-limiting reaction in the translocation of activated fatty acids into the mitochondria (McGarry *et al.* 1989). In the heart, CPT1 is sensitive to cytosolic malonyl-CoA levels, with increasing malonyl-CoA levels inhibiting CPT1 activity and therefore decreasing the rate of fatty acid oxidation (reviewed in Lopaschuk *et al.* 1994a).

Acetyl-CoA carboxylase (ACC) is the enzyme responsible for converting acetyl-CoA to malonyl-CoA through the incorporation of CO_2 from bicarbonate into the acetyl-CoA molecule. In fatty acid synthesizing tissue, this reaction is the first committed step of fatty acid synthesis; however, in the heart the primary role of ACC is to regulate fatty acid oxidation (Saddik *et al.* 1993). ACC exists in three isoforms: 280 kDa, 265 kDa, and 273 kDa. The 280 kDa isoform predominates in the heart, although the 265 kDa isoform is present as a minor component. As acetyl-CoA levels increase, ACC280 is activated. This results in increased malonyl-CoA levels. Since ACC controls the production of malonyl-CoA, it clearly plays a role in regulating CPT1 activity and hence fatty acid oxidation (Fig. 1-3). ACC is primarily regulated by substrate availability (acetyl-CoA) as well as phosphorylation.

A novel stress kinase, AMP-activated protein kinase (AMPK), also regulates fatty acid oxidation by phosphorylating and inactivating acetyl-CoA carboxylase (Kudo *et al.*

1996). Activity of this kinase is stimulated by the AMP/ATP ratio as well as the CrP/Cr ratio and is thus responsive to the energy status of the cell (Hardie & Carling, 1997). AMPK may influence myocardial energy substrate metabolism in additional ways besides activation of fatty acid oxidation during reperfusion. Interestingly, AMPK may also be involved in regulation of glycogen metabolism, as glycogen phosphorylase activity is stimulated by AICAR, an activator of AMPK (Young *et al.* 1996). As adenosine A_1-receptor agonists alter glycogen metabolism, the involvement of AMPK in adenosine A_1-receptor mediated cardioprotection is investigated as a component of the experimental work in this thesis.

Intracellular Ca^{2+} concentrations may also play a role in stimulating fatty acid oxidation by accelerating enzymes of the TCA cycle. In addition, regulatory control of the TCA cycle by NADH/NAD^+ has been discussed above. A more elaborate discussion of the regulatory mechanisms of fatty acid oxidation is beyond the scope of this thesis (see reviews by Lopaschuk *et al.* 1994a; Grynberg & Demaison, 1996; Hendrickson *et al.* 1997).

1.4.3.2) Regulation of Glycolysis

Regulation of glycolysis occurs at many points in the enzymatic conversion of glucose to pyruvate. Each of these points of regulation will be discussed in order in the pathway (Fig. 1-2). The first point of control in glycolysis is at hexokinase (HK). This enzyme converts glucose to glucose-6-phosphate, hydrolyzing one ATP molecule. Both insulin and ischemia stimulate HK (Kashiwaya *et al.* 1994). HK is also controlled by

feedback inhibition from glucose-6-phosphate (Hers, 1976). Phosphofructokinase (PFK), that catalyzes the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate and hydrolyzes one ATP molecule, is also subject to regulation. This enzyme normally exists in an inhibited state due to adequate ATP and citrate level and is considered the rate-limiting enzyme of glycolysis. PFK can be stimulated when ATP, CrP or citrate levels decrease, or when intracellular pH increases. All of these modulators change during ischemia. PFK is activated during low-flow ischemia and initially during no-flow ischemia although it is inhibited during severe ischemia as pH decreases. Fructose 2,6-bisphosphate, produced when glycolysis proceeds at high rates in the presence of glucose and insulin, is another potent stimulator of PFK and glycolysis (Depre *et al.* 1993). During severe ischemia, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can become a rate-limiting enzyme in glycolysis. GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate with the conversion of NAD^- to $NADH + H^-$. Both NADH and lactate (from pyruvate) inhibit GAPDH by feedback inhibition during ischemia.

1.4.3.3) Regulation of Glucose Oxidation

Glucose oxidation is primarily controlled by the rate of entry of pyruvate into the TCA cycle by the pyruvate dehydrogenase complex (PDC) which is located in the mitochondria (Fig. 1-1) (reviewed in Salway, 1994). This enzyme converts pyruvate to acetyl-CoA while reducing NAD^+ to $NADH + H^+$ with the release of CO_2. PDC exists in both an active and inactive form, the balance of which is regulated by phosphorylation

and dephosphorylation. The enzyme is inactive when phosphorylated by PDC kinase, and active when dephosphorylated by PDC phosphatase (Patel & Roche, 1990). PDC kinase is activated by acetyl-CoA and NADH (such as in ischemia), and inhibited by CoA, NAD^-, pyruvate and dichloroacetate (discussed in Section 1.5.1). Inhibition of PDC kinase allows PDC to remain in the dephosphorylated (active) form thereby stimulating glucose oxidation. Conversely, PDC phosphatase is inhibited by NADH (ischemia) and stimulated by Ca^{2+}. Inhibition of PDC phosphatase prevents the dephosphorylation and activation of PDC therefore slowing the rate of glucose oxidation.

Competition between fatty acids and glucose as fuels in the heart is well-established (Randle *et al.* 1963) as is the competition between fatty acids and ketone bodies (Vanoverschelde *et al.* 1993). The regulation of energy substrate preference is very complex. The Randle cycle, published in 1963, was the first description of the reciprocal interaction between fatty acid oxidation and glucose oxidation. As fatty acid oxidation increases, glucose oxidation decreases. The key regulators of this interaction are the acetyl-CoA/CoA and the NADH/NAD^+ ratios. When fatty acids are the primary substrate, an increase in acetyl-CoA/CoA ratio leads to the activation of PDH kinase and phosphorylation of PDC to the inactive form (Patel & Roche, 1990). On the other hand, an increase in carbohydrate metabolism leads to an increased production of malonyl-CoA from ACC and inhibition of CPT1 (Fig 1-3). Thus, reciprocal interactions between fatty acids and carbohydrates are controlled by alterations in the oxidation rates of individual substrates.

The inhibition of glucose oxidation by the products of fatty acid oxidation contributes to the metabolic uncoupling of glycolysis from glucose oxidation. Complete

metabolic coupling would exist if every molecule of pyruvate produced from glycolysis of glucose were subsequently oxidized. In contrast, complete uncoupling of glycolysis from glucose oxidation would occur if pyruvate were not oxidized. This uncoupling of glycolysis from glucose oxidation can occur under normal physiological conditions where O_2 and substrate availability are not limited. Under physiological conditions, the rate of glycolysis exceeds the rate of glucose oxidation by ~6–times. The detrimental effects of metabolic uncoupling of glycolysis from glucose oxidation occur when the ATP generated from glycolysis is hydrolyzed to produce protons. Proton production resulting from uncoupling of glycolysis from glucose oxidation can be considerable (Finegan *et al.* 1993). Improved coupling between rates of glycolysis and glucose oxidation reduces the rate of proton production from glycolytically derived ATP.

1.4.3.4) Regulation of Glycogen Metabolism

Although the pathways of glycogen synthesis and degradation (glycogen turnover) were characterized in the liver some time ago (Hers, 1976), the regulation of glycogen metabolism in the heart is not well understood. Several reviews on glycogen, its components and its metabolism (Whelan, 1986; Smythe & Cohen, 1991; Alonso *et al.* 1995) describe how previously accepted dogma regarding glycogen is outdated. These papers suggest that glycogen is a much more complex molecule than previously believed, with an equally complex regulation.

Glycogen metabolism is regulated by many factors (Fig. 1-4). The two key enzymes, glycogen synthase and glycogen phosphorylase are regulated primarily by

phosphorylation and dephosphorylation. These two enzymes exist in two forms: active and inactive and coordinated regulation of glycogen turnover occurs when one enzyme is activated and the other is inactivated. Glycogen synthase in the heart is extensively phosphorylated at multiple sites (McCullough & Walsh, 1979), and like skeletal muscle, inactivation is governed by the phosphorylation state of the enzyme. Several kinases (PKA, cAMP-independent protein kinases and Ca^{2+}-dependent protein kinases) are able to phosphorylate and inactivate glycogen synthase (Grekinis *et al.* 1995). Dephosphorylation by protein phosphatases leads to activation of glycogen synthase. Glucose-6-phosphate is believed to act on a glycogen synthase phosphatase increasing the activation of glycogen synthase during ischemia in rat hearts (McNulty & Luba, 1995).

Insulin is also involved in the regulation of glycogen synthase. Insulin through its actions on one or more phosphoprotein phosphatases activates glycogen synthase (Denton *et al.* 1986). Insulin also stimulates glucose uptake providing more substrate for glycogen synthesis; however, the presence of alternate substrates may be a more powerful regulator of glycogen synthase than insulin (Laughlin *et al.* 1994). In order to have a significant and sustained rate of glycogen synthesis, the presence of lactate, pyruvate or β-hydroxybutyrate is necessary even *in vivo* (Laughlin *et al.* 1994). Moreover, the oxidation of alternative substrates, even in the absence of alterations in glucose uptake and phosphorylation results in the inhibition of glycolysis causing glucose to be shuttled into glycogen (Laughlin *et al.* 1994). It is well recognized that the presence of fatty acids in the blood inhibits glycolysis, a condition that will promote glucose storage in the form of glycogen. Furthermore, glucose-6-phosphate accelerates the formation of UDP-glucose that is the substrate for glycogen synthase. Glycogen synthesis in the heart is

also activated after intense work or ischemia where glycogen stores have been depleted (Camici *et al.* 1989).

Glycogen phosphorylase is also controlled by phosphorylation, however, in contrast to glycogen synthase, phosphorylation of the enzyme results in activation. Activated glycogen phosphorylase occurs from phosphorylation by phosphorylase kinase, which is Ca^{2+}-dependent. β-Adrenoceptor agonists, e.g. epinephrine, stimulate glycogen phosphorylase through the cAMP pathway. Thus, the cAMP-dependent protein kinase plays a critical role in regulating glycogen turnover. It not only phosphorylates and activates glycogen phosphorylase, it concurrently phosphorylates and inactivates glycogen synthase. When the energy status of the cell is low (decreased ATP and increased AMP) as observed during hypoxia, ischemia or periods of increased workload, glycogen phosphorylase is activated and glycogen is degraded to glucose-1-phosphate that is then available to enter glycolysis.

Although fatty acid metabolism will not be measured as part of the work described in this thesis, fatty acid was present in all studies because of the critical interactions between fatty acids and glycogen metabolism, glycolysis and glucose oxidation.

1.5) Cardioprotection and Proposed Mechanisms of Action

Cardioprotection refers to protection of the heart from ischemic damage. This may be manifest as increased recovery of post-ischemic mechanical function, decreased infarct size or reduced incidence of dysrhythmias. In this thesis, cardioprotection will be

assessed by recovery of post-ischemic mechanical function. Many substances have been shown to act as cardioprotective agents, and those that are relevant to this thesis and their mechanisms of action are discussed in the following sections.

1.5.1) Metabolic Modulators

Metabolic uncoupling of glycolysis from glucose oxidation in the setting of ischemia and reperfusion can lead to substantial proton production and subsequent impairment of mechanical function. During moderate ischemia, fatty acid oxidation is decreased (but is still the major source of acetyl CoA); however, during reperfusion, rates of fatty acid oxidation quickly recover to pre-ischemic levels (reviewed in Lopaschuk *et al.* 1994a). Under clinical conditions, fatty acid oxidation rates often are further increased because of higher circulating levels of fatty acids (reviewed in Lopaschuk *et al.* 1994a). This increased reliance on fatty acids for oxidative phosphorylation leads to a reduction in glucose oxidation, and an uncoupling of glycolysis from glucose oxidation. The increased ATP production from anaerobic glycolysis rather than from glucose oxidation, followed by the hydrolysis of that ATP results in this substantial proton production and decreased myocardial efficiency (Finegan *et al.* 1995; Liu *et al.* 1996). Improvement of coupling between glycolysis and glucose oxidation is typically achieved by two strategies: indirectly by decreasing of the rate of fatty acid oxidation or directly by increasing the rate of glucose oxidation. Several pharmacological approaches have been utilized to achieve these ends.

One approach is to increase glucose oxidation directly. Dichloroacetate (DCA)

increases PDC (the rate-limiting enzyme of glucose oxidation) activity by inhibiting PDC kinase resulting in a more active PDH (Stacpoole, 1989). This results in a stimulation of glucose oxidation and a subsequent inhibition of fatty acid oxidation. DCA has proven efficacy in *in vitro* models of ischemia reperfusion. It has had a more limited success in clinical trials, which may be due to the high dose required and the short plasma half-life of the drug (Stacpoole, 1989).

Stimulation of glucose oxidation also may be achieved using such drugs as ranolazine. Ranolazine is cardioprotective both *in vivo* and *in vitro* (reviewed in McCormack *et al.* 1996). In addition, ranolazine decreases the incidence of anginal attacks in humans (Hayashida *et al.* 1994). The precise mechanism of action of ranolazine is unknown, but it stimulates the activity of PDC, possibly by an indirect mechanism resulting from the inhibition of the β-oxidation of fatty acids (Clarke *et al.* 1996). Trimetazidine, a compound structurally related to ranolazine, also inhibits fatty acid oxidation and has proven efficacy in clinical trials in Europe (Libersa *et al.* 1990; Dalla-Volta *et al.* 1990; Fabiani *et al.* 1992).

A second approach to improve metabolic coupling of glycolysis to glucose oxidation is to inhibit the rate of fatty acid oxidation that results in a stimulation of the rate of glucose oxidation. Fatty acids are known to increase the severity of injury during acute myocardial ischemia (reviewed in Lopaschuk *et al.* 1994a). Etomoxir inhibits entry of fatty acids into the mitochondria by inhibiting the carnitine palmitoyl transferase 1 (CPT1) and has anti-ischemic efficacy (Lopaschuk *et al.* 1994a). Lowering fatty acid concentrations in the perfusate is also beneficial.

It is evident through the work of various groups over the last two decades that

drug-induced alterations of myocardial metabolism have clear therapeutic benefits. Development of new agents that modify these or other targets in cellular metabolism may provide new treatment strategies for patients suffering from myocardial ischemia.

1.5.2) Adenosine

Adenosine is produced through *de novo* synthesis and from the breakdown of adenosine monophosphate (AMP) or S-adenosyl homocystine (SAH). Most cells produce adenosine. The physiological blood concentration of adenosine is 0.2-1 μM and it has a rapid plasma elimination half-life of 1-5 sec. Adenosine, when present in the extracellular space binds to and activates any or all 4 adenosine receptor subtypes (A_1, A_{2a}, A_{2b} and A_3).

In the cardiovascular system, adenosine A_1-receptors are primarily located on the myocyte and adenosine A_2 receptors are primarily located in the vasculature. Adenosine A_3 receptors are a newly defined subtype and are present in the heart and elsewhere. Adenosine receptors belong to the G-protein coupled receptor superfamily and have been referred to as promiscuous because of their interaction with many transduction systems (reviewed in Mubagwa *et al.* 1996).

In the heart, adenosine A_1-receptor activation decreases heart rate (negative chronotropic; slows depolarization of the SA nodal cells), decreases conduction (negative dromotropic; slows/blocks AV node), and decreases force generation in the atria (negative inotropic; via K^+ channel activation). These actions are associated with G_i proteins (reviewed in Ohyanagi & Iwasaki, 1996).

In the coronary circulation, adenosine receptor stimulation increases coronary blood flow (vasodilation, autoregulation of coronary blood flow) via adenosine A_2 receptor mechanisms (Mubagwa *et al.* 1996). In addition, adenosine can cause the phenomenon of coronary steal where vasodilation occurs in normal vessels with less occurring in the already dilated, ischemic vessels. Therefore, flow shifts from ischemic areas to normal areas. This and other potential adverse effects have limited the use of adenosine for stress echocardiography (Marwick, 1997).

In the systemic vasculature, adenosine receptor stimulation causes widespread vasodilation (mainly adenosine A_2 receptor-mediated), and reduced systemic vascular resistance. The associated decrease in afterload and reflex sympathetic activation increases cardiac output.

The cardioprotective actions of adenosine are manifest as improvement in the recovery of post-ischemic mechanical function, reduction in infarct size (independent of changes in coronary flow) and antidysrhythmic effects (atrial and ventricular). Furthermore, adenosine is effective when co-administered with cardioplegia or after transplantation (Lawton *et al.* 1996; Mentzer, Jr. *et al.* 1996; Dorman *et al.* 1997). Initially, controversy existed concerning the mechanism of adenosine-induced improvements in post-ischemic mechanical function. One proposed mechanism was that adenosine simply acted as a substrate for ATP regeneration (Takeo *et al.* 1988). However, this is unlikely. Adenosine can increase ATP content under some conditions without improving mechanical function, and more importantly, the beneficial effects of adenosine are mimicked by N^6-cyclohexyladenosine, an adenosine A_1-receptor agonist that is not a substrate for ATP generation. A receptor-mediated mechanism has also been

proposed (Mustafa, 1980), and is now supported by numerous studies that show that the cardioprotective action of adenosine can be blocked with selective adenosine receptor antagonists.

Protection may arise from either an increased delivery of energy substrates and O_2 due to coronary hyperemia and/or to a decreased energy demand due to bradycardia and afterload reduction. Furthermore, adenosine can induce angiogenesis (Morris *et al.* 1989). Angiogenesis itself may be responsible for the increased delivery of energy substrates to the heart and therefore be protective following chronic long-term ischemia rather than from acute administration.

Other proposed mechanisms for the anti-ischemic actions of adenosine involve actions on the cellular elements of blood. Although low concentrations of adenosine can facilitate chemotaxis in neutrophils (a source of free radicals and capillary plugging), high concentrations of adenosine can also decrease neutrophil adherence and prevent release of superoxide anions (Cronstein *et al.* 1985). Adenosine decreases platelet aggregation and prevents microthrombosis (Olsson & Pearson, 1990). Since the studies in this thesis involve the use of *ex vivo* isolated perfused working rat hearts in the absence of blood or its components, these mechanisms can not play a role in the present studies.

In addition, adenosine-induced cardioprotection in isolated perfused working rat hearts has been shown to occur by improving the metabolic coupling of glycolysis to glucose oxidation (Section 1.4.1). Adenosine and adenosine A_1-receptor agonists improve coupling between the rates of glycolysis and glucose oxidation during the critical early period of reperfusion, and so reduce the rate of proton production from glycolytically derived ATP (Finegan *et al.* 1993). The resulting reduction in Na^+/H^-

exchange prevents Na^+ and Ca^{2+} overload, thereby allowing a more rapid and complete recovery of mechanical and metabolic function during reperfusion (Finegan *et al.* 1996b). Such a mechanism of protection is supported by the observation that ischemia-induced increases in intracellular H^+, Na^+ and Ca^{2+} (measured by nuclear magnetic resonance) are attenuated by adenosine (Steenbergen *et al.* 1993). Ca^{2+} overload has been shown to cause increased contractile activity, resulting in excessive hydrolysis of ATP and eventually myocardial damage (Fleckenstein, 1971). However, since ATP content can be normal even in the presence of excess Ca^{2+} other mechanisms (for example, increased ATP turnover) must be involved in acidosis-induced injury. Other proposed mechanisms of the deleterious effects of Ca^{2+} overload include activation of proteases that disrupt cell function, activation of phospholipases that disrupt cell membranes, development of contracture (sustained excess contraction), mitochondrial oxygen wasting and arrhythmia (reviewed in Opie, 1991).

An alternate mechanism for the cardioprotective actions of adenosine receptor stimulation and ischemic preconditioning (IPC) is the activation of K_{ATP} channels (Kirsch *et al.* 1990; Toombs *et al.* 1993; Van Winkle *et al.* 1994). It has been suggested that K_{ATP} channel activation may be protective due to shortening of the cardiac action potential and a decrease in Ca^{2+} influx (Noma, 1983). However, this mechanism has been disputed and shortened action potential duration may not be involved (Yao & Gross, 1994; Grover *et al.* 1995). Moreover, either a direct activation of K_{ATP} channels (Gross & Auchampach, 1992; Opie, 1993; Grover, 1994), or K_{ATP} channel blockade (Bril *et al.* 1992; Billman *et al.* 1993; Siegl, 1994) is protective to the heart. Others believe that it is not sarcolemmal K_{ATP} but mitochondrial K_{ATP} channels that are responsible for cardioprotection (Garlid *et*

al. 1997; Grover, 1997; Sato *et al.* 1998; Liu *et al.* 1998). In contrast, a recent study suggests that K_{ATP} channel activation is not cardioprotective, and that K_{ATP} channel blockade impairs recovery after ischemia (Ford *et al.* 1998). The authors also demonstrated that A_1-receptor stimulation with N^6-cyclohexyladenosine (CHA) is protective in post-ischemic recovery of mechanical function, but not through activation of K_{ATP} channels (Ford *et al.* 1998).

1.5.3) Glycogen

Glycogen depletion occurs during transient ischemia and may play a role in the cardioprotective effects of IPC. Murray *et al.*, (Murry *et al.* 1986) first described the phenomenon of IPC as the process where brief periods of ischemia rendered the heart resistant to a more prolonged period of ischemia. In 1991, Liu *et al.*, first observed that IPC may be mediated by adenosine released from the heart because the protective effect could be blocked with nonselective adenosine receptor antagonists (Liu *et al.* 1991). IPC has been observed in both animal models and human studies (Meldrum *et al.* 1993; Cleveland, Jr. *et al.* 1997). Adenosine A_1-receptor stimulation mimics IPC, and it has recently been suggested that A_3 receptor activation may also be involved (Liu *et al.* 1994; Wang *et al.* 1997; Tracey *et al.* 1997; Carr *et al.* 1997). Furthermore, adenosine A_1-receptor antagonists (Thornton *et al.* 1993; Walsh *et al.* 1995; Gomoll, 1996) can inhibit IPC. However, since other groups have shown that adenosine A_1-receptor blockade only partially blocks IPC (Tomai *et al.* 1997), and since IPC can occur without adenosine involvement, other mechanisms of cardioprotection must be considered. Both adenosine

and IPC decrease proton production (Finegan *et al.* 1993; Finegan *et al.* 1995; Finegan *et al.* 1996b) and both involve G_i protein pathways (Ravingerova *et al.* 1995; Yabe *et al.* 1995). However, many other agents mimic IPC. Mechanisms of IPC include: activation of protein kinase C (PKC), K_{ATP} channels or α_1 adrenoceptors; reduction of glycogen or intracellular acidosis; or a decrease in energy demand.

Excessive rates of glycogenolysis during ischemia have been considered deleterious to recovery of post-ischemic function due to lactate accumulation (Neely & Grotyohann, 1984), and glycogen depletion before hypothermic ischemic arrest is not beneficial to post-ischemic function (Lagerstrom *et al.* 1988). Conversely, increased glycogen stores prior to ischemia are also believed to be responsible for cardioprotective actions as positive results have been observed with glycogen loading before cardiac surgery, on ischemic tolerance and post-ischemic functional recovery (Hewitt *et al.* 1973; Girard *et al.* 1992). Furthermore, treatments with drugs that are cardioprotective, such as adenosine or adenosine A_1-receptor agonists (CHA) result in a higher level of glycogen relative to untreated hearts after reperfusion of previously ischemic hearts (Finegan *et al.* 1996a; Finegan *et al.* 1996b). Interestingly, glycogen depletion as observed with brief periods of ischemia prior to a prolonged ischemia, (IPC, described above) is believed to be cardioprotective and results in higher myocardial glycogen after reperfusion (Finegan *et al.* 1995; Soares *et al.* 1997). These results clearly suggest that glycogen is associated with cardioprotection; however, it is still unclear exactly what that involvement might be and what role, if any, it plays in adenosine-induced cardioprotection.

Brief periods of ischemia decrease glycogen content; however, whether the amount of glycogenolysis is adequate to protect the heart remains controversial. The

variable levels of glycogen prior to the brief periods of preconditioning ischemia may also alter the outcome from the sustained period of ischemia. IPC results in glycogenolysis, which is associated with cardioprotection. However, as glycogen is allowed to replenish, the cardioprotective effect is diminished (Wolfe *et al.* 1993). Generally, if glycogen content is low (relative to *in vivo* concentrations) prior to ischemia, the outcome is poor, however, if glycogen content was initially close to normal, the outcome is more favorable. This is reinforced by observations that longer periods of IPC prior to a prolonged period of ischemia result in depletion of glycogen beyond "critical" levels, and impairment of recovery of post-ischemic mechanical function (Finegan *et al.* 1996a).

Critical evaluation of the "glycogen literature" is difficult. *Ex vivo* studies of perfused hearts are carried out under various conditions; for example, some studies use perfusates containing only glucose while others use glucose and fatty acids. Studies may also be in the absence or presence of insulin or with various concentrations of Ca^{2+}. Studies performed in the absence of fatty acids result in pre-ischemic glycogen levels that are much lower than *in vivo*, which may be detrimental to the heart. Furthermore, the presence of insulin is also necessary to allow for appropriate glycogen metabolism. The studies presented in this thesis contain both fatty acids and insulin which permit glycogen storage at approximately *in vivo* levels.

1.5.4) Estrogen

1.5.4.1) Types and Functions of Estrogens

At the turn of the century, women rarely lived to menopause (average age 48 years), yet currently, women in Canada live to greater than 80 years (Statistics Canada). This increased life expectancy with concomitant decline in estrogen levels during menopause parallels an increased incidence of cardiovascular disease. In the Western world, coronary heart disease is responsible for killing nearly half of the women over age 60. In this age range women have a greater probability of suffering a silent or unrecognized myocardial infarction than men (Lerner & Kannel, 1986), and this is more often fatal in women than men (Greenland *et al.* 1991). However, prior to menopause, women possess an endogenous cardioprotective factor, which is believed to be estrogen. Indeed estrogen replacement therapy (ERT) has been shown to reduce the risk of cardiovascular disease in postmenopausal females by as much as 50% (Grady *et al.* 1992). Several beneficial mechanisms have been proposed for the protective actions of estrogen (discussed below), however the exact protective mechanism of estrogen remains unclear.

There are two types of estrogens, natural and synthetic. The natural estrogens include: 17β-estradiol (principal ovarian estrogen, most potent), estriol (placental estrogen) and estrone (metabolite of 17β-estradiol). Conjugated equine estrogens (CEEs) are a commonly prescribed estrogen replacement that is extracted from pregnant mare urine. This replacement consists of many components including estrone (40%), 17α-

dihydroequilin, equilin and equilenin (51%) and 17β-estradiol (<4%) (Subbiah, 1998). Synthetic estrogens include ethinyl estradiol and mestranol, which are used in oral contraceptives.

The well-known physiological effects of estrogen are first observed during puberty with the development of secondary sexual characteristics (breasts, reproductive tract, fat, bone mass/closure, lipids) and fluctuations in plasma estrogen concentrations control the ovulatory cycle (Fritsch & Murdoch. 1994). Estrogen also has been implicated in the etiology of estrogen-sensitive breast cancer and uterine cancer. Moreover, estrogen increases Na^+/H_2O retention and decreases bowel motility. The most widely recognized pharmacological use of estrogen is in oral contraceptives. However, other uses include hormone replacement therapy and induction of ovulation. Estrogen replacement therapy has been used successfully for the treatment of menopause, osteoporosis and ovarian failure.

Estrogen has not always been considered favorably as a treatment. When oral contraceptives were first introduced (1960), epidemiological studies indicated a 2 to 4-fold increase in risk of myocardial infarction (reviewed in Thorogood & Vessey, 1990). However, with the newer, low-dose oral contraceptives, the risk of heart disease is not different from the general population (Sidney *et al.* 1996).

Estrogen replacement therapy (ERT) for women with surgically-induced or naturally occurring menopause was first reported in 1967 (Castallo, 1967; McEwen, 1967). Currently, ERT is one of the most commonly prescribed drugs, however it has a relatively low compliance. ERT has proven benefits against osteoporosis (reviewed in Rozenberg *et al.* 1995) and may be beneficial against stroke (Falkeborn *et al.* 1993),

colon cancer (Calle *et al.* 1995) and Alzheimer's disease (reviewed in Beckmann, 1997). However, with the use of unopposed estrogens (estrogens without progestins) there is a clear risk of endometrial cancer and a possible risk of breast cancer. Mortality due to heart disease is 1 in 2, whereas the mortality rate for breast cancer is 1 in 26 (www.americanheart.org/Scientific/Hsstats98/02about.html). Clearly, ERT, by reducing the risk of heart disease, is still beneficial.

The transition through menopause causes many alterations in female physiology, including genitourinary atrophy, osteoporosis and cardiovascular disease. The benefits of ERT are numerous and include a 50-70% reduction in the risk of atherosclerotic cardiovascular disease, improved cholesterol profile, prevention of osteoporosis and alleviation of peri-menopausal symptoms including hot flashes. Because ERT is given in low doses, few side effects such as coagulation disorders and hypertension are observed compared with the higher dose oral contraceptives.

Several studies support the use of hormone replacement therapy, which in contrast to ERT contain both estrogens and progestins. Progestins decrease the risk of uterine and breast cancer, but have been suggested to prevent the cardioprotective benefits of unopposed estrogens (Whitehead, 1988). Other studies dispute this, suggesting that the beneficial cardioprotective effects of estrogen are maintained with the addition of progestins (McCrohon *et al.* 1996; Persson *et al.* 1996). Yet, combination therapy can reduce the risk of heart disease and prevent the increased risk of breast or uterine cancer (which is observed with unopposed estrogens) without losing the cardioprotective actions (Persson *et al.* 1996). This is of critical importance because fear of developing breast cancer is greater than the fear of heart disease even though the odds of dying from either

disease state is clearly in favor of the benefits of estrogen replacement therapy.

Currently, the development of "designer" estrogens (selective estrogen receptor modulators, SERMs) is actively being pursued as a form of pharmacological intervention. These drugs typically have both estrogen agonistic and antagonistic properties. An example is raloxifene (Evista®, Eli Lilly), that was designed for the treatment of osteoporosis. It has the added advantage of reducing the risk of breast cancer (Cummings *et al.* 1998) and it has additional cardioprotective potential because it improves serum lipid profiles (Delmas *et al.* 1997).

In addition to the observational data suggesting beneficial effects of estrogen on the cardiovascular system, other evidence suggests that 17β-estradiol may have a direc. effect on energy substrate metabolism. 17β-Estradiol was observed almost 3 decades ago to alter glycogen metabolism (increase glycogen deposition) in the uterus and other tissues (Demers & Jacobs, 1973; Carrington & Bailey, 1985). Estrogen has also been shown to inhibit glycogen mobilization (Kendrick *et al.* 1987; Rooney *et al.* 1993) and improve exercise performance (Kendrick & Ellis, 1991). There are other indications that glucose metabolism may be altered with 17β-estradiol treatment. 17β-Estradiol treatment stimulates several gluconeogenic enzymes in liver (pyruvate carboxylase, phosphoenolpyruvate carboxykinase and lactate dehydrogenase) (Dahm, Jr. *et al.* 1978), increases glucose uptake in the uterus (Smith & Gorski, 1968), improves glucose tolerance (Carrington & Bailey, 1985) and stimulates glycolytic enzyme activity (hexokinase, phosphofructokinase and pyruvate kinase) in the brain (Kostanyan & Nazaryan, 1992).

The potential that estrogen may be cardioprotective due to alterations of glycogen

and/or glucose metabolism prompted the later investigations described in this thesis.

1.5.4.2) Estrogen Receptor-Mediated Mechanisms

When estrogen enters the cell, it binds to specific estrogen receptors that bind to estrogen response elements (ERE) on DNA (primarily the 5' flanking region of estrogen-responsive genes), stimulating transcription and therefore, protein synthesis (Fig. 1-5) (Scholz *et al.* 1998). Further, the estrogen-receptor complex can bind to additional, non ERE sites. One such region is the cAMP response element, which suggests that estrogen may be involved in protein kinase associated signal transduction traditionally implicated with plasma membrane signaling cascades (Zhou *et al.* 1996).

Two estrogen receptors (ER) have been identified so far. These are the classical ERα and the newly defined ERβ subtypes. In each of these groups there are splice variants with distinct pharmacological properties (Petersen *et al.* 1998). These receptors belong to the superfamily of nuclear receptors that function as ligand-activated transcription factors. When estrogen is present, the majority of receptors are located in the nucleus; however, when estrogen is absent, estrogen receptors are primarily located in the cytoplasm (Lin & Shain, 1985). Cytoplasmic receptors readily translocate to the nucleus when estrogen is reintroduced (Lin & Shain, 1985). The differential localization of ERα and ERβ receptors in the body is currently being investigated, but there appears to be an organ-specific distribution. Some tissues contain both ERα and ERβ receptors whereas others have either primarily ERα or ERβ (Lin *et al.* 1986; Kuiper *et al.* 1998). The heart possesses both ERα (Grohe *et al.* 1997) and ERβ receptors (Grohe *et al.* 1998;

Register & Adams, 1998; Foegh & Ramwell, 1998) and their biosynthesis is upregulated by an increase in estrogen concentration (Grohe *et al.* 1998). 17β-Estradiol binds to both the ERα and the ERβ receptor with equal affinity (Pace *et al.* 1997).

Typically, ER stimulation results in beneficial effects on bone, the cardiovascular system and brain, whereas it results in adverse effects in breast and uterine tissues. The advent of designer estrogens (SERMs) is exciting in that they may be able to provide the beneficial effects on the cardiovascular system and bone, without the negative side-effects of unopposed estrogen therapy on breast and uterus. Furthermore, since males also possess functional ERs in cardiac myocytes (Grohe *et al.* 1997), SERMs may benefit men as well as women by reducing cardiovascular disease. Unfortunately, little data have been gathered to date to support the notion that SERMs prevent heart disease.

1.5.4.3) Nitric Oxide-Mediated Mechanisms of Estrogen

Another proposed mechanism for the direct effect of estrogen is stimulation of nitric oxide synthase (NOS) (Hayashi *et al.* 1995; Darkow *et al.* 1997; Binko & Majewski, 1998). NOS is the rate-limiting enzyme that converts L-arginine and O_2 to L-citrulline and NO. NO has many physiological functions, many of which result from stimulation of soluble guanylyl cyclase and cGMP production.

There are three isoforms of NOS: eNOS, iNOS, and nNOS, (also called NOS3, NOS2 and NOS1, respectively). All 3 isoforms of NOS are present in the heart (Moncada *et al.* 1991). The eNOS and nNOS isoforms are Ca^{2+}-dependent enzymes, whereas iNOS is Ca^{2+}-independent. Their genes exist on different chromosomes and their

protein products share > 50% amino acid sequence homology. Both eNOS and nNOS are constitutive but regulated, whereas iNOS expression is induced by a number of stimuli. Both eNOS and iNOS expression can be stimulated by estrogen (Siminiak *et al.* 1995; Kauser & Rubanyi, 1997; Kleinert *et al.* 1998). Further, the regulation of NOS may be tissue specific as 17β-estradiol down-regulates iNOS expression in macrophages (Hayashi *et al.* 1997).

NO has both beneficial and detrimental effects on cardiac function. Low concentrations of NO, generated from either eNOS or iNOS have several beneficial effects, whereas high concentrations of NO generated from iNOS are primarily associated with deleterious effects. Beneficial effects in the heart include increased blood flow and improved relaxation (Moncada *et al.* 1991). The negative effects of NO occur as a result of an interaction with superoxide anion that produces peroxynitrite (Schulz & Wambolt, 1995; Schulz *et al.* 1997). Peroxynitrite production increases as NOS activity is stimulated and free radical scavengers such as superoxide dismutase do not inactivate the resulting NO. However, since iNOS activity can increase NO production to a much greater extent and is not dependent on Ca^{2+}, induction of this isoform is typically believed to be responsible for peroxynitrite production and cell death (apoptosis).

The sensitivity of vascular smooth muscle to vasodilators and vasoconstrictors is altered by estrogen. Several studies cite an upregulation of eNOS as the mechanism of estrogen-induced vasodilation (Node *et al.* 1997; Rubanyi *et al.* 1997), as well as for the reduction of atherosclerotic plaque progression (reviewed in Nathan & Chaudhuri, 1997) and for protection from osteoporosis (Armour & Ralston, 1998). eNOS-induced generation of NO is involved in blood pressure regulation and may inhibit platelet

aggregation and adhesion, and reduce vascular smooth muscle proliferation (reviewed in Moncada & Higgs, 1995). A decreased endothelial NO production is associated with atherosclerosis, diabetes and hypertension (reviewed in Moncada & Higgs, 1993). Many studies suggest increased eNOS activity as a mechanism of decreased atherosclerosis and improved vascular tone, but there is little evidence to support an alteration in iNOS activity. Interestingly, the eNOS (Weiner *et al.* 1994) and iNOS (Grohe *et al.* 1998) genes were identified as estrogenic downstream targets in the myocardium providing evidence that NOS gene regulation may be responsive to estrogen receptor activation.

1.6) Hypothesis and Objectives

The information presented in this introduction clearly shows that myocardial energy substrate metabolism plays an important role in determining the recovery of post-ischemic mechanical function. Although considerable research on exogenous and endogenous energy substrate utilization, both during and following ischemia, has been conducted, the role of glycogen turnover in cardioprotection remains controversial. Whereas virtually all cardioprotective interventions improve post-ischemic glycogen levels, ischemic preconditioning depletes glycogen stores prior to ischemia and yet is cardioprotective. Furthermore, there remains considerable commercial interest in the discovery of new drugs that alter energy substrate metabolism and are cardioprotective. Elucidation of the role of glycogen turnover in cardioprotection may reveal new targets for pharmacological exploitation. Therefore, the overall hypothesis for the work performed in this thesis was as follows: **Myocardial glycogen metabolism plays an**

important role in energy substrate metabolism and alterations in glycogen turnover contribute to drug-induced cardioprotection. In order to investigate this hypothesis, experiments, which were performed in isolated perfused working rat hearts, had the following objectives.

1. To develop methodologies to measure simultaneously glucose metabolism and glycogen turnover that account for the simultaneous synthesis and degradation of glycogen.
2. To study the time-dependent changes in glycogen and glucose metabolism during low-flow ischemia.
3. To assess the cardioprotective potential of drug-induced alterations in glycogen and glucose metabolism. The effects of 3 distinct classes of drugs (adenosine A_1-receptor agonists, N^6-cyclohexyladenosine; α-glucosidase inhibitors, deoxynojirimycin and N-methyldeoxynojirimycin; and estrogen receptor agonists, 17β-estradiol) were investigated.

Based on the data with 17β-estradiol on the coupling of glycolysis to glucose oxidation discussed later in this thesis (Chapter 6), alternative mechanisms must participate in 17β-estradiol-induced cardioprotection. Therefore, we hypothesized that: **Chronic 17β-estradiol-induced cardioprotection is due to enhanced NO biosignaling.** To investigate this hypothesis, the following objective was tested.

1. To measure indices of NO biosignaling including eNOS and iNOS activity and cGMP content in heart tissue from reperfused 17β-estradiol-treated rats following ischemia.

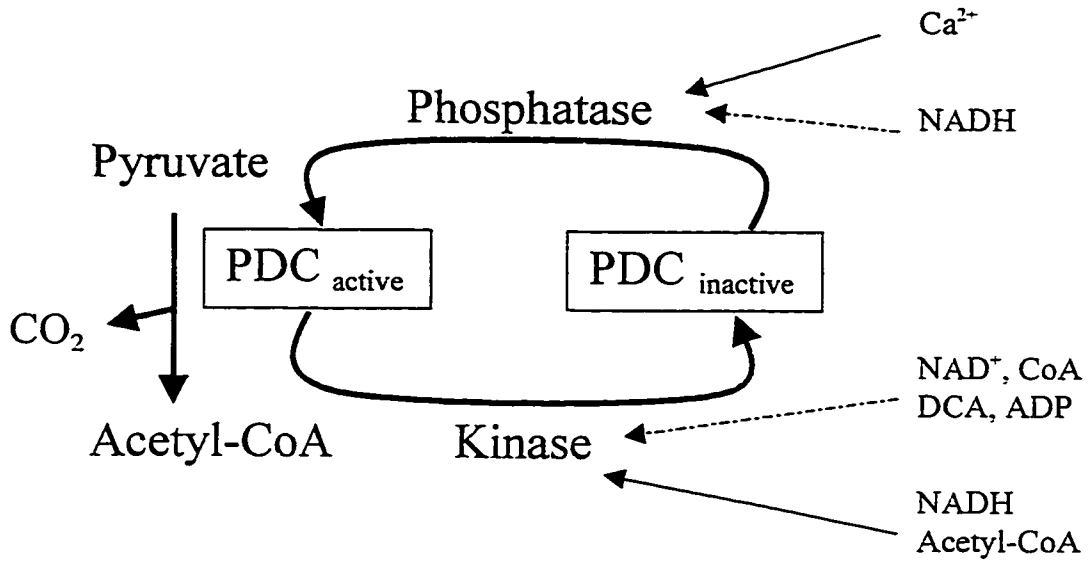


Fig. 1-1. *Regulation of pyruvate dehydrogenase complex (PDC) activity.* Dashed lines represent inhibition of activity.

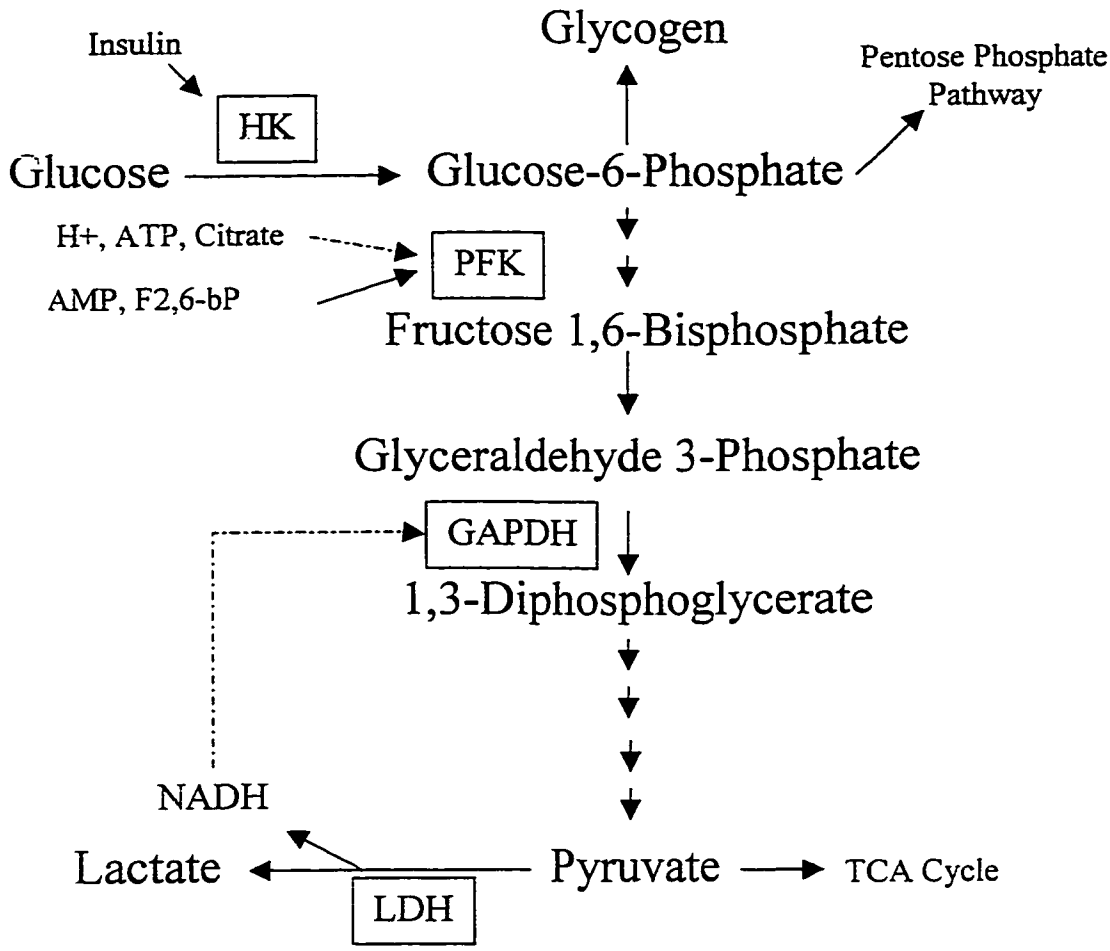


Fig. 1-2. *Points of glycolytic pathway regulation.* Three major enzymes are involved, HK (hexokinase), PFK (phosphofructokinase) and GAPDH (glyceraldehyde3-phosphate dehydrogenase. LDH is lactate dehydrogenase. Dashed lines represent inhibition of activity.

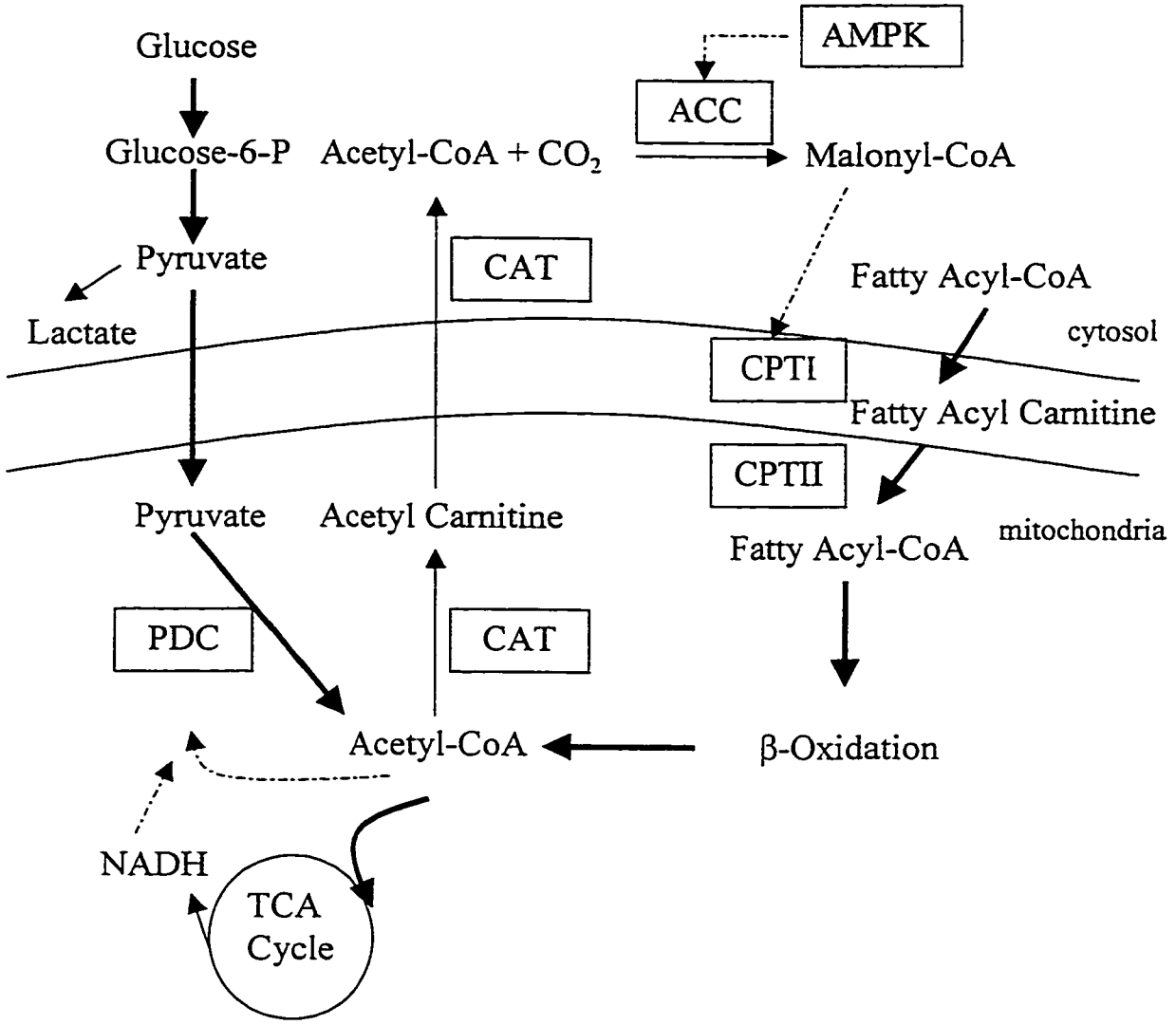


Fig. 1-3. *Coordinated regulation of glucose and fatty acid oxidation.* Boxed letters represent enzymes (PDC, pyruvate dehydrogenase; CAT, carnitine acetyl transferase; AMPK, 5'AMP-activated protein kinase; CPTI, carnitine palmitoyl transferase I; CPTII, carnitine palmitoyl transferase II, ACC, acetyl-CoA carboxylase). Dashed lines represent inhibition of activity. Adapted from Stanley *et al.*, 1997.

