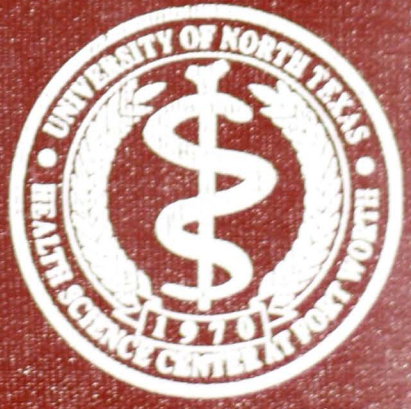
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Knott, E. Marty, The Effects of Pyruvate on Oxidative Stress and Myocardial Energetics During Cardioplegic Arrest and Reperfusion Doctor of Philosophy (Integrative Physiology),October 2005, 118 pp, 1 table, 17 figures, references, 130 titles.

Cardioplegic arrest for bypass surgery imposes global ischemia on the myocardium generating oxyradicals which contribute to post-surgical cardiac dysfunction. Early clinical trials have demonstrated that pyruvate-fortified cardioplegia reduces myocardial energy and improves mechanical recovery in patients undergoing elective cardiopulmonary bypass for coronary artery bypass grafting. This study was designed to determine the effects of the natural carbohydrate, pyruvate, on oxidative stress, myocardial energy state, and activities of myocardial metabolic enzymes during and immediately following cardiopulmonary bypass. In the first set of experiments, *in situ* swine hearts were arrested for 60 min with a 4:1 mixture of blood and crystalloid cardioplegia solution containing 188 mM glucose alone (control) or with additional 23.8 mM lactate or 23.8 mM pyruvate, then reperfused for 3 min with cardioplegia-free blood. Glutathione redox state (GSH/GSSG) and phosphocreatine phosphorylation potential were determined from measurements of myocardial metabolites in left ventricular heart tissue snap frozen at 45 min arrest and 3 min reperfusion. Coronary sinus 8-isoprostane indexed oxidative stress. Pyruvate-fortified cardioplegia decreased oxidative stress, lowering 8-isoprostane content accumulated during arrest and reperfusion. Phosphorylation potential was maintained in all groups during arrest but fell upon reperfusion in the control and lactate cardioplegia groups. Use of pyruvate cardioplegia during arrest prevented the decline in phosphorylation potential during reperfusion.

Pyruvate cardioplegia doubled GSH/GSSG during arrest as compared to lactate, but GSH/GSSG fell during reperfusion in all 3 groups. Pyruvate proved to be an effective antioxidant and energy yielding fuel in the setting of cardioplegic arrest and reperfusion. From these data, we hypothesized that pyruvate would protect oxidant-sensitive enzymes from inactivation. To test this hypothesis, *in situ* swine hearts were arrested for 60 min with control cardioplegia and reperfused for 3 min with cardioplegia-free blood alone or with co-infusion of *c.* 12 mM pyruvate. Activities of oxidant-sensitive enzymes, 8-isoprostane content, and energy and antioxidant metabolites were measured in left ventricular myocardium snap-frozen at 45 min arrest and 3 min reperfusion. At 3 min reperfusion, glutathione redox state fell by 70% while 8-isoprostane content increased 75%. Pyruvate administration during reperfusion suppressed oxidative stress, maintained glutathione redox state, and enhanced phosphocreatine phosphorylation potential. Aconitase and glucose 6-phosphate dehydrogenase activities fell during arrest; creatine kinase and phosphofructokinase were inactivated upon reperfusion. Pyruvate protected creatine kinase and reactivated aconitase, which are at least partially mitochondrial enzymes, but did not modify the cytosolic enzymes glucose 6-phosphate dehydrogenase and phosphofructokinase. We conclude that 1) pyruvate-fortified cardioplegia and administration of pyruvate during early reperfusion increase the antioxidant state of the heart and reduce oxidative stress occurring as a result of cardioplegic arrest and reperfusion; 2) pyruvate bolsters the myocardial energy state during early reperfusion when administered during cardioplegic arrest or during reperfusion; 3) Cardioplegic arrest and reperfusion inactivates several key metabolic enzymes. Pyruvate administration during reperfusion, the period of most intense oxidative stress, increases

the activity of two mitochondrial enzymes during early reperfusion when compared to control. These investigations provide likely mechanisms for the ability of pyruvate-fortified cardioplegia to reduce myocardial injury and improve post-surgical cardiac performance in patients undergoing CPB. More research must be done to solidify pyruvate's role as a cardioprotective intervention during CPB.