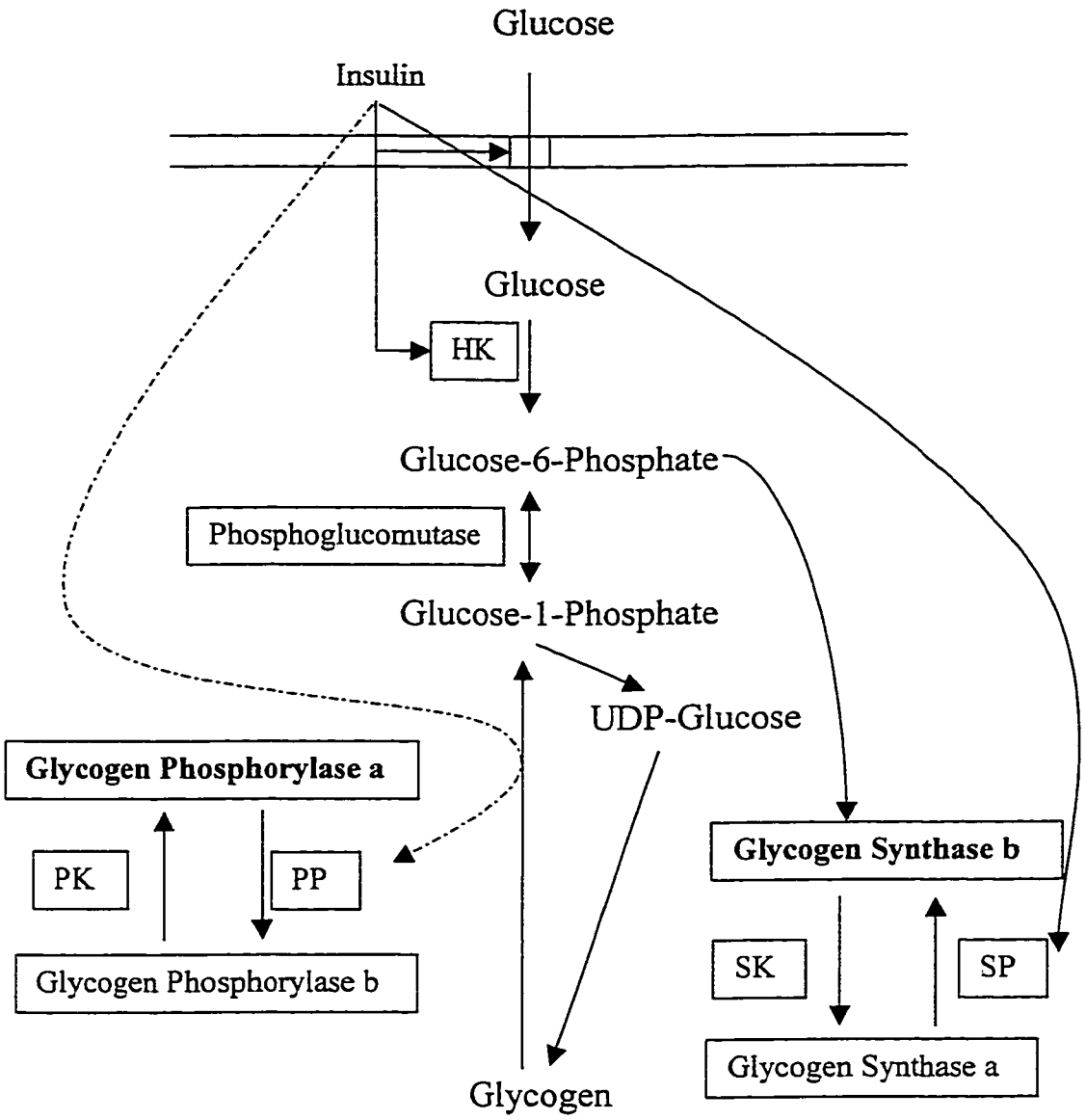


Fig. 1-4. *Glycogen synthesis and degradation (Glycogen turnover).* Enzymes are in boxes. Dashed lines indicate points of enzyme inhibition. Enzymes in bold are those that drive the reaction. Phosphorylation of glycogen phosphorylase activates the enzyme, whereas phosphorylation of glycogen synthase inactivates the enzyme. This reciprocal regulation results in glycogenolysis. Symbols: HK, hexokinase; PK, phosphorylase kinase; PP, phosphorylase phosphatase; SK, synthase kinase; SP, synthase phosphatase.

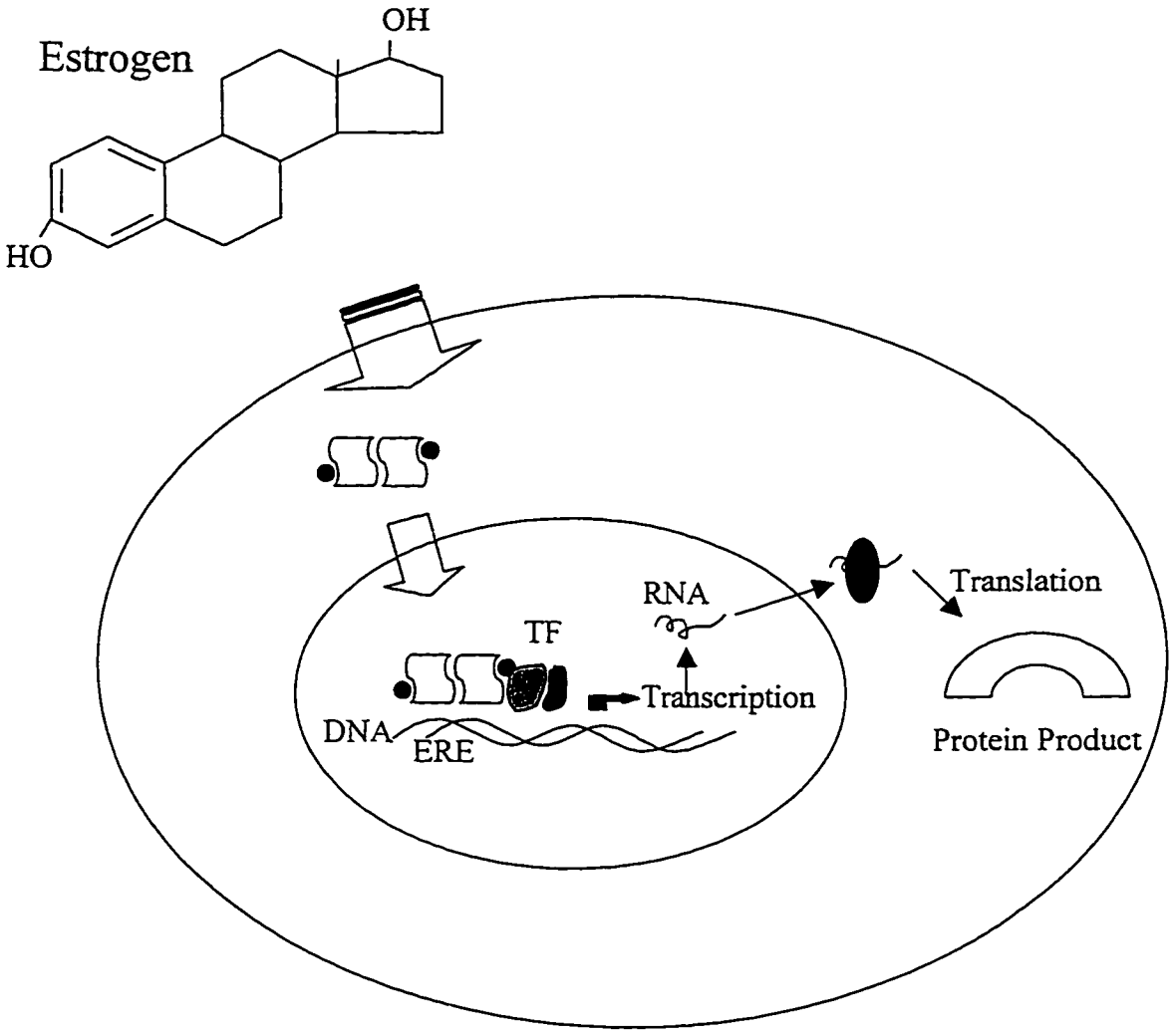


Fig. 1-5. *Diagrammatic representation of estrogen receptor action.* Estrogen enters the cell and binds to a specific receptor. Estrogen receptor dimers bind to estrogen response elements on DNA, which results in transcription and ultimately protein synthesis. TF means transcription factors, ERE means estrogen response element and E-ER Dimers means estrogen-estrogen receptor dimers. Adapted from Kuiper *et al.*, 1998.

CHAPTER 2

Methods

2.1) Animal Models

All rats were housed and treated according to the standards set by the Canadian Council of Animal Care. All procedures received prior approval from the Health Sciences Animal Welfare Committee of the University of Alberta. Rats were fed and watered ad lib and housed two per cage at room temperature (21°C) under a 12-hr light-dark cycle. Post-surgical female rats were housed individually. Female rats were housed separately from male rats.

2.1.1) Studies Using Male Rats

Male Sprague Dawley rats (275-350 g) were obtained from local sources via Health Sciences Lab Animal Sciences (HSLAS) and after 2-7 days of acclimation, killed by anesthetic overdose and without any further intervention except for heart removal for *in vitro* perfusion studies.

2.1.2) Studies Using Female Rats

Female Sprague Dawley rats (275-300 g) were also obtained from local sources. One week after arrival, rats were anesthetized with 50 mg/kg Brietal® and surgical

removal of the ovaries was completed using aseptic technique. Briefly, two incisions were made on either side of the spinal column half way between neck and tail. Blunt dissection through the muscle layer revealed a fat pad containing the ovary. The ovaries were then surgically removed from the animal. The remaining fat pad was reintroduced into the abdomen and the muscle layer closed with sutures. A 17β-estradiol or placebo pellet was placed subcutaneously near one incision site. Finally, the skin was stapled shut, and the area cleaned. The rats were allowed to recover from surgery for two weeks, at which time they were killed and hearts removed for *in vitro* perfusion. In addition, at the time of death, blood was removed for the later determination of serum 17β-estradiol concentrations, and the uterus was removed and weighed to confirm 17β-estradiol therapy.

17β-Estradiol and placebo pellets were obtained from Innovative Research of America, Sarasota, FL. 17β-Estradiol pellets contained 0.25 mg in a 3-week-release formulation. Pellets were left in place for two weeks.

2.2) Isolated Perfused Working Rat Heart Preparation

2.2.1) Perfusate

Langendorff perfusion was conducted using a Krebs-Henseleit solution containing: (mM) NaCl (118.0), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25.0) and glucose (11) (Finegan *et al.* 1996a). The working heart perfusate consisted of a Krebs-Henseleit solution (as above) with the addition of palmitate (1.2

mM) pre-bound to 3% bovine serum albumin (BSA) and insulin (100 μU/ml). Palmitate was initially dissolved in an ethanol:water mixture (40:60%) containing 0.5-0.6 g Na_2CO_3 per g of palmitate. Following heating to evaporate the ethanol, this mixture was then added to the 3% BSA-Krebs-Henseleit mixture (without glucose) and allowed to dialyze (8000 MW cut-off) overnight in 10 vol of glucose-free Krebs-Henseleit solution. The next day, glucose was added to the solution and the mixture was filtered through glass microfiber filters (GF/C, Whatman, Maidstone, England) and kept on ice, or refrigerated, prior to use. The perfusate was continuously oxygenated with carbogen (95% CO_2, 5% O_2) while in the perfusion apparatus.

2.2.2) Heart Perfusion Protocols

Rats were anesthetized with pentobarbital (240 mg/rat, intraperitoneally) and hearts were rapidly removed and placed in ice-cold Krebs-Henseleit solution. The hearts were then rapidly cannulated via the aortic stump and Langendorff perfusion at constant pressure (60 mm Hg) was initiated and continued for a 10-min equilibration period. During this equilibration period, the pulmonary artery was cut, and excess fat and lung tissue removed to reveal the pulmonary vein. The left atrium was cannulated and connected to the preload line originating from the oxygenation chamber. After the 10-min equilibration period, hearts were switched to working mode (by clamping off the Langendorff line and opening the preload and afterload lines) and perfused at 37°C under aerobic conditions at a constant left atrial preload (11.5 mm Hg) and aortic afterload (80 mm Hg). The compliance chamber was filled with air adequate to maintain developed

pressure at 50-60 mm Hg. Aortic compliance was calculated during perfusion as (aortic systolic pressure – preload pressure) / stroke volume (CO x HR). During aerobic perfusion, aortic compliance was approximately 500 mm Hg/ml; however, during reperfusion, compliance varied depending on the level of functional recovery. A diagrammatic representation of the perfusion apparatus is presented in Fig. 2-1. Perfusate was delivered to the oxygenation chamber via a peristaltic pump from the reservoir chamber that collected aortic and coronary flows as well as overflow from the oxygenator.

Typically, hearts were perfused under aerobic conditions for 60 min and then subjected to low-flow ischemia (0.5 ml/min) for 60 min followed by 30 min of reperfusion. Hearts were paced at 300 beats/min throughout each phase of the perfusion protocol (voltage adjusted as necessary) with the exception of the initial 5 min of reperfusion when hearts were allowed to beat spontaneously. Additional groups of hearts were perfused either for only the 10-min Langendorff perfusion phase, until the end of the aerobic phase of perfusion or until the end of the low-flow ischemia phase. Details of perfusion groups are indicated where necessary for each study. Changes to this standard protocol are noted in individual chapters.

Low-flow ischemia was conducted by connecting an additional line from the preload chamber to the aortic cannula. A peristaltic pump was used to control the flow of perfusate, via the compliance chamber, into the aortic cannula and through the coronary circulation at a rate of 0.5 ml/min. There was minimal cooling of the perfusate during transit through this low-flow circuit (temperature ~ 34-35°C). The low-flow ischemia tubing was primed with warm oxygenated perfusate prior to initiation of low-flow

ischemia. Drugs, when required by the experimental protocol, were added 5 min prior to the initiation of low-flow ischemia and circulated throughout the system in order to allow adequate mixing in the perfusate and the tubing of the low-flow circuit.

At the end of each of the phases of the perfusion protocol, hearts were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. Frozen tissues were pulverized and the resulting powders stored at -80°C.

2.2.3) Myocardial Mechanical Function

Aortic systolic and diastolic pressures were measured using a Gould P21 pressure transducer connected to the aortic outflow line. Cardiac output, aortic flow and coronary flow (cardiac output minus aortic flow) were measured (ml/min) using in-line ultrasonic flow probes connected to a Transonic T206 ultrasonic flow meter. Left ventricular minute work (LV work), calculated as cardiac output x left ventricular developed pressure (aortic systolic pressure - preload pressure), was used as a continuous index of mechanical function. Hearts were excluded if LV work decreased more than 20% during the 60-min period of aerobic perfusion.

2.3) Measurement of Glucose Metabolism

Determining the rate of production of $^{3}H_2O$ and $^{14}CO_2$ from [^{3}H/^{14}C]glucose in the isolated working rat model allows a direct and continuous measure of the rates of glycolysis and glucose oxidation. A 3-ml sample of perfusate was taken from the

injection port of the recirculating perfusion apparatus at various time-points throughout the perfusion protocol (10, 20, 40, 60, 65, 70, 80, 100, 120, 130, 140 and 150 min) for analysis of $^{3}H_2O$ and $^{14}CO_2$ and immediately placed under mineral oil until assayed for metabolic product accumulation. Perfusate was supplemented with [^{3}H/^{14}C]glucose to approximate a specific activity of 20,000 dpm/mol glucose. Average rates of glycolysis and glucose oxidation were calculated from linear cumulative time-courses of product accumulation between 10 and 60 min for aerobic perfusion, between 70 and 120 min for low-flow ischemia, and from 140 to 150 min for reperfusion. Rates of glycolysis and glucose oxidation are expressed as μmol glucose metabolized/min/g dry wt.

2.3.1) Measurement of Myocardial Glycolysis

Rates of glycolysis were measured directly as previously described (Saddik & Lopaschuk, 1991) from the quantitative determination of $^{3}H_2O$ liberated from radiolabeled [5-^{3}H]glucose at the enolase step of glycolysis (Fig. 2-2). Perfusate samples were collected at various time-points throughout the perfusion protocol (see above). $^{3}H_2O$ was separated from the perfusate by passing perfusate samples through columns containing Dowex 1-X 4 anion exchange resin (200-400 mesh). A 90 g/L Dowex in 0.4 M potassium tetraborate mixture was stirred overnight after which 2 ml of the suspension was loaded into separation columns and washed extensively with dH_2O to remove the tetraborate. The columns were found to exclude 98-99.6% of the total [^{3}H]glucose (Saddik & Lopaschuk, 1991). Perfusate samples (100 μl) were loaded onto the columns and washed with 1.0 ml dH_2O. Effluent was collected into 5 ml of Ecolite Scintillation

Fluid (ICN, Radiochemicals, Irvine, CA) and counted for 5 min in a Beckman LS 6500 Scintillation Counter with an automatic dual ($^3H/^{14}C$) quench correction program. Average rates of glycolysis for each phase of perfusion are expressed as μmol glucose metabolized/min/g dry wt as described above.

2.3.2) Measurement of Myocardial Glucose Oxidation

Glucose oxidation was also determined directly as previously described (Saddik & Lopaschuk, 1991) by measuring $^{14}CO_2$ from [^{14}C]glucose liberated at the level of pyruvate dehydrogenase and in the Krebs cycle. Both $^{14}CO_2$ gas exiting the oxygenation chamber and [^{14}C]bicarbonate retained in solution were measured (Fig. 2-2). Perfusate samples were collected at various time-points throughout the perfusion protocol (see above). $^{14}CO_2$ gas was collected by passing the gas exiting the oxygenator through a hyamine hydroxide trap (20-50 ml depending on perfusion duration). Perfusate samples (2 x 1 ml), which were stored under oil to prevent the escape of gas by equilibration with atmospheric CO_2, were injected into 16 x 150 mm test tubes containing 1 ml of 9 N H_2SO_4. This process releases $^{14}CO_2$ from the perfusate present as $H^{14}CO_3^-$. These duplicate tubes were sealed with a rubber stopper attached to a 7-ml scintillation vial containing a 2 x 5 cm piece of filter paper saturated with 300 μl of hyamine hydroxide. Tubes were allowed to equilibrate overnight. The scintillation vials with filter papers were then removed and Ecolite Scintillation Fluid (7 ml) added. Samples were counted by standard procedures as described above. Average rates of glucose oxidation for each phase of perfusion are expressed as μmol glucose metabolized/min/g dry wt.

2.3.3) Calculation of Proton Production

Proton production attributable to the hydrolysis of ATP arising from glucose metabolism was calculated as 2 x (rate of glycolysis – rate of glucose oxidation). This accounts for the net production of two protons per molecule of glucose that passes through glycolysis that is not subsequently oxidized (Lopaschuk *et al.* 1993). The rate of proton production arising from glycogen turnover is equivalent to 2 x (rate of glycolysis – rate of glucose oxidation) + (1 x G_{in}). G_{in} is defined as the rate of glycogen synthesis (Chapter 4). The release of one glucosyl unit from glycogen consumes one of the 3 protons produced from ATP hydrolysis, and simultaneous synthesis produces one proton from the addition of each UDP-glucose to glycogen.

2.4) Myocardial Metabolite Measurements

2.4.1) Dry to Wet Ratios

Frozen ventricles were pulverized at the temperature of liquid nitrogen with a mortar and pestle. Dry to wet determinations were made by weighing a small amount of frozen heart tissue and re-weighing that same tissue after 24-48 hr of air drying and taking the ratio of the two weights. From this ratio, total dry tissue could be calculated. This ratio was used to normalize, on a per g dry weight basis, rates of glycolysis, glucose oxidation and glycogen turnover as well as metabolite contents.

2.4.2) Measurement of High Energy Phosphate and their Metabolites[1]

High performance liquid chromatography analysis (HPLC) of neutralized perchloric acid (PCA) extracts of frozen heart tissue was used to measure concentrations of ATP, ADP, AMP, adenosine, inosine, IMP, xanthine, hypoxanthine, Cr, and CrP. Pulverized heart tissue (100 mg) was homogenized with an ice-cold mortar and pestle in 1 ml of 6% PCA and 0.5 mM EGTA. These homogenates were centrifuged (10 min at 1500 x g) and 95 µl of dithiothreitol (0.32 M) was added to the resultant supernatant. The supernatant was neutralized with 5 M K_2CO_3 and centrifuged (10 min at 1500 x g). The supernatant was then analyzed for high energy phosphates and their metabolites according to the HPLC method of Ally and Park (1992).

2.5) Measurement of Glycogen Content

2.5.1) Glycogen Content

Myocardial glycogen content (µmol glucosyl units/g dry wt) was determined following the method of Bergmeyer and Grassl (1983) by measuring the glucose content in samples (150-200 mg) of frozen crushed tissue that were subjected to alkaline extraction (0.3 ml, 30% KOH, 100°C, 60 min) to separate glycogen from exogenous

[1] HPLC analyses were performed by Mr. K. Strynadka.

glucose. This was followed by precipitation of glycogen with acid (0.2 ml, 2% Na_2SO_4) and ethanol (2 ml, absolute ethanol) overnight at –20°C. After centrifugation (5 min, 1500 x g), the precipitate was washed with 2 ml of 66% ethanol and boiled for 3 hr in acid (1 ml, 2N H_2SO_4) to release endogenous glucose (monoglycerides) from glycogen. Thereby, the radiolabeled content of the glycogen pool could be determined accurately without any contamination from free unincorporated glucose. Samples were neutralized and glucose content was measured using a Sigma spectrophotometric kit at a wavelength of 475 nm against a standard curve of 0-20 μg glucose.

2.5.2) Glycogen Labeling

Glycogen extract samples were also analyzed for [^{3}H]glucose and [^{14}C]glucose so that the specific activity of [^{3}H/^{14}C]glycogen and the percentage of glycogen that became labeled with either [^{3}H]glucose or [^{14}C]glucose could be determined in hearts frozen at the end of each phase of the perfusion protocol. This was achieved by counting a 100 μl sample of the perfusate and a 100 μl sample of the glycogen extract in Ecolite following standard counting procedures as described above.

2.6) Glucose Uptake and Extraction

Glucose uptake in aerobic and ischemic hearts was calculated from the sum of the actual rates of glycogen synthesis (G_{in}, see Chapter 4) and glycolysis from exogenous glucose. Glucose extraction (%) under these conditions was calculated from rates of

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

2.7) Myocardial Enzyme Levels

The activities of various enzymes were determined in tissue samples frozen at the end of each phase of the perfusion protocols.

2.7.1) Glycogen Phosphorylase

Glycogen phosphorylase activity, expressed as phosphorylase *a* as a percent of total, was determined as described previously (Dobson & Fenton, 1993) by measuring the formation of glucose-6-phosphate in the presence of excess glycogen and in the absence (active) and presence (total) of AMP. Frozen powdered cardiac tissue (20 mg) was homogenized at 0°C (20 mM KF, 4 mM EDTA, 20 mM β-glycerophosphate and 20 mM β-mercaptoethanol) and mixed with activated charcoal (1 mg/ml) to bind AMP. After mixing and incubating the samples on ice for 15 min, the samples were centrifuged (3000 x g, 20 min, 4°C) and the supernatant was then allowed to react in the absence (active) or presence (total) of 3 mM AMP (30°C, 60 min) to allow the formation of glucose-6-phosphate by the extracted enzyme. The reaction was stopped by adding 60 µl of 0.5 N HCl. The presence of glucose-6-phosphate was then assayed at a wavelength of 340 nm on a spectrophotometer using a standard curve of 0-150 µM glucose-6-phosphate.

2.7.2) Glycogen Synthase

Glycogen synthase activity, expressed as percent active, was determined as previously described (Passonneau & Rottenberg, 1973), by measuring the consumption of UDP-glucose in the absence and presence of glucose-6-phosphate. Tissue (50 mg) was homogenized (50 mM Tris HCl, 5 mM EDTA, 25 mM KF and 5 mM dithiothrietol) and 50 µl samples of this mixture were allowed to react in the absence (active) and presence (total) of 5 mM glucose-6-phosphate (37°C, 60 min). This allowed for the formation of uridine-diphosphate (UDP) by the synthase enzyme. The reaction was stopped by boiling for 2 min. The amount of UDP formed in the mixture was assayed in the resulting supernatants after centrifugation (3000 x g, 5 min, 4°C). Absorbances (340 nm wavelength) were determined on a spectrophotometer 20 min after reagent was added. A mixture of lactate dehydrogenase (1 mg/ml), pyruvate kinase (0.5 mg/ml) and water was then added to the supernatants and read at the same wavelength 20 min later against a standard curve (0-25 nmol) of UDP that was also reacted in the presence and absence of the enzyme mixture.

2.7.3) Pyruvate Dehydrogenase Activity

Total PDH activity (PDH_t) and that in the active dephosphorylated form (PDH_a) (µmol/min/g dry wt) were determined using a radioisotopic coupled enzyme assay (Constantin-Teodosiu *et al.* 1991) as modified by Collins-Nakai et al (Collins-Nakai *et al.*

1994). PDH activity was determined as a function of acetyl CoA production, where acetyl CoA formed from pyruvate via the PDH reaction is condensed with excess [^{14}C]oxaloacetate in the presence of citrate synthase to form [^{14}C]citrate, which is separated from the reaction mixture by ion-exchange (Dowex 50W-X8, 100-200 mesh) chromatography[2].

2.8) Nitric Oxide Synthase (NOS)

2.8.1) NOS Activity (Citrulline Assay)[2]

Frozen heart tissue (150 mg) was homogenized (10 mM Hepes buffer containing sucrose (320 mM), DTT (1 mM), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml) and aprotinin (2 μg/ml), pH 7.4) using an ultrasonic processor and centrifuged at 4°C, 10,000 g for 20 min. Supernatant (40μl) was incubated for 20 min at 37°C with assay buffer (KH_2PO_4 (40 mM) containing $MgCl_2$ (1 mM), $CaCl_2$ (0.2 mM), valine (45.7 mM), L-citrulline (1 mM), L-arginine (20 μM), dithiothrietol (1 mM), NADPH (0.1 mM), BH_4 (0.01 mM), and L-[U-^{14}C]arginine (0.07 μCi), pH 7.4, final concentrations per assay). Three samples were prepared, one with assay buffer alone, the other in the presence of L-NMMA (1mM) and the third in the presence of EGTA (1 mM, pH 7.2). In this way, Ca^{2+}-dependent NOS and Ca^{2+}-independent NOS activities were determined. This step allowed the enzymatic conversion of L-[U-^{14}C]arginine to [^{14}C]citrulline and NO. Separation of [^{14}C]citrulline from [^{14}C]arginine was achieved by incubating the

[2] This assay was performed by Mrs. Barbara Zielnick-Drabik under my supervision.

samples in an ion exchange resin for 45 min followed by determining the radioactivity counts in 500 µl of solution (no resin) using standard scintillation counting procedures described above. NOS activity was expressed as pmol/min/mg protein. Protein concentrations were determined using the Bradford assay against a standard curve of bovine serum albumin (described in Section 2.8.4).

2.8.2) cGMP Content

Tissue preparation involved homogenizing ~200 mg frozen pulverized heart tissue in 500 µl of 2.2% PCA in 0.1 M Hepes buffer (pH 7.4) and 25 µl of 0.1 M Na_2EDTA (final EDTA concentration of 5 mM). After 15 min, the samples were centrifuged at 10,000 x g at 4°C for 2 min. A 250 µl aliquot of supernatant was removed and neutralized with 1.1 M K_3PO_4 (40 µl), after which samples were centrifuged at 10,000 x g at 4°C for 2 min. Two 50 µl samples were used for the cGMP assay.

From the pellet, a protein assay was performed. The pellet from the PCA extraction was solubilized in 0.5 ml of 2 N NaOH with 10 min of boiling. Tissue pellets were subsequently neutralized with 0.5 ml of 2 N HCl (pH= 6.8-7.0) and assayed with BCA reagent (bichinchonic acid with copper (II) sulphate 50:1) for protein content against a standard curve (5 standards) 0-4 mg/ml bovine serum albumin in water at 560 nm.

Myocardial cGMP content was determined using an enzyme immunoassay kit (Cayman Chemical). The immunoassay detects free based on competition with tracer cGMP for cGMP antisera. After washing the microplate (96 well), the wells were

developed with Ellman's Reagent which contains the substrate to acetylcholinesterase and the absorbance measured at 412 nm, against a standard curve (8 standards) of cGMP (0.023 – 3 nM). Data were analyzed by plotting the known concentrations of standards vs absorbance using a curve-fitting program to fit a sigmoid curve (appropriate for competition plots). From this curve, cGMP contents in samples were calculated. The cGMP content was normalized per mg protein (as with NOS activity) and expressed as pmol/mg protein.

2.8.3) Western Blotting for NOS Protein Expression

Frozen, pulverized heart tissue (200-300 mg) was homogenized in buffer (10 mM Hepes, 0.32 M sucrose, 0.1 mM EDTA, 1 mM DTT, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin hemisulfate salt, 2.1 mg/ml aprotinin and 100μg/ml PMSF) (1:4 w/v) for 3 x 5 sec pulses using a polytron apparatus at high speed. Homogenates were centrifuged 100,000 x g at 4°C in an ultracentrifuge for 35 min. Protein content of the supernatant was determined using the Bradford protein assay (as with iNOS activity) and diluted to load 90 μg of protein per lane.

Discontinuous SDS (sodium dodecylsulfate) polyacrylamide (8% running and 3.5% stacking) gels (8.3 cm x 7.4 cm x 0.75 mm) were prepared and used for electrophoresis of protein. Equal volumes of sample and 2x sample buffer (50 μl each) were boiled immediately for 3-5 min. Markers (5 μl) and standards (5 μl + 5 μl of 2x sample buffer) were also boiled for 3-5 min to unfold the proteins. Samples were loaded into wells and run at 120 V constant for ~105 min, or until blue dye crossed the black

zone of the running frame. Protein was transferred to membrane (nylon) using an electroblot system for 2.5 hr at 15 V at room temperature. Once transfer was complete, the remaining gel was stained with 0.1% Comassie Brilliant Blue dye (5 min) and the membrane was blocked in 7% milk solution in Tris-buffered saline (TBS: Tris base, 20 mM; NaCl, 137 mM; HCl, 1M, pH 7.5) overnight with constant rocking. The next day, the membrane was washed with 0.1% tween-TBS and the primary antibody (mouse monoclonal (1:1000 dilution in TBS-tween) was incubated with the membrane for 2 hr at room temperature with constant shaking. After another series of 3 washes, the membrane was incubated with the second antibody (horseradish peroxidase labeled anti-mouse 1:2000 dilution in TBS-tween) at room temperature for 1 hr with constant shaking, then washed again. Antibody detection was done using enhanced chemiluminescence (ECL, Amersham LIFE Sciences) following the instructions in the kit. Briefly, the membrane was incubated with ECL for 15 sec at room temperature and then exposed to X-ray film for 15 sec to 160 min depending on development. The molecular weight of eNOS was 155 kDa and iNOS was 130 kDa. The positive control (standard) for eNOS was human endothelial cell lysates and for iNOS was lysates from mouse macrophage.

2.8.4) Bradford Protein Assay

The Bradford protein assay was utilized to determine protein concentrations for cGMP content and NOS content and activity. Briefly, 10 µl of protein isolate was diluted with 100 µl HEPES (40 mM) and 1.5 N NaOH. Diluted protein isolate (20 µl) was assayed in duplicate with Comassie Brilliant Blue reagent (filtered and stored at 4°C)

against a standard curve of BSA (0-8 μg) at 595 nm 2-30 min after mixing.

2.9) Drugs and Reagents

D-[5-^{3}H]glucose and D-[U-^{14}C]glucose were purchased from Du Pont-New England Nuclear (Boston, MA). Bovine serum albumin (fraction V) was obtained from Boehringer Mannheim (Indianapolis, IN). Hyamine hydroxide (methylbenzethonium hydroxide; 1M in methanol solution) and Ecolite® counting scintillant were obtained from ICN Biomedicals Canada Ltd (Mississauga, Ontario). Dowex 1x4 anion exchange resin (200-400 mesh chloride form) was obtained from Bio-Rad (Richmond, Virginia). Lactate dehydrogenase, pyruvate kinase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim (Indianapolis, IN). Assay kits for glucose, triglycerides and free fatty acids were obtained from Sigma Chemical Company (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN) and Wako Chemicals (Richmond, VA), respectively. Antibodies and standards for Western blotting were purchased from Transduction Laboratories (Mississauga, ON). 17β-Estradiol pellets (0.25 mg, 21-day release formulation) were obtained from Innovative Research of America, (Sarasota, FL). Serum 17β-estradiol levels were determined by radioimmunoassay obtained from Diagnostic Products (Los Angeles, CA). The myocardial contents of cGMP were determined by commercially available enzyme immunoassay kits purchased from Cayman Chemical (Ann Arbor, MI). L-arginine and L-[U-^{14}C]arginine were purchased from Fluka Biochem (Buchs, Switzerland) and Amersham Life Science (Oakville, ON), respectively. The remainder of the chemicals was purchased

from Sigma Chemical Co. Other chemicals were reagent grade.

2.10) Statistical Analysis

Data are expressed as the mean ± standard error of the mean (S.E.). Comparisons between groups were performed using the unpaired Student's t-test. Multiple comparisons were made using analysis of variance followed by the Student-Newman-Keuls post-hoc test. When sample variances were significantly different, a nonparametric test was used (Mann-Whitney U-statistic, unpaired, two-tailed test). Differences were judged to be significant when $P < 0.05$. In Chapter 7 where glucose metabolism was compared at different time-points in hearts from the same animal, data were analyzed by a Two Way Repeated Measures Analysis of Variance.

Fig. 2-1. *Diagrammatic representation of the isolated heart perfusion apparatus.* This diagram shows how the perfusate is recirculated after being ejected from the heart into the afterload line, through the perfusate reservoir and back through the oxygenation chamber and down the preload line into the left atrium of the heart. Langendorff perfusion apparatus is also depicted. After 10 min of Langendorff perfusion, flow was stopped by clamping the Langendorff line and the heart was switched into working mode by opening the clamps on the preload and afterload lines. Preload and afterload were set at 11.5 mm Hg and 80 mm Hg, respectively. A pressure transducer was connected to the aortic outflow line to measure aortic pressures and in-line ultrasonic flow probes were placed in the preload and afterload lines to measure cardiac output and aortic flow respectively. Coronary flow was then calculated from the difference between cardiac output and aortic flow.

Perfusion Apparatus

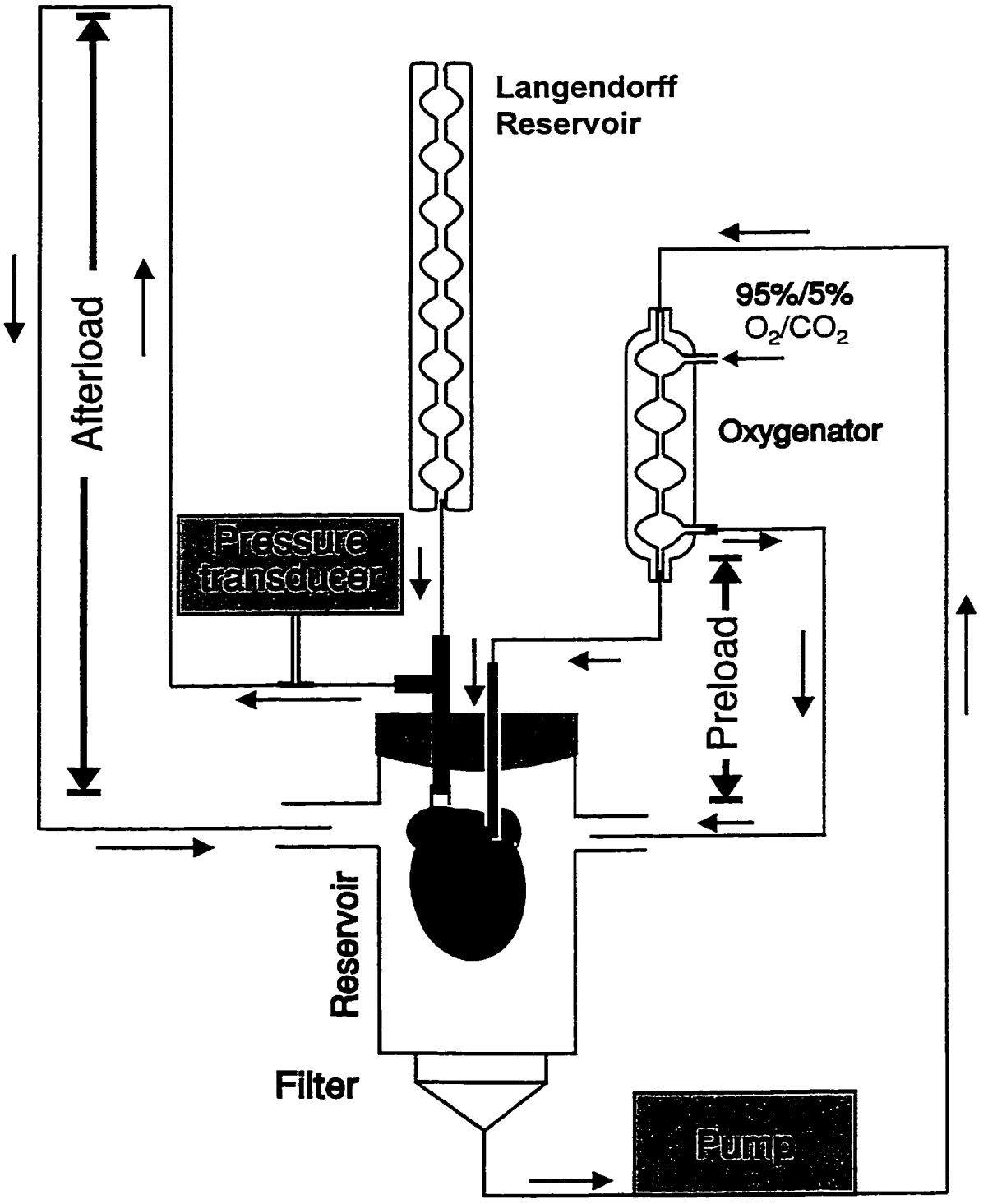
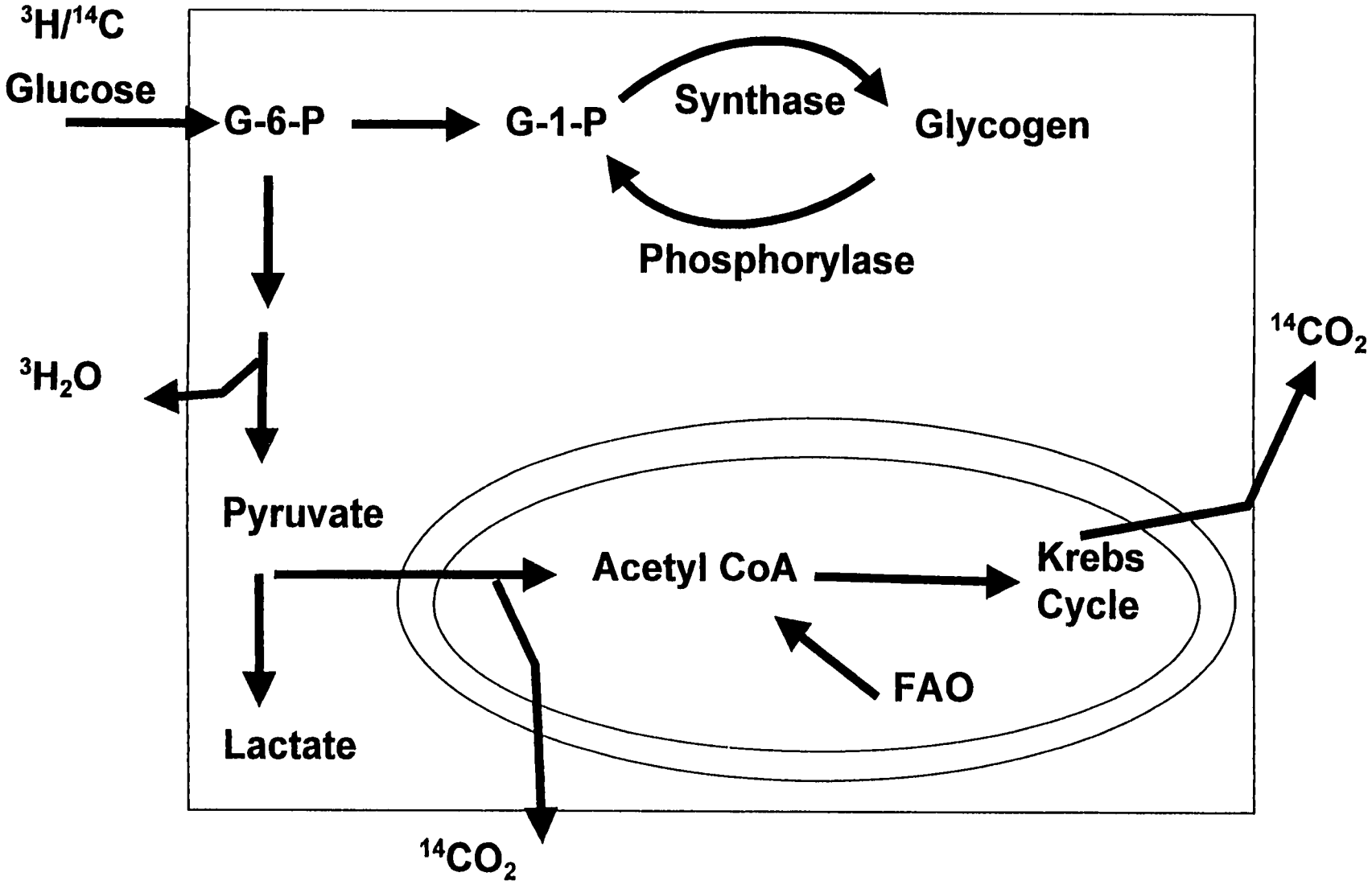


Fig. 2-2. *Diagrammatic representation of glucose metabolism in a myocyte.* Labeling the perfusate with [^{3}H] and [^{14}C]glucose allows for the quantification of labeled byproduct accumulation and the direct measurement of rates of glycolysis (^3H_2O) and glucose oxidation ($^{14}CO_2$). Glycogen turnover is also described in this figure as the simultaneous synthesis and degradation of glycogen that is controlled by two key enzymes (glycogen synthase and glycogen phosphorylase), the activities of which were also measured in frozen heart tissue.

Measurement of Glycolysis and Glucose Oxidation



CHAPTER 3

Assessment of glycogen turnover in aerobic, ischemic and reperfused working rat hearts[1]

[1] *A version of this chapter has been accepted for publication. "Assessment of glycogen turnover in aerobic, ischemic and reperfused working rat hearts". H. Fraser, G.D. Lopaschuk and A.S. Clanachan, 1998. The American Journal of Physiology.*

3.1) Abstract

Glycogen and its turnover are important components of myocardial glucose metabolism that significantly impact on post-ischemic recovery. We developed a method to measure glycogen turnover (rates of glycogen synthesis and degradation) in isolated, working rat hearts using [^{3}H]- and [^{14}C]glucose. In aerobic hearts perfused with 11 mM glucose, 1.2 mM palmitate and 100 μU/ml insulin, rates of glycogen synthesis and degradation were 1.24 ± 0.3 and 0.53 ± 0.25 μmol/min/g dry wt, respectively. Low-flow ischemia (0.5 ml/min, 60 min) elicited a marked glycogenolysis; rates of glycogen synthesis and degradation were 0.54 ± 0.16 and 2.12 ± 0.14 μmol/min/g dry wt, respectively. During reperfusion (30 min), mechanical function recovered to 20% of pre-ischemic values. Rates of synthesis and degradation were 1.66 ± 0.16 and 1.55 ± 0.21 μmol/min/g dry wt, respectively, and glycogen content remained unchanged (25 ± 3 μmol/g dry wt). The assessment of glycogen metabolism needs to take into account the simultaneous synthesis and degradation of glycogen. With this approach, a substantial turnover of glycogen was detectable, not only during aerobic conditions, but also during ischemia as well as reperfusion.

3.2) Introduction

Glycogen is an important source of glucose for energy substrate metabolism and

ATP generation. The contribution of glycogen as an endogenous source of glucose depends on both energy substrate availability as well as the metabolic status of the heart (reviewed in Opie, 1991). The regulation of glycogen synthesis and degradation (turnover) has been extensively studied in liver (Whelan, 1986; Smythe & Cohen, 1991; Alonso *et al.* 1995). Although the heart has a great potential for the synthesis and storage of glycogen (Pitcher *et al.* 1988), myocardial glycogen turnover is less well understood, particularly under ischemic or reperfused conditions.

When cardiac muscle is adequately perfused, exogenous glucose is transported into the myocyte where it primarily either enters the glycolytic pathway or is stored as glycogen. This glycogen can also be subsequently mobilized to provide a source of endogenous glucose for glycolysis. During ischemia, when the supply of O_2 and exogenous substrates are impaired, fatty acid and glucose oxidation are inhibited and ATP generation from anaerobic glycolysis increases. Although glycolysis produces ATP in the absence of O_2, excessive rates of glycolysis may be deleterious during and following severe ischemia due to the production of protons from the hydrolysis of glycolytically derived ATP (Dennis *et al.* 1991). During reperfusion of ischemic hearts, glycolytic rates continue to exceed glucose oxidation rates. This uncoupling of glycolysis from glucose oxidation continues to be an important source of protons; leading to intracellular acidosis, Na^+ accumulation and Ca^{2+} overload (Tani & Neely, 1989; Liu *et al.* 1996). Recent data suggest that glucose, released from glycogen under conditions of glycogenolysis, is preferentially oxidized (Goodwin *et al.* 1995; Henning *et al.* 1996; Schonekess *et al.* 1997). This important finding implies that endogenous glucose contributes less to proton production than exogenous glucose. Thus, the preferential utilization of endogenous

glucose, rather than exogenous glucose, may result in lower rates of proton production and Ca^{2+} overload.

Although the relationships between endogenous and exogenous glucose metabolism, proton production and post-ischemic myocardial function have not been investigated, there is considerable evidence indicating that cardioprotection may arise from interventions that optimize energy substrate utilization, both during and following ischemia. Depletion of glycogen by hypoxic pre-perfusion of hearts limits the potential for glycolysis during ischemia and is cardioprotective (Neely & Grotyohann, 1984). Similarly, ischemic preconditioning (IPC), an adaptive process in which brief periods of ischemia protect the heart from a subsequent period of severe ischemia (Murry *et al.* 1986; Jenkins *et al.* 1995), reduces glycogen content, inhibits glycolysis and reduces proton production arising from glucose metabolism (Finegan *et al.* 1995). Other cardioprotective interventions that improve the coupling of glucose metabolism include stimulation of glucose oxidation by dichloroacetate (McVeigh & Lopaschuk, 1990; Lopaschuk *et al.* 1993) or inhibition of glycolysis by adenosine (Finegan *et al.* 1993). These results support the hypothesis that the metabolic coupling of glycolysis to glucose oxidation is an important determinant of post-ischemic myocardial function.

Substrate availability as well as the activities of glycogen synthase and glycogen phosphorylase control rates of glycogen synthesis and degradation. Although the relative activities of these enzymes are tightly coupled, simultaneous synthesis and degradation of glycogen (turnover) is demonstrable in isolated working rat hearts perfused under aerobic conditions with glucose as the sole exogenous energy substrate (Goodwin *et al.* 1995). Another study examining glycogen turnover under conditions of net glycogenolysis also

showed that simultaneous synthesis and degradation of glycogen is still detectable (Henning *et al.* 1996). A limitation of most studies addressing glycogen metabolism is the inappropriate reliance on net changes in total glycogen content with little consideration for the simultaneous rates of synthesis and degradation that occur under aerobic and ischemic conditions. Consequently, there is a need to clarify the relative activities of glycogen synthase and glycogen phosphorylase and their contribution to actual rates of myocardial glycogen turnover and glucose metabolism. This is particularly true during ischemia and reperfusion where glycogen turnover has not been adequately addressed.

The present study was designed to measure glycogen turnover during aerobic conditions as well as during and following ischemia. The contribution of glycogen turnover to glucose metabolism and mechanical function post-ischemia was also assessed. A perfusion protocol utilizing dual labeled glucose was designed to measure rates of glycogen turnover and the contributions of endogenous and exogenous glucose to glycolysis and glucose oxidation. Studies were performed under appropriate conditions of energy supply and demand in isolated working rat hearts perfused with both glucose and fatty acids.

3.3) Methods

3.3.1) General Methodologies

3.3.1.1) Heart Perfusions

Male Sprague Dawley rats (327 ± 3 g) were anesthetized and the hearts were extracted and prepared for perfusion as described in Chapter 2. Hearts were perfused under aerobic conditions for 60 min and then subjected to low-flow ischemia (0.5 ml/min) for 60 min followed by 30 min of aerobic reperfusion (Fig. 3-1). Mechanical function, glucose metabolism (glycolysis, glucose oxidation and proton production) and enzyme activities (glycogen synthase and glycogen phosphorylase) were determined as well as statistical analyses performed as described in Chapter 2.

3.3.2) Study-Specific Methodologies

4.3.2.2) Measurement of the Sources and Fate of Glucose During Low-flow Ischemia

A dual label ([5-^{3}H]- and/or [U-^{14}C]glucose) protocol was designed to label the glycogen pool and then follow separately the fate of glucose (both glycolysis and glucose oxidation) arising from either exogenous or endogenous sources. Two series of identical perfusions were designed except the order, and timing, of isotope addition differed (Fig.

3-1).

In one series, hearts were perfused with [^{3}H]glucose that was added at the beginning of aerobic perfusion and was present throughout each phase of the perfusion protocol. During aerobic perfusion, a period of net glycogen synthesis, [^{3}H]glucose became incorporated into glycogen, hereafter referred to as endogenous glucose. During low-flow ischemia, hearts underwent net glycogenolysis and the fate of endogenous [^{3}H]glucose liberated from glycogen was followed as described above. As hearts in this series were exposed to both exogenous [^{3}H]glucose delivered in the perfusate and endogenous [^{3}H]glucose liberated from glycogen, the rate of ^3H_2O production represents the rate of total glycolysis occurring during low-flow ischemia. The second isotope, [^{14}C]glucose, was added at the beginning of low-flow ischemia. As this isotope was absent during aerobic perfusion and therefore did not become incorporated into glycogen during the period of glycogen synthesis, the rate of production of $^{14}CO_2$ during low-flow ischemia represents oxidation of only the exogenous source of [^{14}C]glucose.

In a second series of perfusions, the order of isotope addition was reversed. [^{14}C]Glucose, that was present throughout the perfusion protocol, became incorporated into glycogen during aerobic perfusion and was subsequently mobilized during low-flow ischemia. [^{3}H]Glucose was present during only low-flow ischemia and reperfusion. Thus, the rate of production of $^{14}CO_2$ during low-flow ischemia in this series of perfusions represents the rate of oxidation of both exogenous and endogenous sources of [^{14}C]glucose while the rate of ^3H_2O production represents the rate of glycolysis of only the exogenous source of [^{3}H]glucose. Rates of glycolysis of endogenous [^{3}H]glucose and rates of oxidation of endogenous [^{14}C]glucose were then calculated as the difference

between total and exogenous rates obtained from each series of hearts (the mean values from series 2 minus the mean value for series 1) (Fig. 3-1). In order to measure the rates of endogenous and exogenous glucose metabolism during reperfusion 2 additional series of hearts would have to have been perfused in a similar manner but with the isotope additions at the onset of low-flow ischemia and reperfusion.

4.3.2.2) Measurement of Glycogen Content and Rates of Glycogen Turnover

Myocardial glycogen content (μmol glucosyl units/g dry wt) was determined by measuring the glucose content in samples of frozen tissue as described in Chapter 2. Samples were also analyzed for [^{3}H]glucose and [^{14}C]glucose so that the specific activity of [^{3}H/^{14}C]glycogen and the percentage of glycogen that became labeled with either [^{3}H]glucose or [^{14}C]glucose could be determined in hearts frozen at the end of each phase of the perfusion protocol.

Glycogen turnover was assessed by measuring the simultaneous rates (μmol glucose/min/g dry wt) of glycogen synthesis (G_{in}) and degradation (G_{out}). Apparent rates of G'_{in} and G'_{out} were calculated from the difference in glycogen content measured at the beginning and end of each phase of the perfusion protocol (see below). This calculation was performed for unlabeled glycogen as well as for the component of glycogen that became labeled with either [^{3}H]glucose or [^{14}C]glucose.

Apparent rates of G'_{in} and G'_{out} were not equivalent to the actual rates of G_{in} and

G_{out} because it was observed that glycogen synthesis and degradation occurred simultaneously, not only during periods of net glycogen synthesis, but also during periods of marked glycogenolysis. Actual rates of G_{in} and G_{out} were calculated by incorporating values for the actual changes in unlabeled and labeled glycogen during each phase of perfusion. During the 60-min period of aerobic perfusion, the rate of change (dG_{net}/dt) of total glycogen (labeled and unlabeled) between time 0 (G_0) and the end of the 60-min period of aerobic perfusion (G_{60}) is equal to the difference between G_{in} and G_{out} and was calculated as follows:

$$dG_{net}/dt = (G_{60} - G_0)/60 = G_{in} - G_{out} \quad \text{.....Eq. 1}$$

Similarly, the rate of change (dG_{hot}/dt) of labeled glycogen between time 0 (G_0) and the end of aerobic perfusion (G_{60}) is equal to the difference between glycogen synthesis (G_{in}) and the rate of degradation of the labeled component of the glycogen pool. The rate of glycogen degradation (G_{out}) varied according to the proportion of glycogen that was labeled; this proportion was 0% at time 0 and was determined experimentally at time 60. Consequently, at time 0 (just prior to the addition of labeled glucose), the rate of synthesis was:

$$dG_{hot\text{-}0}/dt = G_{in}$$

while the rate of synthesis at time 60,

$$G_{hot\text{-}60}/dt = G_{in} - (G_{out} \cdot (G_{hot}/G_{60})).$$

Thus, the average rate of incorporation of radiolabeled glucose into glycogen ($G_{hot\text{-}avg}/dt$), may be calculated both from the experimentally determined incorporation of radiolabeled glucose, $G_{hot}/60$, as well as from the average of the rates of incorporation at time 0 ($dG_{hot\text{-}0}/dt$) and at time 60 ($G_{hot\text{-}60}/dt$). The equation was as follows:

$$G_{hot}/60 = [(dG_{hot\text{-}0}/dt) + (G_{hot\text{-}60}/dt)] / 2 = [2G_{in} - (G_{out} \cdot (G_{hot}/G_{60}))] / 2 \quad \text{.....Eq. 2}$$

Values for average rates of G_{in} and G_{out} (μmol/min/g dry wt) were calculated from Eq. 1 and Eq. 2. Using a similar approach, average rates of G_{in} and G_{out} were calculated for the periods of low-flow ischemia and reperfusion.

3.4) Results

3.4.1) Mechanical Function of Aerobic, Ischemic and Reperfused Hearts

LV work, which was used as an index of mechanical function, was stable throughout the 60-min period of aerobic perfusion (Fig. 3-2). All measurable LV work ceased during low-flow ischemia and recovered to 20 ± 5% of pre-ischemic levels by the end of the 30-min period of aerobic reperfusion. Other indices of myocardial function, including cardiac output, aortic flow and coronary flow were also constant during aerobic perfusion and recovered with a similar time-course to 25 ± 5%, 9 ± 3%, and 57 ± 3%,

respectively, by the end of reperfusion (Table 3-1).

3.4.2) Content and Percentage Labeling of Glycogen in Aerobic, Ischemic and Reperfused Hearts

Normal levels of glycogen in the heart *in vivo* are 120-150 μmol/g dry wt (Wolfe *et al.* 1993). As expected, removal of the heart and perfusion for a short period (10 min) without fatty acids in the Langendorff mode resulted in a decrease in glycogen to 74 ± 9 μmol/g dry wt. Glycogen content of hearts increased towards *in vivo* levels when perfused for 60 min under aerobic conditions (Fig. 3-3) and during this period 52 ± 3% and 61 ± 3% of the glycogen pool became labeled with [^{3}H]- or [^{14}C]glucose, respectively. As glycogen labeling is independent of the nature of the isotope, values for percentage labeling are presented as an average of values obtained with each isotope in each perfusion series. After 60 min of aerobic perfusion, 56 ± 4% of the glycogen was labeled with [^{3}H]- or [^{14}C]glucose. During low-flow ischemia, there was a predictable glycogenolysis, and after 60 min, glycogen content had markedly decreased relative to values immediately prior to the onset of ischemia (Fig. 3-3). Although total glycogen decreased during low-flow ischemia to values less than those observed prior to exposure to either [^{3}H]- or [^{14}C]glucose, the isotope that was used to label the glycogen during aerobic perfusion was not entirely depleted. In fact, during low-flow ischemia, the percentage of glycogen labeled with this initial isotope increased further to 76 ± 10%. In addition, there was a net incorporation (38 ± 4%) of the second isotope that was added at

the onset of low-flow ischemia. Glycogen contents of hearts following reperfusion were not significantly different from those measured at the end of low-flow ischemia. However, in reperfused hearts, a further loss of unlabeled glycogen was observed, such that the extent of glycogen labeling was 87 ± 16% and 68 ± 9%, respectively, with the first and second isotopes.

3.4.3) Rates of Glycolysis, Glucose Oxidation and Proton Production in Aerobic, Ischemic and Reperfused Hearts

Similar to previous studies in fatty acid perfused working hearts (McVeigh & Lopaschuk, 1990; Finegan *et al.* 1993; Finegan *et al.* 1995), the steady state rate of glycolysis during the initial aerobic perfusion period was greater than that of glucose oxidation (Fig. 3-4). This uncoupling of glycolysis from glucose oxidation resulted in proton production rates attributable to glucose metabolism of 7.5 ± 0.9 μmol/min/g dry wt. During low-flow ischemia, the sum of the rates of glycolysis, glucose oxidation and proton production arising from both exogenous and endogenous sources of glucose (indicative of total rates) were similar to those measured during the initial aerobic perfusion. In the reperfusion period, when LV work had recovered to only 20% of aerobic values, glycolysis of glucose from both endogenous and exogenous sources was slightly depressed. Glucose oxidation was unaffected and this resulted in an improvement in the coupling of glycolysis to glucose oxidation and an inhibition in proton production.

3.4.4) Relative Contributions of Endogenous and Exogenous Glucose to Rates of Glycolysis, Glucose Oxidation and Proton Production During Low-flow Ischemia

Despite the dramatic decrease in coronary flow during low-flow ischemia, rates of glycolysis using exogenous glucose were significantly higher than for glycolysis from endogenous glucose. However, the contributions of exogenous and endogenous glucose to rates of glucose oxidation were not significantly different. This indicates a preferential oxidation of the endogenous glucose liberated from glycogen, resulting in a lower rate of proton production from endogenous, relative to exogenous, sources of glucose (Fig. 3-4).

3.4.5) Glucose Uptake and Extraction

Rates of glucose uptake were inhibited by 32% during ischemia, and further decreased to 53% of aerobic values during the reperfusion period (Table 3-2). Glucose extraction values indicate that glucose availability was not rate-limiting, because even at maximal rates of glucose uptake, that were observed during low-flow ischemia, only 21% of the available glucose was extracted from the low-flow perfusate (11 mM glucose).

3.4.6) Rates of Glycogen Turnover (G_{in} and G_{out}) in Aerobic, Ischemic and Reperfused Hearts

Apparent rates of glycogen synthesis and degradation (G'_{in} and G'_{out}), that were

based on net changes in total glycogen content, indicated that synthesis predominated during aerobic perfusion (Table 3-2). As expected, degradation predominated during low-flow ischemia. During reperfusion, G'_{in} and G'_{out} were essentially similar. Calculations that measured the average rates of the simultaneous synthesis (G_{in}) and degradation (G_{out}) of glycogen, indicated that G_{in} was 2.3-fold higher than G_{out} during the initial aerobic perfusion while during low-flow ischemia, G_{out} was 3.9-fold greater than G_{in}. Although there was no net change in glycogen content during the reperfusion period and the rates of synthesis and degradation were not significantly different, the high values for G_{in} and G_{out} indicated that there was considerable glycogen turnover during this period (Table 3-2). Glycogen synthesis rates were suppressed by 2.3-fold during low-flow ischemia, but recovered to pre-ischemic values during reperfusion. During low-flow ischemia, glycogenolysis was stimulated 4-fold relative to aerobic rates and remained elevated (3-fold) during reperfusion (Table 3-2).

3.4.7) Activities of Glycogen Synthase and Glycogen Phosphorylase in Aerobic, Ischemic and Reperfused Hearts

At the end of the period of low-flow ischemia, glycogen phosphorylase and glycogen synthase activities were increased significantly compared with values measured at the end of aerobic perfusion (Table 3-3). During reperfusion, glycogen phosphorylase activity returned to aerobic values, whereas glycogen synthase activity remained significantly elevated.

3.5) Summary

1. This study developed a new approach to determine directly the contributions of glycogen turnover to overall glucose metabolism in aerobic, ischemic and reperfused working rat hearts.
2. The data demonstrate that there is a substantial turnover of glycogen not only under aerobic conditions, but also during low-flow ischemia, and during aerobic reperfusion.
3. Despite the marked acceleration of glycogenolysis during ischemia, glycogen synthesis was still demonstrable.
4. Furthermore, although the total myocardial glycogen pool was markedly reduced, glycogen turnover persisted during reperfusion.
5. Direct measurements of radiolabeled glucose in the glycogen pool revealed that glucose, which became incorporated during the initial aerobic perfusion, was retained within the glycogen pool following ischemia, despite marked glycogenolysis.
6. Finally, the finding that the preferential oxidation of glucose derived from glycogen during low-flow ischemia was associated with a lower rate of proton production, suggests that optimization of glycogen turnover may be a useful therapeutic strategy for improving the recovery of mechanical function during reperfusion of post-ischemic hearts.

Table 3-1. *Cardiac output, aortic flow and coronary flow in aerobic, ischemic and reperfused rat hearts.*

	Cardiac Output (ml/min)	**Aortic Flow (ml/min)**	**Coronary Flow (ml/min)**	**Coronary Vascular Conductance (ml/min/mm Hg)**
Aerobic	66.3 ± 1.4	43.9 ± 1.4	22.4 ± 1.2	0.28 ± 0.02
LFI	0*	0*	0.5 (fixed)	0
Reperfusion	16.8 ± 4.5*	3.9 ± 3.4*	12.8 ± 3.1*	0.18 ± 0.04*

Values are the means ± S.E. (n=17) measured during the steady state in each phase of the perfusion protocol. Hearts were perfused as described in Fig. 3-1. * $P<0.05$ compared with corresponding aerobic value.

Table 3-2. *Rates of glycogen turnover, glucose uptake and glucose extraction in aerobic, ischemic and reperfused rat hearts.*

	Apparent G_{in}	Apparent G_{out}	Actual G_{in}	Actual G_{out}	Glucose Uptake	Glucose Extraction
	(μmol/min/g dry wt)					%
Aerobic (n=14)	1.08	0.38	1.24 ± 0.30	0.53 ± 0.25	5.86 ± 0.28	0.64 ± 0.08
LFI (n=14)	0.14	1.71	0.54 ± 0.16*#	2.12 ± 0.14*	3.96 ± 0.48*#	21.1 ± 2.1*#
Reperfusion (n=17)	0.16	0.09	1.66 ± 0.16	1.55 ± 0.21*	2.75 ± 0.35*	2.77 ± 0.72

Data are the mean ± S.E. for n hearts. Hearts were perfused as described in Fig. 3-1. Apparent and actual rates of glycogen turnover (synthesis, G_{in}; degradation, G_{out}), during the aerobic, low-flow ischemic and reperfusion periods, were calculated as described in Chapter 3, Section 3.3.2.2. * $P<0.05$ compared with aerobic values; # $P<0.05$ compared with corresponding value measured during reperfusion.

Table 3-3. *Activities of glycogen phosphorylase and glycogen synthase at the end of aerobic, ischemic and reperfusion phases of perfusion.*

	Glycogen Phosphorylase Activity (% active)	**Glycogen Synthase Activity (% active)**
Aerobic (n=7)	13.1 ± 0.7	20.7 ± 1.7
LFI (n=7)	27.2 ± 1.0 *#	32.6 ± 2.4 *
Reperfusion (n=10)	12.8 ± 1.0	36.1 ± 2.3 *

Values are the mean ± S.E. for n hearts measured at the end of the aerobic, low-flow ischemic (LFI) and reperfusion phases of perfusion. * $P<0.05$ compared with corresponding aerobic value; # $P<0.05$ compared with corresponding value measured during reperfusion.

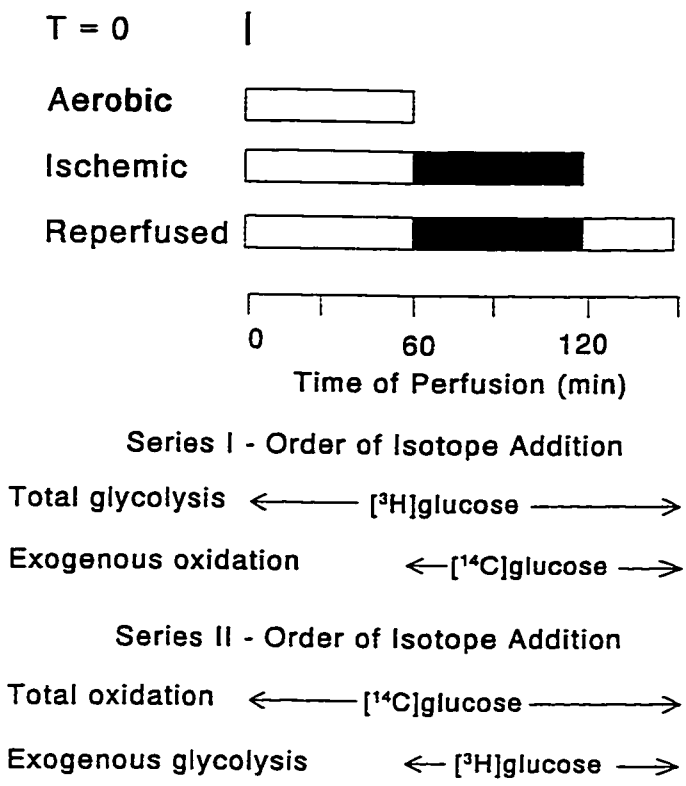


Fig. 3-1. *Heart perfusion protocols for series I and II heart perfusions used to measure glycogen turnover.* In each series, hearts were frozen at the end of the aerobic, low-flow ischemic and reperfusion periods. The two series represent a different order of radiolabeled glucose that was necessary to determine exogenous and endogenous contributions of glucose to glycolysis and glucose oxidation during low-flow ischemia. Calculations and further details are presented in Chapter 3, Section 3.3.2.1.

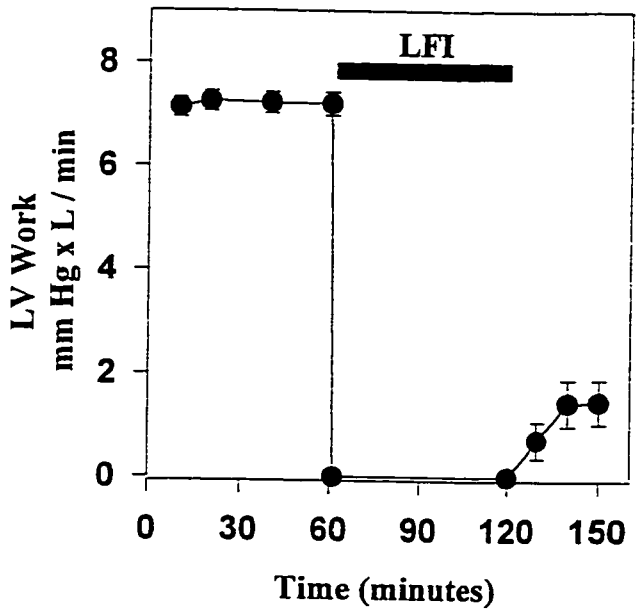


Fig. 3-2. *Left ventricular (LV) work during the aerobic, low-flow ischemic and reperfusion phases of perfusion in isolated working rat hearts.* The solid bar represents the 60-min period of low-flow ischemia (LFI, 0.5 ml/min coronary flow). Data are the mean ± S.E. for 17 hearts.

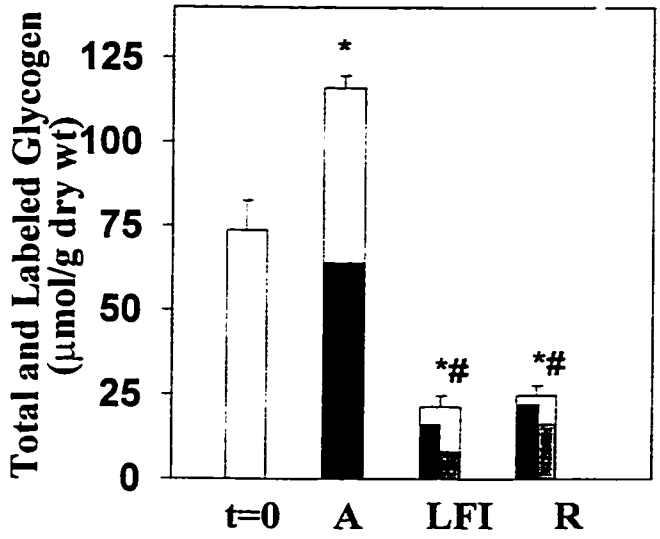


Fig. 3-3. *Glycogen content and percentage labeling of glycogen at the end of aerobic, ischemic and reperfusion periods.* Values are the mean ± S.E. of 7-17 hearts in each group. Working rat hearts were perfused as described in Fig. 3-1. T=0 is glycogen content before the aerobic working period, A is end of aerobic perfusion, LFI is end of low-flow ischemia and R is end of reperfusion. Glycogen labeled with the first isotope is represented by the solid black bars, glycogen labeled with the second isotope is represented by the shaded bars while unlabeled glycogen is shown by the open bars.

* $P<0.05$ compared with T=0 (0 min); # $P<0.05$ compared with aerobic values.

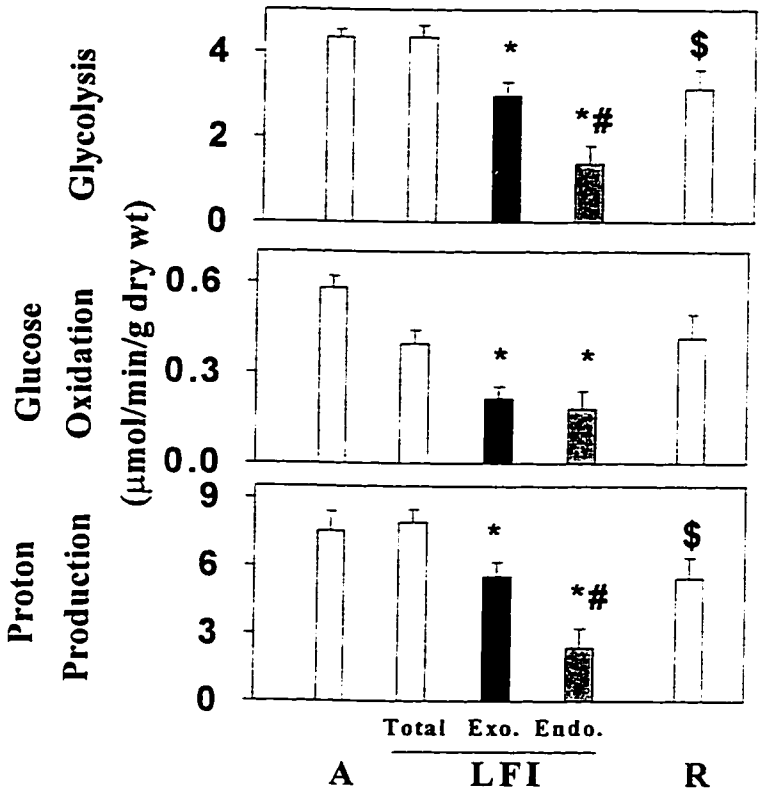


Fig. 3-4. *Steady state rates of glycolysis, glucose oxidation and proton production from glucose metabolism during the aerobic (A), low-flow ischemic (LFI) and reperfusion (R) periods.* Values are the mean ± S.E. of 7-10 hearts in each group. Endogenous (Endo) and exogenous (Exo) contributions of glucose to glycolysis, glucose oxidation, and proton production were determined during low-flow ischemia. Hearts were perfused as described in Fig. 3-1. * $P<0.05$ compared with total during LFI; # $P<0.05$ compared with endogenous during LFI; $ $P<0.05$ compared with aerobic period.

CHAPTER 4

Cardioprotection by adenosine A_1-receptor stimulation alters glucose and glycogen metabolism[1,2]

[1] A version of this chapter has been submitted for publication. H. Fraser, G.D. Lopaschuk and A.S. Clanachan. *The American Journal of Physiology*.

[2] Measurements of high energy phosphates and their metabolites were performed by K. Strynadka, and AMPK activity was determined by L. Atkinson and J. Altarejos.

4.1) Abstract

The well-established cardioprotective efficacy of adenosine A_1-receptor stimulation may be due, in part, to alterations of glucose metabolism that reduce proton production from glycolytically derived ATP. Since glycogen is an important source of glucose, we investigated the effects of the adenosine A_1-receptor agonist, N^6-cyclohexyladenosine (CHA), on glycogen and glucose metabolism during low-flow ischemia as well as during aerobic reperfusion. CHA improved the recovery of mechanical function of isolated working rat hearts following 60 min of low-flow ischemia and decreased rates of glycolysis and proton production from exogenous glucose. CHA also altered glycogen turnover in post-ischemic hearts and accelerated the recovery of glycogen content by stimulating glycogen synthesis while glycogen degradation was unchanged. CHA had no effect on glycogen turnover or glucose metabolism during ischemia. However, glycogen phosphorylase activity, which was elevated at the end of ischemia, was inhibited by CHA, possibly in response to a direct CHA-induced inhibition of AMP-activated protein kinase (AMPK) activity. These results indicate that CHA-induced cardioprotection is associated with alterations of glycogen turnover during reperfusion as well as improved metabolic coupling of glycolysis to glucose oxidation.

4.2) Introduction

The ability of adenosine to improve recovery of myocardial mechanical function

during reperfusion of post-ischemic hearts is well documented, but its mechanism of action remains unclear. The role of adenosine A_1-receptors is clearly supported by the demonstrations that selective adenosine A_1-receptor agonists such as N^6-cyclohexyladenosine (CHA) mimic the protective actions of adenosine (Lasley & Mentzer, Jr. 1993; Finegan *et al.* 1996a; Finegan *et al.* 1996b), while selective adenosine A_1-receptor antagonists inhibit the effects of adenosine and adenosine A_1-receptor agonists (Finegan *et al.* 1996b).

Mechanisms suggested to explain the cardioprotective effects of adenosine receptor stimulation include inhibition of adenylyl cyclase, stimulation of protein kinase C and activation of K_{ATP} channels (reviewed in Mubagwa *et al.* 1996). Alterations in glucose metabolism have also been proposed to contribute to the beneficial effects of adenosine (Finegan *et al.* 1992; Finegan *et al.* 1996a; Finegan *et al.* 1996b). Adenosine inhibits glycolysis in isolated working rat hearts perfused under aerobic conditions and enhances the metabolic coupling between glycolysis and glucose oxidation (Finegan *et al.* 1992). In addition, adenosine administered prior to a 60-min period of severe low-flow ischemia decreases glycolysis during ischemia and enhances the recovery of mechanical function during reperfusion (Finegan *et al.* 1993). Improved coupling between rates of glycolysis and glucose oxidation reduces the rate of proton production from glycolytically derived ATP during the critical early period of reperfusion. The resulting reduction in Na^+/H^+ exchange prevents Na^+ and Ca^{2+} overload, thereby allowing a more rapid and complete recovery of mechanical and metabolic function during reperfusion (Tani & Neely, 1989). Such a mechanism of protection is supported by the observation that ischemia-induced increases in intracellular H^+, Na^+ and Ca^{2+} (measured by nuclear

magnetic resonance) are attenuated by adenosine (Steenbergen *et al.* 1993).

Glycogen is an important energy substrate in the heart, particularly during ischemia when it is readily degraded, following activation of glycogen phosphorylase, to form an endogenous source of glucose phosphate for glycolytic ATP production (anaerobic glucose metabolism). Glycogen synthesis and restoration of glycogen stores occur in response to adequate levels of glucose phosphate and activation of glycogen synthase. Although the relative activities of these enzymes are tightly coupled, simultaneous synthesis and degradation of glycogen (turnover) is demonstrable in working rat hearts perfused under aerobic conditions (Goodwin *et al.* 1995; Henning *et al.* 1996; Chapter 3), as well as during conditions of net glycogenolysis (Schonekess *et al.* 1997; Chapter 3) or during post-ischemic reperfusion (Chapter 3).

Glycogen turnover is regulated by a number of factors and recent data suggest that a novel stress kinase, AMP-activated protein kinase (AMPK) may also be involved. Activity of this kinase is stimulated by increased AMP/ATP ratio and CrP/Cr ratio and is thus responsive to the energy status of the cell (Hardie & Carling, 1997). AMPK influences myocardial energy substrate metabolism in a number of ways, including activation of fatty acid oxidation during reperfusion due to a phosphorylation and inactivation of acetyl CoA carboxylase (Kudo *et al.* 1996). Furthermore, AICAR, an activator of AMPK, has been shown to activate glycogen phosphorylase and glycogenolysis in skeletal muscle (Young *et al.* 1996). In this way, AMPK activity may serve as a link between glycogen turnover and myocardial energy status. However, the role of AMPK on glycogen turnover and glucose metabolism during ischemia and reperfusion has not been defined.

Interestingly, alterations in glycogen content and in the activities of glycogen synthase and phosphorylase also occur in ischemic preconditioned (IPC) hearts and glycogen depletion may contribute to IPC-induced cardioprotection by reducing the potential for the accumulation of glycolytic end-products during ischemia (Wolfe *et al.* 1993). However, the protective roles of glycogen depletion and alterations in glycogen turnover remain controversial, as alterations in glycogen content elicited by non-ischemic mechanisms do not always confer resistance to ischemia (Asimakis, 1996). Moreover, adenosine, which is a putative trigger of IPC, increases glycogen content in aerobic and reperfused ischemic hearts (Finegan *et al.* 1995). However, the effects of adenosine A_1-receptor stimulation on glycogen turnover during ischemia and post-ischemic conditions, and the role of these changes in the recovery of post-ischemic mechanical function have not been addressed.

Endogenous glucose, which is released from glycogen under conditions of glycogenolysis, does not undergo the same metabolic fate as exogenous glucose. Recent data suggest that endogenous glucose is preferentially oxidized (Goodwin *et al.* 1996a; Henning *et al.* 1996; Schonekess *et al.* 1997), a finding that was confirmed in this thesis (Chapter 3). Consequently, rates of glycolysis and oxidation of endogenous glucose are more closely coupled and so lead to lower rates of proton production. Thus, cardioprotection due to attenuation of rates of proton production may arise in response to a switch to the utilization of endogenous rather than exogenous glucose. Although adenosine inhibits the flux of exogenous glucose through glycolysis (Finegan *et al.* 1993; Finegan *et al.* 1996b), thereby reducing proton production, the effects of adenosine on the relative rates of utilization of endogenous and exogenous glucose have not been measured.

In view of the importance of glycogen metabolism in the heart and its potential contribution to anti-ischemic mechanisms, this study was designed to assess the effects of the selective adenosine A_1-receptor agonist, CHA on glycogen turnover in ischemic and post-ischemic hearts. CHA-induced alterations in the relative rates of metabolism of endogenous and exogenous glucose during low-flow ischemia were also measured. Studies were performed under appropriate conditions of energy demand and supply in isolated working rat hearts perfused with both glucose and fatty acids.

4.3) Methods

4.3.1) General Methodologies

4.3.1.1) Heart Perfusions

Male Sprague Dawley rats (300-350 g) were anesthetized and the hearts were extracted and prepared for perfusion as described in Chapter 2. Mechanical function, glucose metabolism (glycolysis, glucose oxidation and proton production) and enzyme activities (glycogen synthase and glycogen phosphorylase) were determined as well as statistical analyses performed as described in Chapter 2. Glycogen turnover was assessed by measuring the simultaneous rates of glycogen synthesis and degradation as described in Chapter 3. Two series of heart perfusions were performed as described in Chapter 3 where the order of isotope addition was reversed (Fig 4-1).

4.3.2) Study-Specific Methodologies

4.3.2.1) Drug Addition

Hearts were perfused under aerobic conditions for 60 min and then subjected to low-flow ischemia (0.5 ml/min) for 60 min followed by 30 min of reperfusion (Fig. 4-1), either in absence (untreated) or presence of CHA (0.5 μM).

4.3.2.2) AMP-Activated Protein Kinase Determinations

AMPK activity (nmol/min/mg protein) was measured in 6% polyethylene glycol fractions extracted from 200 mg of frozen LV tissue by determining the incorporation of [^{32}P] from [γ^{32}P]ATP into a serine-79 phosphorylation site-specific SAMS (HMRSAMSGLHVKRR) peptide as previously described (Kudo *et al.* 1995; Kudo *et al.* 1996). The serine residue corresponding to the phosphorylation site for cyclic AMP-dependent protein kinase (Ser-77) was replaced by alanine (Ala-77) to prevent phosphorylation by cAMP-dependent protein kinase present in assay extracts. SAMS peptide also contained two additional arginine residues at the C-terminus to facilitate its binding to phosphocellulose paper.

4.4) Results

4.4.1) Mechanical Function

LV work, which was used as an index of mechanical function, was stable throughout the initial 60-min period of aerobic perfusion (Fig. 4-2). Coronary flow, aortic flow, cardiac output and coronary vascular conductance were also constant during aerobic perfusion. All measurable LV work ceased during low-flow ischemia (0.5 ml/min) and recovered to 20 ± 5% of pre-ischemic levels by the end of the 30-min period of aerobic reperfusion. During reperfusion, coronary flow, aortic flow, cardiac output and coronary vascular conductance were depressed to 57 ± 3%, 9 ± 3%, 25 ± 5% and 64 ± 4%, respectively, of pre-ischemic values. CHA, added 5 min prior to the start of low-flow ischemia and present throughout reperfusion, significantly improved recovery of LV work to 40 ± 5% of pre-ischemic values. CHA also significantly improved the recovery of coronary flow, aortic flow, cardiac output and coronary vascular conductance, when compared with untreated hearts, to 87 ± 3, 28 ± 3%, 48 ± 5%, and 87 ± 3%, respectively (Table 4-1).

4.4.2) Rates of Glycolysis, Glucose Oxidation and Proton Production

The steady state rate of glycolysis during aerobic perfusion was greater than that of glucose oxidation (Table 4-2). This uncoupling of glycolysis from glucose oxidation resulted in a proton production rate attributable to glucose metabolism of 7.5 ± 0.9

μmol/min/g dry wt. CHA, when added immediately prior to the onset of low-flow ischemia, caused no measurable changes in the relative contributions of endogenous and exogenous glucose to rates of glycolysis and glucose oxidation during low-flow ischemia. Although the rate of glycolysis from exogenous glucose was greater than that from endogenous glucose derived from glycogen, the rates of glucose oxidation arising from exogenous and endogenous sources were similar (Table 4-2). This indicates that glucose arising from endogenous sources was preferentially oxidized. Thus, the coupling ratio of glycolysis to glucose oxidation was 1.8-fold greater for endogenous glucose (7.6) than for exogenous glucose (13.5). Consequently, proton production arising from the metabolism of endogenous glucose was 53% less than that for exogenous glucose. CHA did not alter any of these rates during low-flow ischemia. During reperfusion, CHA inhibited glycolysis but had no effect on glucose oxidation. This improved the metabolic coupling in CHA-treated hearts and resulted in a significantly lower (48%) rate of proton production during reperfusion compared with untreated hearts (Table 4-2).

4.4.3) Metabolite Contents

After aerobic perfusion, the high energy phosphates and their metabolites were similar to values reported previously for aerobic working rat hearts perfused with fatty acids (Finegan *et al.* 1995). ATP and CrP levels decreased during low-flow ischemia by 4.7- and 2.8-fold, respectively, and increased towards pre-ischemic levels by the end of reperfusion. CHA did not alter levels of these metabolites during low-flow ischemia, but it increased ATP and CrP content during reperfusion compared with untreated hearts (1.4-

and 1.5-fold, respectively). During low-flow ischemia, AMP and adenosine levels increased (3.5-fold and 15.1-fold, respectively) and returned toward aerobic levels during reperfusion. Accordingly, the AMP to ATP ratio and the Cr to CrP ratio increased during low-flow ischemia and decreased to near pre-ischemic levels after reperfusion (Table 4-3). ADP, GTP and NAD also decreased during low-flow ischemia (53%, 89%, and 36% respectively, Table 4-4), whereas xanthine and IMP increased during low-flow ischemia (1.4-fold and 8.5-fold respectively, Table 4-4). Myocardial contents of all of these metabolites returned toward pre-ischemic levels during reperfusion. During reperfusion, the AMP to ATP ratio and Cr to CrP ratio were lower in CHA-treated hearts, an effect indicative of an improved energetic state. Further, GTP levels increased to a greater extent in the presence of CHA (Table 4-3).

Glycogen content (μmol/g dry wt) of hearts increased substantially during aerobic perfusion from 74.0 ± 8.8 (at time 0) to 116.2 ± 3.6 (after 60 min) (Fig. 4-3). Low-flow ischemia elicited marked glycogenolysis and the decrease in glycogen content was similar in untreated and CHA-treated hearts. In untreated hearts, glycogen content remained constant during reperfusion, whereas CHA significantly increased glycogen resynthesis by 81% compared with the untreated group (Fig. 4-3).

4.4.4) Rates of Glycogen Turnover (G_{in} and G_{out})

Calculations of glycogen turnover, which incorporate measures of the simultaneous rates of synthesis and degradation of glycogen, indicated that G_{in} was 2.3-fold higher than G_{out} during aerobic perfusion, while during low-flow ischemia G_{out} was

3.9-fold greater than G_{in} (Table 4-5). CHA had no effect on G_{in} or G_{out} during low-flow ischemia. However, while G_{in} recovered to pre-ischemic values in untreated hearts during reperfusion, CHA stimulated G_{in} during reperfusion to rates significantly higher than pre-ischemic values. In contrast, G_{out} remained elevated (3-fold) during reperfusion compared with aerobic values and was not altered by CHA. Although there was no net change in glycogen content during reperfusion in untreated hearts as the simultaneous rates of synthesis and degradation were not significantly different, the high values for G_{in} and G_{out} indicated that there was considerable glycogen turnover during this period (Table 4-5). CHA stimulated glycogen resynthesis by increasing G_{in}, as G_{out} was unchanged.

4.4.5) Glucose Uptake and Extraction

Glucose uptake during aerobic perfusion was 5.86 ± 0.28 μmol/min/g dry wt and was inhibited by 32% during low-flow ischemia. In CHA-treated hearts, glucose uptake was not inhibited during low-flow ischemia. During reperfusion, glucose uptake was similar in untreated and CHA-treated hearts, but rates in both groups were inhibited compared with pre-ischemic values (Table 4-5).

Glucose extraction during aerobic perfusion was 0.64 ± 0.08%. Extraction significantly increased during low-flow ischemia to 21 ± 2% and 27 ± 3% in untreated and CHA-treated hearts, respectively. Glucose extraction returned to pre-ischemic levels during reperfusion in both untreated and CHA-treated hearts (Table 4-5).

4.4.6) Activities of Glycogen Synthase and Glycogen Phosphorylase

Myocardial glycogen synthase and glycogen phosphorylase activities after aerobic perfusion were similar to those reported previously for rat hearts (Passonneau & Rottenberg, 1973). At the end of low-flow ischemia, glycogen synthase and glycogen phosphorylase activities were increased significantly compared with aerobic values (Fig 5-4). CHA inhibited glycogen phosphorylase during low-flow ischemia, but had no effect on glycogen synthase. During reperfusion, glycogen phosphorylase activity returned to aerobic values in untreated hearts, whereas glycogen synthase activity remained significantly elevated. The activities of glycogen synthase and glycogen phosphorylase at the end of reperfusion were not affected by CHA (Fig. 4-4).

4.4.7) AMPK Activity

AMPK activity in hearts frozen at the end of aerobic perfusion was similar to that reported previously for fatty acid perfused working rat hearts (Kudo *et al.* 1995). AMPK activity increased significantly during low-flow ischemia then partially recovered during reperfusion (Fig. 4-4). Relative to untreated hearts, CHA inhibited AMPK activity both during low-flow ischemia as well as reperfusion.

4.5) Summary

1. In agreement with previously published results (Finegan *et al.* 1996a; Finegan *et al.* 1996b), CHA improved the recovery of post-ischemic mechanical functional in association with increased cardiac output.
2. CHA-treated hearts had a higher rate of glycogen synthesis, an increased glycogen content and increased glycogen turnover during reperfusion. These data indicate an association between glycogen turnover and cardioprotection elicited by adenosine A_1-receptor stimulation.
3. These data also confirm that CHA improved myocardial post-ischemic mechanical function in association with improvement in metabolic coupling of glycolysis to glucose oxidation and a subsequent reduction in proton production.
4. AMP-activated protein kinase (AMPK) activity was inhibited by CHA both at the end of low-flow ischemia and after reperfusion. Furthermore, evidence presented here supports a link during ischemia between AMPK activity and glycogen phosphorylase. During reperfusion, although it had no effect on glycogen synthase or phosphorylase activities, CHA inhibited AMPK activity and stimulated glycogen synthesis.
5. CHA-induced alterations in glycogen and glucose metabolism are elicited during the critical early period of reperfusion, rather than during ischemia.

Table 4-1. *Effect of N^6-cyclohexyladenosine (CHA) on mechanical function during aerobic perfusion, low-flow ischemia and reperfusion.*

	Aerobic	Low-flow Ischemia	Reperfusion
Cardiac Output (ml/min)			
Untreated	66.3 ± 1.4 (46)	0 (31)	16.8 ± 4.5 (17)
CHA	66.1 ± 1.4 (32)	0 (32)	31.8 ± 4.8* (18)
Aortic Flow (ml/min)			
Untreated	43.9 ± 1.4 (46)	0 (31)	3.9 ± 2.4 (17)
CHA	45.6 ± 1.2 (32)	0 (32)	12.6 ± 3.0 *(18)
Coronary Flow (ml/min)			
Untreated	22.4 ± 1.2 (46)	0.5 (31)	12.8 ± 3.0 (17)
CHA	22.0 ± 1.0 (32)	0.5 (32)	19.2 ± 2.4 (18)
Coronary Vascular Conductance (ml/min/mmHg)			
Untreated	0.28 ± 0.01 (46)	0 (31)	0.18 ± 0.04 (17)
CHA	0.27 ± 0.01 (32)	0 (32)	0.24 ± 0.03 (18)

Cardiac output, aortic flow, coronary flow and coronary vascular conductance were determined at regular intervals and averaged for each perfusion phase (see Chapter 2). CHA (0.5 μM) was added 5-min prior to low-flow ischemia and was present in the perfusate throughout low-flow ischemia and reperfusion. Data are mean ± S.E. (n observations); * $P<0.05$ compared with untreated group.

Table 4-2. *Effect of N^6-cyclohexyladenosine (CHA) on the steady state rates of glucose metabolism and proton production during aerobic perfusion, low-flow ischemia and reperfusion.*

	Aerobic	Low-flow Ischemia		Reperfusion
		Exogenous Glucose	Endogenous Glucose	
Glycolysis (μmol/min/g dry wt)				
Untreated	4.34 ± 0.19 (42)	2.98 ± 0.32 (7)	1.37 ± 0.42$ (7)	3.14 ± 0.45# (10)
CHA		3.57 ± 0.52 (7)	1.41 ± 0.66$ (7)	1.96 ± 0.35* (11)
Glucose Oxidation (μmol/min/g dry wt)				
Untreated	0.58 ± 0.04 (36)	0.22 ± 0.04 (7)	0.18 ± 0.06 (7)	0.41 ± 0.08 (7)
CHA		0.26 ± 0.04 (7)	0.25 ± 0.06 (7)	0.65 ± 0.12 (7)
Proton Production (μmol/min/g dry wt)				
Untreated	7.52 ± 0.87 (36)	5.53 ± 0.64 (7)	2.92 ± 0.86$ (7)	5.45 ± 0.91 (7)
CHA		6.62 ± 1.00 (7)	2.85 ± 1.35$ (7)	2.63 ± 0.74* (7)

Rates of glycolysis, glucose oxidation and proton production (μmol/min/g dry wt) were measured throughout aerobic perfusion, low-flow ischemia and reperfusion. Contributions of glucose from endogenous and exogenous sources were determined during low-flow ischemia as described in Chapter 2, Section 2.3. Data represent mean ± S.E. (n observations); *$P<0.05$ compared with untreated group, # $P<0.05$ compared with aerobic groups, $ $P<0.05$ compared with exogenous glucose.

Table 4-3. *High energy phosphate levels in heart frozen at the end of aerobic, ischemic and reperfusion phases of perfusion.*

	Aerobic	Low-flow Ischemia	Reperfusion
ATP (μmol/g dry wt)			
Untreated	32.0 ± 1.5 (6)	6.9 ± 1.7# (7)	16.8 ± 1.7 #(9)
CHA		10.1 ± 3.1# (7)	23.13 ± 1.49*# (11)
AMP (μmol/g dry wt)			
Untreated	5.7 ± 0.6 (6)	20.0 ± 3.8 # (7)	3.4 ± 0.7 (9)
CHA		23.6 ± 6.9 # (7)	2.3 ± 0.5 (11)
AMP/ATP			
Untreated	0.18 ± 0.02 (6)	4.8 ± 1.6 #(7)	0.23 ± 0.04 (9)
CHA		5.8 ± 2.2 #(7)	0.10 ± 0.02 *(11)
Cr (μmol/g dry wt)			
Untreated	200.9 ± 7.2 (6)	195.4 ± 8.3 (7)	138.7 ± 17.5 #(9)
CHA		212.9 ± 12.9 (7)	130.0 ± 9.9 #(11)
CrP (μmol/g dry wt)			
Untreated	55.6 ± 7.5 (6)	19.6 ± 3.2 #(7)	63.7 ± 9.0 #(9)
CHA		17.3 ± 4.1 #(7)	93.5 ± 5.7 *#(11)
Cr/CrP			
Untreated	4.0 ± 0.7(6)	11.4 ± 1.6 #(7)	2.3 ± 0.3 #(9)
CHA		16.5 ± 3.1 #(7)	1.5 ± 0.2 *#(11)

Data are mean ± S.E. (n observations); * $P<0.05$ compared with untreated group, # $P<0.05$ compared with aerobic group.

Table 4-4. *Cellular metabolite levels in heart frozen at the end of aerobic, ischemic and reperfusion phases of perfusion*

	Aerobic	Low-flow Ischemia	Reperfusion
ADP (μmol/g dry wt)			
Untreated	23.3 ± 0.9 (6)	11.1 ± 2.0 # (7)	12.2 ± 1.2 # (9)
CHA		13.7 ± 2.6 # (7)	13.1 ± 0.9 # (11)
Adenosine (μmol/g dry wt)			
Untreated	0.08 ± 0.03 (6)	1.15 ± 0.30# (7)	0.12 ± 0.02 (9)
CHA		0.92 ± 0.24# (7)	0.11 ± 0.03 (11)
IMP (μmol/g dry wt)			
Untreated	0 (6)	8.5 ± 2.2# (7)	0.4 ± 0.4 (9)
CHA		10.6 ± 3.9# (7)	0.5 ± 0.5 (11)
Inosine (μmol/g dry wt)			
Untreated	0.2 ± 0.1	2.4 ± 1.1 (7)	1.7 ± 0.8 (9)
CHA		3.6 ± 2.5 (7)	0.8 ± .04 (11)
Xanthine (μmol/g dry wt)			
Untreated	0 (6)	1.4 ± 0.4# (7)	0.14 ± 0.06 (9)
CHA		2.2 ± 0.7# (7)	0.08 ± 0.03 (11)
Hypoxanthine (μmol/g dry wt)			
Untreated	0.52 ± 0.16 (6)	11.5 ± 4.5 (7)	8.9 ± 4.3 (9)
CHA		6.6 ± 2.8 (7)	2.3 ± 1.4 (11)
NAD (μmol/g dry wt)			
Untreated	14.6 ± 0.3 (6)	9.3 ± 0.6 # (7)	12.5 ± 1.2 (9)
CHA		10.6 ± 1.1 # (7)	15.1 ± 0.8 (11)
GTP (μmol/g dry wt)			
Untreated	2.0 ± 0.1 (6)	0.2 ± 0.1 #(7)	0.8 ± 0.1 #(9)
CHA		0.3 ± 0.1 #(7)	1.4 ± 0.1 *#(11)

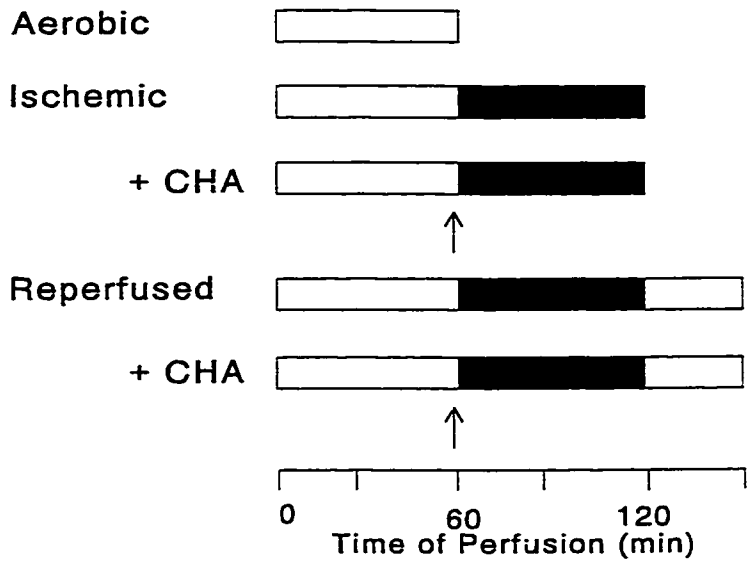
Data are mean ± S.E. (n observations); * $P<0.05$ compared with untreated group, # $P<0.05$ compared with aerobic group.

Table 4-5. *Effect of N^6-cyclohexyladenosine (CHA) on rates of glycogen turnover, glucose uptake and extraction in aerobic, low-flow ischemic and reperfused rat hearts.*

	Aerobic	Low-flow Ischemia	Reperfusion
Glycogen Degradation: G_{out} (μmol/min/g dry wt)			
Untreated	0.53 ± 0.25 (14)	2.12 ± 0.14# (14)	1.55 ± 0.21# (17)
CHA		2.05 ± 2.0# (14)	1.70 ± 0.28# (18)
Glycogen Synthesis: G_{in} (μmol/min/g dry wt)			
Untreated	1.24 ± 0.30 (14)	0.54 ± 0.16# (14)	1.66 ± 0.16# (14)
CHA		0.53 ± 0.21# (14)	2.48 ± 0.34*# (14)
Glucose Uptake (μmol/min/g dry wt)			
Untreated	5.86 ± 0.28 (7)	3.96 ± 0.48# (7)	2.75 ± 0.35# (17)
CHA		5.03 ± 0.87 (7)	2.94 ± 0.31#$ (18)
Glucose Extraction (%)			
Untreated	0.64 ± 0.08 (7)	21 ± 2# (7)	2.8 ± 0.7$ (17)
CHA		27 ± 3# (7)	1.5 ± 0.5$ (18)

Data are presented as mean ± S.E. (n observations). G_{in}, G_{out}, glucose uptake and glucose extraction were measured as described in Chapter 3, Section 3.3.2.2. * $P<0.05$ compared with untreated groups, # $P<0.05$ compared with aerobic groups, $ $P<0.05$ compared with values during low-flow ischemia.

Fig. 4-1. *Perfusion protocol used to measure glycogen turnover in the absence and presence of N^6-cyclohexyladenosine (CHA).* Hearts were subjected to 60 min of aerobic working perfusion, 60 min of low-flow ischemia and 30 min of aerobic working reperfusion. Hearts were perfused with modified Krebs-Henseleit solution containing 1.2 mM palmitate, 11 mM glucose, 2.5 mM Ca^{2+}, 100 μU/ml insulin and 3% BSA. During low-flow ischemia, coronary flow was reduced to 0.5 ml/min. During aerobic perfusion and reperfusion, coronary flow was not restricted. Hearts were paced at 5 Hz throughout the protocol except the first 5 min of reperfusion. Hearts were frozen at the end of each perfusion period with Wollenberger clamps cooled to the temperature of liquid nitrogen. Parallel series of hearts were also frozen at time zero, and at the beginning or end of low-flow ischemia for determination of metabolic parameters. Series 1 and 2 depict groups of hearts that were divided into groups based on the order of isotope addition. CHA, (0.5 μM) was added 5 min prior to low-flow ischemia and remained in the perfusate throughout the remainder of the protocol. For further information, see Chapter 3 Section 3.3.2.1.