PYRUVATE MITIGATES OXIDATIVE STRESS

AND ENHANCES MYOCARDIAL ENERGETICS DURING

CARDIOPLEGIC ARREST AND REPERFUSION

E. Marty Knott, B.A.

APPROVED:

Robin T. Mallet

Major Professor

Committee Member

H. Fred Downey

Committee Member

James L. Caffrey

Committee Member

Committee Member

Committee Member

University Member

Michael L. Smith

Chair, Department of Integrative Physiology

Dean, Graduate School of Biomedical Sciences

THE EFFECTS OF PYRUVATE ON OXIDATIVE STRESS AND MYOCARDIAL ENERGETICS DURING CARDIOPLEGIC ARREST AND REPERFUSION

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

E. Marty Knott

Fort Worth, Texas

October, 2005

ACKNOWLEDGEMENTS

This study was supported by grants from the Osteopathic Heritage Foundation (02-18-522) and the National Heart, Lung and Blood Institute (HL071684).

Working on this project provided me the opportunity to learn science from some great minds. I wish to thank my major professor, Robert T. Mallet, Ph.D., who taught me to think, write and enjoy research. I also thank my co-major professor, Albert H. Olivencia-Yurvati, D.O., for mentoring me as I pursue my goal of becoming a cardiothoracic surgeon that is actively involved in research. Without the guidance and encouragement of H. Fred Downey, Ph.D., I would not have discovered my love for research and the pursuit of scientific knowledge. In addition, I appreciate the commitment of my committee members: James Caffrey, Ph.D., Jerry Simecka, Ph.D., Johnathan Tune, Ph.D., and Neeraj Agarwal, Ph.D. This project could not have been completed without the excellent technical assistance of Jie Sun, Myoung-Gwi Ryou, Abraham Heymann, Arti Sharma, Yu Lei, Mirza Baig, and Arthur Williams.

Finally, I would like thank my wife and sons, who have shown me patience, support, and love as I have completed this project.

TABLE OF CONTENTS

ABBREVIATIONS

BL	baseline
CPB	cardiopulmonary bypass
Cr	creatine
ΔG_{ATP}	Gibb's free energy of ATP hydrolysis
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
IL	interleukin
LDH	lactate dehydrogenase
NF-κB	nuclear factor kappaB
$O_2^{\bullet -}$	superoxide
$ONOO^-$	peroxinitrite
PCr	phosphocreatine
PFK	phosphofructokinase
P_i	inorganic phosphate
ROS	reactive oxygen species
TNF-α	tumor necrosis factor-alpha

PEER-REVIEWED PUBLICATIONS

Knott, EM, M-G Ryou, J Sun, A Heymann, AB Sharma, Y Lei, M Baig, RT Mallet, AH Olivencia-Yurvati. (2005) Pyruvate-fortified cardioplegia suppresses oxidative stress and enhances phosphorylation potential of arrested myocardium. *Am J Physiol Heart Circ Physiol.* 289:H1123-1130.

Knott, EM, J Sun, Y Lei , M-G Ryou, AH Olivencia-Yurvati , RT Mallet (2006) Pyruvate mitigates oxidative stress during reperfusion of cardioplegia-arrested myocardium. *Ann Thor Surg* 81: 928-34.

Knott, EM, JD Tune, S Stoll, HF Downey (2005) Lymphatic pump treatments increase thoracic duct flow. *JAOA* 105: 447-456.