Series I - Order of Isotope Addition

Total glycolysis ⟵ [^{3}H]glucose ⟶

Exogenous oxidation ⟵[^{14}C]glucose ⟶

Series II - Order of Isotope Addition

Total oxidation ⟵ [^{14}C]glucose ⟶

Exogenous glycolysis ⟵ [^{3}H]glucose ⟶

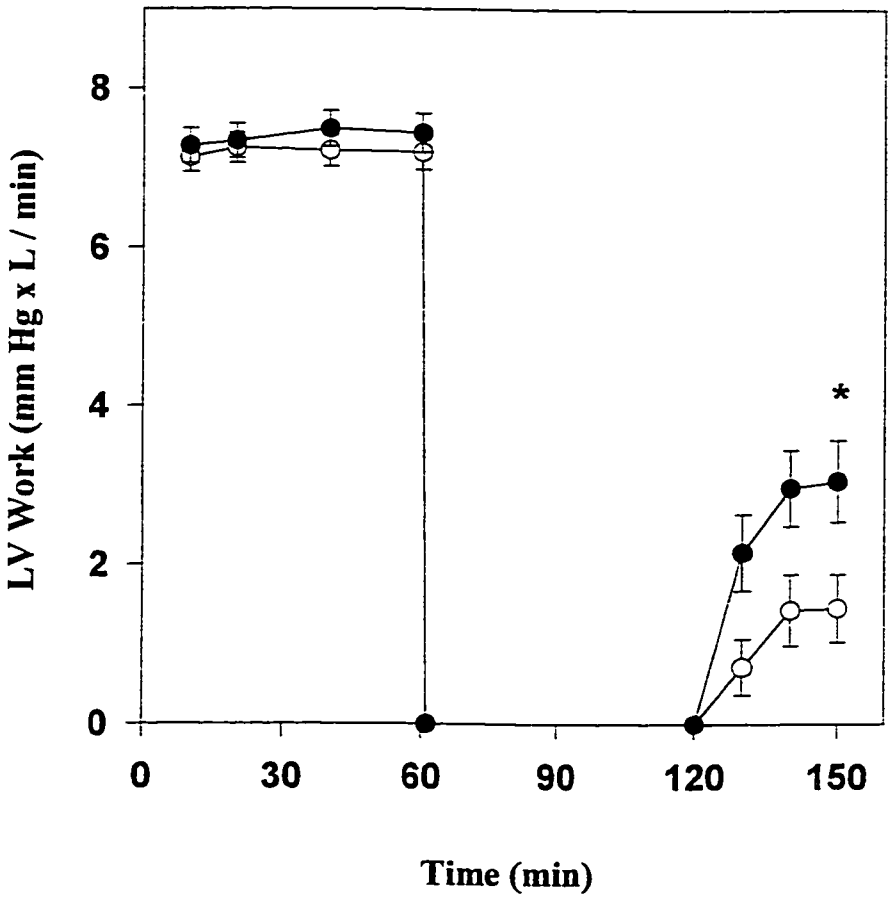


Fig. 4-2. *Effect of N^6-cyclohexyladenosine (CHA) on left ventricular work of isolated rat hearts*. Values are means (± S.E.) for hearts during aerobic perfusion and during low-flow ischemia and reperfusion in the absence (Untreated, O, n=14) or presence of CHA (0.5 μM, ●, n=17). * $P<0.05$ compared with untreated group.

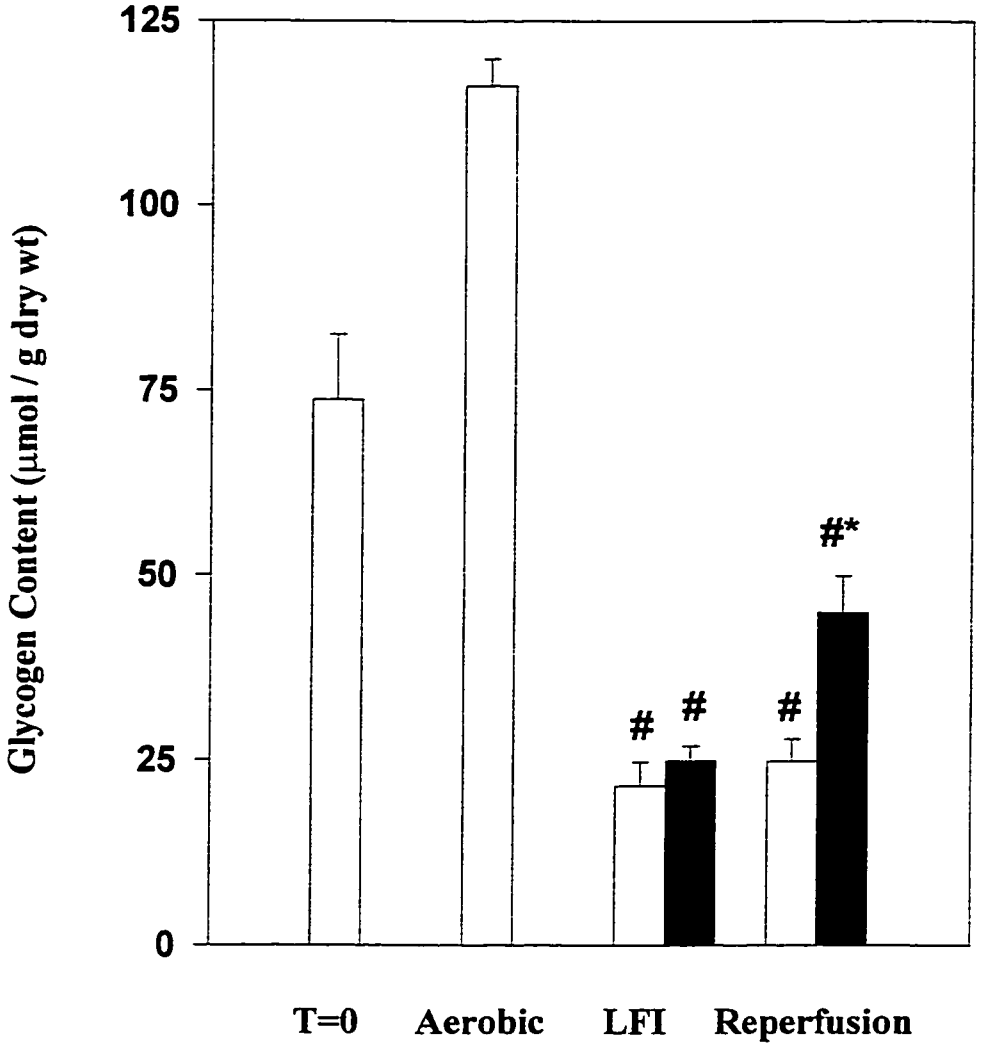
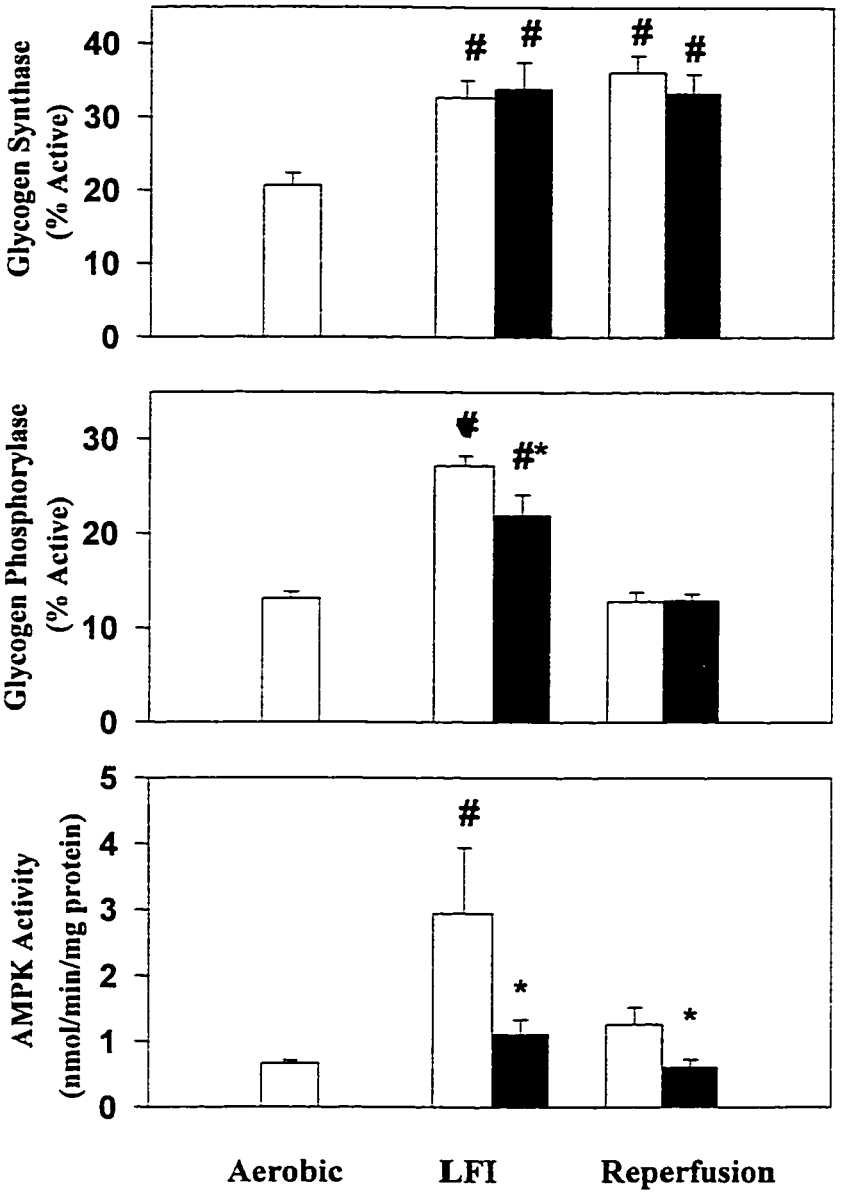


Fig. 4-3. Effect of N^6-cyclohexyladenosine (CHA) on glycogen content of isolated rat hearts. Values are means (± S.E.) for hearts frozen either at the start (T=0) or at the end of aerobic perfusion (Aerobic, n=14) and in hearts perfused the absence or presence of CHA following low-flow ischemia (LFI, Untreated, □, n=14; CHA, 0.5 µM, ■, n=14) and reperfusion (Untreated, □, n=17; CHA, 0.5 µM, ■, n=18). * $P<0.05$ compared with untreated group, # $P<0.05$ compared with aerobic values.

Fig. 4-4. *Effect of N^6-cyclohexyladenosine (CHA) on glycogen synthase, glycogen phosphorylase and AMPK activities.* Activities were measured at the end of aerobic perfusion (n=6) and in hearts perfused in the absence or presence of CHA (0.5μM) following low-flow ischemia (LFI, Untreated, □, n=5; CHA, 0.5 μM, ■, n=7) and reperfusion (Untreated, □, n=10; CHA, 0.5 μM, ■, n=11). * $P<0.05$ compared with untreated group, # $P<0.05$ compared with aerobic values.


Glycogen Synthase
(% Active)
40
30
20
10
0
#
#
#
#
Glycogen Phosphorylase
(% Active)
30
20
10
0
#
#*
AMPK Activity
(nmol/min/mg protein)
5
4
3
2
1
0
#
*
*
Aerobic
LFI
Reperfusion

CHAPTER 5

Time-dependent changes in glycogen and glucose metabolism during low-flow ischemia[1]

[1] Measurements of high energy phosphates and their metabolites were performed by K. Strynadka.

5.1) Abstract

The time-dependent changes in glycogen and glucose metabolism during low-flow ischemia (60 min, 0.5 ml/min coronary flow) were followed in order to: 1) assess the linearity of these changes during low-flow ischemia, and 2) to investigate the effect of potential metabolic modulators on glycogen and glucose metabolism. These changes were studied in untreated hearts and in the presence of N^6-cyclohexyladenosine (CHA, 0.5 μM) and the debranching enzyme inhibitors, deoxynojirimycin (DNJ, 0.5 μM) and N-methyl-deoxynojirimycin (mDNJ, 100 μM). Glycogenolysis during the first 5 min of low-flow ischemia resulted in a 43% decrease in the glycogen pool that was attributed to a 12-fold increase in G_{out} relative to G_{in} (12.1 ± 1.6 and 1.0 ± 0.1 μmol/min/g dry wt, respectively). Glycogenolysis continued during the 60-min period of low-flow ischemia and resulted in an 81% decrease in glycogen content. The rate of glycogenolysis during low-flow ischemia decreased over time (G_{out}=2.1 ± 0.1 at t=60 min compared with 12.1 ± 1.6 at t=5 min). Synthesis and degradation occurred simultaneously during the 60-min period of low-flow ischemia. The rapid rate of glycogenolysis during the initial 5 min of low-flow ischemia did not correlate with glycogen phosphorylase activity which only increased after 60 min of ischemia. The rate of glycolysis was constant during low-flow ischemia; however, glucose oxidation decreased within the first 10 min and remained low throughout the remainder of the ischemic period. Neither CHA nor DNJ affected the rate or extent of glycogenolysis or influenced glucose metabolism. mDNJ had no effect on G_{out} but inhibited G_{in} (51%), glycolysis (57%) and glucose oxidation (80%), effects

suggestive of a decrease in glucose uptake. In conclusion, glycogenolysis, that was not constant during ischemia, is not solely regulated by glycogen phosphorylase. Interestingly, time-dependent changes in glycogen labeling revealed that glycogenolysis is not simply a random process, but may involve both random and ordered processes. Moreover, the rate of glycogenolysis is not influenced by CHA, further supporting the finding that CHA exerts its cardioprotective effects predominantly during reperfusion. The debranching enzyme inhibitor, DNJ, did not alter glycogenolysis, however, mDNJ had widespread effects on glycogen and glucose metabolism, possibly due to alterations in glucose uptake.

5.2) Introduction

Glycogen is an important energy substrate for the heart during ischemia; however, its involvement in decreasing ischemic damage remains controversial. It is now accepted that glycogen turnover is the simultaneous synthesis and degradation of glycogen, and the experimental work performed for this thesis demonstrates that glycogen turnover occurs not only during aerobic perfusion, but also during ischemia and during reperfusion (Chapter 3). The methodology and calculation of rates of glycogen synthesis and degradation developed in Chapter 3 is based on the average of the changes occurring throughout any given perfusion period. This assumes that the changes in glycogen metabolism are linear over the entire perfusion period. Moreover, although *in vivo* studies show that glycogenolysis proceeds rapidly in the heart (Laughlin *et al.* 1993; Wolfe *et al.* 1993; McNulty & Luba, 1995) other studies of glycogenolysis in isolated hearts have

been performed in the absence of physiologically relevant concentrations of energy substrates (Vander Heide *et al.* 1993; Schaefer *et al.* 1995; Asimakis, 1996). As discussed previously (Chapters 3 and 4), the presence of physiologically relevant energy substrates, particularly fatty acids, in the perfusate of isolated working rat hearts allows for appropriate maintenance of the biochemical pathways that allow normal glycogen metabolism.

Further, several studies have implied that changes in glucose metabolism early during ischemia are responsible for cardioprotection caused by ischemic preconditioning (IPC) or by adenosine-receptor stimulation (Rehring *et al.* 1997). Studies using the adenosine receptor agonist N^6-cyclohexyladenosine (CHA) indicate that cardioprotection is not associated with changes in glucose metabolism during low-flow ischemia (Chapter 4); however, that conclusion was based on measurements of glycogen and glucose metabolism that were dependent on values at the beginning and end of the entire period of low-flow ischemia rather than at time-points within the ischemic period. Possible alterations in glycogen and glucose metabolism early during ischemia may be averaged into the net effect occurring over the entire period of ischemia. Consequently, major changes occurring over shorter periods of ischemia may be obscured.

IPC is the process whereby brief periods of ischemia render the heart resistant to subsequent prolonged periods of ischemia (Murry *et al.* 1986). Preconditioned hearts exhibit lower rates of glycolysis post-ischemia, which has been attributed to reduced rates of glycogenolysis (Asimakis *et al.* 1992; Bhavnani, 1998). Furthermore, as discussed in Chapter 4, CHA-induced cardioprotection is associated with a stimulation of glycogen synthesis and an inhibition of glycolysis during reperfusion. Therefore, pharmacological

inhibition of glycogenolysis may also protect ischemic hearts. The methodologies developed to measure glycogen turnover in perfused hearts allows for the detailed examination of drug effects on each component of glycogen and glucose metabolism.

One approach for the pharmacological modulation of glycogenolysis is to inhibit the glycogen debranching enzyme (α-glucosidase), and two drugs were investigated for their potential to alter myocardial glycogen metabolism, deoxynojirimycin (DNJ) and N-methyl-deoxynojirimycin (mDNJ). As debranching enzyme inhibitors, DNJ and mDNJ, are expected to limit the extent of glycogenolysis. As a component of carbohydrate digestion, α-glucosidase degrades dietary carbohydrates to monosaccharides that can be absorbed by the gut. Inhibitors of this enzyme slow the digestion and absorption of complex carbohydrates resulting in decreased post-prandial blood glucose and serum insulin peaks after carbohydrate meals. These agents have been effective in diabetic patients for stabilizing blood glucose levels (Bischoff, 1994). DNJ is taken up into cells and rapidly equilibrates between extracellular and intracellular compartments. Its activity as an α-glucosidase inhibitor is not dependent upon metabolic transformation into an active product (Bollen & Stalmans, 1989).

α-Glucosidase inhibitors are effective at reducing glycogenolysis in the liver (Bollen & Stalmans, 1989) and intestine (Yoshikuni *et al.* 1988), but their effects in heart are not well understood. One study using Langendorff-perfused rat hearts showed that DNJ inhibits hormone-sensitive lipolysis, suggesting that glycogen metabolism may be involved with the control of myocardial triglyceride turnover (Hulsmann *et al.* 1990). A more recent study using working rat hearts subjected to ischemia and reperfusion,

examined the effects of a glucose analog, BAYo1248 (similar in structure to DNJ) that inhibits α-amylo-1,6-glucosidase. BAYo1248 inhibited glycogenolysis and impaired recovery of mechanical function during reperfusion (Depre & Hue, 1997). Although that study found no benefit from inhibition of glycogenolysis, it does not negate the possibility that inhibition of glycogenolysis could be protective. The absence of fatty acids from the perfusate may have prevented any beneficial effects as it is well established that fatty acids directly influence glycogen metabolism. In order to delineate more precisely glycogen and glucose metabolism during ischemia and their perturbations by drugs, this series of experiments investigated the time-dependent changes in glycogen and glucose metabolism during 60 min of low-flow ischemia. The effects of the cardioprotective drug CHA and the inhibitors of α-glucosidase, DNJ and mDNJ on glucose and glycogen metabolism were also investigated in the isolated perfused working rat heart subjected to low-flow ischemia. In addition, a group of hearts was subjected to prolonged aerobic perfusion in the absence and presence of CHA in order to compare with the alterations to glycogen and glucose metabolism during low-flow ischemia.

5.3) Methods

5.3.1) General Methodologies

5.3.1.1) Heart Perfusions

Male Sprague Dawley rats (300-350g) were anesthetized and perfused for 10 min

in Langendorff mode, then switched to paced working mode for 60 min of aerobic perfusion as described in the Chapter 2. All indices of mechanical function were measured as described in Chapter 2. Measures of glycogen and glucose metabolism were determined as described in Chapters 3 and 2, respectively.

5.3.2) Study-Specific Methodologies

5.3.2.1) Protocols

After 60 min of aerobic perfusion, hearts were subjected to low-flow ischemia (0.5 ml/min) for 5, 10, 15 or 60 min (Fig. 5-1). At the end of each period of perfusion, hearts were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. An additional group of hearts was frozen at the end of the aerobic perfusion period. Frozen tissues were pulverized and the resulting powders were stored at -80°C. A prolonged aerobic control was also performed where hearts were perfused for 90 min under aerobic conditions without being subjected to low-flow ischemia.

The effects of three drugs were evaluated on glycogen and glucose metabolism during low-flow ischemia. First, the effects of CHA were measured at 5, 10, 15 and 60 min of low-flow ischemia. The effects of the α-glucosidase inhibitor, DNJ and mDNJ were assessed only after 15 min of low-flow ischemia. CHA (0.5 μM) and DNJ (0.5 μM) were added 5 min prior to ischemia and were present throughout ischemia. In the case of mDNJ, as this drug was expensive, tissue exposure was maximized by adding a high

concentration (100 μM) 30-min prior to the onset of ischemia and was present throughout ischemia. The 15-min time-point was chosen in order to maximize the chances of observing changes in glycogen and glucose metabolism that might be missed early or late after severe ischemia.

A 90-min prolonged aerobic perfusion period was also evaluated for changes in glycogen and glucose metabolism in the absence and presence of CHA. This group of hearts served two purposes. First, it provided a partial time control for the changes occurring to glycogen and glucose metabolism during ischemia. Secondly, it provided the opportunity to investigate changes to glycogen and glucose metabolism induced by CHA during aerobic perfusion.

5.3.2.2) Measurement of the Sources and Fate of Glucose during Low-Flow Ischemia

A dual label ([5-^{3}H]- and/or [U-^{14}C]glucose) protocol was designed to label the glycogen pool and then follow separately the fate (rate of glycolysis and glucose oxidation) of glucose arising from either exogenous or endogenous sources. Hearts were perfused with [^{3}H]glucose that was added at the beginning of aerobic perfusion and was present during each phase of the perfusion protocol. The second isotope, [^{14}C]glucose, was added at the beginning of low-flow ischemia. As this isotope was absent during aerobic perfusion, and therefore did not become incorporated into glycogen during the period of glycogen synthesis, the rate of production of $^{14}CO_2$ during low-flow ischemia represents oxidation of only the exogenous source of [^{14}C]glucose with no contribution

from endogenous [^{14}C]glucose. The reverse series of perfusions described in Chapter 3 was not performed and thus proton production could not be calculated in this study. In the group of hearts perfused under aerobic conditions for 90 min, [^{3}H]glucose was administered at the beginning of aerobic perfusion and [^{14}C]glucose was added after 60 min of perfusion.

5.4) Results

5.4.1) Time-dependent Changes in Glucose and Glycogen Metabolism During Low-flow Ischemia

5.4.1.1) Mechanical Function

LV work during the 60 min aerobic baseline period was stable and LV work was similar between untreated and each of the drug-treated (CHA, DNJ and mDNJ) groups, as were all measurements of pressures and flows. Pre-ischemic functional parameters are shown in Table 5-1. In order to prevent excessive duplication of data presentation, this table represents indices of mechanical function measured under aerobic conditions from the group of hearts that were subjected to 60-min of aerobic perfusion followed by 15 min of low-flow ischemia. During low-flow ischemia, LV work is not detectable.

5.4.1.2) Glycogen Turnover

Time-dependent changes in myocardial glycogen content indicated a rapid glycogenolysis in the first 5 min of low-flow ischemia and a slower rate of glycogenolysis during the subsequent 10-60 min low-flow ischemic period (Fig. 5-2). The rapid rate of glycogenolysis during the initial 5 min of low-flow ischemia did not correlate with either glycogen synthase or glycogen phosphorylase activity. In fact, significant increases in glycogen phosphorylase activity were only detected after 60 min of low-flow ischemia. Furthermore, the time-course for G_{out} (Fig. 5-3) indicated a rapid and significant stimulation in the first 5 min of low-flow ischemia. Rates of G_{out} measured during the other time intervals displayed a gradual decline where at longer ischemic times, G_{out} decreased to aerobic values (Fig. 5-3). On the other hand, rates of G_{in} are not significantly different from pre-ischemic values until 60 min of low-flow ischemia. In addition, glycogen synthase activity was not altered during early low-flow ischemia, but was significantly increased after 60 min of low-flow ischemia (Fig. 5-2).

CHA had no effect on glycogen content, glycogen synthase activity (Fig 5-2) or rates of glycogen turnover (G_{in} and G_{out}, Fig. 5-3) during low-flow ischemia. In fact, the only parameter of glycogen metabolism affected by CHA during low-flow ischemia was glycogen phosphorylase activity (which was inhibited) but this was observed only after 60 min of low-flow ischemia (Fig. 5-2).

DNJ and mDNJ also had no effect on glycogen content, glycogen phosphorylase activity or glycogen synthase activity after 15 min of low-flow ischemia (Table 5-2). Rates of G_{out} for values measured at 15 min of low-flow ischemia in the presence of DNJ

or mDNJ were also similar to untreated and CHA-treated hearts. Although DNJ did not alter the rate of G_{in}, a significant inhibition of G_{in} was observed with mDNJ (Table 5-1).

The percentage of the glycogen pool that became labeled with the second isotope (^{14}C) during low-flow ischemia increased throughout the 60-min period of ischemia (Fig. 5-4). This isotope was added just prior to low-flow ischemia and was incorporated into the diminishing glycogen pool in the face of rapid glycogenolysis. Only 5% of the pool was labeled at 5 min which increased to ~35% by the end of low-flow ischemia. The time-course of ^{3}H content in the glycogen pool was somewhat different. This isotope was added at the beginning of aerobic perfusion and was incorporated to approximately 50% at the end of aerobic perfusion. During the period of rapid glycogenolysis that occurred in the first 5 min of low-flow ischemia, the percentage of glycogen that was labeled with ^{3}H decreased. During the subsequent 55 min of low-flow ischemia, ^{3}H incorporation into the glycogen pool increased to account for a greater percentage (~65%) than that observed prior to the onset of low-flow ischemia (~50%). CHA did not alter the pattern of glycogen labeling during low-flow ischemia. The percentage of glycogen labeled with ^{14}C during low-flow ischemia was decreased with mDNJ, but not with DNJ (data not shown).

5.4.1.3) Myocardial Metabolism

The rates of glycolysis during 60 min of low-flow ischemia were similar at all time-intervals. Rates of exogenous glucose oxidation were lower during low-flow ischemia than rates measured under aerobic conditions (T=0). Proton production could

not be calculated in this experiment because the protocol design did not permit calculation of total rates of glucose oxidation.

CHA significantly increased glycolysis at the 15 min time-point, compared with the untreated group but there were no differences at the other time-points during low-flow ischemia (Fig. 5-5). CHA had no effect on rates of exogenous glucose oxidation (Fig. 5-5). When all drug-treatment groups were compared over the 15-min interval of low-flow ischemia, the significance of the increased glycolysis with CHA is lost and total rates of glycolysis are similar between untreated, CHA, and DNJ groups. On the other hand, the rates of glycolysis and exogenous glucose oxidation were significantly inhibited with mDNJ treatment (Table 5-1).

5.4.1.4) Myocardial High Energy Phosphates and their Metabolites

ATP levels significantly decreased by 25% during the first 5 min of low-flow ischemia and continued to fall slowly (to 80% decrease) over the 60 min of low-flow ischemia. Conversely, AMP levels increased 3.5-fold by 60 min of low-flow ischemia resulting in a 16-fold increase in the AMP/ATP ratio (Fig. 5-6). ADP levels remained at normal levels until 15 min of low-flow ischemia, and their levels decreased by 95% at 60 min. Creatine levels remained stable during ischemia; however, CrP levels decreased early in low-flow ischemia (64% decrease after 5 min), then remained stable during the remainder of low-flow ischemia. Thus, ATP and CrP are early indicators of ischemia whereas AMP, ADP, AMP/ATP ratio and Cr are poor indicators as their levels only change after prolonged periods of ischemia.

Adenosine and hypoxanthine levels increased (15-fold and 22-fold, respectively) by the end of low-flow ischemia (Fig. 5-7) whereas levels of xanthine did not change during ischemia. Moreover, GTP and NAD levels decreased within the first 5 min of low-flow ischemia. NAD levels remained stable thereafter but GTP levels continued to decrease during low-flow ischemia. Interestingly, inosine levels increased early during ischemia then declined to aerobic values by the end of ischemia.

CHA resulted in a further decrease in ATP levels compared with untreated hearts but only at the initial time-point (5-min). Although CHA did not significantly affect AMP content, it caused a significant decrease in the AMP/ATP ratio by 60 min of low-flow ischemia compared to the untreated group. CHA did not alter the level of any other cellular metabolite (Fig. 5-6 and Fig. 5-7). Nucleotides were not measured in hearts perfused in the presence of DNJ or mDNJ.

5.4.2) Time-dependent Changes in Glucose and Glycogen Metabolism During Prolonged Aerobic Perfusion

5.4.2.1) Mechanical Function During Prolonged Aerobic Perfusion

LV work measured during the 90-min of aerobic perfusion was stable and values were not different between 10-60 min and 65-90 min of perfusion. LV work was not altered by CHA (Fig. 5-8).

5.4.2.2) Glycogen Turnover During Prolonged Aerobic Perfusion

Glycogen content increased during the 65-90 min of aerobic perfusion and was comparable with glycogen content determined at the end of the 10-60 min period of aerobic perfusion (Fig. 5-9). Net glycogen synthesis was slower in the 65-90 min period compared with the previous 10-60 min period. This slower rate of glycogen accumulation corresponds to a significant decrease in the rates of glycogen synthesis (G_{in}). There was no change in the rate of glycogen degradation (G_{out}) during the subsequent 30 min period of prolonged aerobic perfusion (Fig. 5-10). CHA had no effect on glycogen turnover during prolonged aerobic perfusion.

5.5) Summary

1. The measurements of glycogen and glucose metabolism described in Chapters 2 and 3 were dependent on averages from the entire 60-min period of low-flow ischemia. In order to define more precisely the time-course of the changes in glycogen and glucose metabolism within the 60-min period, a study was designed in which hearts were subjected to a perfusion protocol similar to that presented for Chapters 2 and 3, except that groups of hearts were frozen after 5, 10, 15 and 60 min of low-flow ischemia. With this approach, the rates of glycogen turnover (G_{in} and G_{out}), glycolysis and glucose oxidation were measured during each of these perfusion intervals. The time-courses of the changes in the content of glycogen, ATP, CrP and metabolites were also

determined.

2. Although CHA has no demonstrable effect on glycogen and glucose metabolism, when averaged over the entire 60-min period of low-flow ischemia, the possibility that CHA could exert effects early in low-flow ischemia was investigated by comparing the time-dependent changes in glycogen and glucose metabolism in untreated hearts with those measured in hearts exposed to CHA (0.5 μM) 5 min prior to the onset of low-flow ischemia. In an attempt to inhibit glycogen degradation during ischemia, the effects of the debranching enzyme inhibitors, DNJ (0.5 μM) and mDNJ (100 μM), on glycogen and glucose metabolism and metabolite contents were explored using hearts frozen after 15 min of low-flow ischemia.
3. Glycogenolysis increased rapidly during the first 5 min of low-flow ischemia, which resulted in a 43% decrease in glycogen content in response to a marked increase in G_{out}. Thereafter, the rates of glycogenolysis and G_{out} gradually declined to close to aerobic values after 60 min by which time glycogen content had decreased by 81% of pre-ischemic levels. Rates of glycogenolysis did not correlate with glycogen phosphorylase activity.
4. Simultaneous synthesis and degradation of glycogen occurred throughout all the time-intervals of low-flow ischemia. Rates of G_{in} remained stable throughout the ischemic period despite marked increases in G_{out}. Glycogen synthase activity remained stable at the earlier time-points during ischemia and increases were only detectable at the end of the 60-min period of low-flow

ischemia.

5. The % labeling of the glycogen pool that had occurred during aerobic perfusion declined rapidly in association with the ischemia-induced glycogenolysis suggesting that an entirely random process can not explain release of glucosyl units from the glycogen molecule. Instead, glucosyl units that were added last appeared to be the first to be released. During low-flow ischemia, there was a gradual and similar rate of incorporation of both isotopes equivalent to the measured rate of G_{in}.
6. Glycolysis remained stable throughout low-flow ischemia, whereas glucose oxidation declined rapidly within the first 5 min and remained depressed throughout the ischemic period.
7. ATP and CrP declined rapidly in association with increases in AMP, ADP and adenosine occurring after prolonged low-flow ischemia. The AMP/ATP and Cr/CrP ratios, which have been used as markers of the degree of ischemic stress, were not affected by the shorter phases (0-15 min) of low-flow ischemia and were significantly increased only after 60 min.
8. CHA did not affect any of the above indices of glycogen and glucose metabolism measured during low-flow ischemia, except that, after 60 min, CHA improved the AMP/ATP ratio and inhibited the activation of glycogen phosphorylase activity.
9. Although DNJ had no effects on any of the indices of glycogen and glucose metabolism, mDNJ inhibited glycolysis, glucose oxidation and glycogen turnover.

10. Prolonged aerobic perfusion exhibited stable mechanical function throughout the entire 90 min of aerobic working heart perfusion. During prolonged aerobic perfusion, glycogen content increased beyond that observed after 60 min of aerobic perfusion in association with a reduced G_{in} and no change in G_{out} compared to the initial 60 min of perfusion.
11. These data indicate that CHA does not alter glycogen and glucose metabolism during ischemia and confirms that its cardioprotective and metabolic effects are manifest during the critical early period of reperfusion. DNJ and mDNJ did not alter ischemia-induced glycogenolysis indicating that these debranching enzyme inhibitors may not be useful tools with which to explore the cardioprotective efficacy of inhibitors of glycogenolysis.

Table 5-1. *Time-dependent changes in pre-ischemic functional parameters*

	Untreated (n=9)	**CHA (n=7)**	**DNJ (n=6)**	**MDNJ (n=5)**
Aortic Systolic Pressure (mm Hg)	118.3 ± 1.9	115.8 ± 1.4	120.2 ± 2.0	119.6 ± 2.5
Developed Pressure (mm Hg)	55.3 ± 1.7	52.7 ± 1.8	55.8 ± 1.4	59.4 ± 4.0
Cardiac Output (ml/min)	64.6 ± 2.4	59.9 ± 2.8	68.9 ± 1.9	68.4 ± 2.9
Coronary Flow (ml/min)	21.6 ± 1.3	23.3 ± 1.5	24.8 ± 1.6	19.7 ± 0.7
LV Work (mm Hg.L/min)	6.9 ± 0.3	6.5 ± 0.2	7.5 ± 0.3	7.4 ± 0.5

Values are averaged over 10-55 min of aerobic perfusion prior to drug addition. Data are from the groups of hearts subjected to 15 min of low-flow ischemia after 60 min of aerobic perfusion. Data are means ± S.E.

Hearts were perfused for 60 min in aerobic working mode, then subjected to 15 min of low-flow ischemia in the absence and presence of N^6-cyclohexyladenosine (CHA, 0.5 μM), deoxynojirimycin (DNJ, 0.5 μM) or N-methyl-deoxynojirimycin (mDNJ, 100 μM). Data are means ± S.E. * $P<0.05$ compared with untreated. ND means not done.

Table 5-2. *Indices of glycogen and glucose metabolism in hearts subjected to 15 min of low-flow ischemia.*

	Untreated (n=9)	**CHA (n=7)**	**DNJ (n=6)**	**MDNJ (n=5)**
Total Glycolysis (μmol/min/g dry wt)				
	5.69 ± 0.53	7.02 ± 0.69	5.94 ± 0.76	2.43 ± 0.55*
Exogenous Glucose Oxidation (μmol/min/g dry wt)				
	0.21 ± 0.07	0.23 ± 0.04	0.22 ± 0.06	0.04 ± 0.01*
Glycogen Content (μmol/g dry wt)				
	58.4 ± 4.6	53.7 ± 3.78	53.7 ± 4.1	64.8 ± 5.1
Glycogen Synthesis (G_{in}) (μmol/min/g dry wt)				
	1.05 ± 0.09	1.12 ± 0.05	1.14 ± 0.56	0.49 ± 0.05*
Glycogen Degradation (G_{out}) (μmol/min/g dry wt)				
	4.64 ± 0.50	5.19 ± 0.32	5.31 ± 0.33	4.21 ± 0.38
Glycogen Synthase (% Active)				
	14.0 ± 3.4	23.2 ± 1.8	ND	23.5 ± 3.5
Glycogen Phosphorylase (% Active)				
	13.0 ± 1.7	11.5 ± 1.4	ND	14.3 ± 1.8

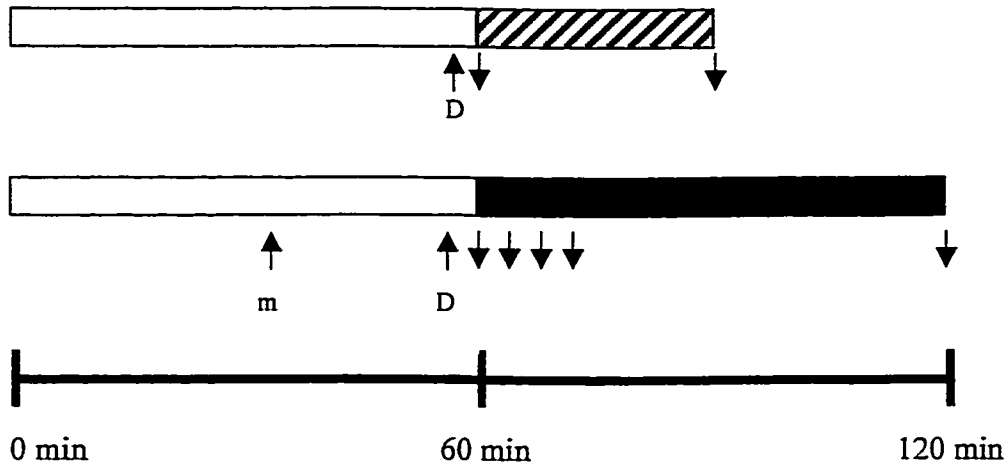
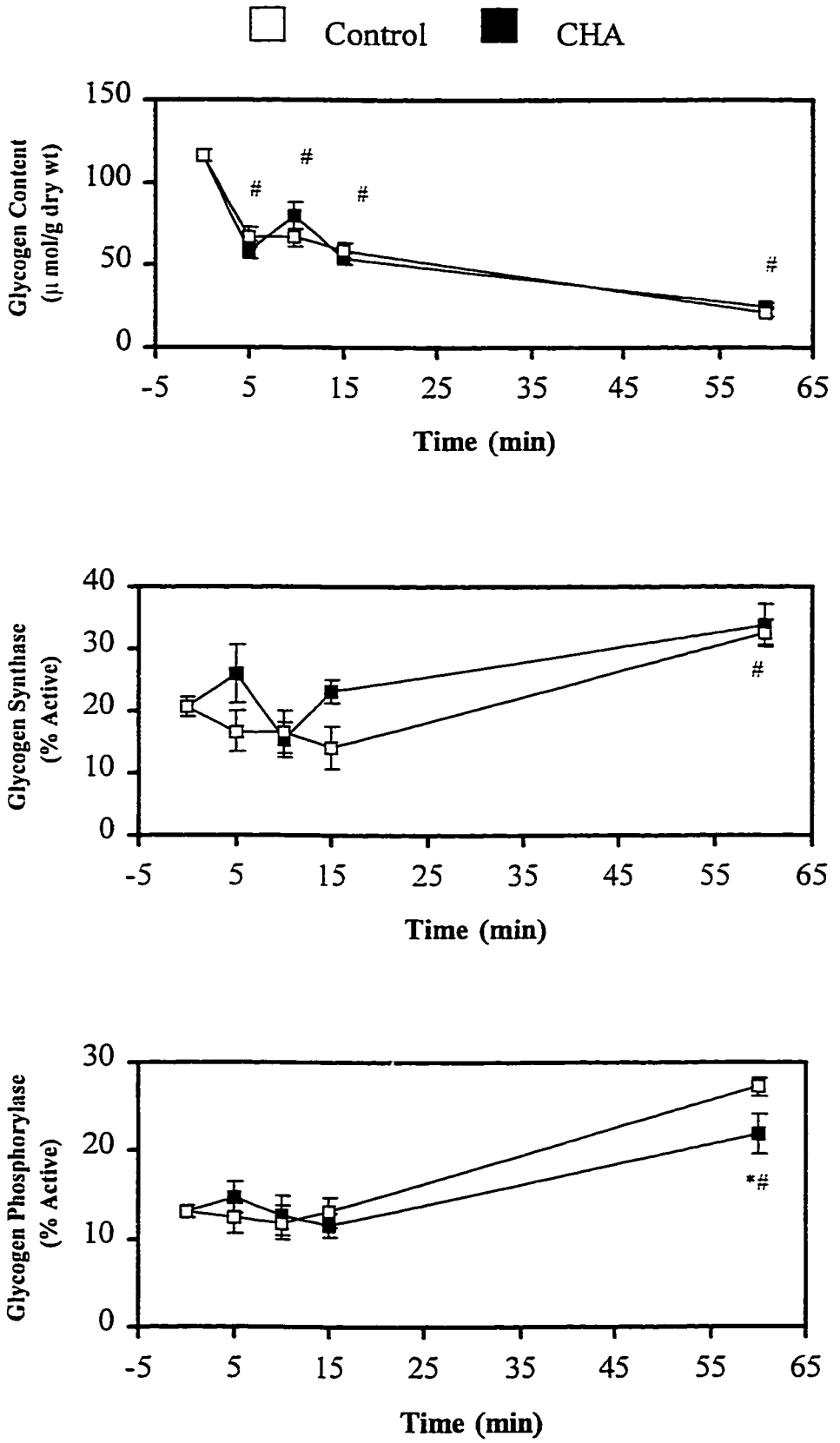


Fig. 5.1. *Protocol groups for measurement of time-dependent changes in glycogen and glucose metabolism.* White bars represent 60 min of aerobic perfusion, striped bars represent further aerobic perfusion and black bars represent low-flow ischemia (LFI). Hearts were frozen after graded times in low-flow ischemia (5, 10, 15 or 60 min) as indicated by the south facing arrows. N^6-Cyclohexyladenosine (CHA, 0.5 μM) and deoxynojirimycin (DNJ, 0.5 μM) were added 5 min before the onset of low-flow ischemia (indicated by D). N-Methyl-deoxynojirimycin (mDNJ, 100 μM) was added 30 min prior to ischemia (indicated by m). In all experiments, [^{3}H]glucose was added at the beginning of aerobic perfusion and [^{14}C]glucose was added before starting low-flow ischemia. Hearts were freeze-clamped at the end of each time-period with tongs cooled to the temperature of liquid nitrogen and stored at –80°C for later analysis.

Fig. 5.2. *Time-course of myocardial glycogen content, glycogen synthase activity and glycogen phosphorylase activity.* Determinations were made throughout low-flow ischemia in the absence (n=7-14) and presence (n=6-14) of 0.5 μM CHA. Separate groups of hearts were frozen at each time-point. Data are means ± S.E. for each time-point. * $P<0.05$ compared with untreated; # $P<0.05$ compared with T=0.


Control
CHA
Glycogen Content
(μ mol/g dry wt)
150
100
50
0
-5
5
15
25
35
45
55
65
Time (min)
Glycogen Synthase
(% Active)
40
30
20
10
0
Time (min)
Glycogen Phosphorylase
(% Active)
30
20
10
0
*#
Time (min)

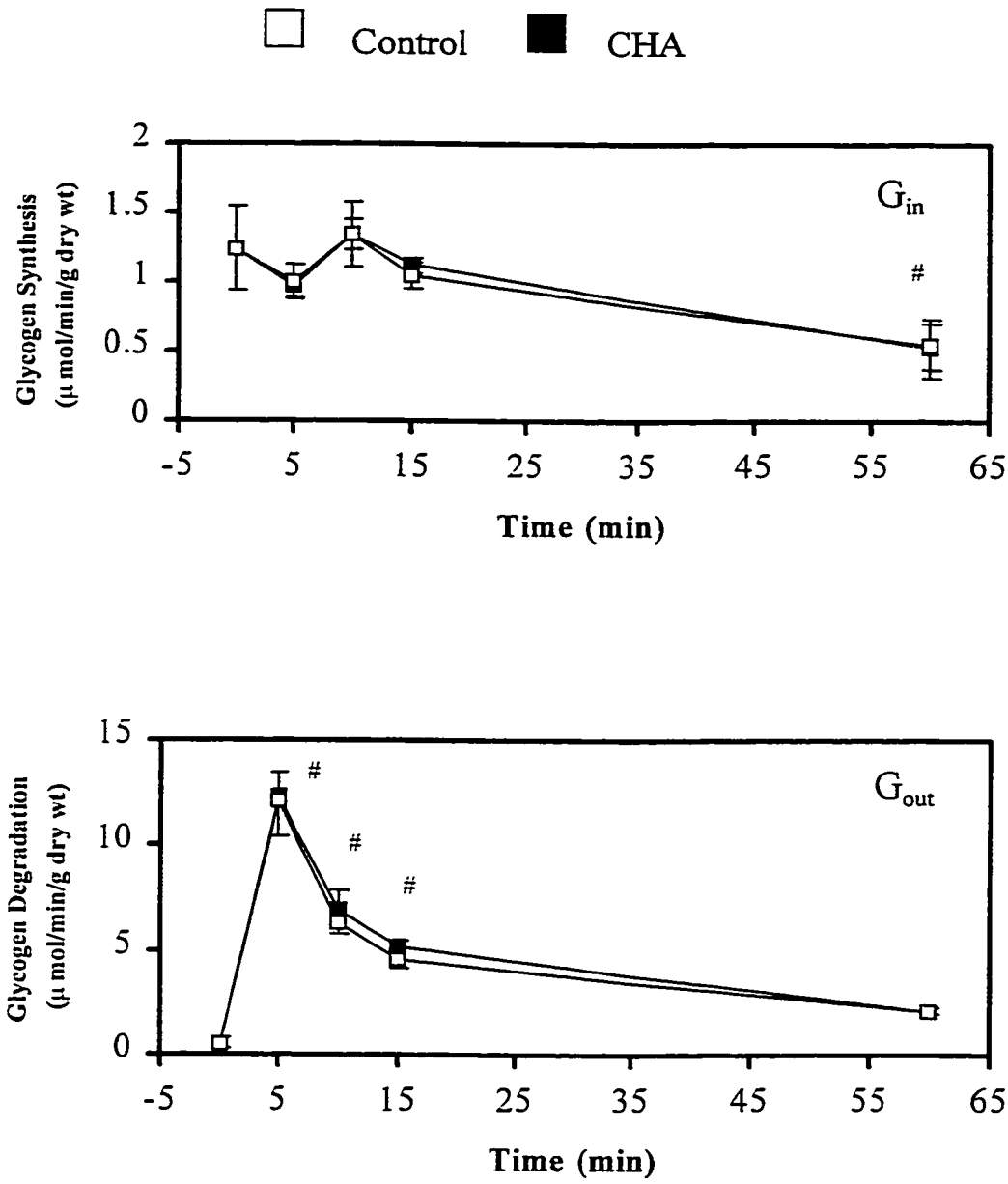


Fig. 5-3. *Time-course of glycogen turnover.* Values were measured during low-flow ischemia in the absence (n=7-14) and presence (n=6-14) of 0.5 μM CHA. Separate groups of hearts were frozen at each time-point. Data are means ± S.E. # $P<0.05$ compared with T=0.

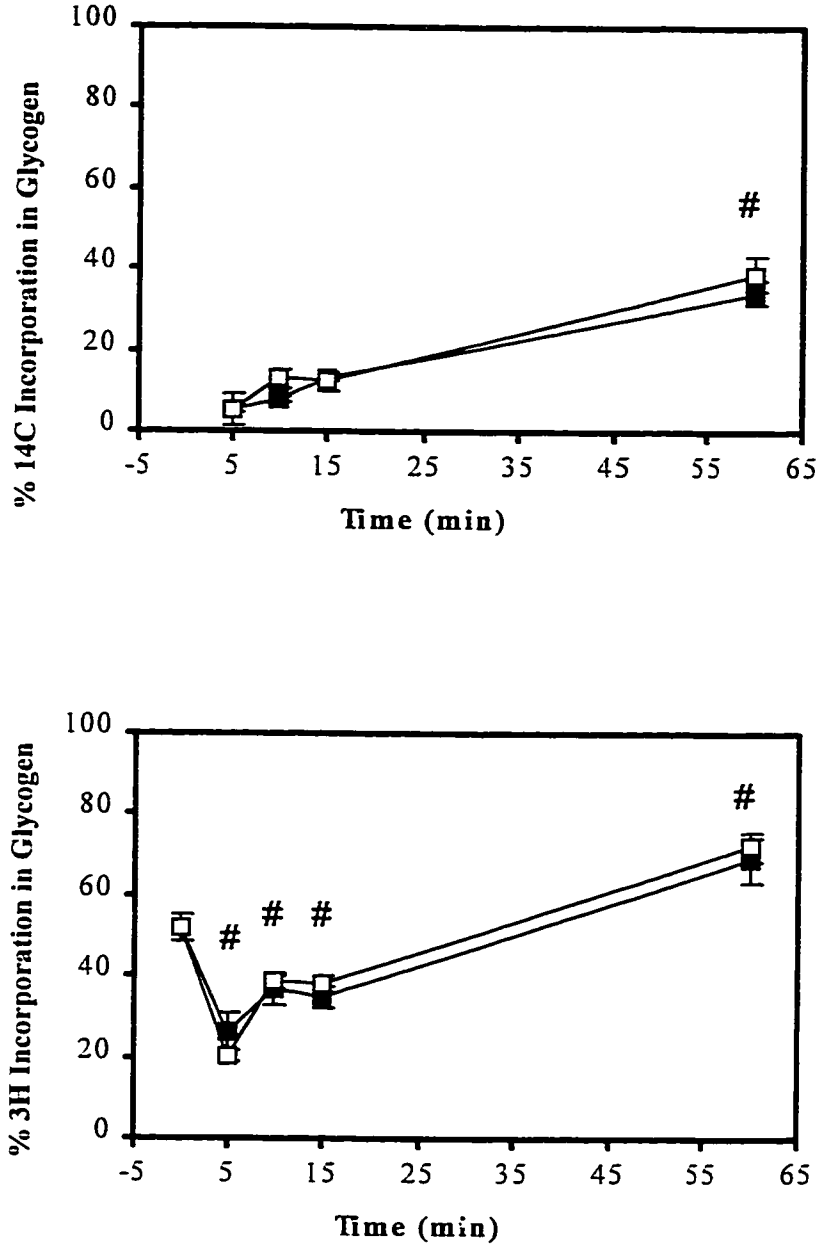


Fig. 5-4. *Time-course of radiolabel incorporation into the glycogen pool during low-flow ischemia in the absence and presence of 0.5 μM N^6-cyclohexyladenosine (CHA).* ^3H was present from the onset of working heart perfusion (t=0). ^{14}C was added just prior to the onset of low-flow ischemia. #P<0.05 compared with initial time-point.

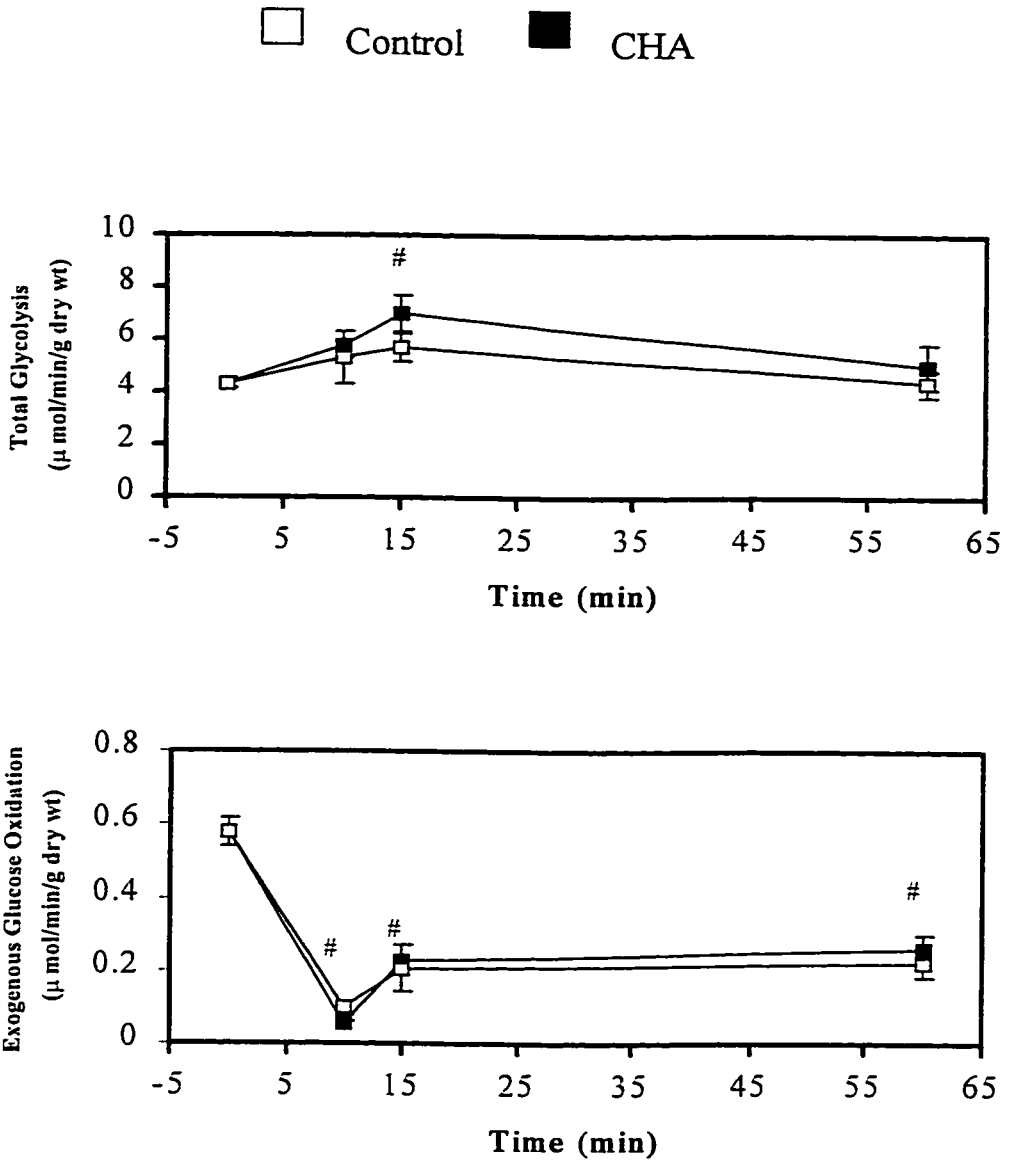


Fig. 5-5. *Time-course of total glycolysis and exogenous glucose oxidation in the absence and presence of 0.5 μM N^6-cyclohexyladenosine (CHA).* Measurements were taken during low-flow ischemia in the absence (n=7-14) and presence (n=6-14) of 0.5 μM CHA. Separate groups of hearts were frozen at each time-point. Data are means ± S.E. # $P<0.05$ compared with T=0.

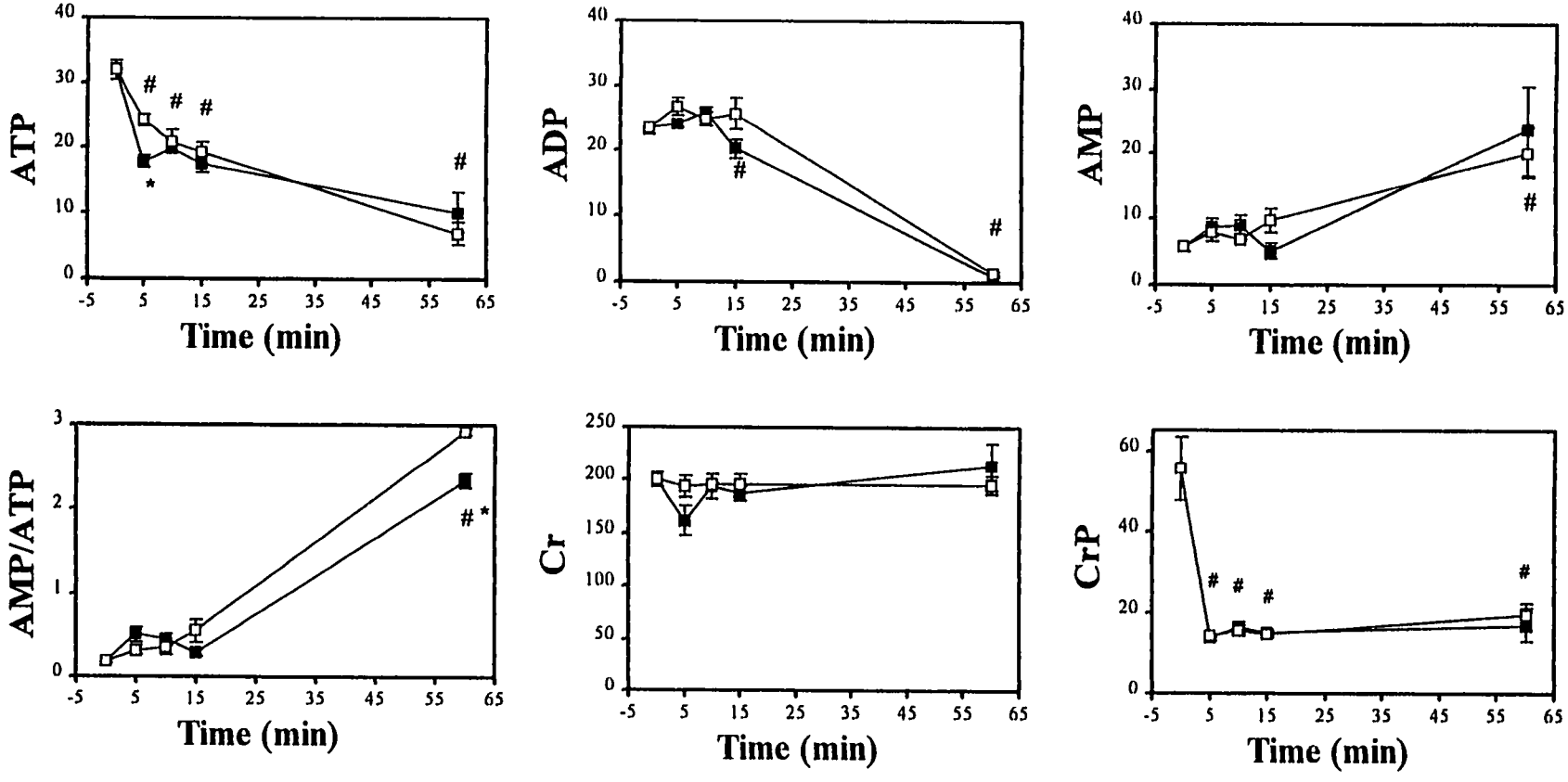


Fig. 5-6. *Time-course of high energy phosphate levels during low-flow ischemia in the absence and presence of 0.5 μM N^6-cyclohexyladenosine (CHA).* Data are means ± S.E. for 5-7 hearts per group. * P<0.05 compared with untreated; # P<0.05 compared with 0 min.

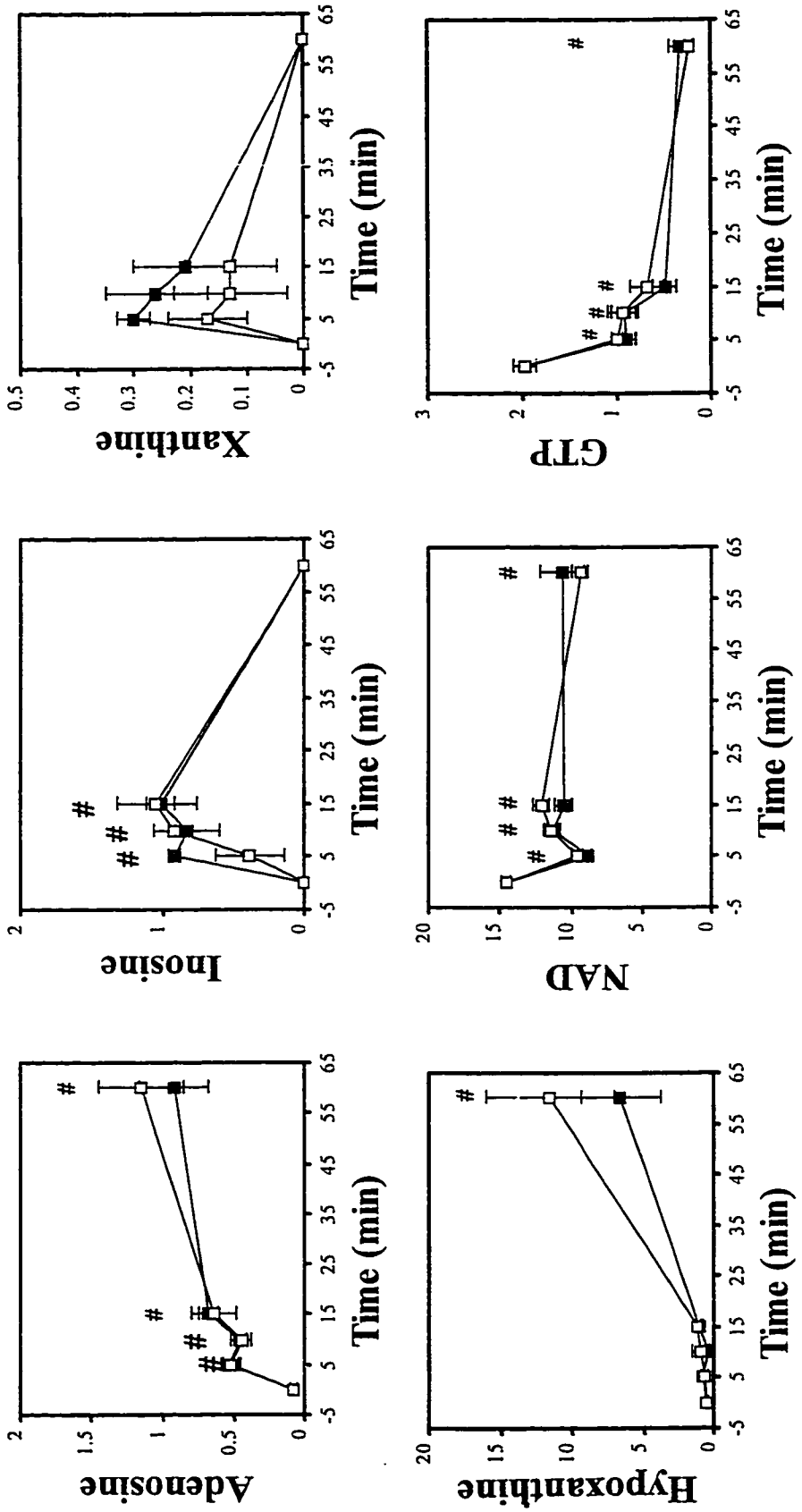


Fig. 5-7. *Time-course of cellular metabolite levels during low-flow ischemia in the absence and presence of 0.5 μM N6-cyclohexyladenosine (CHA).* Data are means ± S.E. for 5-7 hearts per group. # P<0.05 compared with 0 min.

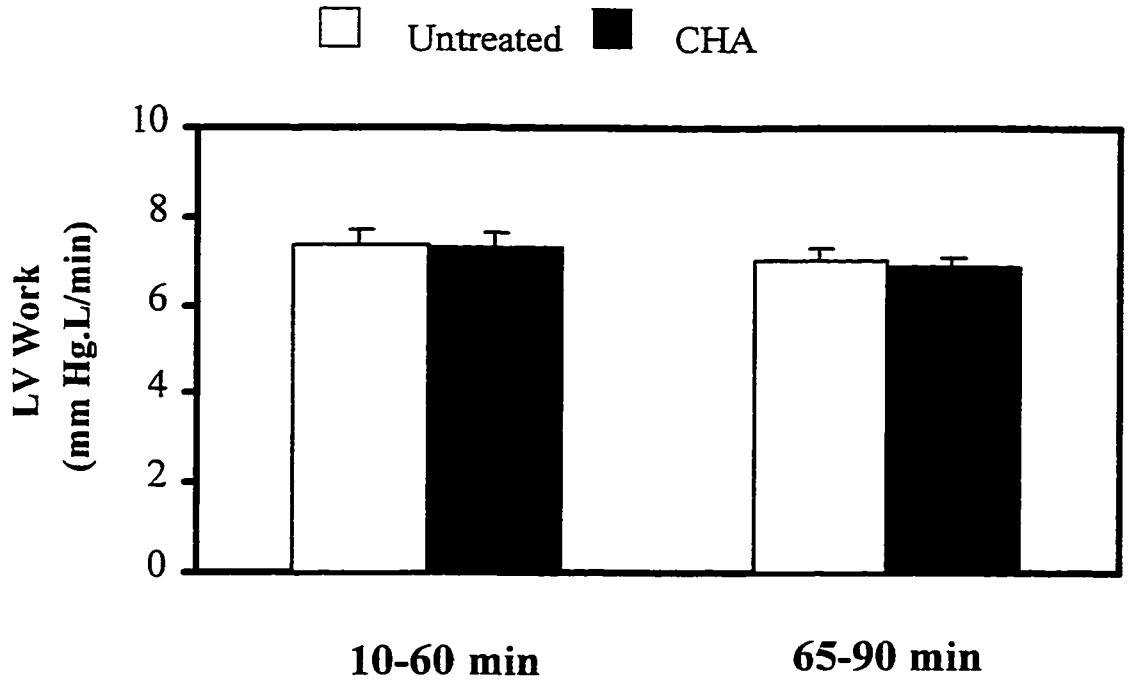
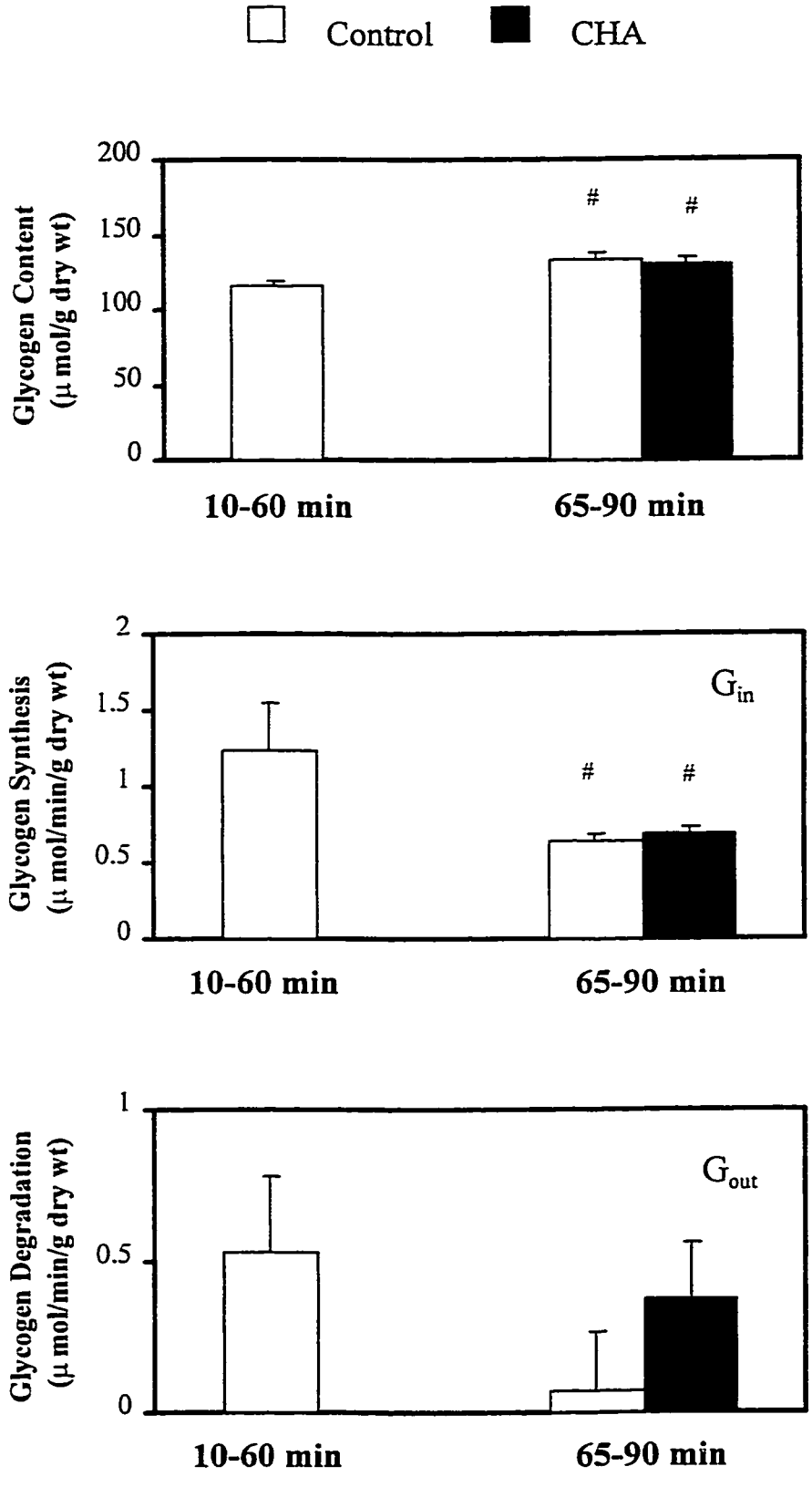


Fig. 5-8. *Steady state values for LV work during 90 min of prolonged aerobic perfusion.* Values were averaged during 60 min (10-60 min) of aerobic perfusion and during the subsequent 30 min of aerobic perfusion (65-90 min) in the absence (n=13) and presence (n=12) of 0.5 μM CHA. Groups of hearts were frozen after each perfusion phase. Data are means ± S.E.

Fig. 5-9. *Myocardial glycogen turnover during 90 min of prolonged aerobic perfusion.* Values are averaged during 60 min (10-60 min) of aerobic perfusion and during the subsequent 30 min of aerobic perfusion (65-90 min) in the absence (n=13) and presence (n=12) of 0.5 μM CHA. Data are means ± S.E. # $P<0.05$ compared with respective 60-min aerobic group.


Control
CHA
Glycogen Content
(μ mol/g dry wt)
200
150
100
50
0
#
#
10-60 min
65-90 min
Glycogen Synthesis
(μ mol/min/g dry wt)
2
1.5
1
0.5
0
G_{in}
#
#
10-60 min
65-90 min
Glycogen Degradation
(μ mol/min/g dry wt)
1
0.5
0
G_{out}
10-60 min
65-90 min

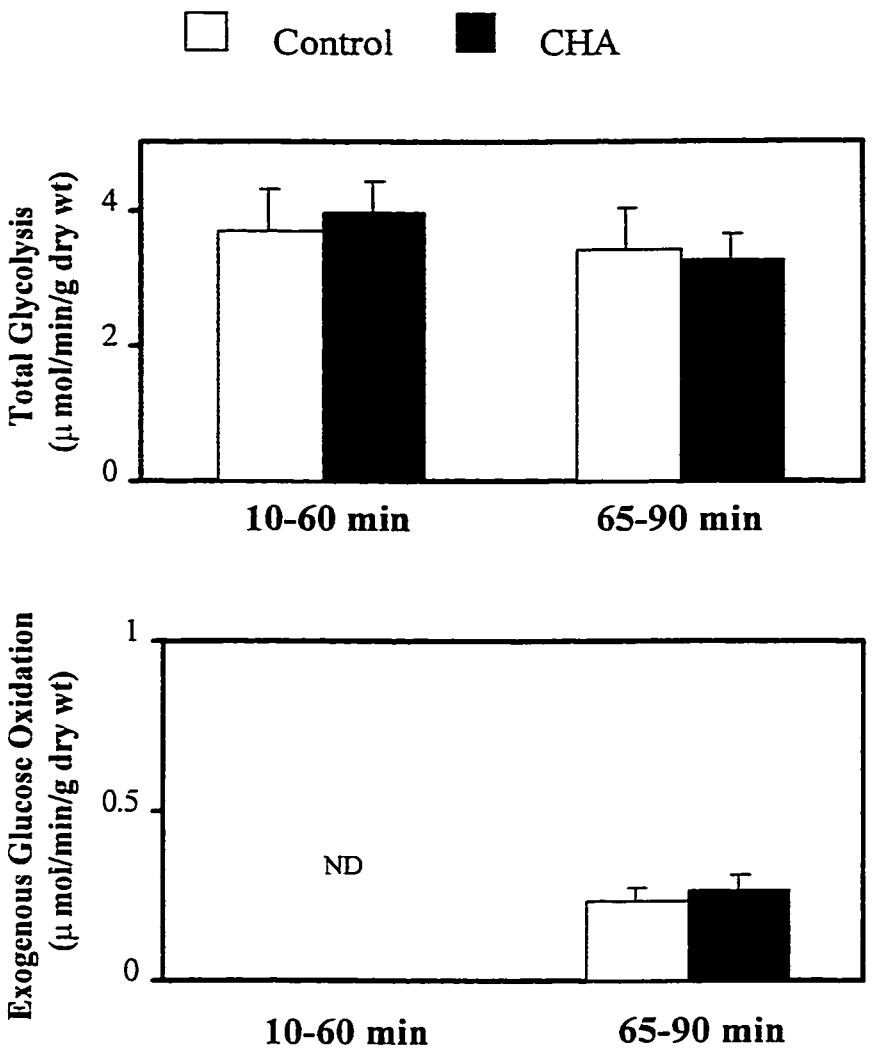


Fig. 5-10. *Steady state glucose metabolism during 90 min of prolonged aerobic perfusion.* Values are averaged during 60 min (10-60 min) of aerobic perfusion and during the subsequent 30 min of aerobic perfusion (65-90 min) in the absence and presence of 0.5 μM CHA. Values are shown for total glycolytic rates and rates of exogenous glucose oxidation; therefore, proton production could not be calculated in this series of hearts. Exogenous glucose oxidation was determined only during the 65-90 min perfusion period (ND means not determined). Data are means ± S.E.

CHAPTER 6

Chronic 17β–estradiol therapy causes critical alterations in myocardial glucose utilization[1,2]

[1] A version of this chapter has been submitted for publication. H. Fraser, S.T. Davidge and A.S. Clanachan. *The Journal of Molecular and Cellular Cardiology.*

[2] Serum 17β-estradiol levels were measured by Dr. Y. Zhang and pyruvate dehydrogenase activity was measured by B. Zielnick-Drabik.

6.1) Abstract

This study was designed to assess the effects of chronic therapy with 17β-estradiol on myocardial glucose utilization. Female rats were ovariectomized and pellets containing either 17β-estradiol (0.25 mg) or placebo were implanted subcutaneously. After 14 days, serum concentrations of 17β-estradiol were 100 ± 15 and 8 ± 2 pg/ml, (369 and 28 pM) respectively. Hearts were isolated and perfused aerobically in working mode with Krebs-Henseleit solution containing 1.2 mM palmitate, 11 mM [5-^{3}H/U-^{14}C]glucose, 2.5 mM Ca^{2-} and 100 μU/ml insulin. 17β–Estradiol decreased the rate of glycogen degradation by 69% and increased the rate of glucose oxidation by 40%. 17β-Estradiol did not affect rates of glycogen synthesis or glycolysis. 17β-Estradiol also increased left ventricular minute work by 11%. These data show that chronic 17β-estradiol elicits critical alterations in myocardial glucose metabolism. As stimulation of glucose oxidation and inhibition of glycogenolysis enhance recovery of post-ischemic hearts, these changes in glucose utilization may contribute to the cardioprotective effects of chronic estrogen therapy.

6.2) Introduction

Cardiovascular disease is a major cause of death of both men and women over the age of 60 years. However, at age 40 (prior to menopause) there is a 6-fold lower

incidence of death due to ischemic heart disease in females than in males (Furman, 1968). These data indicate premenopausal females possess an endogenous protective mechanism. Further, estrogen replacement therapy (ERT) has been shown to reduce the risk of cardiovascular disease in postmenopausal females (Stampfer & Colditz, 1991; Grady *et al.* 1992). The cardioprotective efficacy of 17β-estradiol is also observed as a reduction in myocardial necrosis after ischemia and reperfusion in rabbits (Hale *et al.* 1996) or as an improvement in mechanical function following global ischemia in isolated rat hearts (Kolodgie *et al.* 1997). The beneficial mechanisms proposed for the protective actions of estrogen include enhanced vascular smooth muscle relaxation (Williams *et al.* 1990), improved plasma lipid profile (Stevenson *et al.* 1993), reduced atheroma formation (Williams *et al.* 1990), and antioxidant activity (Delyani *et al.* 1996). In addition there is an, as yet, unidentified direct effect of estrogen on cardiac muscle as an improvement in mechanical function following global ischemia in isolated rat hearts has been observed independent of the aforementioned mechanisms (Kolodgie *et al.* 1997).

Energy substrate metabolism is an important determinant of myocardial mechanical function. Interestingly, estrogen-induced regulation of carbohydrate metabolism is demonstrable in a number of tissues. Effects include a stimulation of glucose uptake (rat uterus) (Smith, 1967) and activation of several gluconeogenic (rat liver) (Dahm, Jr. *et al.* 1978) and glycolytic enzymes (rat brain) (Bianchi *et al.* 1995). Increased glycogen deposition by estrogen has been observed in uterus, skeletal muscle, heart and liver (Carrington & Bailey, 1985). Moreover, estrogen inhibits exercise-induced glycogenolysis in liver and skeletal muscle (Rooney *et al.* 1993), improves exercise tolerance and prevents depletion of myocardial glycogen (Kendrick *et al.* 1987).

Consequently, estrogen may affect myocardial function through alterations in glucose metabolism.

The present study was designed to measure directly the effects of chronic treatment with 17β-estradiol on myocardial glycogen turnover, glucose utilization and mechanical function. Studies were performed in isolated working hearts obtained from ovariectomized female rats that had been treated for 14 days with either 17β-estradiol or placebo.

6.3) Methods

6.3.1) General Methodologies

6.3.1.1) Heart Perfusions

After 14 days of treatment, animals were anesthetized with pentobarbital and hearts were perfused as described in Chapter 2. After a 10-min equilibration period, hearts were either rapidly frozen (T=0) or perfused in paced (5 Hz) working mode (recirculating volume 100 ml) for 60 min and then frozen (T=60). Hearts were perfused at 37°C under aerobic conditions at a constant left atrial preload (11.5 mm Hg) and aortic afterload (80 mm Hg). A standard perfusate containing both glucose and fatty acid was used (see Chapter 2). Measures of flow, pressure and LV work were determined as described in Chapter 2.

6.3.1.2) Biochemical Determinations

Glycogen content (μmol glucosyl units/g dry wt) was determined in hearts that were rapidly freeze-clamped, either at the beginning or at the end of working perfusion (Chapter 2) and glycogen turnover was assessed by measuring the rates (μmol glucose/min/g dry wt) of glycogen synthesis and degradation as outlined in Chapter 3.

Activities of glycogen synthase and glycogen phosphorylase were determined from heart tissue frozen at the end of aerobic perfusion as outlined in Chapter 2.

Rates of glycolysis, glucose oxidation and proton production were determined directly using standard procedures described in Chapter 2. Rates were calculated from the linear time-course of product accumulation obtained between 10 and 60 min of perfusion.

Glucose uptake (μmol/min/g dry wt) and % glucose extraction were determined as described in Chapter 2.

6.3.2) Study-Specific Methodologies

6.3.2.1) Animal Treatments

Female Sprague Dawley rats (n=28, 275 to 300 g), that were treated according to standards of the Canadian Council of Animal Care, were anesthetized with Brietal® (50

mg/kg, intraperitoneally) and ovariectomized. Rats were then divided into two groups that were treated with either placebo (n=14) or 17β-estradiol (n=14) subcutaneously (17β-estradiol, 0.25 mg, and placebo pellets, 21-day release formulation; Innovative Research of America, Sarasota, Fl).

6.3.2.2) Heart Groups

Hearts from placebo- and 17β-estradiol-treated animals were further subdivided into a 10-min Langendorff-perfused group (T=0 min: placebo, n=7; 17β-estradiol, n=7) and a 60-min aerobic working perfusion group (T=60 min; placebo, n=7; 17β-estradiol, n=7). One heart was removed from the T=0, 17β-estradiol-treated group because subsequent analysis of the concentration (12 pg/ml) of serum 17β-estradiol and lack of increased uterine weight indicated a non-functioning pellet. A second 17β-estradiol-treated heart was removed from the T=60 perfusion group because mechanical function decreased by more than 20% over the 60-min period of perfusion. This resulted in 7 hearts in each for the two placebo groups and 6 in each of the two 17β-estradiol groups.

6.3.2.3) Pyruvate Dehydrogenase Activity

Total PDH activity (PDH_t) and that in the active dephosphorylated form (PDH_a) (μmol/min/g dry wt) were determined using a radioisotopic coupled enzyme assay (Constantin-Teodosiu *et al.* 1991) as modified by Collins-Nakai *et al* (Collins-Nakai *et*

al. 1994) (Chapter 2).

6.3.2.4) Validation of 17β-Estradiol Therapy

The concentrations of 17β-estradiol in serum samples collected at the time of heart removal were determined by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Body, heart and uterine weights were also determined at the time of death.

6.4) Results

6.4.1) Effect of 17β-Estradiol on Physiological Parameters and LV Work

The serum concentration of 17β-estradiol was 13-fold higher and uterine weight was 3-fold greater in rats treated with 17β-estradiol compared with placebo (Table 6-1). 17β-Estradiol also significantly inhibited body weight gain, had no measurable effect on heart weight and therefore increased heart weight to body weight ratio.

During working mode perfusion, LV work was significantly higher in hearts from the 17β-estradiol-treated group (Table 6-2). This was associated with an increased cardiac output and aortic flow compared with placebo-treated animals. The increased LV work in 17β-estradiol-treated hearts was not due to changes in coronary flow, aortic compliance, coronary vascular conductance or aortic pressures as these parameters were

similar in the two groups (Table 6-2).

6.4.2) Effect of 17β-Estradiol on Glycogen Turnover

During working mode perfusion, a difference in glycogen turnover between groups was demonstrable as evinced by values of the simultaneous rates of glycogen synthesis and degradation. In the placebo group, the rate of glycogen synthesis exceeded glycogen degradation by 1.8-fold. However, in the 17β-estradiol-treated group, the rate of glycogen synthesis exceeded glycogen breakdown by 5-fold due to a significant inhibition (by 69%) in the rate of glycogen degradation (Table 6-3). Rates of glycogen synthesis were similar in both groups (Table 6-3). As a result, glycogen content (μmol/g dry wt) increased during aerobic perfusion by only 32.4 ± 6.9 in the placebo group compared with 58.9 ± 9.4 in the 17β-estradiol-treated group (Table 6-3). Furthermore, the percentage of glycogen labeled, glucose uptake and glucose extraction were similar in hearts from 17β-estradiol-treated and placebo-treated groups (Table 6-3). Glycogen synthase and phosphorylase activities measured at the start of working mode perfusion were similar in both groups. However, after 60 min of perfusion, glycogen synthase activity was significantly higher in the 17β-estradiol-treated group (Fig 6-1). Glycogen phosphorylase activity was similar in both groups after 60 min but was significantly lower than at the start of working mode perfusion.

6.4.3) Effect of 17β-Estradiol on Glucose Metabolism

Rates of glucose oxidation were several-fold less than rates of glycolysis. 17β-Estradiol treatment stimulated the rate of glucose oxidation but had no effect on the rate of glycolysis or the calculated rate of proton production arising from glucose metabolism (Fig 6-2). Thus, relative to the placebo group, 17β-estradiol treatment increased the ratio of glucose oxidation to glycolysis from 0.17 ± 0.02 to 0.28 ± 0.05.

6.4.4) Effect of 17β-Estradiol on Pyruvate Dehydrogenase Activity

Activity of the rate-limiting enzyme in glucose oxidation, PDH, was not different between placebo- and 17β-estradiol-treated groups (Table 6-3) suggesting that the increase in the rate of glucose oxidation is controlled by another mechanism.

6.5) Summary

1. The objective of this study was to determine whether alterations in myocardial glucose metabolism may contribute to the beneficial direct cardioprotective actions of estrogen. This study evaluated the effects of chronic 17β-estradiol therapy on glycogen turnover, glucose utilization and cardiac function.
2. The data indicate that chronic 17β-estradiol elicits two important alterations in carbohydrate metabolism that comprise a marked inhibition of glycogen

degradation and a stimulation of glucose oxidation. Each of these effects has been shown previously to increase the recovery of mechanical function of the post-ischemic heart.

3. Increased rates of glucose oxidation were not due to either activation of PDH or increased glucose uptake in the 17β-estradiol group.

Table 6-1. *Characteristics of placebo- and 17β-estradiol-treated groups of rats*

	Placebo (n=14)	**17β-Estradiol (n=12)**
Serum 17β-Estradiol (pg/ml)	7.7 ± 1.9	100.5 ± 14.6*#
Uterine Weight (g)	0.21 ± 0.01	0.69 ± 0.06*
Heart Weight (g)	1.41 ± 0.04	1.36 ± 0.03
Body Weight (g)	332.5 ± 5.0	285.6 ± 5.1*
Heart Weight:Body Weight	4.21 ± 0.07	4.68 ± 0.05*

Data represent means ± S.E. * $P<0.05$ compared with placebo. # Variances were significantly different, consequently a nonparametric test was used.

Table 6-2. *Hemodynamic measurements during 60 min of aerobic perfusion of hearts from placebo- and 17β-estradiol-treated rats.*

	Placebo (n=7)	**17β-Estradiol (n=6)**
Systolic Pressure (mm Hg)	120.8 ± 0.9	121.8 ± 1.4
Developed Pressure (mm Hg)	50.6 ± 1.2	52.6 ± 1.4
LV Work (mm Hg · L/min)	6.54 ± 0.27	7.33 ± 0.15*
Cardiac Output (ml/min)	59.9 ± 2.4	66.4 ± 0.7*
Aortic Flow (ml/min)	37.5 ± 2.2	45.4 ± 1.4*
Coronary Flow (ml/min)	22.4 ± 0.6	21.0 ± 0.9
Coronary Vascular Conductance (ml/min/mm Hg)	0.26 ± 0.01	0.24 ± 0.01
Aortic Compliance (mm Hg/ml)	553 ± 22	499 ± 5

Data represent means ± S.E. Hemodynamic measurements were averaged between 10 and 60 min of aerobic perfusion in placebo- and 17β-estradiol-treated animals. * $P<0.05$ compared with placebo.

Table 6-3. *Average rates of glycogen and glucose metabolism in aerobic-perfused hearts from placebo- and 17β-estradiol-treated rats.*

	Placebo (n=7)	**17β-Estradiol (n=6)**
Glycogen Synthesis (μmol/min/g dry wt)	1.24 ± 0.12	1.11 ± 0.12
Glycogen Degradation (μmol/min/g dry wt)	0.70 ± 0.07	0.22 ± 0.16*
Change in Glycogen Content (μmol/g dry wt)	32.4 ± 6.9	58.9 ± 9.4*
Glycogen Labeling (%)	40.6 ± 6.0	44.8 ± 2.7
Glucose Uptake (μmol/min/g dry wt)	3.63 ± 0.24	3.81 ± 0.49
Glucose Extraction (%)	0.33 ± 0.02	0.37 ± 0.05
Pyruvate Dehydrogenase Activity (% active)	13.7 ± 0.8	12.3 ± 0.2

Data represent means ± S.E. Measurements were determined after aerobic perfusion in placebo- and 17β-estradiol-treated hearts. * $P<0.05$ compared with placebo.

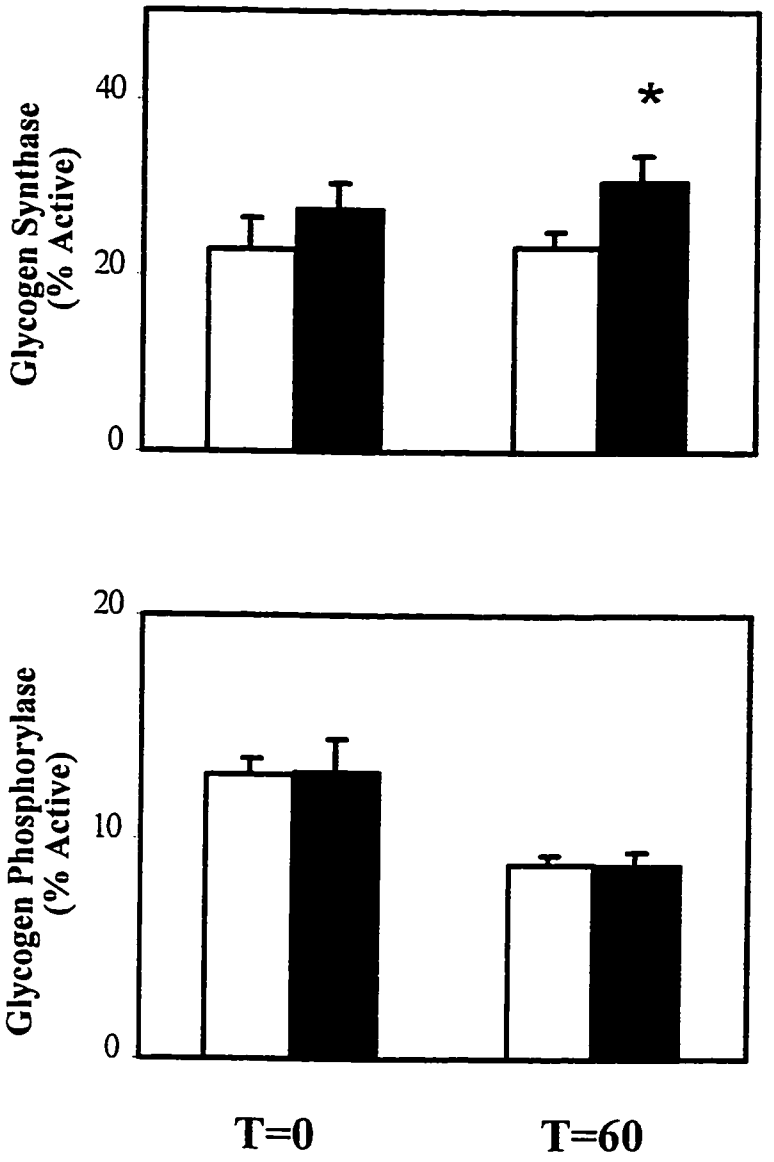


Fig. 6-1. *Myocardial glycogen synthase and glycogen phosphorylase activity in hearts from placebo- and 17β-estradiol-treated rats after aerobic perfusion.* Data are means ± S.E. of values measured before (T=0) and after 60 min of aerobic perfusion (T=60) for 7 placebo- (□) and 6 17β-estradiol-treated (■) hearts. * $P<0.05$ compared with placebo group. # $P<0.05$ compared with respective T=0 group.

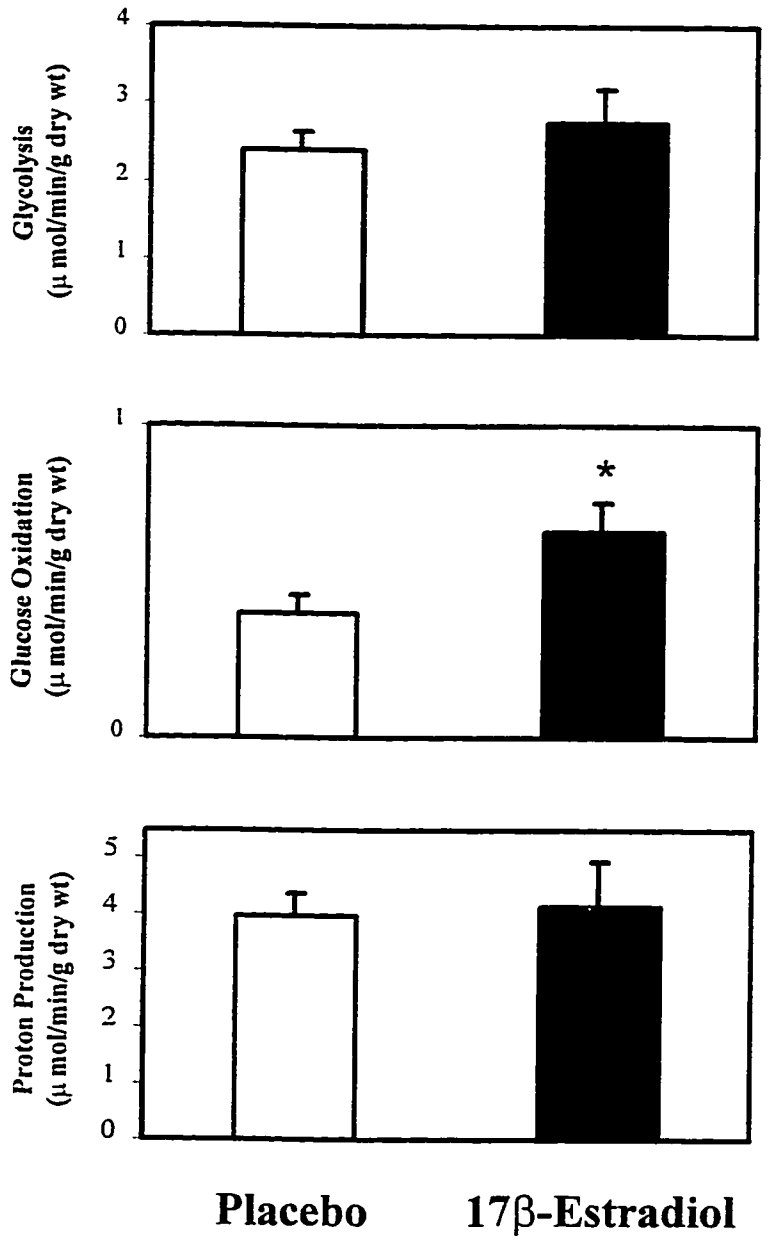


Fig. 6-2. *Rates of glycolysis, glucose oxidation and calculated proton production from glucose metabolism in hearts from placebo- and 17β-estradiol-treated rats during aerobic perfusion.* Data are means ± S.E. of values averaged between 10 and 60 min of aerobic perfusion for 7 placebo- (□) and 6 17β-estradiol-treated (■) hearts. * $P< 0.05$ compared with placebo.

CHAPTER 7

Cardioprotection by chronic 17β-estradiol therapy: enhanced NO biosignaling in the post-ischemic rat heart[1,2]

[1] A version of this chapter is currently in preparation. H. Fraser, S.T. Davidge and A.S. Clanachan.
[2] Measurements of high energy phosphates and their metabolites were performed by K. Strynadka, PDH and NOS activity were determined by B. Zielnick-Drabik

7.1) Abstract

The risk of cardiovascular disease in postmenopausal females is lowered by estrogen replacement therapy but the precise mechanisms are still unknown. This study determined whether the cardioprotective effects of estrogen might involve alterations in either myocardial glucose metabolism and/or nitric oxide (NO) biosignaling. Female rats were ovariectomized and half were implanted subcutaneously with 17β-estradiol (0.25 mg, 21-day release formula) for 14 days. Isolated hearts were perfused aerobically for 60 min, and then subjected to low-flow ischemia for 60 min followed by aerobic reperfusion for 30 min. Chronic treatment with 17β-estradiol significantly improved recovery of post-ischemic mechanical function. This occurred in association with increased glycogen content and stimulated glycogen synthase activity compared with hearts from untreated rats. Glucose oxidation was higher in hearts from 17β-estradiol-treated rats during aerobic perfusion and reperfusion, an observation often associated with improvement in metabolic coupling of glycolysis to glucose oxidation. 17β-Estradiol treatment, however, did not alter rates of glycolysis and proton production, suggesting that the observed improvement in post-ischemic mechanical function was not related to improved metabolic coupling of glycolysis to glucose oxidation. After 30 min of reperfusion, hearts from 17β-estradiol-treated rats had elevated Ca^{2+}-independent NOS activity and higher cGMP content compared with hearts from untreated rats. As NO donors have been shown to be cardioprotective, these data suggesting that induction of NOS and enhanced NO biosignaling may contribute to the cardioprotective efficacy of 17β-

estradiol.

7.2) Introduction

Heart disease is the primary cause of death in both men and women over the age of 60 in Canada. While pre-menopausal women have a low incidence of cardiovascular disease compared with men, after menopause mortality rates are similar in men and women (Furman, 1968). Studies describing improved cholesterol profiles (Godsland *et al.* 1987), decreased insulin resistance and enhanced glucose tolerance (Bailey & Ahmed-Sorour, 1980) with estrogen replacement therapy (ERT) in menopausal women have provided more direct evidence that estrogen is protective against coronary heart disease. Estrogen also stops the progression of atherosclerosis in cynomolgus monkeys (Williams *et al.* 1990) and increases blood flow in many vessels including the carotid and coronary arteries (Killam *et al.* 1973; Volterrani *et al.* 1995). In addition, 17β-estradiol also reduces myocardial necrosis in rabbits after ischemia and reperfusion (Hale *et al.* 1996) and improves recovery of mechanical function following global ischemia in isolated rat hearts (Kolodgie *et al.* 1997).

Although the mechanism of the beneficial effects of 17β-estradiol in the heart has not yet been determined, considerable evidence indicates that cardioprotection may arise from interventions that optimize energy substrate utilization, both during and following ischemia. Glycogen metabolism appears to be altered in many organs by estrogen. For instance, estrogen therapy prevents exercise-induced glycogenolysis in liver and skeletal

muscle (Rooney *et al.* 1993), increases exercise tolerance, spares myocardial glycogen (Kendrick *et al.* 1987) and increases glycogen deposition in liver, uterus, heart and skeletal muscle (Carrington & Bailey, 1985). The consequences of ERT-induced alterations in glycogen metabolism may be more significant than previously considered. Glycogen is an important source of energy during brief periods of ischemia (Taegtmeyer *et al.* 1997; Chapter 3), and the preferential oxidation of glucose released from glycogen may limit the extent of tissue acidosis, which is believed to lead to Ca^{2-} overload and poor recovery of post-ischemic mechanical function (Tani & Neely, 1989; Goodwin *et al.* 1996a; Chapter 3). Glycogen turnover, which is the simultaneous synthesis and degradation of glycogen, may play a critical role in cardioprotection. Previous experiments investigating the effects of chronic 17β-estradiol treatment on glucose metabolism during aerobic perfusion discovered that glycogen degradation is significantly reduced in hearts from 17β-estradiol-treated rats (Chapter 6). This has the potential of allowing glycogen accumulation and hence increased substrate availability during ischemia and reperfusion and thus may contribute to improved recovery of post-ischemic mechanical function. Furthermore, the detailed study of glycogen turnover indicated that the cardioprotective adenosine A_1-receptor agonist, N^6-cyclohexyladenosine, improves recovery of post-ischemic mechanical function in conjunction with increasing rates of glycogen synthesis during reperfusion (Chapter 4), further suggesting the importance of glycogen in cardioprotection. No information exists regarding the role of glycogen in the cardioprotective actions of estrogen.

Energy substrate preference is an important determinant of the recovery of post-

ischemic myocardial function. Interestingly, 17β-estradiol alters glucose metabolism in a number of tissues. For example, glycolytic enzymes including hexokinase, phosphofructokinase and pyruvate kinase are induced in the brain (Kostanyan & Nazaryan, 1992), and glucose transport is stimulated in the uterus (Smith & Gorski, 1968) with 17β-estradiol treatment. Consequently, the cardioprotective efficacy of ERT may involve alterations in glucose metabolism. During ischemia, ATP production from glycolysis is important for maintaining the energy status of the cell. However, when ischemia is severe, hydrolysis of glycolytically derived ATP leads to glycolytic end-product accumulation. This metabolic uncoupling of rates of glycolysis from glucose oxidation leads to excessive proton production, which is detrimental to post-ischemic myocardial mechanical function (Finegan *et al.* 1995; Liu *et al.* 1996). In a previous study, we determined that rates of glucose oxidation are increased in 17β-estradiol-treated, ovariectomized rats during aerobic perfusion (Chapter 6), suggesting a potential for improvement in metabolic coupling during ischemia and reperfusion. This study directly addresses whether improvement in metabolic coupling during reperfusion is associated with cardioprotection with chronic 17β-estradiol treatment.

Nitric oxide (NO) may also be involved in 17β-estradiol-induced cardioprotection. Many physiological and pharmacological actions have been attributed to NO, some of which may be beneficial to cardiac function such as the stimulation of soluble guanylyl cyclase (sGC) leading to elevation of cGMP and coronary smooth muscle dilation (Smith *et al.* 1996), and enhanced ventricular relaxation (Paulus *et al.* 1994; Shah *et al.* 1995). Other effects that occur in response to higher concentrations of

NO may be detrimental and include inhibition of Ca^{2+} entry during cardiac failure (Campbell *et al.* 1996) or generation of hydroxyl radicals via the formation of peroxynitrite (Schulz *et al.* 1992; Yasmin *et al.* 1997). NO-mediated biosignaling has been implicated in ischemia reperfusion injury (Ma *et al.* 1993; Lefer, 1995) and enhancement of NO generation reduces post-ischemic dysfunction (Pinsky *et al.* 1994; Lefer & Lefer, 1996; Zhao *et al.* 1997). The cardioprotective efficacy of NO donors (Ali *et al.* 1998; Du Toit *et al.* 1998) provides direct support for the notion that an enhancement of NO biosignaling, possibly via a myocardial cGMP mechanism, is beneficial for the ischemic heart. Interestingly, elevation of cGMP content has been shown to influence favorably myocardial energy substrate metabolism by reducing indirect indices of myocardial glycolysis (Metsa-Ketela *et al.* 1981; Laustiola *et al.* 1983) that in turn may improve the coupling between glycolysis and glucose oxidation and attenuate proton production and acidosis (Finegan *et al.* 1996a). In addition, monophosphoryl lipid A, an endotoxin derivative that causes low-level induction of either iNOS or eNOS, is also protective in rabbit and canine models of ischemia and reperfusion by reducing myocardial infarct size by 50 to 70% (Elliott *et al.* 1996; Mei *et al.* 1996; Przyklenk *et al.* 1996) and to improve the recovery of mechanical function of post-ischemic hearts. Consequently, cardioprotection may result from a correction in the deficiency in NO biosignaling in the post-ischemic heart.