Sharma, AB, **EM Knott**, J Bi, RR Martinez, J Sun, RT Mallet. (2005) Pyruvate improves cardiac electromechanical and metabolic recovery from cardiopulmonary arrest and resuscitation. *Resuscitation* 66:71-81.

Mallet, RT, J Sun, **EM Knott**, AB Sharma, AH Olivencia-Yurvati (2005) Metabolic cardioprotection by pyruvate: recent progress. *Exp Biol Med.* 230: 435-443.

ABSTRACTS

Knott, EM, MG Ryou, J Sun, Heymann A, RR Martinez, AB Sharma, Y Lei, RT Mallet, AH O-Yurvati (2005) Pyruvate cardioplegia reduces oxidative stress and improves myocardial energy state *FASEB J* 19:A690.

Sharma, AB, J Sun, **EM Knott**, RR Martinez, RT Mallet (2005) Antioxidants protect myocardial enzymes during cardiac arrest. *FASEB J* 19: A689.

Mallet, RT, J Sun J, **EM Knott**, R Martinez, AB Sharma (2004) Intravenous pyruvate therapy improves myocardial electromechanical recovery from cardiac arrest. *Circulation* 110:III-1099.

Mallet, RT, AB Sharma, **EM Knott**, J Bi, RR Martinez, J Sun (2004) Pyruvate augments phosphorylation potential and glutathione redox state of arrested myocardium. *J Mol Cell Cardiol* 37:284.

Sun, J, AB Sharma, **EM Knott,** J Bi, R Martinez, RT Mallet (2004) Cardiac Function and phosphorylation potential during cardiac resuscitation: pyruvate therapy. *FASEB J* 18:A261.

AWARDS

- 2006 George W. Northup, DO, Medical Writing Award
- 2006 Texas College of Osteopathic Medicine 2005-2006 Student D.O. of the Year
- 2006 Member of American Association for the Advancement of Science
- 2005 National Student Research Forum: Surgery Award – 'Pyruvate Cardioplegia Suppresses Oxidative Stress and Bolsters Phosphorylation Potential of Arrested Myocardium'
- 2005 Outstanding Graduate Student of the Year – Department of Integrative Physiology, University of North Texas Health Science Center
- 2005 Sigma Xi Member – National Research Honors Society
- 2005 OCCTIC Conference – Student Essay Contest Award
- 2005 UNTHSC Research Appreciation Day: 2nd Prize, poster presentation – 'Pyruvate Cardioplegia Suppresses Oxidative Stress and Preserves Phosphorylation Potential of Arrested Myocardium'
- 2005 Frontiers in Cardiology Conference – Best Research Paper by a Graduate Student
- Young Investigator Award – 2004 International Conference on Advances in Osteopathic Research

- 2004 Burnett Student Osteopathic Research Award
- 2003 UNTHSC Research Appreciation Day: 1st Prize, oral presentation – 'Lymphatic Pump Treatments Increase Thoracic Duct Flow'
- 2002 American Heart Association Summer Grant – Student Scholar in Cardiovascular Disease and Stroke

TABLE

CHAPTER II

FIGURES

CHAPTER I.

Figure

CHAPTER II.

Figure

CHAPTER IV.

Figure

CHAPTER I

INTRODUCTION

The great 19th century surgeon, Theodore Billroth, once said that "any surgeon who operates on the heart, should lose the respect of his colleagues." Much has changed from that time as cardiac surgery has evolved from a taboo to a well-respected and masterful art of cardiac protection and preservation.

History of cardiopulmonary bypass

One of the first men to consider extracorporeal circulation as a means of assisting in cardiac operation was John Heysham Gibbon, Jr. His thought process likely began in October of 1930, when, during his fellowship at Harvard, he watched a patient slowly die from a massive pulmonary embolus. His account of that afternoon follows. "...Helplessly watching the patient struggle for life as her blood became darker and her veins more distended, the idea naturally occurred to me that if it were possible to remove continuously some of the blue blood from the patient's swollen veins, put oxygen into that blood and allow carbon dioxide to escape from it, and then to inject continuously the now-red blood back into the patient's arteries, we might have saved her life.... we would have bypassed the obstructing embolus and performed part of the work of the patient's heart and lungs outside the body (22)."