Of particular interest to the present study is the observation that estrogen may enhance NOS expression and enzyme activity and so elevate endogenous NO production. Estrogen receptors exist on cardiac myocytes and when stimulated induce eNOS (Kleinert *et al.* 1998) and iNOS (Grohe *et al.* 1997) gene expression. Females possess an enhanced

capacity to produce NO that reduces vascular smooth muscle tone (Kauser & Rubanyi, 1994). The role of myocardial NO biosignaling in direct cardioprotective actions of 17β-estradiol has not yet been addressed.

Thus, we hypothesized that estrogen may exert its cardioprotective effects by altering glucose metabolism and/or NO-mediated biosignaling. The present study investigated the effects of chronic 17β-estradiol treatment (0.25 mg pellet, 14 d) in ovariectomized female Sprague-Dawley rats on post-ischemic mechanical function, glucose metabolism and NO signaling.

7.3) Methods

7.3.1) General Methodologies

7.3.1.1) Animal Groups

Female Sprague Dawley rats (275 to 300 g) were anesthetized and ovariectomized as described in Chapter 6. Rats were then divided into two groups: untreated (n=16) or 17β-estradiol-treated (n=14). 17β-Estradiol-treated rats received a time-release pellet subcutaneously for 14 days (17-β estradiol, 0.25 mg, 21-d release formula; Innovative Research of America, Sarasota, Fl).

7.3.1.2) Heart Perfusions

Heart perfusions were essentially the same as those described in Chapter 6 with the exceptions noted under Study-Specific Methodologies (Section 7.3.2.1).

7.3.1.3) Validation of 17β-Estradiol Therapy

The concentration of serum 17β-estradiol and uterine and heart weights were determined as described in Chapter 6.

7.3.1.4) Biochemical Determinations

Rates of glycolysis, glucose oxidation and proton production were determined directly using standard procedures as previously described in Chapter 2. Glycogen content, glycogen synthase activity and glycogen phosphorylase activity were determined at the end of reperfusion following the methods described in Chapter 2. Adenosine, ATP, AMP, creatine (Cr), creatine phosphate (CrP) and GTP were determined in neutralized, 6% perchloric acid extractions (Chapter 2). Total PDH activity (PDH_t) and that in the active dephosphorylated form (PDH_a) were determined using the radioisotopic coupled enzyme assay described in Chapter 2.

7.3.2) Study-Specific Methodologies

7.3.2.1) Heart Perfusions

Two series of perfusions were performed. Hearts from half the treated and untreated groups were paced (5 Hz) throughout the perfusion protocol. The remainder of the hearts in each group were paced only through the first 20 min of low-flow ischemia. These two series of heart perfusions were performed in order to investigate the mechanism of increased rates of glucose oxidation. Ongoing studies in our lab recognize the difficulties associated with measuring pyruvate dehydrogenase (PDH, rate-limiting enzyme in glucose oxidation) activity in paced hearts. Therefore PDH activity was assessed in an unpaced group of hearts as well as the paced group of hearts. Since left ventricular minute work (LV work) was similar in hearts from the two series of perfusions (Table 7-1), they were grouped for data analysis.

A additional series of hearts were perfused in order to determine the acute effect of 17β-estradiol on post-ischemic mechanical function. In this series, hearts from female rats that had been ovariectomized for one week (n=6) were perfused under identical conditions to those described for the chronic-treated animals with the exception that the perfusate was devoid of radiolabeled glucose. In these perfusions, 17β-estradiol (369 pM, water soluble) was added to the perfusate 30 min prior to the onset of low-flow ischemia. The concentration of 17β-estradiol was chosen to be the same as that observed in serum from female rats following chronic treatment with 17β-estradiol (Chapter 6).

7.3.2.2) Serum Lipid Levels

Serum free fatty acids (Wako Chemicals, Richmond, VA) and triglycerides (Boehringer Mannheim, Laval, PQ) were measured using standard spectrophotometric kits.

7.3.2.3) NOS Activity (Citrulline Assay) and Expression (Western Blot) and cGMP Content

Myocardial NOS activities and expression and cGMP content were determined from frozen heart tissue obtained at the end of reperfusion following the methods described in Chapter 2.

7.4) Results

7.4.1) Validation of 17β-Estradiol Therapy

Successful 17β-estradiol treatment was confirmed by significant increases in serum 17β-estradiol levels (175.5 ± 16.1 vs 3.02 ± 0.04 pg/ml; equivalent to 644 and 11 pM, respectively) and uterine weight (4-fold higher) (Table 7-2). Hearts would have been excluded from analysis if their respective serum 17β-estradiol levels were below 12

pg/ml and low uterine weight, an indication that the pellet did not dissolve. No hearts had to be removed from this study due to inactive pellets. In addition, although heart weight was not different, 17β-estradiol-treated rats had significantly lower body weights, and thus, greater, heart weight to body weight ratios (Table 7-2). Serum free fatty acid and triglyceride levels were also significantly elevated (2-fold and 3-fold, respectively) in 17β-estradiol-treated rats compared with untreated rats (Table 7-2).

7.4.2) Mechanical Function of Paced and Unpaced Hearts

Two series of hearts were perfused. In the first, heart rate was controlled by pacing, while in the second group, hearts were allowed to beat spontaneously. Under aerobic conditions, spontaneous heart rate was 257 ± 10 and 245 ± 10 for untreated and 17β-estradiol-treated, respectively, compared with 300 for the paced groups. However, LV work was not significantly different between these two series of hearts (Table 7-1). Furthermore, no correlation existed between heart rate and recovery of post-ischemic function (data not shown). Therefore, the two series were grouped together for further analysis.

Cardioprotection in 17β-estradiol-treated hearts was evinced by significantly higher recovery of LV work (2-fold) during reperfusion following 60 min of low-flow ischemia (Table 7-3). The time-courses of recovery of LV work showed higher and more stable values during the 30-min reperfusion period in hearts from 17β-estradiol-treated rats (Fig. 7-1). In conjunction with improved recovery of LV work, 17β-estradiol

significantly improved recovery of cardiac output (1.7-fold) and coronary flow (1.6-fold).

Acute administration of 17β-estradiol (100 pg/ml or 369 pM) had no effect on mechanical function either during aerobic or reperfusion conditions (data not shown).

7.4.3) Effect of 17β-Estradiol on Myocardial Metabolism

The rate of glucose oxidation was significantly reduced during low-flow ischemia and returned to pre-ischemic values during reperfusion. 17β-Estradiol treatment elevated rates of glucose oxidation during aerobic perfusion (35%) as well as during reperfusion (28%) compared with rates from untreated hearts, but had no effect during low-flow ischemia (Fig. 7-2).

During low-flow ischemia, there was a significant increase in rate of glycolysis only in the estradiol-treated group. However, comparison of the rates of glycolysis between treatments during low-flow ischemia indicated that there was no treatment effect (Fig. 7-2).

The calculated rates of proton production from the uncoupling of rates of glycolysis from glucose oxidation were similar among the three phases of perfusion in hearts from untreated rats (Fig. 7-2). Proton production increased during low-flow ischemia in the presence of 17β-estradiol compared with its respective value during aerobic perfusion. Proton production returned to pre-ischemic levels during reperfusion.

7.4.4) Effect of 17β-Estradiol on Myocardial Glycogen Metabolism

17β-Estradiol treatment significantly increased myocardial glycogen content (1.6-fold) in reperfused hearts in association with increased glycogen synthase activity (1.3-fold), but had no effect on glycogen phosphorylase activity (Fig. 7-3).

7.4.5) Effect of 17β-Estradiol on Myocardial Nucleotide Levels

17β-Estradiol treatment had no effect on myocardial nucleotide levels (ATP, AMP, ADP and adenosine) measured at the end of reperfusion even though improved recovery of mechanical function was observed. 17β-Estradiol also did not alter myocardial content of Cr and CrP and GTP measured at the end of reperfusion (Table 7-4).

7.4.6) Effect of 17β-Estradiol on Myocardial Pyruvate Dehydrogenase Activity

Pyruvate dehydrogenase (PDH) activity was investigated in order to determine the mechanism of action for the increase in glucose oxidation in 17β-estradiol-treated hearts. PDH activity was not different between the two groups at the end of reperfusion (Table 7-5). Furthermore, PDH activity was not different in paced vs unpaced hearts (data not shown).

7.4.7) Effect of 17β-Estradiol on Nitric Oxide Synthase Activity and cGMP Content

To determine whether changes in NO biosignaling were involved in the improved recovery of post-ischemic mechanical function, Ca^{2+}-dependent NOS and Ca^{2+}-independent NOS activities were determined by citrulline assays. At the end of 60-min of aerobic perfusion, Ca^{2+}-dependent NOS activity exceeded Ca^{2+}-independent NOS activity by 3.3-fold and 2.7-fold in hearts from untreated and 17β-estradiol-treated rats, respectively (Fig. 7-4). Neither Ca^{2+}-dependent NOS nor Ca^{2+}-independent NOS activity was altered in hearts from 17β-estradiol-treated rats during aerobic perfusion. Ca^{2+}-dependent NOS activity was also similar between 17β-estradiol and untreated groups at the end of reperfusion. However, 17β-estradiol treatment increased Ca^{2+}-independent NOS activity 3.5-fold compared with untreated hearts (Fig. 7-5). Furthermore, 17β-estradiol also significantly increased cGMP content of reperfused hearts (Fig. 7-5), whereas cGMP content was similar in aerobically perfused hearts from placebo- and 17β-estradiol-treated animals after aerobic perfusion.

7.4.8) Effect of 17β-Estradiol on NOS Expression

Western blotting for NOS protein showed no change in eNOS protein content after 60 min of aerobic perfusion (Fig. 7-6). iNOS protein was not detectable in 90 μg samples of total myocardial protein even after 30 min of development, the point when

background became significant and bands were difficult to differentiate (data not shown).

7.5) Summary

1. The objective of this study was to investigate potential mechanisms of 17β-estradiol-induced cardioprotection. We hypothesized that 17β-estradiol may alter glucose metabolism and/or NO biosignaling.
2. Chronic 17β-estradiol treatment was cardioprotective and increased 2-fold the recovery of post-ischemic mechanical function compared with untreated hearts.
3. Hearts from 17β-estradiol-treated rats had higher glycogen contents and glycogen synthase activity after reperfusion.
4. Although 17β-estradiol treatment increased rates of glucose oxidation during aerobic perfusion and reperfusion, it had no effect on rates of glycolysis or proton production. Thus, it is unlikely that improved metabolic coupling of glycolysis and glucose oxidation during reperfusion is responsible for 17β-estradiol-induced cardioprotection. During ischemia, 17β-estradiol treatment stimulated glycolysis.
5. Ca^{2+}-independent NOS (iNOS) activity determined after post-ischemic reperfusion was elevated in hearts from 17β-estradiol-treated animals. Induction of iNOS occurred as a result of 17β-estradiol treatment in combination with ischemia as values of iNOS activity obtained prior to

ischemia were not elevated. Ca^{2+}-dependent NOS activities were similar after aerobic perfusion and reperfusion in both untreated and 17β-estradiol-treated groups.

6. Further, enhanced NO biosignaling in hearts from 17β-estradiol-treated rats was confirmed by the finding of elevated content of cGMP at the end of reperfusion, but not at the end of aerobic perfusion.

Table 7-1. *Steady state LV work during aerobic perfusion in hearts for Series 1 and 2.* Series 1 hearts were not paced throughout perfusion, whereas Series 2 hearts were paced (300 bpm).

	LV Work (mm Hg . L/min)
Series 1 - Paced	
Untreated (n=8)	6.57 ± 0.39
17β-Estradiol (n=7)	7.45 ± 0.65
Series 2 - Unpaced	
Untreated (n=7)	6.60 ± 0.33
17β-Estradiol (n=8)	7.24 ± 0.29

Table 7-2. *Physiological parameters of untreated and 17β-estradiol-treated ovariectomized rats*

	Untreated (n=16)	**17β-Estradiol (n=15)**
Body Weight (g)	350.9 ± 4.4	302.4 ± 3.8 *
Heart Weight (g)	1.67 ± 0.05	1.74 ± 0.05
Heart Weight:Body Weight (x 10^{-3})	4.77 ± 0.14	5.75 ± 0.18 *
Uterine Weight (g)	0.23 ± 0.02	0.98 ± 0.04 *
Serum 17β-Estradiol (pg/ml)	3.02 ± 0.04	175.5 ± 16.1 *
Serum Triglycerides (mM)	1.4 ± 0.2	4.0 ± 0.4*
Serum Free Fatty Acids (mM)	0.13 ± 0.01	0.27 ± 0.04*

Data represent mean ± S.E. * $P<0.05$ compared with untreated.

Table 7-3. *Indices of myocardial function during aerobic perfusion and reperfusion in hearts from untreated and 17β-estradiol-treated rats.*

	Untreated (n=15)	**17β-Estradiol (n=15)**
LV work (mm Hg . L/min)		
Aerobic	6.58 ± 0.25	7.34 ± 0.33
Reperfusion	0.99 ± 0.28 #	2.11 ± 0.38 *#
% Recovery	13.36 ± 3.76	27.42 ± 5.28 *
HR x PSP (bpm.mm Hg x 10^{-3})		
Aerobic	31.86 ± 0.88	32.15 ± 0.97
Reperfusion	13.10 ± 2.62 #	19.33 ± 2.08 #
% Recovery	39.10 ± 9.22	61.06 ± 6.56 *
Cardiac Output (ml/min)		
Aerobic	57.0 ± 1.9	62.3 ± 2.2
Reperfusion	13.0 ± 2.6 #	24.0 ± 3.3 *#
% Recovery	20.7 ± 4.2	35.4 ± 5.4 *
Coronary Flow (ml/min)		
Aerobic	15.8 ± 0.8	17.3 ± 0.9
Reperfusion	10.0 ± 1.6 #	15.2 ± 2.0
% Recovery	54.6 ± 8.9	85.8 ± 10.8 *

Data represent means ± S.E. * $P < 0.05$ compared with untreated. # $P < 0.05$ compared with corresponding aerobic value.

Table 7-4. *Myocardial nucleotide and metabolite levels in reperfused hearts from untreated and 17β-estradiol-treated rats.*

	Untreated (n=8)	**17β-Estradiol (n=7)**
ATP (μmol/g dry wt)	9.17 ± 1.31	12.17 ± 1.25
AMP (μmol/g dry wt)	5.29 ± 1.03	4.78 ± 0.84
AMP/ATP ratio	0.65 ± 0.14	0.44 ± 0.12
Adenosine (μmol/g dry wt)	0.21 ± 0.03	0.23 ± 0.03
GTP (μmol/g dry wt)	0.39 ± 0.07	0.40 ± 0.08
Cr (μmol/g dry wt)	99.2 ± 11.8	81.3 ± 8.5
CrP (μmol/g dry wt)	15.7 ± 1.9	17.4 ± 1.4
Cr/CrP ratio	6.65 ± 0.81	4.59 ± 0.86

Values are means ± S.E.

Table 7-5. *Myocardial PDH activity in reperfused hearts from untreated and 17β-estradiol-treated rats.*

	Untreated (n=15)	**17β-Estradiol (n=14)**
PDH_a (μmol/min/g dry wt)	2.67 ± 0.58	1.61 ± 0.35
PDH_t (μmol/min/g dry wt)	7.72 ± 0.54	7.66 ± 0.35
% Active	31.6 ± 5.5	20.7 ± 3.7

Values are means ± S.E.

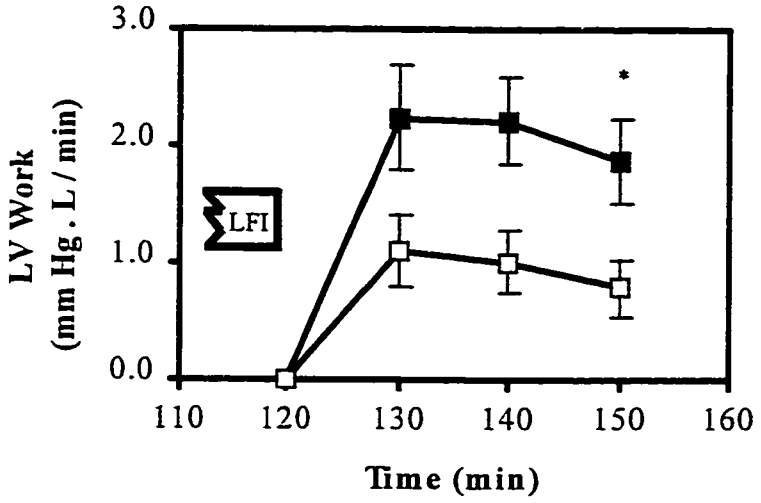


Fig. 7-1. *Recovery of LV work after 60 min of low-flow ischemia in hearts from untreated- and 17β-estradiol-treated rats.* Data are means ± S.E. for time-points during reperfusion for placebo (□, n=15) and estradiol-treated (■, n=15) rat hearts. LFI means low-flow ischemia. * $P < 0.05$ compared with hearts from untreated rats.

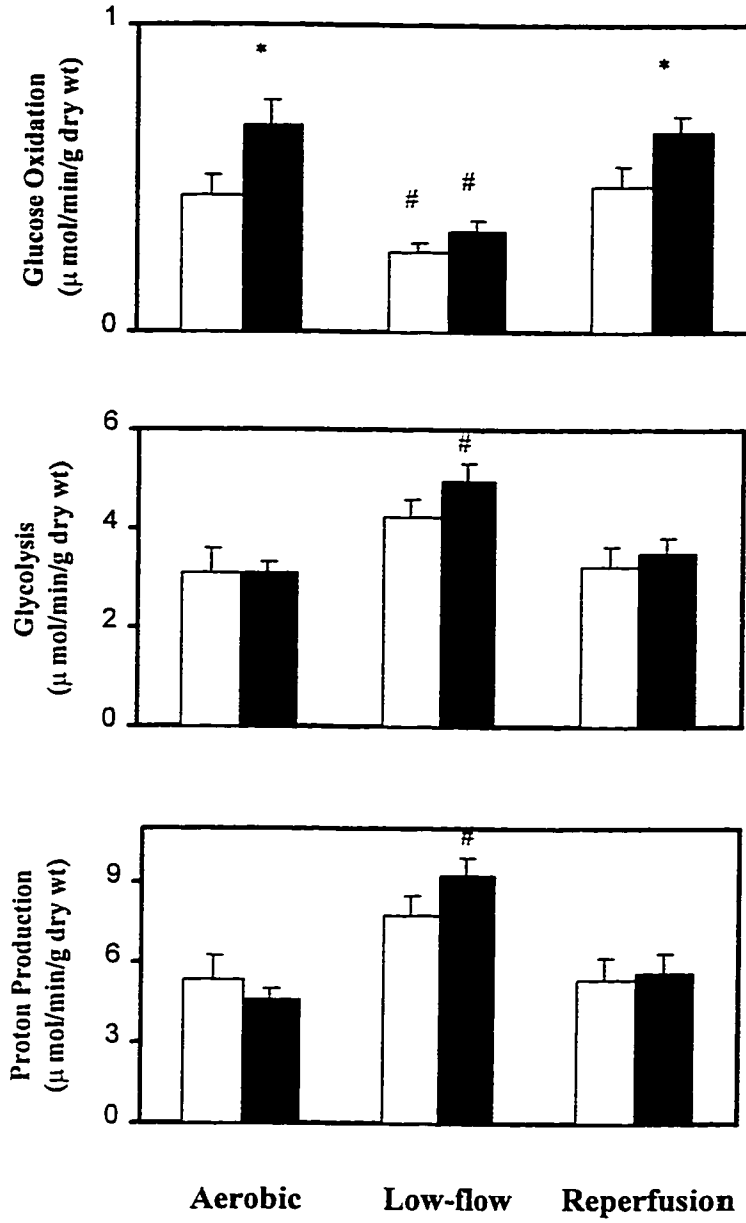


Fig. 7-2. *Rates of myocardial glucose metabolism measured during aerobic perfusion, low-flow ischemia and reperfusion in hearts from untreated and 17β-estradiol-treated rats.* Rates of glucose oxidation, glycolysis and proton production (μmol/min/g dry wt) were measured simultaneously throughout the perfusion protocol. Data are means ± S.E. for untreated, □, n=13 and 17β-estradiol-treated ■, n=15 rats. * $P < 0.05$ compared with untreated; # $P < 0.05$ compared with corresponding aerobic level. Statistical analysis was performed using a Two Way Repeated Measures Analysis of Variance test followed by the Tukey post-hoc test.

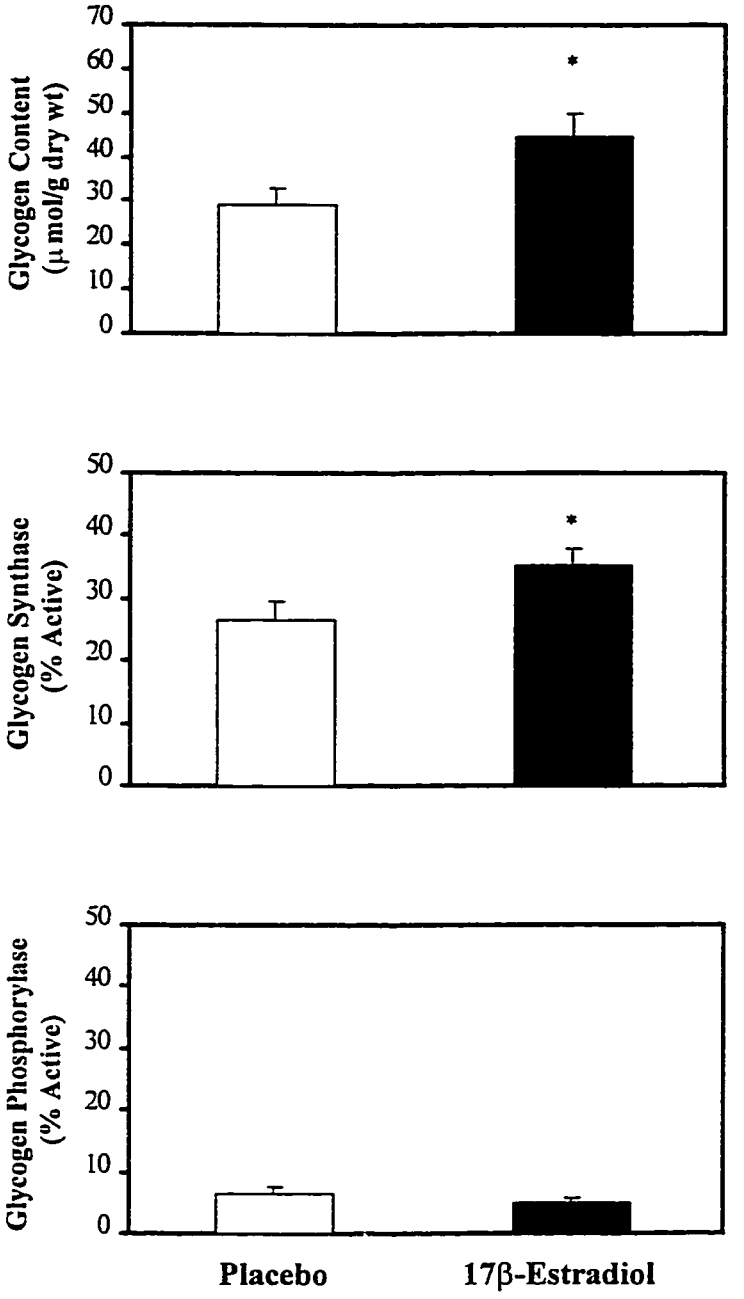


Fig. 7-3. *Myocardial glycogen content, glycogen synthase and phosphorylase activities after reperfusion of hearts from untreated and 17β-estradiol-treated rats.* Values were determined at the end of reperfusion in hearts from untreated (□, n=15) and 17β-estradiol-treated (■, n=15) rats. Data are means ± S.E. * $P < 0.05$ compared with untreated.

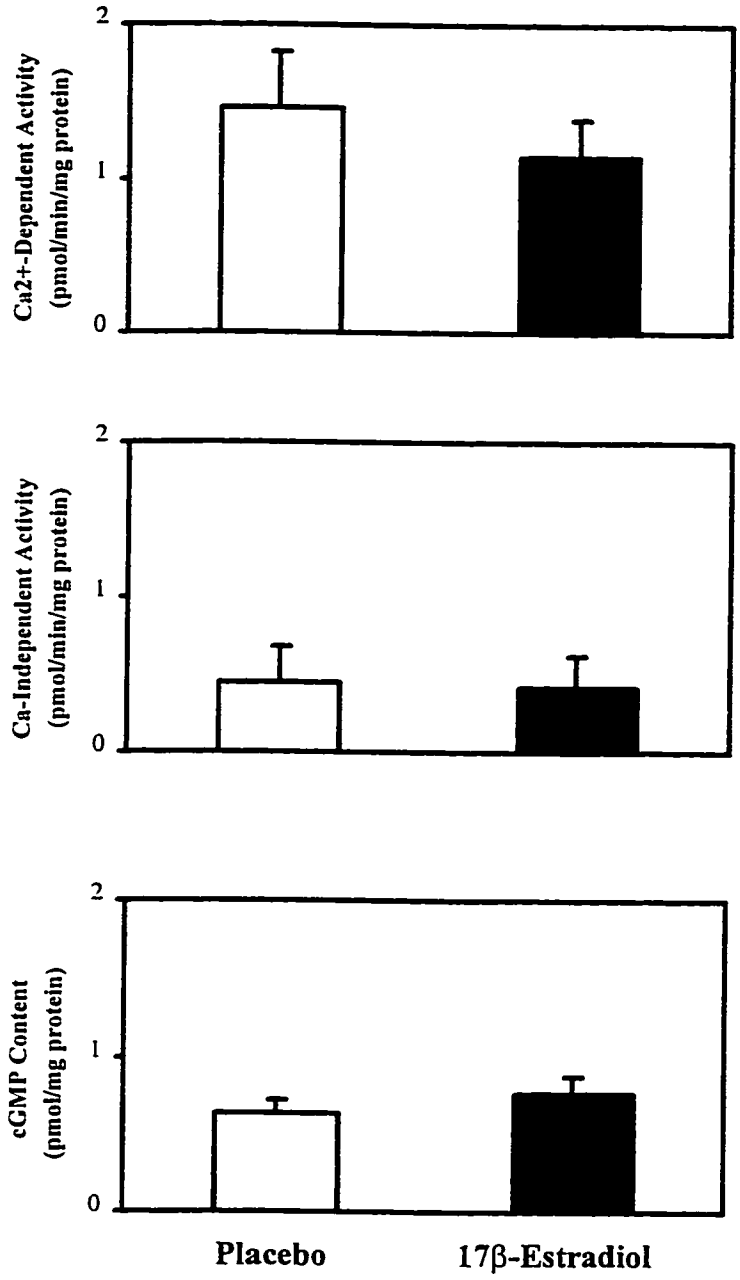


Fig. 7-4. *Aerobic levels of myocardial Ca^{2+}-dependent (A), Ca^{2+}-independent NOS activities (B) and cGMP content (C) after reperfusion of hearts from untreated and 17β-estradiol-treated rats.* Measurements were made in heart tissue frozen after 60 min of aerobic perfusion from ovariectomized female rats treated for 14 days with either placebo or 17β-estradiol. Data represent mean ± S.E.

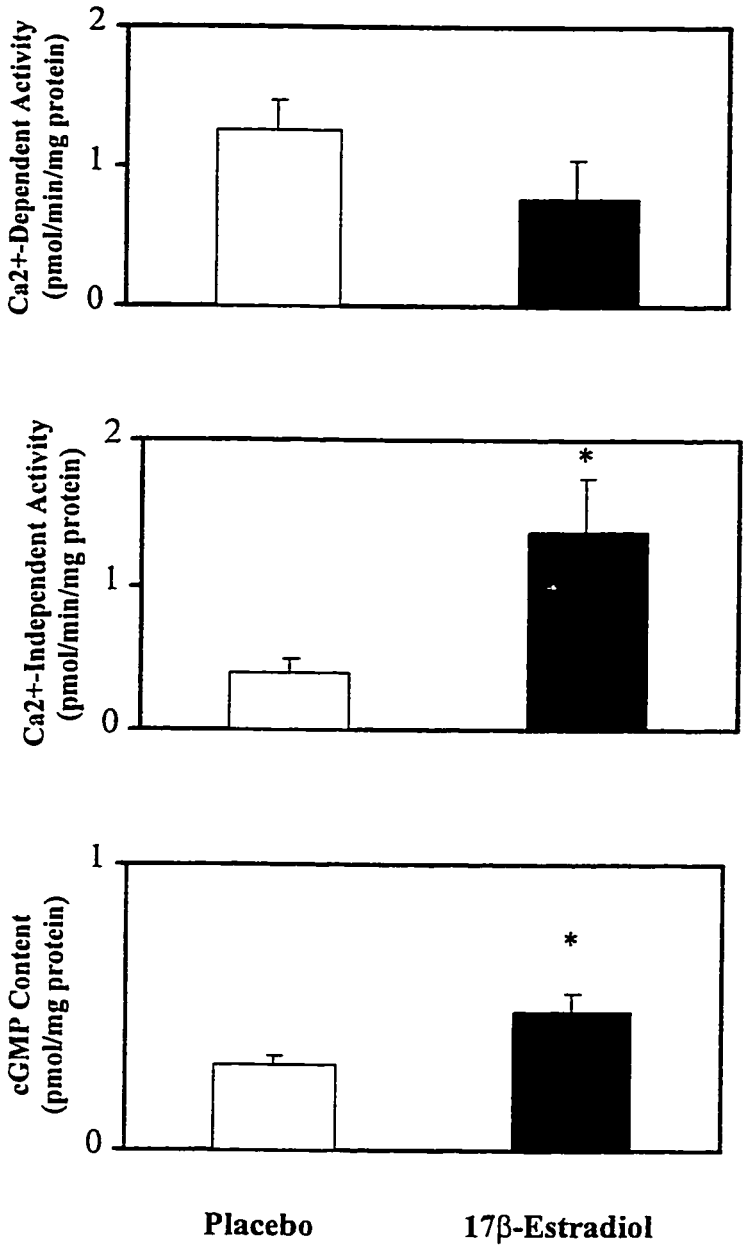


Fig. 7-5. *Reperfusion levels of myocardial Ca^{2+}-dependent and Ca^{2+}-independent NOS activities and cGMP content after reperfusion of hearts from untreated and 17β-estradiol-treated rats.* Values were measured after 30 min of reperfusion in hearts from untreated (□, n=8) and 17β-estradiol-treated (■, n=6) rats. Data are means ± S.E. * $P<0.05$ compared with untreated.

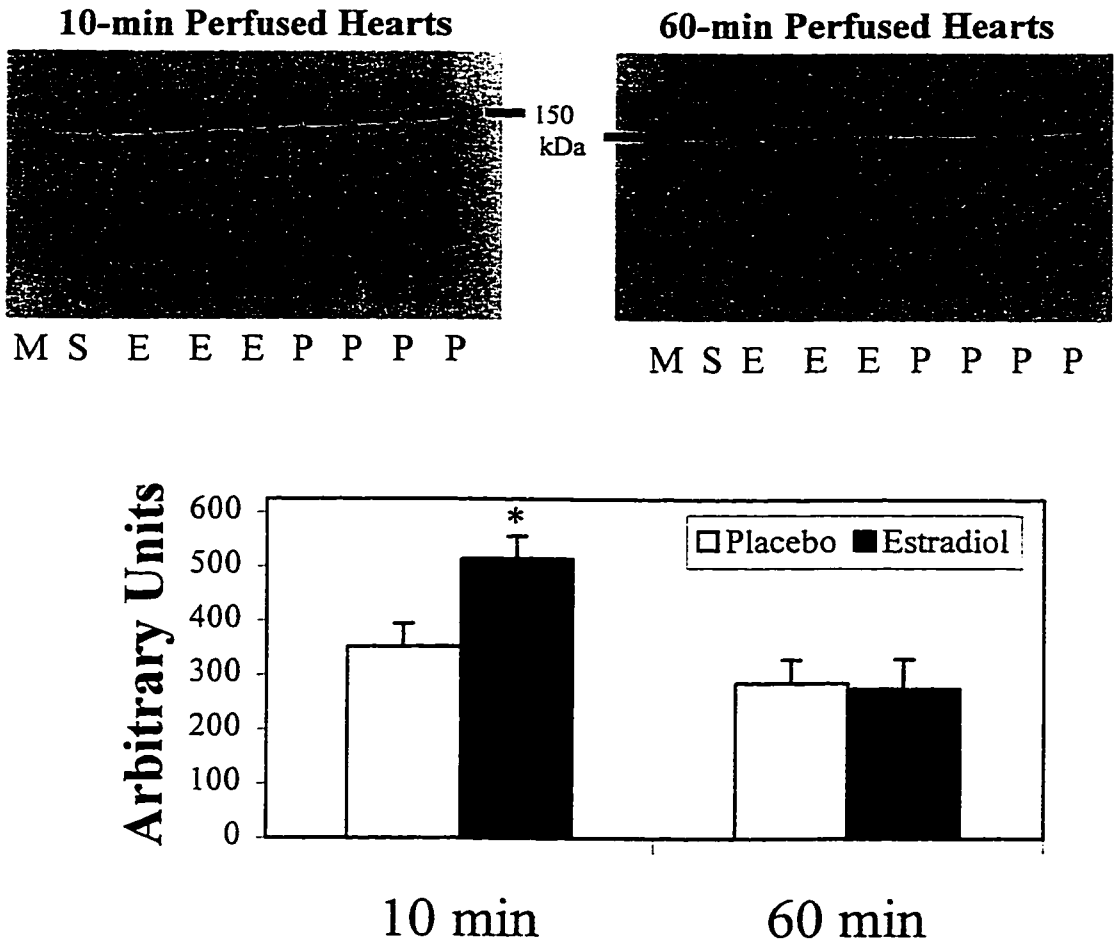


Fig. 7-6. *Western blot analysis of eNOS expression in hearts from placebo- and 17β-estradiol-treated rats after aerobic perfusion.* eNOS expression was measured in heart tissue frozen after 10 min of Langendorff perfusion or after 60 min of aerobic perfusion from ovariectomized female rats treated for 14 days with either placebo or 17β-estradiol. The upper panel represents the autoradiography of the eNOS immunoblots. M is molecular markers, S is standard, E is 17β-estradiol-treated heart, P is placebo-treated heart. The lower panel is the densitometric semi-quantitative analysis of the Western blots. Data represent mean ± S.E. * $P<0.05$ compared with placebo.

CHAPTER 8

Discussion

8.1) Main Findings

Although suggested to possess only a minor role in myocardial energy substrate metabolism (Neely & Morgan, 1974), glycogen metabolism has recently been shown to contribute significantly to overall myocardial ATP production (Goodwin *et al.* 1995; Henning *et al.* 1996). Experimental work described in this thesis developed a new approach to determine directly the contributions of glycogen turnover (simultaneous synthesis and degradation) to overall glucose metabolism in aerobic, ischemic and reperfused working rat hearts. The data demonstrated that there is a substantial turnover of glycogen not only under aerobic conditions, but also during low-flow ischemia, and during aerobic reperfusion. Despite the marked acceleration of glycogenolysis during ischemia, glycogen synthesis was still demonstrable. Furthermore, although the total myocardial glycogen pool was markedly reduced, glycogen turnover persisted during reperfusion. Moreover, the finding that the preferential oxidation of glucose derived from glycogen during low-flow ischemia was associated with a lower rate of proton production, suggests that optimization of glycogen turnover may be a useful therapeutic strategy for improving the recovery of mechanical function during reperfusion of post-ischemic hearts.

To investigate further the role of glycogen in cardioprotection, glycogen turnover was studied in the presence of the cardioprotective agent, N^6-cyclohexyladenosine (CHA,

0.5 μM), an adenosine A_1-receptor agonist whose cardioprotective efficacy is well established. Although the underlying mechanisms of this cardioprotection remain unclear, alterations in the metabolism of exogenous glucose may contribute to enhanced recovery of mechanical function by reducing proton production from glycolytically derived ATP (Finegan *et al.* 1996a; Finegan *et al.* 1996b; Askenasy & Navon, 1997). In agreement with previous reports (Finegan *et al.* 1996a; Finegan *et al.* 1996b), CHA improved the recovery of mechanical function during reperfusion and decreased the rate of glycolysis and proton production from the hydrolysis of glycolytically derived ATP. CHA also altered glycogen turnover during reperfusion by stimulating glycogen synthesis (G_{in}), an effect that accelerated the recovery of post-ischemic glycogen content. CHA had no effect on glycogen or glucose metabolism at any time-point during ischemia. However, glycogen phosphorylase activity at the end of ischemia was inhibited by CHA, and this was associated with inhibition of AMPK activity. These results indicate that the CHA-induced changes in glucose metabolism, which consist of an improvement in the metabolic coupling of glycolysis to glucose oxidation, are manifest primarily during reperfusion. CHA also altered glycogen turnover during reperfusion, possibly in response to the improved functional and energetic state and/or inhibition of glycolysis.

Time-dependent changes in glucose and glycogen metabolism during 60 min of low-flow ischemia were evaluated to determine whether these processes were linear and whether they provided insight into cardioprotective events occurring during ischemia. Time-course data revealed a rapid rate of glycogenolysis during the first 5 min of low-flow ischemia that corresponded to a rapid rate of glycogen degradation with no change in glycogen synthesis. These changes in glycogen turnover were not reflective of the

activities of the two key enzymes controlling glycogen turnover, glycogen synthase and glycogen phosphorylase.

Direct measurements of radiolabeled glucose in the glycogen pool revealed that glucose, which became incorporated during the initial aerobic perfusion, was retained within the glycogen pool following ischemia, despite marked glycogenolysis and glycogen depletion. This result suggests that the "last on, first off" hypothesis of glycogen metabolism (Laughlin *et al.* 1988; Brainard *et al.* 1989) at the molecular level does not apply to the intact organ. However, the time-dependent changes in the percent labeling of the glycogen pool indicated rapid depletion of the labeled component of glycogen in conjunction with rapid rates of overall glycogenolysis. This suggests a more ordered removal of glucosyl units, and therefore, that glycogenolysis is not simply a random process.

The potential for cardioprotection was also evaluated with a second class of drugs, the α-glucosidase inhibitors that potentially inhibit glycogenolysis. Unexpectedly, deoxynojirimycin (DNJ, 0.5 μM), did not alter glycogen turnover or its components. However, N-methyl-deoxynojirimycin (mDNJ, 100 μM), another α-glucosidase inhibitor, inhibited both glycogen turnover and glucose metabolism, a spectrum of activities suggestive of an inhibition of glucose uptake. The lack of expected effect on glycogenolysis as well as the cost of the drug precluded further study of the potential cardioprotective actions of this important class of drugs.

A member of a third class of drugs, estrogen receptor agonists, was tested for its potential to alter glycogen and or glucose metabolism. Chronic 17β-estradiol treatment

elicited two important alterations in carbohydrate metabolism in the heart during aerobic perfusion. These comprised a marked inhibition of glycogen degradation and a stimulation of glucose oxidation. These effects may contribute to cardioprotection by inhibition of glycolytic end-product accumulation during myocardial ischemia and reperfusion. Each of these effects has been shown previously to increase the recovery of mechanical function of the post-ischemic heart (Finegan *et al.* 1995; Liu *et al.* 1996; Lopaschuk & Stanley, 1997) and may, therefore, contribute to the beneficial actions of estrogen replacement therapy.

Chronic, but not acute, 17β-estradiol treatment improved the recovery of post-ischemic mechanical function by 2-fold. After reperfusion, hearts from 17β-estradiol-treated rats exhibited increased glycogen content and glycogen synthase activity compared with untreated hearts. It remains to be determined whether 17β-estradiol-induced changes in glycogen turnover cause improved post-ischemic functional recovery or whether they are simply a consequence of improved mechanical function. Contrary to the predicted improvement of metabolic coupling between glycolysis and glucose oxidation and the reduction in the production of protons and Ca^{2+} overload, this mechanism did not appear to play a role in the cardioprotective actions of 17β-estradiol.

NO biosignaling was enhanced in response to chronic 17β-estradiol treatment. Two important changes occurred in NO biosignaling after reperfusion of ischemic hearts that included an increase in Ca^{2+}-independent NOS activity and an increase in cGMP content. These changes were not observed in hearts after aerobic perfusion. Therefore, these alterations in NO biosignaling occurred only in chronically 17β-estradiol-treated

hearts in combination with low-flow ischemia, suggesting the involvement of both in NOS-induced cardioprotection.

8.2) Experimental Approaches and Study Design

The isolated perfused working rat heart provided an ideal system for studying myocardial energy metabolism because hearts can be exposed to an appropriate array of energy substrates (fatty acid and carbohydrates), as well as to a physiological workload and energy demand. LV work was used in this thesis as an index of mechanical function. During aerobic perfusion of isolated working rat hearts, LV work was stable, thereby facilitating measurement of glycogen and exogenous glucose metabolism under constant levels of energy demand. Total myocardial glycogen content increased during aerobic perfusion, indicative of net glycogen synthesis in response to abundant energy supply. During low-flow ischemia, all measurable LV work ceased and glycogen content decreased. Hearts were perfused at a low coronary flow (0.5 ml/min) during ischemia to mimic ischemia due to coronary occlusion with some residual perfusion as might be associated with collateral flow. In contrast to no-flow ischemia, the use of low-flow ischemia had the added advantage that it allowed the collection of coronary perfusate that was required for the determination of glycogen turnover and rates of glycolysis and glucose oxidation during ischemia. The duration of low-flow ischemia (60-min) was chosen to produce period a gradual and only partial recovery of LV work (20-40% of pre-ischemic levels), indicative of severe ischemia. This provided conditions for the study of

glycogen turnover and glucose metabolism in the post-ischemic heart where mechanical function was severely compromised by ischemia-induced myocardial damage. Relative to baseline aerobic conditions, coronary flow, and hence delivery of energy substrates (O_2, glucose, fatty acids), was more than adequate for the level of LV work during reperfusion.

There are several reasons why the presence of exogenous fatty acid was critical for the studies performed in this thesis. First, when present at concentrations (1.2 mM) observed in patients suffering myocardial ischemia or undergoing cardiac surgery (Lopaschuk *et al.* 1994b), fatty acid oxidation contributes up to 90% of total myocardial ATP production (Saddik & Lopaschuk, 1991). Fatty acids also influence glucose and glycogen metabolism and their presence in the perfusate of isolated hearts maintains myocardial glycogen contents close to levels measured *in vivo* (Wolfe *et al.* 1993; Laughlin *et al.* 1994). This is in marked contrast to the majority of studies using isolated hearts in which the absence of fatty acids in the perfusate leads to abnormally low glycogen levels (Lagerstrom *et al.* 1988; Schaefer *et al.* 1995; Goodwin *et al.* 1995; Goodwin *et al.* 1996a). Furthermore, due to their marked inhibition of glucose oxidation (Randle *et al.* 1963), fatty acids result in a metabolic uncoupling between rates of glycolysis and glucose oxidation (Wisneski *et al.* 1990; Saddik & Lopaschuk, 1992), a condition that leads to a significant rate of proton production (Finegan *et al.* 1996a; Liu *et al.* 1996).

The measurement of time-dependent changes in total glycogen content only provides information on the relative rates of glycogen synthesis and degradation. Consequently, a perfusion protocol was designed that utilized both [^{3}H]- and [^{14}C]-labeled glucose to enable the measurement of the average rates of the simultaneous synthesis and

degradation of glycogen during each phase of the perfusion protocol. This allowed for a detailed assessment of glycogen turnover during aerobic, ischemic and post-ischemic conditions. Another advantage of the dual labeling protocol was that rates of glycolysis and glucose oxidation for endogenous, as well as for exogenous, glucose during low-flow ischemia, could be determined.

Alterations to the general experimental approach were made for the 17β-estradiol studies, where a modified dual label protocol was utilized. Both ^{3}H and ^{14}C-labeled glucose were included in the perfusate from the beginning of perfusion and remained present throughout ischemia and reperfusion. Although this prevented the evaluation of contributions from endogenous (glycogen) or exogenous glucose to glucose metabolism, it did provide a continuous index of total glycolysis and glucose oxidation throughout all phases of perfusion.

A second modification was made only to the series of hearts that were subjected to low-flow ischemia and reperfusion. While one of the two groups of hearts in the 17β-estradiol study was allowed to beat spontaneously, all other groups of hearts were paced at 300 bpm. Pacing was used to avoid marked changes in heart rate, particularly in the CHA-treated group. In the second 17β-estradiol study, an effort was made to determine the mechanism responsible for the increased rates of glucose oxidation. Preliminary data in our laboratory indicated that measurements of the activity of the rate-limiting enzyme of glucose oxidation, PDH, may be affected by pacing. Consequently, a second group was perfused where hearts were allowed to beat spontaneously. There were no

differences in LV work between paced and unpaced groups; consequently, the two groups were combined for subsequent data analysis.

8.3) Glucose Metabolism and Glycogen Turnover in Aerobic, Ischemic and Reperfused Rat Heart: Role in Cardioprotective Actions of Adenosine A_1-Receptor Stimulation

8.3.1) Glucose Metabolism

The rate of glycolysis in working hearts perfused with glucose and fatty acids normally exceeds that of glucose oxidation even under aerobic conditions (Saddik & Lopaschuk, 1991; Finegan *et al.* 1993; Finegan *et al.* 1996b). This so-called "metabolic uncoupling of glucose metabolism" is an important source of protons (Dennis *et al.* 1991) that, in the absence of adequate perfusion, leads to acidosis and impaired mechanical function upon reperfusion. This is supported by studies showing that improvement in the coupling between glycolysis and glucose oxidation caused either by inhibition of glycolysis (Finegan *et al.* 1992; Finegan *et al.* 1996a; Finegan *et al.* 1996b), or by stimulation of glucose oxidation (McVeigh & Lopaschuk, 1990; Lopaschuk *et al.* 1993), will attenuate proton production and acidosis. This effect is associated with an improved recovery of mechanical function and cardiac efficiency in the post-ischemic heart (Liu *et al.* 1996), possibly due to a decrease in the supply of protons for Na^+-H^+ exchange that limits the potential for Ca^{2+} overload (Tani & Neely, 1989). Recently, it has been shown that endogenous glucose, namely that derived from glycogen, is preferentially oxidized in aerobic hearts (Goodwin *et al.* 1996a; Henning *et al.* 1996; Schonekess *et al.* 1997). In

the work described in this thesis, we demonstrate that this preferential oxidation of endogenous glucose also occurs during low-flow ischemia, in agreement with recently published data (Schonekess *et al.* 1997). This improvement in the metabolic coupling of glycolysis to glucose oxidation resulted in a lower rate of proton production from endogenous (glycogen), relative to exogenous sources. Preferential oxidation of glycogen may ensure ATP supply from a limited supply of endogenous substrate or limit lactate accumulation during glycogenolysis (Goodwin *et al.* 1996).

In ischemic hearts, the decrease in the total rate of glucose oxidation, i.e., from both endogenous and exogenous sources, in combination with an unaltered rate of total glycolysis, increased the degree of metabolic uncoupling and so further increased proton production. As there is preferential oxidation of endogenous glucose, proton production from the metabolism of exogenous glucose was significantly greater than that from endogenous sources. The lower rate of metabolism of exogenous glucose was not due to a limitation in glucose delivery as glucose extraction was only 21% during low-flow ischemia. In addition, ischemia did not cause a complete inhibition of glucose oxidation suggesting that the availability of O_2 and glucose was sufficient to maintain a degree of oxidative metabolism.

The calculated rate of glycolysis from endogenous sources (1.4 μmol/min/g dry wt) is less than the rate of glycogenolysis (G_{out} 2.1 μmol/min/g dry wt). The apparent mismatch in these rates may be due to the simultaneous synthesis of glycogen (G_{in}, 0.54 μmol/min/g dry wt) that consumes some of the glucose-6-phosphate liberated by glycogen breakdown. If one assumes that glycogen synthesis consumes equal proportions

of glucose-6-phosphate derived from hexokinase (exogenous glucose) and from glycogen breakdown (endogenous glucose), then the calculated rates of G_{out} are not significantly different from the rate of glycolysis from endogenous glucose.

During reperfusion, the uncoupling of glycolysis from glucose oxidation persisted. This continued production of protons during the critical early period of reperfusion may limit the recovery of mechanical function. The importance of proton production during reperfusion is supported by previous studies in which inhibition of proton production only during the reperfusion phase results in an improved recovery of post-ischemic mechanical function (Finegan *et al.* 1996b). This finding highlights the need to study glycogen turnover in the actual reperfusion period. The relative contributions of the metabolism of endogenous and exogenous sources of glucose to proton production during reperfusion could not be determined in these experiments, since both isotopic labels had been incorporated into glycogen by the end of ischemia.

The cardioprotective efficacy of adenosine A_1-receptor stimulation was not only confirmed in this thesis (Lasley & Mentzer, Jr. 1993; Finegan *et al.* 1996b), but it was also demonstrated that the improvement of post-ischemic mechanical function is associated with an inhibition of glycolysis and proton production arising from glucose metabolism (Finegan *et al.* 1996a; Finegan *et al.* 1996b). Rather than occurring during ischemia, this beneficial effect of CHA occurs only during reperfusion. As inhibition of glycolysis and proton production is also demonstrable in aerobic hearts (Finegan *et al.* 1996b), the CHA-induced inhibition of proton production during reperfusion was not a consequence of improved post-ischemic function. Instead, these data suggest that the CHA-induced alterations in glucose metabolism contribute to the improved recovery of

mechanical function (Finegan *et al.* 1996a; Finegan *et al.* 1996b) by decreasing proton production during the critical early period of reperfusion, that limits the potential for intracellular Na^+ and Ca^{2+} accumulation. Moreover, the causal relationship between proton production arising from glycolysis uncoupled from glucose oxidation and the extent of recovery of post-ischemic function is further supported by recent data showing that an increase in proton production from glucose metabolism worsens the recovery of post-ischemic mechanical function (Finegan *et al.* 1996a).

Although this data also confirmed the preferential oxidation of endogenous glucose (Henning *et al.* 1996; Goodwin *et al.* 1996a; Schonekess *et al.* 1997) and indicated that the metabolism of endogenous glucose results in a lower rate of proton production, CHA had no effect on the metabolic fate of either endogenous or exogenous glucose during ischemia. Consequently, the main effects of CHA on glucose metabolism are manifest during reperfusion. The importance of the reperfusion period for the cardioprotective activity of adenosine A_1-receptor agonists is further supported by the equivalent efficacy of CHA when administered prior to ischemia or only at the onset of reperfusion (Finegan *et al.* 1996b).

8.3.2) Glycogen Turnover

Glycogen turnover has been studied previously in aerobic hearts in the absence and presence of catecholamines and other hormones (McElroy *et al.* 1989; Goodwin *et al.* 1996a; Henning *et al.* 1996; Schonekess *et al.* 1997). However, this study is the first to investigate directly glycogen turnover under aerobic conditions as well as under

pathophysiological conditions of low-flow ischemia and reperfusion in the presence of glucose and fatty acids. In addition, previous studies have failed to account for any simultaneous synthesis and degradation of glycogen or have studied hearts using perfusates devoid of fatty acids (Goodwin *et al.* 1995; Bolukoglu *et al.* 1996). Rates of glycogen synthesis and degradation were calculated in two ways. First, apparent rates (G'_{in} and G'_{out}) were calculated simply on the relative, time-dependent changes in total glycogen content. However, these apparent rates represent an underestimation of the true rates of glycogen synthesis and degradation, as they do not account for any simultaneous synthesis and degradation of glycogen. Consequently, a new calculation was adopted that accounted for the potential of radiolabeled glucose to be released following its incorporation into glycogen. With this approach, higher rates of glycogen synthesis (G_{in}) and degradation (G_{out}), indicative of substantial turnover, were observed for periods of aerobic perfusion, low-flow ischemia, and aerobic reperfusion compared with calculated apparent rates. A limitation of both these calculations of glycogen turnover is the assumption that the rates of synthesis and degradation are constant throughout each phase of perfusion. Although rates of myocardial glycolysis and glucose oxidation were indeed stable throughout each phase, further studies evaluating other time intervals were designed to assess time-dependent alterations in glycogen turnover within each phase of perfusion. These time-dependent alterations in glycogen metabolism are discussed below.

Interestingly, during reperfusion, rates of glycogen synthesis as well as degradation were high, indicating a marked increase in glycogen turnover in the post-ischemic heart. Measurements of glycogen content alone would have suggested that glycogen turnover did not occur during reperfusion since total glycogen content did not

change. However, direct measurements of the rates of synthesis and degradation determined that this was not the case, and that glycogen did not recover during reperfusion because rates of synthesis and degradation were equal. This result emphasizes the need to measure directly glycogen turnover when studying glucose metabolism in the post-ischemic heart.

Glycogen turnover is tightly controlled by the activities of two key enzymes, glycogen synthase and glycogen phosphorylase. At the end of low-flow ischemia, a marked increase in glycogen phosphorylase activity was detectable and this correlated with the stimulation of glycogen degradation that occurred during this period. Although an inverse relationship between the activities of glycogen synthase and phosphorylase has been reported (Cohen, 1983), a decrease in glycogen synthase activity was not detected in hearts at the end of ischemia. This result agrees with that of McNulty *et al.*, who showed that glycogen mobilization early in ischemia also activates glycogen synthase (McNulty *et al.* 1995), and that of Huang *et al.*, who showed that epinephrine stimulated glycogenolysis and activated both glycogen phosphorylase and glycogen synthase (Huang *et al.* 1997). During reperfusion, net glycogen synthesis did not occur even in the presence of decreased glycogen phosphorylase activity and increased glycogen synthase activity. Both G_{in} and G_{out} increased during reperfusion to a similar extent, explaining the lack of any net change in glycogen content. This suggests that it is not simply the activity of these two enzymes that control glycogen turnover during reperfusion. For example, substrate supply may influence glycogen synthesis in addition to the activity of glycogen synthase. Alternatively, this apparent discrepancy may be explained by the fact that the rates of G_{in} and G_{out} are averages based on the entire period of reperfusion, while the

activities of these two enzymes are determined at a single time-point (end of reperfusion). Furthermore, assays of glycogen synthase and glycogen phosphorylase activities *in vitro,* that are conducted under controlled substrate and cofactor conditions, may not reflect accurately the activities of these enzymes under in situ conditions.

The ability to radiolabel glycogen stores also enabled study of the effects of the adenosine A_1-receptor agonist, CHA, on hitherto unexplored aspects of glycogen and glucose metabolism. This extends the knowledge of glycogen turnover (Chapter 3) by measuring rates of the simultaneous G_{in} and G_{out} of glycogen in the absence and presence of CHA during ischemic and post-ischemic conditions. Results presented in this thesis showed that while glycogen turnover was still detectable during low-flow ischemia despite an acceleration of glycogenolysis, CHA did not affect rates of glycogen synthesis or degradation. This indicates that the cardioprotective effects of CHA do not involve beneficial alterations in glycogen turnover during ischemia.

Interestingly, CHA had a small but significant inhibitory effect on glycogen phosphorylase activity measured in hearts frozen at the end of low-flow ischemia. At this time, the AMP/ATP and Cr/CrP ratios, both of which are indicative of cellular energetic state and which activate AMPK (Hardie & Carling, 1997; Ponticos *et al.* 1998), were increased by a similar extent in both untreated and CHA-treated groups. As expected, AMPK activity was also increased in response to the ischemic stress. However, the lower activation in CHA-treated hearts suggests that CHA may have exerted a direct inhibitory action on AMPK activity. As AICAR, an activator of AMPK, can activate glycogen phosphorylase and glycogenolysis in skeletal muscle (Young *et al.* 1996), CHA-mediated inhibition of AMPK may have contributed to the lower activity of glycogen

phosphorylase in CHA-treated hearts. The CHA-induced change in glycogen phosphorylase activity did not affect glycogen content at the end of ischemia or the sources and fates of glucose during ischemia, and so it is unlikely that it is involved in the cardioprotective action of CHA.

By the end of reperfusion, CHA-treated hearts had an improved energetic state as indicated by lower AMP/ATP and Cr/CrP ratios as well as an improved recovery of glycogen content. To determine mechanisms for the accelerated recovery of glycogen content, we examined rates of glycogen turnover during reperfusion. One possibility for the enhanced rate of glycogen resynthesis is a lower rate of glycogen degradation. Although AMPK and glycogen phosphorylase activities were lower in CHA-treated hearts at the onset of reperfusion, by the end of reperfusion both AMPK and glycogen phosphorylase activities had recovered to aerobic values in both untreated and CHA-treated groups. Consequently, it appears that glycogen phosphorylase activity measured at the onset of reperfusion is not predictive of glycogenolysis during reperfusion. The average rate of glycogen degradation (G_{out}) during reperfusion, although higher than in aerobic hearts, was not affected by CHA.

Alternatively, facilitation of recovery of glycogen content may have occurred in response to stimulation of glycogen synthesis (G_{in}). CHA did indeed stimulate G_{in} during reperfusion, but this was not in association with altered activities of AMPK or glycogen synthase. Thus, the recovery of glycogen content may have been due to the CHA-mediated inhibition of glycolysis that would have increased the availability of substrate (glucose-6-phosphate) for glycogen synthase.

8.3.3) Time-Dependent Changes in Glucose Metabolism and Glycogen Turnover

The measurement of glycogen turnover described in Chapters 3 was dependent on averages based on the entire 60-min period of low-flow ischemia. In order to define more precisely the time-course of the changes in glycogen and glucose metabolism within the 60-min ischemic period, hearts were subjected to a perfusion protocol similar to that presented for Chapters 3 and 4 except that groups of hearts were frozen after 5, 10, 15 and 60 min of low-flow ischemia. With this approach, the rates of glycogen turnover (G_{in} and G_{out}), glycolysis and glucose oxidation were measured during each of these perfusion intervals. The time-courses of the changes in the content of glycogen, high energy phosphates and their metabolites were also determined.

The rapid progression of glycogenolysis during the first 5 min of low-flow ischemia followed by slower rates of glycogenolysis during the subsequent 55 min of low-flow ischemia paralleled changes in G_{out}, but not glycogen phosphorylase activity. Glycogen phosphorylase activity increased only after 60 min of low-flow ischemia. Although this suggests that glycogen phosphorylase is not the sole regulator of glycogenolysis, the lack of correlation may simply be due to the method of determining glycogen phosphorylase activity. The assay used in the present studies was performed under ideal conditions of substrate and AMP, which may not be appropriate to the condition of the heart during ischemia. For example, Ca^{2+} and AMP concentrations may be different *in situ* than *in vitro*. Furthermore, the observation in Chapter 4 that glycogen phosphorylase activity is highest when AMPK activity is elevated suggests that AMPK-induced phosphorylation of glycogen phosphorylase mediated by phosphorylation of

phosphorylase kinase may be necessary for activation. Thus, multiple mechanisms may be involved in regulating glycogenolysis, such that regulation of glycogenolysis occurs by different mechanisms in early, compared with late, ischemia. Furthermore, glycogen phosphorylase activity measured in tissue extracts is normally higher than activity observed *in vivo*, suggesting that additional factors participate in the control of glycogenolysis in the heart (Laughlin *et al.* 1993). The possibility that the assay was simply "not working" is unlikely since positive controls were performed with each assay, that consisted of re-assaying heart tissue of known high phosphorylase activity. Alternatively, glycogenolysis may be stimulated by mechanisms that do not require covalent modification of glycogen phosphorylase (as discussed above).

The relatively constant rate of G_{in} corresponded with unaltered glycogen synthase activity until 60 min of low-flow ischemia when glycogen synthase is activated along with glycogen phosphorylase. The enhanced glycogen synthase activity may have resulted form an attempt by the heart to limit further glycogen depletion. This is not a unique concept. During epinephrine-induced glycogenolysis, both glycogen synthase and glycogen phosphorylase are activated in the heart, but not in skeletal muscle or liver (Huang *et al.* 1997). This dual activation confirms that glycogen synthesis and glycogen degradation are simultaneous processes that control glycogen turnover.

As expected, during ischemia, rates of glucose oxidation decreased immediately but were not completely inhibited. Contrary to an expected increase in rates of glycolysis (Camici *et al.* 1989), glycolysis remained stable throughout low-flow ischemia. These results may be explained by the conditions associated with low-flow ischemia. Substrate supply was not limited (glucose extraction is only 21%) and O_2 delivery was reduced, but

not absent. Thus, it is expected that glucose oxidation will continue, albeit at a reduced rate. This limited rate of glucose oxidation may then have supplied sufficient energy to prevent stimulation of glycolysis.

An interesting observation from the time-courses of changes in high energy phosphates and their metabolites was that the AMP/ATP ratio, which is a marker of the degree of ischemic damage, was not elevated during the first 15 min of ischemia and increased only after 60 min of low-flow ischemia. This suggests that the AMP/ATP ratio serves only as an index of severe ischemic stress. Prolonged ischemia was also accompanied by increased AMP, ADP and adenosine. Depression of high energy phosphates, ATP, CrP and GTP, seems to be a better indicator of early ischemic stress.

Although CHA had no demonstrable effects on glycogen and glucose metabolism when averaged over the entire 60-min period of low-flow ischemia (Chapter 4), the possibility that CHA could exert effects early in low-flow ischemia was investigated by comparing the time-dependent changes in glycogen and glucose metabolism in untreated hearts with those measured in hearts exposed to CHA 5 min prior to the onset of low-flow ischemia. CHA did not alter any of the indices of glycogen or glucose metabolism measured during low-flow ischemia, except that after 60 min, CHA improved the AMP/ATP ratio and inhibited glycogen phosphorylase activity. These data confirm that CHA-induced cardioprotection and metabolic effects are manifest during the critical early period of reperfusion.

8.3.4) Drug-induced Alterations in Glycogen Turnover: Inhibitors of Glycogenolysis

It still remains unresolved whether alterations in glycogen turnover, before (IPC), during or after ischemia, affect ischemic injury. One approach used to explore this issue was to alter, pharmacologically, glycogen turnover and determine the effect on recovery of post-ischemic mechanical function. We attempted to alter glycogen metabolism by limiting the extent of glycogenolysis with the α-glucosidase inhibitors, DNJ and mDNJ. The α-glucosidase inhibitors, by blocking the debranching of glycogen, would be expected to limit the extent of glycogenolysis to that only from glycogen phosphorylase activity. Unexpectedly, DNJ did not alter glycogen turnover or its components. mDNJ inhibited both glycogen turnover and glucose metabolism suggesting that it inhibited glucose uptake, possibly because the drug is a glucose analog. The lack of expected effect on glycogen metabolism precluded further study of the potential cardioprotective actions of this important class of drugs. It has subsequently been shown that mDNJ does indeed inhibit the rate of glycogenolysis in rabbit heart and is cardioprotective (Arai *et al.* 1998). It would be interesting to determine the actual rates of glycogen turnover in these rabbit hearts as only net changes in glycogen content were reported. Possible explanations for the disparity of results include species differences or the *in vivo* administration of mDNJ in the rabbit study. Furthermore, α-glucosidase activity was only inhibited by 20%, a response that may have been difficult to detect in the system. A trend for higher glycogen content in the mDNJ-treated hearts compared with untreated

hearts was observed (65 vs 58 μmol/g dry). A 20% difference in glycogen degradation may be beyond the power of the statistical analyses due to the inherent variability in the measurement of glycogenolysis and the relatively low number of replicates.

8.3.5) "Last On, First Off" Hypothesis of Glycogen Turnover

During low-flow ischemia, despite net glycogenolysis and marked glycogen depletion, radiolabeled glucose that was added to the perfusate only at the onset of ischemia, became incorporated into glycogen. Thus, even during severe ischemia, a significant rate of glycogen synthesis was still detectable. Glycogen synthesis has been hypothesized to proceed by the successive additions of glucosyl units to an internal protein core while degradation releases these most recently incorporated glucosyl units. This concept has been termed the "last on, first off" hypothesis (Laughlin *et al.* 1988; Brainard *et al.* 1989). Interestingly, although ischemia stimulated the depletion of glycogen to a level below that measured in aerobic hearts, the proportion of glycogen labeled with this isotope was greater than that labeled with the isotope that was added only at the onset of ischemia. Thus, a component of the radiolabeled glucose that had become incorporated in glycogen during aerobic perfusion was retained at the end of ischemia. This result suggests that the "last on, first off" hypothesis of glycogen metabolism at the molecular level does not apply to the intact working heart during conditions of marked glycogenolysis.

However, the time-course of incorporation of labeled glucose into glycogen indicates that during the first 5 min of low-flow ischemia, approximately half of the

labeled glycogen pool was lost. It was then resynthesized during the subsequent 55 min of low-flow ischemia to levels higher than those at the beginning of ischemia. The rate of accumulation of label is similar for newly labeled glycogen (second isotope) and for previously labeled glycogen (first isotope). This suggests that there is a large component of the previously labeled glycogen pool that is removed first. Therefore, although several studies suggest an ordered removal of glucosyl units (Laughlin *et al.* 1988; Brainard *et al.* 1989), these results are in agreement with those of Goodwin *et al.* (Goodwin *et al.* 1995) and Henning *et al.*, (Henning *et al.* 1996) who suggest that glycogenolysis is neither simply an ordered or a random process, but a combination of the two. Rapid glycogenolysis may proceed via an ordered mechanism, whereas glycogen turnover, with simultaneous synthesis and degradation, may proceed by a more random mechanism. Potential mechanisms to explain the observation concerning the order of glycogen metabolism include the presence of multiple intracellular compartments for glycogen, different cell populations and the existence of two forms of glycogen (proglycogen and macroglycogen).

8.4) 17β-Estradiol-Induced Alterations in Glucose and Glycogen Metabolism

In order to explore further the relationships between glycogen and glucose metabolism and cardioprotection, we sought to study an agent that potentially affected both of these processes. 17β-Estradiol plays a major role in the prevention of coronary artery disease, the mechanisms of which are multi-factorial (Lobo, 1990; Stampfer &

Colditz, 1991; Keaney, Jr. *et al.* 1994). Furthermore, the cardioprotective efficacy of 17β-estradiol has been observed in isolated rat and rabbit models of ischemia and reperfusion (Hale *et al.* 1996; Kolodgie *et al.* 1997), suggesting that a component of the protection is mediated directly on the heart. 17β-Estradiol has been shown to preserve glycogen content in skeletal muscle and reproductive organs (Carrington & Bailey, 1985), but it is unknown if it affects myocardial glycogen turnover. Thus, a series of experiments were performed to elucidate 17β-estradiol-induced alterations in glycogen and glucose metabolism during aerobic perfusion and during reperfusion of ischemic hearts, and to investigate the potential mechanisms of cardioprotection.

8.4.1) Chronic 17β-Estradiol Therapy Causes Critical Alterations in Myocardial Glucose Utilization

To determine whether alterations in myocardial glucose metabolism may contribute to the direct actions of estrogen in the heart, we evaluated the effects of chronic 17β-estradiol therapy on glycogen turnover, glucose utilization and cardiac function. The data indicate that chronic 17β-estradiol treatment elicits two important alterations in carbohydrate metabolism during aerobic perfusion that comprise a marked inhibition of glycogen degradation and a stimulation of glucose oxidation, which may result in lower adverse effects due to an inhibition of glycolytic end-product accumulation during myocardial ischemia and reperfusion. Each of these effects has been shown previously to increase the recovery of mechanical function of the post-ischemic heart (Finegan *et al.*

1995; Liu *et al.* 1996; Lopaschuk & Stanley, 1997) and thus may contribute to the direct cardioprotective action of estrogen replacement therapy.

LV work was stable throughout the period of aerobic perfusion and was higher in the 17β-estradiol-treated group. This effect was apparently due to an increase in cardiac output and aortic flow as there were no differences in any of the other measured indices of cardiac function that potentially could have influenced mechanical function (coronary flow, coronary vascular conductance, aortic pressures or aortic compliance). However, this small (11%) increase in LV work was only observed in one group of hearts. LV work in the second group of hearts, which underwent ischemia and reperfusion, trended to be higher, but the difference was not significant.

Substantial glycogen turnover was observed during aerobic perfusion of hearts from placebo-treated ovariectomized animals. Simultaneous synthesis and degradation of glycogen was demonstrable, but as the rate of G_{in} exceeded G_{out}, there was net glycogen synthesis. The rate of G_{out} in hearts from placebo-treated animals (similar to rate measured in hearts from male animals) represents a substantial proportion (30%) of the rate of glycolysis. 17β-Estradiol treatment exerted marked alterations in myocardial glycogen turnover and inhibited G_{out} so that its rate was equivalent to only 8% of the rate of glycolysis. The pronounced inhibition of glycogen degradation is in agreement with other data showing that physiological concentrations of 17β-estradiol inhibit glycogen mobilization induced by exercise in skeletal muscle and heart (Kendrick *et al.* 1987). Inhibition of glycogenolysis, in the absence of alterations in glycogen synthesis, glucose uptake or glucose extraction, suggests that hearts from 17β-estradiol-treated animals may

possess an enhanced ability to preserve energy, in the form of glycogen, for periods of ischemia. Moreover, a slowed rate of glycogenolysis in 17β-estradiol-treated hearts during ischemia may reduce the deleterious consequences arising from the rapid accumulation of glycolytic end-products. Similar mechanisms including the inhibition of glycolysis and proton production from glucose metabolism have been invoked to account for the cardioprotective efficacy of ischemic preconditioning (Finegan *et al.* 1995) and adenosine (Finegan *et al.* 1993) (also see Chapter 4). Alternatively, a slowed rate of glycogenolysis may be cardioprotective by prolonging the availability of glycogen for glycolysis and anaerobic ATP production (Finegan *et al.* 1995).

A second beneficial alteration in glucose metabolism elicited by chronic 17β-estradiol was stimulation of glucose oxidation. The magnitude of the increase in glucose oxidation was similar to that observed for therapeutic concentrations of dichloroacetate, a drug that owes its cardioprotective efficacy to a stimulation of glucose oxidation in response to activation of PDH, the rate-limiting enzyme of glucose oxidation (Liu *et al.* 1996; Lopaschuk & Stanley, 1997). It is noteworthy that the effect of 17β-estradiol was associated with a serum concentration in the physiological range (proestrous levels) (Mora *et al.* 1994). As stimulation of glucose oxidation and improvement in post-ischemic mechanical function by dichloroacetate required a 4000-fold higher concentration (Liu *et al.* 1996; Lopaschuk & Stanley, 1997), elucidation of the pharmacological mechanism of action of 17β-estradiol in the regulation of glucose oxidation may help identify novel targets for therapeutic exploitation. Mechanisms for the increased rates of glucose oxidation may be due to an insulin-mimetic action (Puah &

Bailey, 1985) or an enhancement of insulin biosignaling. This is unlikely under the present conditions where a relatively high concentration of insulin was included in the perfusate and rates of glucose uptake were similar in hearts from placebo- and 17β-estradiol-treated rats. An alternate mechanism may involve activation of PDH, either directly on the enzyme or indirectly in response to lower rates of β-oxidation of free fatty acids (Randle Cycle) (Randle *et al.* 1963). Deterioration of glucose tolerance has been observed in humans with high free fatty acid levels (Charles *et al.* 1997) and high free fatty acid levels late in pregnancy alter glucose utilization in cardiac and skeletal muscle (Sugden & Holness, 1994). Indirect regulation of PDH by serum lipid levels is unlikely as serum free fatty acids and triglycerides were elevated in 17β-estradiol-treated rats, which would have tended to decrease, rather than increase rates of glucose oxidation. Moreover, PDH activity was similar in both groups suggesting that 17β-estradiol-induced stimulation of glucose oxidation under the present conditions was unrelated to changes in PDH activity.

The cardioprotective activity of estrogen has been observed in both animal studies (Hale *et al.* 1996; Kolodgie *et al.* 1997) and in clinical trials (Stampfer & Colditz, 1991; Grady *et al.* 1992). Although the precise mechanism(s) of these salutary actions have not been defined, a component of the cardioprotective effect of 17β-estradiol is elicited directly on the myocardium as shown by an improvement in mechanical function following global ischemia in isolated hearts from 17β-estradiol-treated rats (Kolodgie *et al.* 1997). These experiments suggest that estrogen-induced alterations in myocardial

glucose metabolism may contribute to the direct cardioprotective efficacy of estrogen, and prompted the study of the cardioprotective actions of 17β-estradiol.

8.4.2) 17β-Estradiol-Induced Cardioprotection and Glucose Metabolism

To investigate further the cardioprotective actions of 17β-estradiol, ovariectomized, female rats were chronically treated for 14 days and their hearts perfused following the low-flow ischemia and reperfusion protocol described in Chapter 7. As anticipated, 17β-estradiol-induced protection was manifest in isolated perfused working rat hearts as an enhanced recovery of LV work (2-fold) following low-flow ischemia. This protective action is similar to that reported in other models of ischemia and reperfusion (Hale *et al.* 1996; Kolodgie *et al.* 1997). Interestingly, this observation suggests an ability to reduce damage associated with ischemia and reperfusion, rather than preventing the incidence of coronary artery disease as suggested by observational studies of women taking estrogen replacement therapy. However, 17β-estradiol, when administered acutely to the perfusate of isolated working rat hearts, was not cardioprotective (Kolodgie *et al.* 1997). Therefore, this suggests that females undergoing ischemia would not benefit from acute 17β-estradiol treatment, as this would not enhance post-ischemic mechanical function. Moreover, the requirement for chronic therapy with 17β-estradiol for the induction of cardioprotection suggests that the underlying mechanism may involve alterations in gene regulation.

This study was designed to measure alterations in glucose metabolism that might be associated with cardioprotection, and therefore, did not permit the evaluation of rates of glycogen turnover during the perfusion protocol. However, total glycogen content did increase in the 17β-estradiol-treated group in association with increased glycogen synthase activity. Increased myocardial glycogen content following reperfusion is a phenomenon observed with IPC (Moolman *et al.* 1995; Finegan *et al.* 1995; Doenst *et al.* 1996) and other agents that are also cardioprotective such as CHA (Chapter 4), suggesting that glycogen turnover does indeed play a role in drug-induced cardioprotection.

In a manner similar to that described above, chronic 17β-estradiol treatment increased the rates of glucose oxidation during aerobic perfusion and during aerobic reperfusion following 60 min of low-flow ischemia. The rates of glycolysis were not altered during any of the three phases of perfusion. Although increased rates of glucose oxidation generally improve metabolic coupling of glycolysis to glucose oxidation (Lopaschuk *et al.* 1993; Finegan *et al.* 1996b; Liu *et al.* 1996), this was not observed in the presence of 17β-estradiol and the calculated rate of proton production was not altered. Had proton production been reduced during reperfusion, that mechanism would have been consistent with that described for cardioprotective agents such as CHA (Finegan *et al.* 1996b) and metabolic modulators such as DCA (Liu *et al.* 1996). This lack of improvement in metabolic coupling between glycolysis and glucose oxidation may have been due to a small, insignificant increase in rate of glycolysis during reperfusion, which contributes more to proton production than can be compensated for by increases in glucose oxidation. The absence of any change in proton production suggests an, as yet,

unidentified mechanism that accounts for the direct cardioprotective effect of chronic 17β-estradiol treatment.

The mechanism of the 17β-estradiol-induced acceleration of glucose oxidation could not be identified, despite best efforts (as with during aerobic perfusion, see above). PDH activity was not different between the two groups of hearts. Interestingly, in contrast to preliminary data described above there was no consistent difference in PDH activity between paced and unpaced hearts.

8.4.3) 17β-Estradiol-Induced Cardioprotection Involves NO Biosignaling

Another potential mechanism for 17β-estradiol-induced cardioprotection involves the nitric oxide (NO) pathway. In endothelium, estrogen receptor activation can result in an upregulation of the constitutive endothelial NOS (eNOS or NOS III) (Hayashi *et al.* 1995). Ligand binding studies, that show that estrogen receptors are present in the heart (McGill, Jr. & Sheridan, 1981), are supported by additional studies that confirm the functional integrity of estrogen receptors in cardiomyocytes by the demonstration of estrogen-mediated gene activation (Grohe *et al.* 1997). Estrogen-mediated upregulation of both eNOS (Weiner *et al.* 1994) and iNOS (Grohe *et al.* 1998) in cardiomyocytes has also been reported. Although such data identify the heart as a target organ for the direct effects of estrogen, the role of estrogen in modulating NO biosignaling and its contribution to cardioprotection have not been determined. Furthermore, impaired

bioavailability of endothelial NO and coronary vasomotor dysfunction have been implicated in ischemia reperfusion injury (Ma *et al.* 1993; Lefer, 1995).

Two types of evidence are apparent from the present experiments for enhanced NO biosignaling in hearts from 17β-estradiol-treated rats. First, in hearts frozen at the end of reperfusion following 60 min of low-flow ischemia, Ca^{2+}-independent NOS activity was enhanced 3.5-fold by 17β-estradiol treatment whereas Ca^{2+}-dependent NOS activity was unchanged. Second, cGMP content, a marker of NO-mediated stimulation of sGC, was also elevated in hearts from 17β-estradiol-treated animals. Thus, it appears that estrogen treatment may have enhanced NOS activity and elicited a cardioprotective effect. The induction of Ca^{2+}-independent NOS was only apparent in hearts from rats treated chronically with 17β-estradiol and only after reperfusion following 60-min of low-flow ischemia. This suggests that Ca^{2+}-independent NOS activity was altered as a result of post-translational events such as phosphorylation, rather than by increase in the expression of the enzyme. After 60 min of aerobic perfusion, neither Ca^{2+}-independent nor Ca^{2+}-dependent NOS activities were different from untreated animals. The content of cGMP was also similar after aerobic perfusion. Furthermore, to confirm the specificity of the observed change in Ca^{2+}-independent NOS activity, the myocardial Ca^{2+}-dependent NOS and Ca^{2+}-independent NOS activities were determined in CHA-treated hearts that exhibited enhanced recovery of post-ischemic mechanical function (Chapter 4). CHA-treated hearts did not have elevated NOS activity indicating that the increase in Ca^{2+}-independent NOS activity was indeed a direct effect of 17β-estradiol. Induction of Ca^{2+}-independent NOS is analogous to the protection observed with chronic therapy with

monophosphoryl lipid A (Zhao *et al.* 1997) and in association with the second window of preconditioning (Bolli *et al.* 1997).

NOS induction has also been cited as the mechanism for many of the beneficial actions of 17β-estradiol in the vasculature. A recent study evaluating the vascular responsiveness of endothelium denuded rat aortas to constrictor substances determined the involvement of an iNOS mechanism (Binko & Majewski, 1998). Furthermore, iNOS upregulation has been associated with other beneficial actions of 17β-estradiol including prevention of platelet aggregation at sites of vascular injury (Hansson *et al.* 1994).

Although data presented in this thesis suggest that induction of NOS plays a role in the cardioprotective actions of 17β-estradiol, the idea that iNOS can be beneficial is controversial. Induction of iNOS can result in significant and rapid production of NO which can react with superoxide anion to produce peroxynitrite (Dusting & Macdonald, 1995; Schulz *et al.* 1997) that, in turn, causes adverse effects on myocardial function. The formation of peroxynitrite undoubtedly occurs when free radical scavengers can not deal with and inactivate increased NO production. However, iNOS induction, if of a limited magnitude, may not result in excess NO production. NO, in low concentrations, is beneficial to the vasculature (Binko & Majewski, 1998) as well as the heart (Zhao *et al.* 1997; Bolli *et al.* 1997).

NO, produced from either eNOS or iNOS, is a potent stimulator of sGC that results in the conversion of GTP to cGMP. The NO-sGC-cGMP transduction system is involved in mediating numerous physiological effects of NO including vascular and non-vascular smooth muscle relaxation. Although these results, and those of others (White *et*

al. 1995; Darkow *et al.* 1997), indicate involvement of the NO-sGC-cGMP transduction system, we did not address the consequences of enhanced NO production. Inhibition of Ca^{2+} activated K^+ channels (Hampl *et al.* 1995), inhibition of L-type Ca^{2+} channels (Quignard *et al.* 1997), activation of large-conductance K^+ channels (Darkow *et al.* 1997) and phosphorylation of small heat shock proteins (Beall *et al.* 1997) by a cGMP-mediated mechanism all result in vasorelaxation. Whether these, or other mechanisms, exist in cardiomyocytes remains to be determined.

The question arises as to whether these changes in glucose utilization and NO-biosignaling in 17β-estradiol-treated ovariectomized rats are due to a correction of the deleterious effects resulting from a loss of estrogen due to ovariectomy. Data suggest that this is not the case as the values obtained for glycogen turnover in placebo-treated ovariectomized rats are similar to those measured using similar methodology in male rats (Chapter 3). This suggests that 17β-estradiol does indeed modify glycogen turnover rather than correcting for loss of endogenous estrogen. However, a properly controlled direct comparison between males and females (both cycling and ovariectomized) is necessary to test this speculation.

8.5) Limitations

As described extensively in the introduction and experimental approach sections of this thesis, the presence of both glucose and fatty acids in the perfusate of isolated perfused working rat hearts is essential to allow for the appropriate regulation of energy

substrate metabolism in the heart. In recognition of this fact, both fatty acids and glucose were present in the perfusate that was used in all of the studies included in this thesis. Fatty acid oxidation was not measured because the major focus of this thesis work was drug-induced alterations in myocardial glycogen and glucose metabolism. Thus, investigation of the potential interactions between fatty acid metabolism and glycogen turnover and glucose metabolism was not performed. It would be useful to complete the profile of energy substrate metabolism during cardioprotection with CHA and 17β-estradiol as these agents may indeed elicit alterations in fatty acid oxidation. Furthermore, the effect of 17β-estradiol on glycogen and/or glucose metabolism should be investigated in the presence of low fatty acid concentrations similar to those observed in *in vivo* untreated rats.

A second limitation to the studies described in this thesis is the potential heterogeneity of the distribution of effects in the myocardium due to an uneven distribution of perfusion during low-flow ischemia. Some areas of the myocardium may have received a constant, albeit low, perfusion with a low O_2 and energy supply, whereas other areas may have been completely devoid of O_2 and energy supply. The effect of this heterogeneity of coronary perfusion on the measurement of glycogen and/or glucose metabolism is uncertain. However, since both ventricles were crushed and mixed together, a random sampling of the tissue is likely to reflect average values for energy substrate metabolism.

A further limitation is the potential change in specific activity glucose-6-phosphate due to dilution by unlabeled products of glycogen breakdown. Such an effect

glycogen turnover occurs via an ordered process of "last on, first off", there would be no dilution effect as the products of glycogen breakdown would have the same specific activity as exogenous glucose. However, if glycogen turnover occurs as a result of completely random synthesis and degradation, a dilution effect may be observed. In the worst case scenario, which would occur early during aerobic perfusion when the glycogen pool is mostly unlabeled, we predict that the maximum effect of dilution would be 10% (calculated from the rate of G_{in} from labeled glycogen (50% of 1.24 μmol/min/g dry wt) as a percentage of that taken up (5.89 μmol/min/g dry wt)). However, since our data suggest that glycogen turnover occurs as a result of both ordered and random processes, the dilution effect would be less than 10% and would not be significant. Furthermore, as the extent of labeling of the glycogen pool becomes greater during more prolonged periods of perfusion, the potential dilution effect becomes even less significant. Another issue related to specific activity and the labeling of exogenous glucose with both ^{3}[H]- and ^{14}[C]-glucose is the apparent extent of labeling of endogenous glycogen during low-flow ischemia and reperfusion. It appears that following perfusion with both [^{3}H]- and [^{14}C]-glucose, that endogenous glycogen became labeled to extents that, in combination, were greater than 100%. However, upon consideration that both labels were present only in tracer amounts, exogenous glucose based on the respective specific activities, was 100% labeled with each isotope. Thus, it should not be unexpected that endogenous glycogen became labeled to an extent that was less than 100% with each isotope, which in combination appeared to label greater than 100%.

Potential differences in the absorption of 17β-estradiol from the pellets in the

animals is another limitation of these studies. Although pellets from the same source were used in all studies, serum concentrations of 17β-estradiol differed in the two series of experiments (100 ± 15 and 175 ± 16 pg/ml, respectively). Given that these were timed-release pellets containing exactly the same amount of 17β-estradiol, and were implanted in rats from the same source for exactly the same length of time, it is unclear why serum 17β-estradiol concentrations were different in the two groups. Subcutaneous injections of 17β-estradiol may be a more reliable means of controlling the chronic administration of 17β-estradiol, but would suffer from greater oscillations in drug levels.

A further limitation of the design of the 17β-estradiol studies is the age of the rats at which ovariectomization and treatment were initiated. The study was designed to mimic the use of 17β-estradiol for estrogen replacement therapy. Thus, 17β-estradiol was administered to adult rats following ovariectomization. Additional studies would be required to investigate the effects of 17β-estradiol in naïve, immature rats, or in older rats that may have depressed serum concentrations of estrogen.

8.6) Conclusions and Clinical Relevance

There are important relationships between glycogen turnover, glycolysis, glucose oxidation and post-ischemic mechanical function (Cross *et al.* 1996). Glycogen depletion by anoxic pre-perfusion (Neely & Grotyohann, 1984) or ischemic preconditioning (Wolfe *et al.* 1993; Murry *et al.* 1986; Cohen & Downey, 1996) improves recovery of post-ischemic hearts and reduces infarct size (Barbosa *et al.* 1996). Conversely, elevation of pre-ischemic glycogen content is protective under some conditions by enhancing substrate

availability for glycolysis (McElroy *et al.* 1989; Goodwin & Taegtmeyer, 1994). Thus, the demonstration that the metabolism of endogenous glucose generates fewer protons than the metabolism of exogenous glucose indicates that regulation of the source of glucose for glycolysis and glucose oxidation may be an important target for strategies to reduce acidosis and so elicit cardioprotective actions. The demonstration that there is substantial turnover of glycogen during ischemia and reperfusion suggests that strategies designed to alter turnover and, in particular, increase the oxidation of glucose from glycogen, as opposed to extracellular glucose, may reduce ischemia-induced acidosis and provide therapeutic benefit. CHA and 17β-estradiol are both cardioprotective and alterations in glycogen and/or glucose metabolism may contribute to their beneficial effects.

Since adenosine also alters the NO biosignaling pathway (Woolfson *et al.* 1995), the possibility existed that 17β-estradiol, adenosine and perhaps other cardioprotective agents participate in the coordinated regulation of glycogen turnover and NO biosignaling. This however, is unlikely as CHA was cardioprotective in the absence of changes in NO biosignaling.

Whether there is an optimal rate of glycogen turnover is difficult to assess. Both stimulation of glycogen synthesis during reperfusion and inhibition of glycogen degradation during aerobic perfusion and ischemia may benefit the heart and improve post-ischemic functional recovery. Optimal glycogen turnover that elicits cardioprotection may be situation-specific. Furthermore, since the preferential oxidation of glycogen may also be beneficial to the heart, but as this requires the availability of O_2,

glycogen turnover may be different under conditions of no-flow ischemia compared with low-flow ischemia as described in this thesis. This means that the ischemic conditions will also affect the role of glycogen turnover in cardioprotection. It remains unclear whether alterations in glycogen turnover are a cause or a consequence of cardioprotection, irrespective of an optimal rate of glycogen turnover that elicits cardioprotection.

Although it is not clear from the work presented in this thesis whether modifying pre-ischemic glycogen content is beneficial to recovery of post-ischemic mechanical function, several aspects of this thesis have direct clinical relevance. The beneficial effects of adenosine A_1-receptor stimulation on recovery of post-ischemic mechanical function via alterations in glycogen and glucose metabolism suggest that adenosine A_1-receptor agonists may have clinical utility in patients suffering from myocardial ischemia. Further, it supports the development and clinical utility of other agents that effect downstream signaling pathways that modifying glycogen and glucose metabolism *in vivo*. Adenosine itself is useful for the acute treatment of supraventricular tachycardia (DiMarco *et al.* 1983), and has been used successfully in cardioplegia solutions during cardiopulmonary bypass (Mentzer, Jr. *et al.* 1996). However, chronic therapy with adenosine is contraindicated as adenosine may cause AV block.

The usefulness of acute 17β-estradiol in limiting myocardial ischemic damage is not supported by work presented in this thesis. However, the results support the use of chronic treatment with 17β-estradiol in postmenopausal women or women who have had their ovaries surgically removed and indicate that 17β-estradiol exerts a direct cardioprotective effect and so limits ischemia-induced myocardial damage. Treatment of

women who are at risk of developing heart disease may be a particularly relevant use. A recent clinical study with hormone replacement therapy in women with existing coronary artery disease showed an increased risk early in the treatment protocol, but showed benefit after several years of therapy (Hully *et al.* 1998). These data suggest that there may be selected groups of women who would benefit more from hormone replacement therapy. It would be interesting to follow a similar group of women who were receiving estrogen replacement therapy as opposed to hormone replacement therapy. The development of selective estrogen receptor modulators has even greater potential clinical relevance as both females and males may benefit from their actions on bone and the cardiovascular system without the increased likelihood of cancer. However, these agents may also only be efficacious with chronic treatment. Nevertheless, acute treatment with estrogen receptor agonists *in vivo* may prove effective in the treatment of other conditions, such as stroke as 17β-estradiol has acute effects in the vasculature.

8.7) Future Directions

Several issues raised by the experimental work described in this thesis deserve further consideration.

Determination of the precise underlying mechanisms of cardioprotection would have enormous clinical implications. Alteration of glycogen turnover may prove to be a critical component of the mechanism; however, in light of the available information this may be difficult to determine. An attempt was made in the studies described in this thesis to measure the cardioprotective potential of drugs that alter glycogen metabolism.

Unfortunately, the negative results obtained with α-glucosidase inhibitors preempted further investigation of this group of drugs. Inhibitors of α-glucosidase have subsequently proven to be cardioprotective. However, as simultaneous rates of glycogen synthesis and degradation (glycogen turnover) were not measured, it remains to be determined whether rates of glycogen turnover are altered with chronic *in vivo* treatment with α-glucosidase inhibitors. This would provide more insight to how glycogen turnover is involved with cardioprotection.

From the extensive literature on IPC, it is now recognized that brief periods of ischemia, that deplete glycogen, render the heart resistant to a prolonged period of ischemia. Thus, studies on IPC-induced alterations in glycogen turnover may provide insight into the cardioprotective potential of changes in glycogen turnover.

It would also be interesting to determine the contribution of exogenous and endogenous glucose sources to the rates of glycolysis and glucose oxidation during reperfusion. Preferential oxidation of glycogen has been observed under aerobic and low-flow ischemic conditions, and it is possible that the same preferential oxidation of endogenous glucose also exists during aerobic reperfusion. Furthermore, there are clinical implications to the preferential oxidation of glycogen. During global, no-flow ischemia, when no O_2 is present in the heart, preferential oxidation of glycogen would not be possible. Yet, preferential oxidation of glycogen during low-flow ischemia and the potential for improved metabolic coupling of glycolysis and glucose oxidation would be useful to patients undergoing angina or MI when collateral circulation may provide some

substrates and O_2 to the heart. Moreover, drugs that could stimulate further the preferential oxidation of glycogen may provide therapeutic benefit.

It remains to be investigated whether glycogen turnover is affected by chronic therapy with 17β-estradiol, and whether the changes in glycogen turnover observed during aerobic perfusion remain during low-flow ischemia and reperfusion. Further, it would be worthwhile to pursue the role of changes in NO biosignaling as a mechanism of cardioprotection by studying 17β-estradiol-induced effects in the absence and presence of NOS inhibitors. Such an approach would also address the potential link between glycogen turnover and NO biosignaling.

Another question raised by the data presented in this thesis is whether differences in glycogen and glucose metabolism are a result of 17β-estradiol treatment or of estrogen deficiency due to removal of the ovaries. A properly controlled direct comparison between males and females (both cycling and ovariectomized) is necessary to address this question. This may also determine potential benefits of 17β-estradiol treatment in males.

Finally, of potentially great clinical and economic benefit is the development of selective estrogen receptor modulators (SERMs) for cardioprotection. This class of estrogen receptor agonist has selective and beneficial actions on bone and the cardiovascular system without the adverse effects associated with unopposed estrogen therapy (breast and uterine cancer). These drugs were designed to treat osteoporosis, and to date these drugs have not been tested for their direct cardioprotective effects. If SERMs prove to be cardioprotective, their utility may also benefit men.

References

ALI IS, GANDHI M, FINEGAN BA, KOSHAL A, CLANACHAN AS, 1998. Cardioprotection by activation of NO/cGMP pathway after cardioplegic arrest and 8-hour storage. *Annals of Thoracic Surgery* **65**: 1303-1309.

ALLY A, PARK G, 1992. Rapid determination of creatine, phosphocreatine, purine bases and nucleotides (ATP, ADP, AMP, GTP, GDP) in heart biopsies by gradient ion-pair reversed-phase liquid chromatography. *Journal of Chromatography* **575**: 19-27.

ALONSO MD, LOMAKO J, LOMAKO WM, WHELAN WJ, 1995. A new look at the biogenesis of glycogen. *FASEB J* **9**: 1126-1137.

ANDING JD, KUBENA KS, MCINTOSH WA, O'BRIEN B, 1996. Blood lipids, cardiovascular fitness, obesity, and blood pressure: the presence of potential coronary heart disease risk factors in adolescents. *Journal of the American Dietetic Association* **96**: 238-242.

ARAI M, MINATOGUCHI S, TAKEMURA G, UNO Y, KARIYA T, TAKATSU H, FUJIWARA T, HIGASHIOKA M, YOSHIKUNI Y, FUJIWARA H, 1998. *N*-methyl-1-deoxynojirimycin (MOR-14), an α-glucosidase inhibitor, markedly reduced infarct size in rabbit hearts. *Circulation* **97**: 1290-1297.

ARMOUR KE, RALSTON SH, 1998. Estrogen upregulates endothelial constitutive nitric oxide synthase expression in human osteoblast-like cells. *Endocrinology* **139**: 799-802.

ASIMAKIS GK, INNERS-MCBRIDE K, MEDELLIN G, CONTI VR, 1992. Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *American Journal of Physiology* **263**: H887-94.

ASIMAKIS GK, 1996. Myocardial glycogen depletion cannot explain the cardioprotective effects of ischemic preconditioning in the rat heart. *Journal of Molecular & Cellular Cardiology* **28**: 563-570.

ASKENASY N, NAVON G, 1997. Intermittent ischemia: energy metabolism, cellular volume regulation, adenosine and insights into preconditioning. *Journal of Molecular & Cellular Cardiology* **29**: 1715-1730.

BAILEY CJ, AHMED-SOROUR H, 1980. Role of ovarian hormones in the long-term control of glucose homeostasis. Effects of insulin secretion. *Diabetologia* **19**: 475-481.

BARBOSA V, SIEVERS RE, ZAUGG CE, WOLFE CL, 1996. Preconditioning ischemia time determines the degree of glycogen depletion and infarct size reduction in rat hearts.

American Heart Journal **131**: 224-230.

BEALL AC, KATO K, GOLDENRING JR, RASMUSSEN H, BROPHY CM, 1997. Cyclic nucleotide-dependent vasorelaxation is associated with the phosphorylation of a small heat shock-related protein. *Journal of Biological Chemistry* **272**: 11283-11287.

BECKMANN CR, 1997. Alzheimer's disease: an estrogen link? *Current Opinion in Obstetrics & Gynecology* **9**: 295-299.

BERGMEYER, J. & GRASSL, M. (1983). *Methods of enzymatic analysis*, ed. CHIMIE, V. New York: Academic Press.

BHAVNANI BR, 1998. Pharmacokinetics and pharmacodynamics of conjugated equine estrogens: chemistry and metabolism. *Proceedings of the Society for Experimental Biology & Medicine* **217**: 6-16.

BIANCHI S, BIGAZZI R, QUINONES GALVAN A, MUSCELLI E, BALDARI G, PECORI N, CIOCIARO D, FERRANNINI E, NATALI A, 1995. Insulin resistance in microalbuminuric hypertension. Sites and mechanisms. *Hypertension* **26**: 789-795.

BILLMAN GE, AVENDANO CE, HALLIWILL JR, BURROUGHS JM, 1993. The effects of the ATP-dependent potassium channel antagonist, glyburide, on coronary blood flow and susceptibility to ventricular fibrillation in unanesthetized dogs. *Journal of Cardiovascular Pharmacology* **21**: 197-204.

BINKO J, MAJEWSKI H, 1998. 17 beta-Estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS. *American Journal of Physiology* **274**: H853-9.

BISCHOFF H, 1994. Pharmacology of alpha-glucosidase inhibition. *European Journal of Clinical Investigation* **24**: Suppl 3:3-10.

BOLLEN M, STALMANS W, 1989. The antiglycogenolytic action of 1-deoxynojirimycin results from a specific inhibition of the alpha-1,6-glucosidase activity of the debranching enzyme. *European Journal of Biochemistry* **181**: 775-780.

BOLLI R, BHATTI ZA, TANG XL, QIU Y, ZHANG Q, GUO Y, JADOON AK, 1997. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circulation Research* **81**: 42-52.

BOLUKOGLU H, GOODWIN GW, GUTHRIE PH, CARMICAL SG, CHEN TM, TAEGTMEYER H, 1996. Metabolic fate of glucose in reversible low-flow ischemia of the isolated working rat heart. *American Journal of Physiology* **270**: H817-26.

BRAINARD JR, HUTSON JY, HOEKENGA DE, LENHOFF R, 1989. Ordered

synthesis and mobilization of glycogen in the perfused heart. *Biochemistry* **28**: 9766-9772.

BRIL A, LAVILLE MP, GOUT B, 1992. Effects of glibenclamide on ventricular arrhythmias and cardiac function in ischaemia and reperfusion in isolated rat heart. *Cardiovascular Research* **26**: 1069-1076.

CALLE EE, MIRACLE-MCMAHILL HL, THUN MJ, HEATH CW, JR. 1995. Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. *Journal of the National Cancer Institute* **87**: 517-523.

CAMICI P, FERRANNINI E, OPIE LH, 1989. Myocardial metabolism in ischemic heart disease: basic principles and application to imaging by positron emission tomography. *Progress in Cardiovascular Diseases* **32**: 217-238.

CAMPBELL DL, STAMLER JS, STRAUSS HC, 1996. Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and S-nitrosothiols. *Journal of General Physiology* **108**: 277-293.

CARR CS, HILL RJ, MASAMUNE H, KENNEDY SP, KNIGHT DR, TRACEY WR, YELLON DM, 1997. Evidence for a role for both the adenosine A1 and A3 receptors in protection of isolated human atrial muscle against simulated ischaemia. *Cardiovascular Research* **36**: 52-59.

CARRINGTON LJ, BAILEY CJ, 1985. Effects of natural and synthetic estrogens and progestins on glycogen deposition in female mice. *Hormone Research* **21**: 199-203.

CASTALLO MA, 1967. Modern management of the menopause; this deficiency disease, caused by lack of ovarian hormone, should be treated throughout life by estrogen replacement therapy. *Pennsylvania Medicine* **70**: 80-81.

CHARLES MA, ESCHWEGE E, THIBULT N, CLAUDE JR, WARNET JM, ROSSELIN GE, GIRARD J, BALKAU B, 1997. The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study. *Diabetologia* **40**: 1101-1106.

CLARKE B, WYATT KM, MCCORMACK JG, 1996. Ranolazine increases active pyruvate dehydrogenase in perfused normoxic rat hearts: evidence for an indirect mechanism. *Journal of Molecular & Cellular Cardiology* **28**: 341-350.

CLEVELAND JC, JR., MELDRUM DR, ROWLAND RT, BANERJEE A, HARKEN AH, 1997. Preconditioning and hypothermic cardioplegia protect human heart equally against ischemia. *Annals of Thoracic Surgery* **63**: 147-152.

COHEN MV, DOWNEY JM, 1996. Myocardial preconditioning promises to be a novel

approach to the treatment of ischemic heart disease. *Annual Review of Medicine* **47**: 21-29.

COHEN P, 1983. Protein phosphorylation and the control of glycogen metabolism in skeletal muscle. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **302**: 13-25.

COLLINS-NAKAI RL, NOSEWORTHY D, LOPASCHUK GD, 1994. Epinephrine increases ATP production in hearts by preferentially increasing glucose metabolism. *American Journal of Physiology* **267**: H1862-71.

CONSTANTIN-TEODOSIU D, CEDERBLAD G, HULTMAN E, 1991. A sensitive radioisotopic assay of pyruvate dehydrogenase complex in human muscle tissue. *Analytical Biochemistry* **198**: 347-351.

CRONSTEIN BN, KRAMER SB, ROSENSTEIN ED, WEISSMANN G, HIRSCHHORN R, 1985. Adenosine modulates the generation of superoxide anion by stimulated human neutrophils via interaction with a specific cell surface receptor. *Annals of the New York Academy of Sciences* **451**: 291-301.

CROSS HR, OPIE LH, RADDA GK, CLARKE K, 1996. Is a high glycogen content beneficial or detrimental to the ischemic rat heart? A controversy resolved. *Circulation Research* **78**: 482-491.

CUMMINGS, S. R., NORTON, L., ECKERT, S., GRADY, D., CAULEY, J., KNICKERBOCKER, R., BLACK, D. M., NICKELSEN, J., GLUSMAN, J., KRUEGER, K. & THE MORE INVESTIGATORS (1998). Raloxifene reduces the risk of breast cancer and may decrease the risk of endometrial cancer in post-menopausal women. Two-year findings from the multiple outcomes of raloxifene evaluation (MORE) trial. *Proceedings of the American Society of Clinical Oncology* **17**, (Abstract).

DAHM CH, JR., JELLINEK M, MINAGAWA J, RAO PS, 1978. Effects of estradiol-17β on some gluconeogenic enzymes in rat liver. *Biology of Reproduction* **18**: 734-735.

DALLA-VOLTA S, MARAGLINO G, DELLA-VALENTINA P, VIENA P, DESIDERI A, 1990. Comparison of trimetazidine with nifedipine in effort angina: a double-blind, crossover study. *Cardiovascular Drugs & Therapy* **4**: Suppl 4:853-9.

DARKOW DJ, LU L, WHITE RE, 1997. Estrogen relaxation of coronary artery smooth muscle is mediated by nitric oxide and cGMP. *American Journal of Physiology* **272**: H2765-73.

DELMAS PD, BJARNASON NH, MITLAK BH, RAVOUX AC, SHAH AS, HUSTER WJ, DRAPER M, CHRISTIANSEN C, 1997. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal

women. *New England Journal of Medicine* **337**: 1641-1647.

DELYANI JA, MUROHARA T, NOSSULI TO, LEFER AM, 1996. Protection from myocardial reperfusion injury by acute administration of 17 β-estradiol. *Journal of Molecular & Cellular Cardiology* **28**: 1001-1008.

DEMERS LM, JACOBS RD, 1973. Hormonal regulation of rat uterine glycogen metabolism. *Biology of Reproduction* **9**: 272-278.

DENNIS SC, GEVERS W, OPIE LH, 1991. Protons in ischemia: where do they come from; where do they go to? *Journal of Molecular & Cellular Cardiology* **23**: 1077-1086.

DENTON RM, MCCORMACK JG, THOMAS AP, 1986. Mechanisms whereby insulin and other hormones binding to cell surface receptors influence metabolic pathways within the inner membrane of mitochondria. *Annals of the New York Academy of Sciences* **488**: 370-384.

DEPRE C, VEITCH K, HUE L, 1993. Role of fructose 2,6-bisphosphate in the control of glycolysis. Stimulation of glycogen synthesis by lactate in the isolated working rat heart. *Acta Cardiology* **48**: 147-164.

DEPRE C, HUE L, 1997. Inhibition of glycogenolysis by a glucose analogue in the working rat heart. *Journal of Molecular & Cellular Cardiology* **29**: 2253-2259.

DIMARCO JP, SELLERS TD, BERNE RM, WEST GA, BELARDINELLI L, 1983. Adenosine: electrophysiologic effects and therapeutic use for terminating paroxysmal supraventricular tachycardia. *Circulation* **68**: 1254-1263.

DOENST T, GUTHRIE PH, CHEMNITIUS JM, ZECH R, TAEGTMEYER H, 1996. Fasting, lactate, and insulin improve ischemia tolerance in rat heart: a comparison with ischemic preconditioning. *American Journal of Physiology* **270**: H1607-15.

DORMAN BH, HEBBAR L, CLAIR MJ, HINTON RB, ROY RC, SPINALE FG, 1997. Potassium channel opener-augmented cardioplegia: protection of myocyte contractility with chronic left ventricular dysfunction. *Circulation* **96**: Suppl II-253-9.

DU TOIT EF, MCCARTHY J, MIYASHIRO J, OPIE LH, BRUNNER F, 1998. Effect of nitrovasodilators and inhibitors of nitric oxide synthase on ischemic and reperfusion function of rat isolated hearts. *British Journal of Pharmacology* **123**: 1159-1167.

DUSTING GJ, MACDONALD PS, 1995. Endogenous nitric oxide in cardiovascular disease and transplantation. *Annals of Medicine* **27**: 395-406.

ELLIOTT GT, COMERFORD ML, SMITH JR, ZHAO L, 1996. Myocardial ischemia/reperfusion protection using monophosphoryl lipid A is abrogated by the ATP-

sensitive potassium channel blocker, glibenclamide. *Cardiovascular Research* **32**: 1071-1080.

FABIANI JN, PONZIO O, EMERIT I, MASSONET-CASTEL S, PARIS M, CHEVALIER P, JEBARA V, CARPENTIER A, 1992. Cardioprotective effect of trimetazidine during coronary artery graft surgery. *Journal of Cardiovascular Surgery* **33**: 486-491.

FALKEBORN M, PERSSON I, TERENT A, ADAMI HO, LITHELL H, BERGSTROM R, 1993. Hormone replacement therapy and the risk of stroke. Follow-up of a population-based cohort in Sweden. *Archives of Internal Medicine* **153**: 1201-1209.

FINEGAN BA, CLANACHAN AS, COULSON CS, LOPASCHUK GD, 1992. Adenosine modification of energy substrate use in isolated hearts perfused with fatty acids. *American Journal of Physiology* **262**: H1501-7.

FINEGAN BA, LOPASCHUK GD, COULSON CS, CLANACHAN AS, 1993. Adenosine alters glucose use during ischemia and reperfusion in isolated rat hearts. *Circulation* **87**: 900-908.

FINEGAN BA, LOPASCHUK GD, GANDHI M, CLANACHAN AS, 1995. Ischemic preconditioning inhibits glycolysis and proton production in isolated working rat hearts. *American Journal of Physiology* **269**: H1767-75.

FINEGAN BA, GANDHI M, LOPASCHUK GD, CLANACHAN AS, 1996a. Antecedent ischemia reverses effects of adenosine on glycolysis and mechanical function of working hearts. *American Journal of Physiology* **271**: H2116-25.

FINEGAN BA, LOPASCHUK GD, GANDHI M, CLANACHAN AS, 1996b. Inhibition of glycolysis and enhanced mechanical function of working rat hearts as a result of adenosine A1 receptor stimulation during reperfusion following ischaemia. *British Journal of Pharmacology* **118**: 355-363.

FLECKENSTEIN, A. (1971). Specific inhibitors and promoters of calcium action in the excitation-contraction coupling of heart muscle and their role in the prevention or production of myocardial lesions. In *Calcium and the heart*, eds. HARRIS, P. & OPIE, L. H. pp. 135-188. New York: Academic Press.

FOEGH ML, RAMWELL PW, 1998. Cardiovascular effects of estrogen: implications of the discovery of the estrogen receptor subtype beta. *Current Opinion in Nephrology & Hypertension* **7**: 83-89.

FORD WR, LOPASCHUK GD, SCHULZ R, CLANACHAN AS, 1998. KATP-channel activation: effects on myocardial recovery from ischaemia and role in the cardioprotective response to adenosine A1-receptor stimulation. *British Journal of Pharmacology* in press.

FRITSCH, MK & MURDOCH FE. (1994). Estrogens, progestins, and oral contraceptives. In Human Pharmacology Molecular to Clinical, eds. BRODY, TM, LARNER J, MINNEMAN KP & NEU HC. pp. 482-500. St. Louis: Mosby-Year Book, Inc.

FURMAN RH, 1968. Are gonadal hormones (estrogens and androgens) of significance in the development of ischemic heart disease. *Annals of the New York Academy of Sciences* **149**: 822-833.

GARLID KD, PAUCEK P, YAROV-YAROVOY V, MURRAY HN, DARBENZIO RB, D'ALONZO AJ, LODGE NJ, SMITH MA, GROVER GJ, 1997. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circulation Research* **81**: 1072-1082.

GIRARD C, QUENTIN P, BOUVIER H, BLANC P, BASTIEN O, LEHOT JJ, MIKAELOFF P, ESTANOVE S, 1992. Glucose and insulin supply before cardiopulmonary bypass in cardiac surgery: a double-blind study. *Annals of Thoracic Surgery* **54**: 259-263.

GODSLAND IF, WYNN V, CROOK D, MILLER NE, 1987. Sex, plasma lipoproteins, and atherosclerosis: prevailing assumptions and outstanding questions. *American Heart Journal* **114**: 1467-1503.

GOMOLL AW, 1996. Cardioprotection associated with preconditioning in the anesthetized ferret. *Basic Research in Cardiology* **91**: 433-443.

GOODWIN GW, ARTEAGA JR, TAEGTMEYER H, 1995. Glycogen turnover in the isolated working rat heart. *Journal of Biological Chemistry* **270**: 9234-9240.

GOODWIN GW, AHMAD F, TAEGTMEYER H, 1996. Preferential oxidation of glycogen in isolated working rat heart. *Journal of Clinical Investigation* **97**: 1409-1416.

GOODWIN GW, TAEGTMEYER H, 1994. Metabolic recovery of isolated working rat heart after brief global ischemia. *American Journal of Physiology* **267**: H462-70.

GRADY D, RUBIN SM, PETITTI DB, FOX CS, BLACK D, ETTINGER B, ERNSTER VL, CUMMINGS SR, 1992. Hormone therapy to prevent disease and prolong life in postmenopausal women. *Annals of Internal Medicine* **117**: 1016-1037.

GREENLAND P, REICHER-REISS H, GOLDBOURT U, BEHAR S, 1991. In-hospital and 1-year mortality in 1,524 women after myocardial infarction. Comparison with 4,315 men. *Circulation* **83**: 484-491.

GREKINIS D, REIMANN EM, SCHLENDER KK, 1995. Phosphorylation and

inactivation of rat heart glycogen synthase by cAMP-dependent and cAMP-independent protein kinases. *International Journal of Biochemistry & Cell Biology* **27**: 565-573.

GROHE C, KAHLERT S, LOBBERT K, STIMPEL M, KARAS RH, VETTER H, NEYSES L, 1997. Cardiac myocytes and fibroblasts contain functional estrogen receptors. *FEBS Letters* **416**: 107-112.

GROHE C, KAHLERT S, LOBBERT K, VETTER H, 1998. Expression of oestrogen receptor a and b in rat heart: role of local oestrogen synthesis. *Journal of Endocrinology* **156**: R1-R7.

GROSS GJ, AUCHAMPACH JA, 1992. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circulation Research* **70**: 223-233.

GROVER GJ, 1994. Protective effects of ATP-sensitive potassium-channel openers in experimental myocardial ischemia. *Journal of Cardiovascular Pharmacology* **24**: Suppl 4:S18-27.

GROVER GJ, D'ALONZO AJ, PARHAM CS, DARBENZIO RB, 1995. Cardioprotection with the KATP opener cromakalim is not correlated with ischemic myocardial action potential duration. *Journal of Cardiovascular Pharmacology* **26**: 145-152.

GROVER GJ, 1997. Pharmacology of ATP-sensitive potassium channel (KATP) openers in models of myocardial ischemia and reperfusion. *Canadian Journal of Physiology & Pharmacology* **75**: 309-315.

GRYNBERG A, DEMAISON L, 1996. Fatty acid oxidation in the heart. *Journal of Cardiovascular Pharmacology* **28**: Suppl 1:S11-7.

HALE SL, BIRNBAUM Y, KLONER RA, 1996. beta-Estradiol, but not alpha-estradiol, reduced myocardial necrosis in rabbits after ischemia and reperfusion. *American Heart Journal* **132**: 258-62.

HAMPL V, HUANG JM, WEIR EK, ARCHER SL, 1995. Activation of the cGMP-dependent protein kinase mimics the stimulatory effect of nitric oxide and cGMP on calcium-gated potassium channels. *Physiological Research* **44**: 39-44.

HANSSON GK, GENG YJ, HOLM J, HARDHAMMAR P, WENNMALM A, JENNISCHE E, 1994. Arterial smooth muscle cells express nitric oxide synthase in response to endothelial injury. *Journal of Experimental Medicine* **180**: 733-738.

HARDIE DG, CARLING D, 1997. The AMP-activated protein kinase--fuel gauge of the mammalian cell? *European Journal of Biochemistry* **246**: 259-273.

HAYASHI T, YAMADA K, ESAKI T, KUZUYA M, SATAKE S, ISHIKAWA T, HIDAKA H, IGUCHI A, 1995. Estrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochemical & Biophysical Research Communications* **214**: 847-855.

HAYASHI T, YAMADA K, ESAKI T, MUTOH E, IGUCHI A, 1997. Effect of estrogen on isoforms of nitric oxide synthase: possible mechanism of anti-atherosclerotic effect of estrogen. *Gerontology* **43**: Suppl 1:24-34.

HAYASHIDA W, VAN EYLL C, ROUSSEAU MF, POULEUR H, 1994. Effects of ranolazine on left ventricular regional diastolic function in patients with ischemic heart disease. *Cardiovascular Drugs & Therapy* **8**: 741-747.

HENDRICKSON SC, ST.LOUIS JD, LOWE JE, ABDEL-ALEEM S, 1997. Free fatty acid metabolism during myocardial ischemia and reperfusion. *Molecular & Cellular Biochemistry* **166**: 85-94.

HENNING SL, WAMBOLT RB, SCHONEKESS BO, LOPASCHUK GD, ALLARD MF, 1996. Contribution of glycogen to aerobic myocardial glucose utilization. *Circulation* **93**: 1549-1555.

HERS HG, 1976. The control of glycogen metabolism in the liver. *Annual Review of Biochemistry* **45**: 167-189.

HEWITT RL, LOLLEY DM, ADROUNY GA, DRAPANAS T, 1973. Protective effect of myocardial glycogen on cardiac function during anoxia. *Surgery* **73**: 444-453.

HUANG MT, LEE CF, DOBSON GP, 1997. Epinephrine enhances glycogen turnover and depresses glucose uptake in vivo in rat heart. *FASEB Journal* **11**: 973-980.

HULLY S, GRADY D, BUSH T, FURBERG C, HERRINGTON D, RIGGS B, VITTINGHOFF E, FOR THE HEART AND ESTROGEN/PROGESTIN REPLACEMENT STUDY (HERS) RESEARCH GROUP, 1998. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *The Journal of the American Medical Association* **280**: 605-613.

HULSMANN WC, DE WIT LE, STAM H, SCHOONDERWOERD K, 1990. Hormonal control of cardiac lipolysis by glyco(geno)lysis. *Biochimica et Biophysica Acta* **1055**: 189-192.

JENKINS DP, BAXTER GF, YELLON DM, 1995. The pathophysiology of ischaemic preconditioning. *Pharmacological Research* **31**: 219-224.

KASHIWAYA Y, SATO K, TSUCHIYA N, THOMAS S, FELL DA, VEECH RL, PASSONNEAU JV, 1994. Control of glucose utilization in working perfused rat heart. *Journal of Biological Chemistry* **269**: 25502-25514.

KAUSER K, RUBANYI GM, 1994. Gender difference in bioassayable endothelium-derived nitric oxide from isolated rat aorta. *American Journal of Physiology* **267**: H2311-7.

KAUSER K, RUBANYI GM, 1997. Potential cellular signaling mechanisms mediating upregulation of endothelial nitric oxide production by estrogen. *Journal of Vascular Research* **34**: 229-236.

KEANEY JF, JR., SHWAERY GT, XU A, NICOLOSI RJ, LOSCALZO J, FOXALL TL, VITA JA, 1994. 17 beta-estradiol preserves endothelial vasodilator function and limits low-density lipoprotein oxidation in hypercholesterolemic swine. *Circulation* **89**: 2251-2259.

KENDRICK ZV, STEFFEN CA, RUMSEY WL, GOLDBERG DI, 1987. Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. *Journal of Applied Physiology* **63**: 492-496.

KENDRICK ZV, ELLIS GS, 1991. Effect of estradiol on tissue glycogen metabolism and lipid availability in exercised male rats. *Journal of Applied Physiology* **71**: 1694-1699.

KILLAM AP, ROSENFELD CR, BATTAGLIA FC, MAKOWSKI EL, MESCHIA G, 1973. Effect of estrogens on the uterine blood flow of oophorectomized ewes. *American Journal of Obstetrics & Gynecology* **115**: 1045-1052.

KIRK-GARDNER R, CROSSMAN J, EYJOLFSSON K, 1992. Modifiable cardiac risk factors of obesity, inactivity, and stress: a community survey. *Canadian Journal of Cardiovascular Nursing* **3**: 25-29.

KIRSCH GE, CODINA J, BIRNBAUMER L, BROWN AM, 1990. Coupling of ATP-sensitive K+ channels to A1 receptors by G proteins in rat ventricular myocytes. *American Journal of Physiology* **259**: H820-6.

KLEINERT H, WALLERATH T, EUCHENHOFER C, IHRIG-BIEDERT I, LI H, FORSTERMANN U, 1998. Estrogens increase transcription of the human endothelial NO synthase gene: analysis of the transcription factors involved. *Hypertension* **31**: 582-588.

KOLODGIE FD, FARB A, LITOVSKY SH, NARULA J, JEFFERS LA, LEE SJ, VIRMANI R, 1997. Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovariectomized rat. *Journal of Molecular & Cellular Cardiology* **29**: 2403-2414.

KOSTANYAN A, NAZARYAN K, 1992. Rat brain glycolysis regulation by estradiol-17 beta. *Biochimica et Biophysica Acta* **1133**: 301-306.

KUDO N, BARR AJ, BARR RL, DESAI S, LOPASCHUK GD, 1995. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *Journal of Biological Chemistry* **270**: 17513-17520.

KUDO N, GILLESPIE JG, KUNG L, WITTERS LA, SCHULZ R, CLANACHAN AS, LOPASCHUK GD, 1996. Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. *Biochimica et Biophysica Acta* **1301**: 67-75.

KUIPER GG, CARLQUIST M, GUSTAFSSON JA, 1998. Estrogen is a male and female hormone. *Science and Medicine* **5**: 36-45.

LAGERSTROM CF, WALKER WE, TAEGTMEYER H, 1988. Failure of glycogen depletion to improve left ventricular function of the rabbit heart after hypothermic ischemic arrest. *Circulation Research* **63**: 81-86.

LASLEY RD, MENTZER RM, JR. 1993. Pertussis toxin blocks adenosine A1 receptor mediated protection of the ischemic rat heart. *Journal of Molecular & Cellular Cardiology* **25**: 815-821.

LAUGHLIN MR, PETIT WA, JR., DIZON JM, SHULMAN RG, BARRETT EJ, 1988. NMR measurements of in vivo myocardial glycogen metabolism. *Journal of Biological Chemistry* **263**: 2285-2291.

LAUGHLIN MR, PETIT WA, JR., BARRETT EJ, 1993. The time course of myocardial glycogenolysis stimulated by glucagon. *Journal of Molecular & Cellular Cardiology* **25**: 175-183.

LAUGHLIN MR, TAYLOR J, CHESNICK AS, BALABAN RS, 1994. Nonglucose substrates increase glycogen synthesis in vivo in dog heart. *American Journal of Physiology* **267**: H219-23.

LAUSTIOLA K, VUORINEN P, VAPAATALO H, METSA-KETELA T, 1983. Sodium nitroprusside inhibits lactate formation in rat atria: is cyclic GMP involved? *Acta Pharmacologica et Toxicologica* **52**: 195-200.

LAWTON JS, HARRINGTON GC, ALLEN CT, HSIA PW, DAMIANO RJ, JR. 1996. Myocardial protection with pinacidil cardioplegia in the blood-perfused heart. *Annals of Thoracic Surgery* **61**: 1680-1688.

LEFER AM, 1995. Role of selectins in myocardial ischemia-reperfusion injury. *Annals of Thoracic Surgery* **60**: 773-777.

LEFER AM, LEFER DJ, 1996. The role of nitric oxide and cell adhesion molecules on

the microcirculation in ischaemia-reperfusion. *Cardiovascular Research* **32**: 743-751.

LERNER DJ, KANNEL WB, 1986. Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. *American Heart Journal* **111**: 383-390.

LIBERSA C, HONORE E, ADAMANTIDIS M, ROUET E, DUPUIS B, 1990. Antiischemic effect of trimetazidine: enzymatic and electric response in a model of in-vitro myocardial ischemia. *Cardiovascular Drugs & Therapy* **4**: Suppl 4:808-9.

LIEDTKE AJ, 1981. Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. *Progress in Cardiovascular Diseases* **23**: 321-336.

LIN AL, GONZALEZ R, JR., CAREY KD, SHAIN SA, 1986. Estradiol-17 beta affects estrogen receptor distribution and elevates progesterone receptor content in baboon aorta. *Arteriosclerosis* **6**: 495-504.

LIN AL, SHAIN SA, 1985. Estrogen-mediated cytoplasmic and nuclear distribution of rat cardiovascular estrogen receptors. *Arteriosclerosis* **5**: 668-677.

LIU B, CLANACHAN AS, SCHULZ R, LOPASCHUK GD, 1996. Cardiac efficiency is improved after ischemia by altering both the source and fate of protons. *Circulation Research* **79**: 940-948.

LIU GS, THORNTON J, VAN WINKLE DM, STANLEY AW, OLSSON RA, DOWNEY JM, 1991. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation* **84**: 350-356.

LIU GS, RICHARDS SC, OLSSON RA, MULLANE K, WALSH RS, DOWNEY JM, 1994. Evidence that the adenosine A3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovascular Research* **28**: 1057-1061.

LIU Y, SATO T, O'ROURKE B, MARBAN E, 1998. Mitochondrial ATP-dependent potassium channels Novel effectors of cardioprotection? *Circulation* **97**: 2463-2469.

LOBO RA, 1990. Estrogen and cardiovascular disease. *Annals of the New York Academy of Sciences* 334-45.

LOPASCHUK GD, WAMBOLT RB, BARR RL, 1993. An imbalance between glycolysis and glucose oxidation is a possible explanation for the detrimental effects of high levels of fatty acids during aerobic reperfusion of ischemic hearts. *Journal of Pharmacology & Experimental Therapeutics* **264**: 135-144.

LOPASCHUK GD, BELKE DD, GAMBLE J, ITOI T, SCHONEKESS BO, 1994a. Regulation of fatty acid oxidation in the mammalian heart in health and disease.

Biochimica et Biophysica Acta **1213**: 263-276.

LOPASCHUK GD, COLLINS-NAKAI R, OLLEY PM, MONTAGUE TJ, MCNEIL G, GAYLE M, PENKOSKE P, FINEGAN BA, 1994b. Plasma fatty acid levels in infants and adults after myocardial ischemia. *American Heart Journal* **128**: 61-67.

LOPASCHUK GD, STANLEY WC, 1997. Glucose metabolism in the ischemic heart. *Circulation* **95**: 313-315.

MA XL, WEYRICH AS, LEFER DJ, LEFER AM, 1993. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circulation Research* **72**: 403-412.

MARWICK TH, 1997. Adenosine echocardiography in the diagnosis of coronary artery disease. *European Heart Journal* **18**: Suppl D:D31-6.

MCCORMACK JG, BARACOS VE, BARR R, LOPASCHUK GD, 1996. Effects of ranolazine on oxidative substrate preference in epitrochlearis muscle. *Journal of Applied Physiology* **81**: 905-910.

MCCROHON JA, ADAMS MR, MCCREDIE RJ, ROBINSON J, PIKE A, ABBEY M, KEECH AC, CELERMAJER DS, 1996. Hormone replacement therapy is associated with improved arterial physiology in healthy post-menopausal women. *Clinical Endocrinology* **45**: 435-441.

MCCULLOUGH TE, WALSH DA, 1979. Phosphorylation of glycogen synthase in the perfused rat heart. *Journal of Biological Chemistry* **254**: 7336-7344.

MCELROY DD, WALKER WE, TAEGTMEYER H, 1989. Glycogen loading improves left ventricular function of the rabbit heart after hypothermic ischemic arrest. *Journal of Applied Cardiology* **4**: 455-465.

MCEWEN DC, 1967. Estrogen replacement therapy at menopause. *Canadian Nurse* **63**: 34-37.

MCGARRY JD, WOELTJE KF, KUWAJIMA M, FOSTER DW, 1989. Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase. *Diabetes-Metabolism Reviews* **5**: 271-284.

MCGILL HC, JR., SHERIDAN PJ, 1981. Nuclear uptake of sex steroid hormones in the cardiovascular system of the baboon. *Circulation Research* **48**: 238-244.

MCNULTY PH, LIU WX, LUBA MC, VALENTI JA, LETSOU GV, BALDWIN JC, 1995. Effect of nonworking heterotopic transplantation on rat heart glycogen metabolism. *American Journal of Physiology* **268**: E48-54.

MCNULTY PH, LUBA MC, 1995. Transient ischemia induces regional myocardial glycogen synthase activation and glycogen synthesis in vivo. *American Journal of Physiology* **268**: H364-70.

MCVEIGH JJ, LOPASCHUK GD, 1990. Dichloroacetate stimulation of glucose oxidation improves recovery of ischemic rat hearts. *American Journal of Physiology* **259**: H1079-85.

MEI DA, ELLIOTT GT, GROSS GJ, 1996. KATP channels mediate late preconditioning against infarction produced by monophosphoryl lipid A. *American Journal of Physiology* **271**: H2723-9.

MELDRUM DR, MITCHELL MB, BANERJEE A, HARKEN AH, 1993. Cardiac preconditioning. Induction of endogenous tolerance to ischemia-reperfusion injury. *Archives of Surgery* **128**: 1208-1211.

MENTZER RM, JR., RAHKO PS, CANVER CC, CHOPRA PS, LOVE RB, COOK TD, HEGGE MO, LASLEY RD, 1996. Adenosine reduces postbypass transfusion requirements in humans after heart surgery. *Annals of Surgery* **224**: 523-529.

METSA-KETELA T, LAUSTIOLA K, LILIUS EM, VAPAATALO H, 1981. On the role of cyclic nucleotides in the regulation of cardiac contractility and glycolysis during hypoxia. *Acta Pharmacologica et Toxicologica* **48**: 311-319.

MONCADA S, PALMER RM, HIGGS EA, 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**: 109-142.

MONCADA S, HIGGS A, 1993. The L-arginine-nitric oxide pathway. *New England Journal of Medicine* **329**: 2002-2012.

MONCADA S, HIGGS EA, 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB Journal* **9**: 1319-1330.

MOOLMAN JA, GENADE S, WINTERBACH R, HARPER IS, WILLIAMS K, LOCHNER A, 1995. Preconditioning with a single short episode of global ischemia in the isolated working rat heart: effect on structure, mechanical function, and energy metabolism for various durations of sustained global ischemia. *Cardiovascular Drugs & Therapy* **9**: 103-115.

MORA OA, CABRERA MM, SANCHEZ-CRIADO JE, 1994. Hormonal pattern of the pheromonal restoration of cyclic activity in aging irregularly cycling and persistent-estrus female rats. *Biology of Reproduction* **51**: 920-925.

MORRIS PB, ELLIS MN, SWAIN JL, 1989. Angiogenic potency of nucleotide

metabolites: potential role in ischemia-induced vascular growth. *Journal of Molecular & Cellular Cardiology* **21**: 351-358.

MUBAGWA K, MULLANE K, FLAMENG W, 1996. Role of adenosine in the heart and circulation. *Cardiovascular Research* **32**: 797-813.

MURRY CE, JENNINGS RB, REIMER KA, 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**: 1124-1136.

MUSTAFA SJ, 1980. Cellular and molecular mechanism(s) of coronary flow regulation by adenosine. *Molecular & Cellular Biochemistry* **31**: 67-87.

NATHAN L, CHAUDHURI G, 1997. Estrogens and atherosclerosis. *Annual Review of Pharmacology and Toxicology* **37**: 477-515.

NEELY JR, GROTYOHANN LW, 1984. Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circulation Research* **55**: 816-824.

NEELY JR, MORGAN HE, 1974. Relationship between carbohydrate and lipid metabolism and the energy balance of the heart. *Annual Review of Physiology* **36**: 413-459.

NODE K, KITAKAZE M, KOSAKA H, MINAMINO T, SATO H, KUZUYA T, HORI M, 1997. Roles of NO and Ca2+-activated K+ channels in coronary vasodilation induced by 17beta-estradiol in ischemic heart failure. *FASEB Journal* **11**: 793-799.

NOMA A, 1983. ATP-regulated K+ channels in cardiac muscle. *Nature* **305**: 147-148.

OHYANAGI M, IWASAKI T, 1996. The guanine nucleotide-binding regulatory proteins (G proteins) in myocardium with ischemia. *Molecular & Cellular Biochemistry* **160-161**: 153-158.

OLSSON RA, PEARSON JD, 1990. Cardiovascular purinoceptors. *Physiological Reviews* **70**: 761-845.

OPIE, L. H. (1991). *The Heart Physiology and Metabolism*, New York: Raven Press.

OPIE LH, 1993. Modulation of ischemia by regulation of the ATP-sensitive potassium channel. *Cardiovascular Drugs & Therapy* **7**: Suppl 3:507-13.

PACE P, TAYLOR J, SUNTHARALINGAM S, COOMBES RC, ALI S, 1997. Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha. *Journal of Biological Chemistry* **272**: 25832-25838.

PASSONNEAU JV, ROTTENBERG DA, 1973. An assessment of methods for measuremennt of glycogen synthetase activity including a new direct one-step assay. *Analytical Biochemistry* **51**: 528-541.

PATEL MS, ROCHE TE, 1990. Molecular biology and biochemistry of pyruvate dehydrogenase complexes. *FASEB Journal* **4**: 3224-3233.

PAULUS WJ, VANTRIMPONT PJ, SHAH AM, 1994. Acute effects of nitric oxide on left ventricular relaxation and diastolic distensibility in humans. Assessment by bicoronary sodium nitroprusside infusion. *Circulation* **89**: 2070-2078.

PERSSON I, YUEN J, BERGKVIST L, SCHAIRER C, 1996. Cancer incidence and mortality in women receiving estrogen and estrogen-progestin replacement therapy--long-term follow-up of a Swedish cohort. *International Journal of Cancer* **67**: 327-332.

PETERSEN DN, TKALCEVIC GT, KOZA-TAYLOR PH, TURI TG, BROWN TA, 1998. Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. *Endocrinology* **139**: 1082-1092.

PINSKY DJ, OZ MC, KOGA S, TAHA Z, BROEKMAN MJ, MARCUS AJ, LIAO H, NAKA Y, BRETT J, CANNON PJ, ET AL, 1994. Cardiac preservation is enhanced in a heterotopic rat transplant model by supplementing the nitric oxide pathway. *Journal of Clinical Investigation* **93**: 2291-2297.

PITCHER J, SMYTHE C, COHEN P, 1988. Glycogenin is the priming glucosyltransferase required for the initiation of glycogen biogenesis in rabbit skeletal muscle. *European Journal of Biochemistry* **176**: 391-395.

PONTICOS M, LU QL, MORGAN JE, HARDIE DG, PARTRIDGE TA, CARLING D, 1998. Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *The EMBO Journal* **17**: 1688-1699.

PRZYKLENK K, ZHAO L, KLONER RA, ELLIOTT GT, 1996. Cardioprotection with ischemic preconditioning and MLA: role of adenosine-regulating enzymes? *American Journal of Physiology* **271**: H1004-14.

PUAH JA, BAILEY CJ, 1985. Effect of ovarian hormones on glucose metabolism in mouse soleus muscle. *Endocrinology* **117**: 1336-1340.

QUIGNARD JF, FRAPIER JM, HARRICANE MC, ALBAT B, NARGEOT J, RICHARD S, 1997. Voltage-gated calcium channel currents in human coronary myocytes. Regulation by cyclic GMP and nitric oxide. *Journal of Clinical Investigation* **99**: 185-193.

RANDLE PJ, HALES CN, GARLAND PB, NEWSHOLME EA, 1963. The glucose

fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* **1**: 785-789.

RAVINGEROVA T, PYNE NJ, PARRATT JR, 1995. Ischaemic preconditioning in the rat heart: the role of G-proteins and adrenergic stimulation. *Molecular & Cellular Biochemistry* **147**: 123-128.

REGISTER TC, ADAMS MR, 1998. Coronary artery and cultured aortic smooth muscle cells express mRNA for both the classical estrogen receptor and the newly described estrogen receptor beta. *Journal of Steroid Biochemistry & Molecular Biology* **64**: 187-191.

REHRING TF, BENDER PR, CAIRNS CB, JOO K, FRIESE RS, SHAPIRO JI, CLEVELAND JC, JR., BANERJEE A, 1997. Different preconditioning stimuli invoke disparate electromechanical and energetic responses to global ischemia in rat hearts. *Canadian Journal of Physiology & Pharmacology* **75**: 335-342.

ROONEY TP, KENDRICK ZV, CARLSON J, ELLIS GS, MATAKEVICH B, LORUSSO SM, MCCALL JA, 1993. Effect of estradiol on the temporal pattern of exercise-induced tissue glycogen depletion in male rats. *Journal of Applied Physiology* **75**: 1502-1506.

ROZENBERG S, KROLL M, PASTIJN A, VANDROMME J, 1995. Osteoporosis prevention and treatment with sex hormone replacement therapy. *Clinical Rheumatology* **14**: Suppl 3:14-7.

RUBANYI GM, FREAY AD, KAUSER K, SUKOVICH D, BURTON G, LUBAHN DB, COUSE JF, CURTIS SW, KORACH KS, 1997. Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *Journal of Clinical Investigation* **99**: 2429-2437.

SADDIK M, GAMBLE J, WITTERS LA, LOPASCHUK GD, 1993. Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. *Journal of Biological Chemistry* **268**: 25836-25845.

SADDIK M, LOPASCHUK GD, 1991. Myocardial triglyceride turnover and contribution to energy substrate utilization in isolated working rat hearts. *Journal of Biological Chemistry* **266**: 8162-8170.

SADDIK M, LOPASCHUK GD, 1992. Myocardial triglyceride turnover during reperfusion of isolated rat hearts subjected to a transient period of global ischemia. *Journal of Biological Chemistry* **267**: 3825-3831.

SADDIK M, LOPASCHUK GD, 1994. Triacylglycerol turnover in isolated working

hearts of acutely diabetic rats. *Canadian Journal of Physiology & Pharmacology* **72**: 1110-1119.

SALWAY, J. G. (1994). *Metabolism at a Glance*, Oxford: Blackwell Scientific Publications.

SATO T, O'ROURKE B, MARBAN E, 1998. Modulation of mitochondrial ATP-dependent K+ channels by protein kinase C. *Circulation Research* **83**: 110-114.

SCHAEFER S, CARR LJ, PRUSSEL E, RAMASAMY R, 1995. Effects of glycogen depletion on ischemic injury in isolated rat hearts: insights into preconditioning. *American Journal of Physiology* **268**: H935-44.

SCHOLZ A, TRUSS M, BEATO M, 1998. Hormone-induced recruitment of Sp1 mediates estrogen activation of the rabbit uteroglobin gene in endometrial epithelium. *Journal of Biological Chemistry* **273**: 4360-4366.

SCHONEKESS BO, ALLARD MF, HENNING SL, WAMBOLT RB, LOPASCHUK GD, 1997. Contribution of glycogen and exogenous glucose to glucose metabolism during ischemia in the hypertrophied rat heart. *Circulation Research* **81**: 540-549.

SCHULZ R, NAVA E, MONCADA S, 1992. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *British Journal of Pharmacology* **105**: 575-580.

SCHULZ R, DODGE KL, LOPASCHUK GD, CLANACHAN AS, 1997. Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *American Journal of Physiology* **272**: H1212-9.

SCHULZ R, WAMBOLT R, 1995. Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. *Cardiovascular Research* **30**: 432-439.

SHAH AM, SILVERMAN HS, GRIFFITHS EJ, SPURGEON HA, LAKATTA EG, 1995. cGMP prevents delayed relaxation at reoxygenation after brief hypoxia in isolated cardiac myocytes. *American Journal of Physiology* **268**: H2396-204.

SIDNEY S, PETITTI DB, QUESENBERRY CP, JR., KLATSKY AL, ZIEL HK, WOLF S, 1996. Myocardial infarction in users of low-dose oral contraceptives. *Obstetrics & Gynecology* **88**: 939-944.

SIEGL P, 1994. Blockers of ATP sensitive potassium current are of potential benefit in ischaemic heart disease. *Cardiovascular Research* **28**: 31-32.

SIMINIAK T, FLORES NA, SHERIDAN DJ, 1995. Neutrophil interactions with

endothelium and platelets: possible role in the development of cardiovascular injury. *European Heart Journal* **16**: 160-170.

SMITH DE, 1967. Location of the estrogen effect on uterine glucose metabolism. *Proceedings of the Society for Experimental Biology and Medicine* **124**: 746-749.

SMITH DE, GORSKI J, 1968. Extrogen control of uterine glucose metabolism. An analysis based on the transport and phosphorylation of 2-deoxyglucose. *Journal of Biological Chemistry* **243**: 4169-4174.

SMITH TW, BALLIGAND JL, KAYE DM, WIVIOTT SD, SIMMONS WW, HAN X, MICHEL T, SINGH K, KELLY RA, 1996. The role of the NO pathway in the control of cardiac function. *Journal of Cardiac Failure* **2**: S141-7.

SMYTHE C, COHEN P, 1991. The discovery of glycogenin and the priming mechanism for glycogen biogenesis. *European Journal of Biochemistry* **200**: 625-631.

SOARES PR, DE ALBUQUERQUE CP, CHACKO VP, GERSTENBLITH G, WEISS RG, 1997. Role of preischemic glycogen depletion in the improvement of postischemic metabolic and contractile recovery of ischemia-preconditioned rat hearts. *Circulation* **96**: 975-983.

STACPOOLE PW, 1989. The pharmacology of dichloroacetate. *Metabolism: Clinical & Experimental* **38**: 1124-1144.

STAMPFER MJ, COLDITZ GA, 1991. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Preventative Medicine* **20**: 47-63.

STEENBERGEN C, FRALIX TA, MURPHY E, 1993. Role of increased cytosolic free calcium concentration in myocardial ischemic injury. *Basic Research in Cardiology* **88**: 456-470.

STEVENSON JC, CROOK D, GODSLAND IF, 1993. Influence of age and menopause on serum lipids and lipoproteins in healthy women. *Atherosclerosis* **98**: 83-90.

SUBBIAH MT, 1998. Mechanisms of cardioprotection by estrogens. *Proceedings of the Society for Experimental Biology & Medicine* **217**: 23-29.

SUGDEN MC, HOLNESS MJ, 1994. The role of the glucose/fatty acid cycle in the selective modulation of non-oxidative and oxidative glucose disposal by oxidative muscle in late pregnancy. *Biol Chem Hoppe Seyler* **375**: 141-147.

TAEGTMEYER H, GOODWIN GW, DOENST T, FRAZIER OH, 1997. Substrate metabolism as a determinant for postischemic functional recovery of the heart. *American*

Journal of Cardiology **80**: 3A-10A.

TAKEO S, TANONAKA K, MIYAKE K, IMAGO M, 1988. Adenine nucleotide metabolites are beneficial for recovery of cardiac contractile force after hypoxia. *Journal of Molecular & Cellular Cardiology* **20**: 187-199.

TANI M, NEELY JR, 1989. Role of intracellular Na+ in Ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H+-Na+ and Na+-Ca2+ exchange. *Circulation Research* **65**: 1045-1056.

THORNTON JD, THORNTON CS, DOWNEY JM, 1993. Effect of adenosine receptor blockade: preventing protective preconditioning depends on time of initiation. *American Journal of Physiology* **265**: H504-8.

THOROGOOD M, VESSEY MP, 1990. An epidemiologic survey of cardiovascular disease in women taking oral contraceptives. *American Journal of Obstetrics & Gynecology* **163**: 274-81.

TOMAI F, CREA F, DANESI A, PERINO M, GASPARDONE A, GHINI AS, RUGGERI G, CHIARIELLO L, GIOFFRE PA, 1997. Effects of A1 adenosine receptor blockade on the warm-up phenomenon. *Cardiologia* **42**: 385-392.

TOOMBS CF, MCGEE DS, JOHNSTON WE, VINTEN-JOHANSEN J, 1993. Protection from ischaemic-reperfusion injury with adenosine pretreatment is reversed by inhibition of ATP sensitive potassium channels. *Cardiovascular Research* **27**: 623-629.

TRACEY WR, MAGEE W, MASAMUNE H, KENNEDY SP, KNIGHT DR, BUCHHOLZ RA, HILL RJ, 1997. Selective adenosine A3 receptor stimulation reduces ischemic myocardial injury in the rabbit heart. *Cardiovascular Research* **33**: 410-415.

VAN WINKLE DM, CHIEN GL, WOLFF RA, SOIFER BE, KUZUME K, DAVIS RF, 1994. Cardioprotection provided by adenosine receptor activation is abolished by blockade of the KATP channel. *American Journal of Physiology* **266**: H829-39.

VANDER HEIDE RS, REIMER KA, JENNINGS RB, 1993. Adenosine slows ischaemic metabolism in canine myocardium in vitro: relationship to ischaemic preconditioning. *Cardiovascular Research* **27**: 669-673.

VANOVERSCHELDE JL, WIJNS W, KOLANOWSKI J, BOL A, DECOSTER PM, MICHEL C, COGNEAU M, HEYNDRICKX GR, ESSAMRI B, MELIN JA, 1993. Competition between palmitate and ketone bodies as fuels for the heart: study with positron emission tomography. *American Journal of Physiology* **264**: H701-7.

VOLTERRANI M, ROSANO G, COATS A, BEALE C, COLLINS P, 1995. Estrogen acutely increases peripheral blood flow in postmenopausal women. *American Journal of*

Medicine **99**: 119-122.

WALSH RS, BORGES M, THORNTON JD, COHEN MV, DOWNEY JM, 1995. Hypoxia preconditions rabbit myocardium by an adenosine receptor-mediated mechanism. *Canadian Journal of Cardiology* **11**: 141-146.

WANG J, DRAKE L, SAJJADI F, FIRESTEIN GS, MULLANE KM, BULLOUGH DA, 1997. Dual activation of adenosine A1 and A3 receptors mediates preconditioning of isolated cardiac myocytes. *European Journal of Pharmacology* **320**: 241-248.

WEINER CP, LIZASOAIN I, BAYLIS SA, KNOWLES RG, CHARLES IG, MONCADA S, 1994. Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 5212-5216.

WHELAN WJ, 1986. The initiation of glycogen synthesis. *Bioessays* **5**: 136-140.

WHITE RE, DARKOW DJ, LANG JL, 1995. Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. *Circulation Research* **77**: 936-942.

WHITEHEAD MI, 1988. Effects of hormone replacement therapy on cardiovascular disease: an interview. *American Journal of Obstetrics & Gynecology* **158**: 1658-9.

WILLIAMS JK, ADAMS MR, KLOPFENSTEIN HS, 1990. Estrogen modulates responses of atherosclerotic coronary arteries. *Circulation* **81**: 1680-1687.

WISNESKI JA, STANLEY WC, NEESE RA, GERTZ EW, 1990. Effects of acute hyperglycemia on myocardial glycolytic activity in humans. *Journal of Clinical Investigation* **85**: 1648-1656.

WOLFE CL, SIEVERS RE, VISSEREN FL, DONNELLY TJ, 1993. Loss of myocardial protection after preconditioning correlates with the time course of glycogen recovery within the preconditioned segment. *Circulation* **87**: 881-892.

WOOLFSON RG, PATEL VC, NEILD GH, YELLON DM, 1995. Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation* **91**: 1545-1551.

YABE K, NASA Y, TAKEO S, 1995. Hypoxic preconditioning in isolated rat hearts: non-involvement of activation of adenosine A1 receptor, Gi protein, and ATP-sensitive K+ channel. *Heart & Vessels* **10**: 294-303.

YAO Z, GROSS GJ, 1994. The ATP-dependent potassium channel: an endogenous cardioprotective mechanism. *Journal of Cardiovascular Pharmacology* **24**: S28-34.

YASMIN W, STRYNADKA KD, SCHULZ R, 1997. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovascular Research* **33**: 422-432.

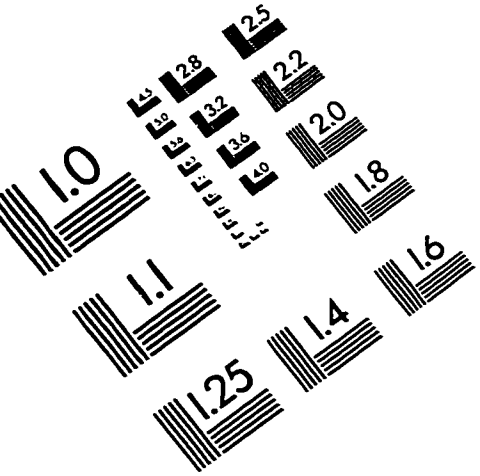
YOSHIKUNI Y, EZURE Y, AOYAGI Y, ENOMOTO H, 1988. Inhibition of intestinal alpha-glucosidase and postprandial hyperglycemia by N-substituted moranoline derivatives. *Journal of Pharmacobio-Dynamics* **11**: 356-362.

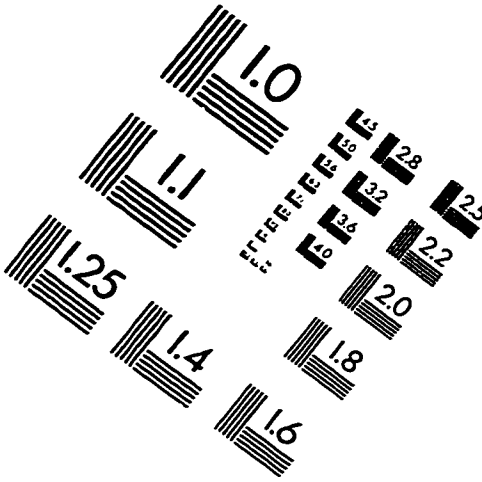
YOUNG ME, RADDA GK, LEIGHTON B, 1996. Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR--an activator of AMP-activated protein kinase. *FEBS Letters* **382**: 43-47.

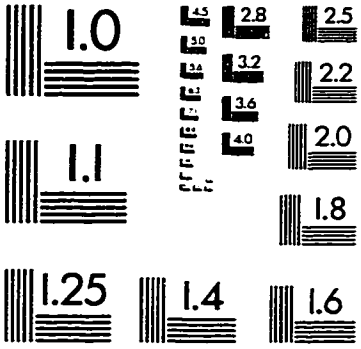
ZHAO L, WEBER PA, SMITH JR, COMERFORD ML, ELLIOTT GT, 1997. Role of inducible nitric oxide synthase in pharmacological "preconditioning" with monophosphoryl lipid A. *Journal of Molecular & Cellular Cardiology* **29**: 1567-1576.

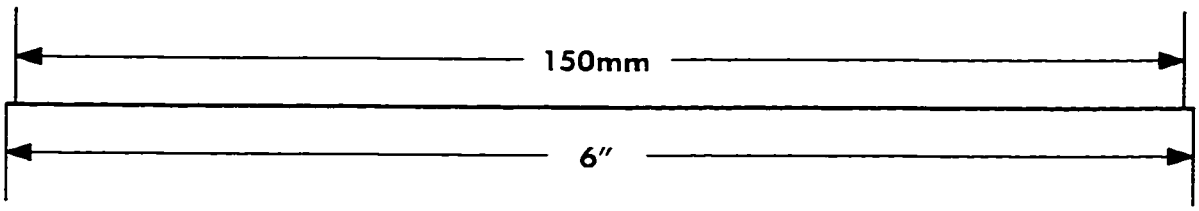
ZHOU Y, WATTERS JJ, DORSA DM, 1996. Estrogen rapidly induces the phosphorylation of the cAMP response element binding protein in rat brain. *Endocrinology* **137**: 2163-2166.

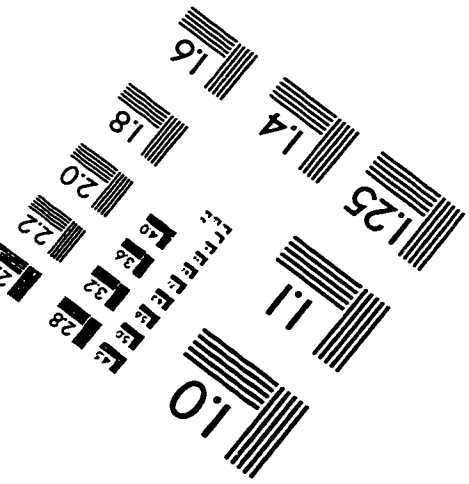
IMAGE EVALUATION
TEST TARGET (QA-3)

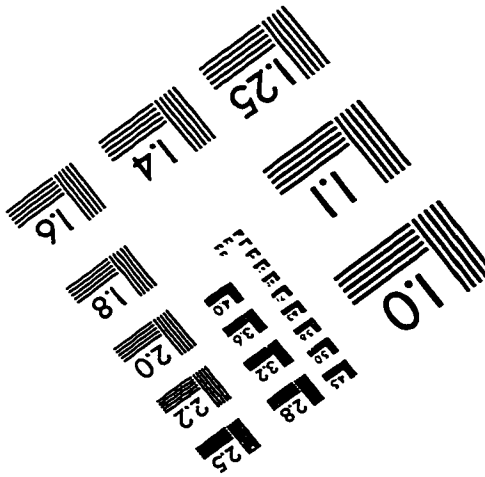












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989