A wealth of knowledge had been gathered prior to Gibbon's thought of extracorporeal circulation. In the late 18th century, Lavoisier discovered the role of gas

exchange by the lungs and, thus, the essential role of oxygen in sustaining life. In 1812, LeGallois first proposed the idea of artificial circulation to deliver arterial blood to organs. The discovery of heparin as an anticoagulant by McLean in 1916 was also an important step. Gibbon understood these concepts as well as the need to prevent hemolysis, avoid air embolus, maintain temperature, and provide a pump for reperfusion. After 20 years of investigation, Gibbon performed the first cardiopulmonary bypass (CPB) in February 1952 on a 15-month-old girl diagnosed with a large atrial septal defect. The baby died during exploration, revealing that the defect was actually a patent ductus arteriosus. The second attempt was successful; in May 1953, 26 minutes of CPB allowed Gibbon and colleagues to repair an atrial septal defect in an 18-year-old girl. The model II oxygenator that was used is shown in Figure 1 (56). The evolution of the CPB circuit has continued as the size and ease of use have improved (Figure 2).

One of the major complications of early CPB was the introduction of deadly air emboli into the circulation (57). It was also difficult to operate on the blood filled beating heart. In 1955, Melrose was the first to "electively" arrest the heart. He termed the technique "cardioplegia (47)." The potassium based solution used by Melrose was designed to reduce air emboli and was not intended to protect the heart. The use of Melrose cardioplegia lost favor when surgeons found that there was late vascular and myocardial injury (35,71). Patchy necrosis was discovered in up to 30% of the hearts after cardiac surgery (49). Investigations later revealed that the high concentration of KCl in Melrose solution, not the arrest itself, was the cause of myocardial damage (65,67). In 1973, crystalloid cardioplegia solutions with much lower concentrations of

KCl were used to more safely achieve electromechanical arrest (21). Alterations in Melrose cardioplegia lead to the development of potassium chloride solutions such as St. Thomas's solution (3,24). First used clinically in 1976 (3), St. Thomas's solution is still used today. The delicate balance of myocardial protection versus efficient, reversible cardiac arrest has been studied since its initial use in 1955. Even today, ongoing studies are attempting to optomize temperature, route of delivery, and metabolic components to protect the myocardium more effectively and ultimately improve patient recovery from cardiac surgery. Much work has been done to minimize the damaging effects of CPB, but there is still room for improvement.

According to the American Heart Association, coronary artery bypass grafting (CABG) is the most commonly performed surgery in the world, with over 500,000 surgeries performed in 2000. CPB is still the gold standard for coronary revascularization (60). Moreover, CPB is an essential component of many other cardiac surgical procedures, allowing the heart to be arrested without compromising blood flow to the body.

Cardiopulmonary bypass induces oxidative stress

Despite its importance and obvious necessity, CPB is known to increase oxidative stress and elicit a complex systemic inflammatory response. A variety of reactive oxygen species (ROS) are generated through numerous mechanisms. Dröge (16) provides an excellent review of the formation and neutralization of ROS. ROS have the potential to inflict cellular damage by oxidizing and inactivating enzymes and by oxidizing

phospholipids, increasing membrane permeability and impairing ion transport (31). ROS and reactive nitrogen intermediates are thought to be involved in the pathogenesis of ischemic heart disease, myocardial infarction and stunning, cardiac failure and post-bypass myocardial dysfunction. They have also been shown to contribute to arrhythmias (28) and apoptotic signal pathway activation (11). A recent investigation examined oxidative stress and inflammation in 20 children undergoing elective heart surgery (8). Oxidative stress occurred immediately after CPB, while cytokine release peaked 3-12 hr after bypass. Oxidative stress is known to activate phosphorylation of nuclear factor κB, resulting in its translocation to the nucleus where it binds to DNA and induces expression of inflammatory mediators (54). Multiple processes, including neutrophil activation, cytokine release, and oxyradical production characterize the resultant systemic inflammatory response syndrome (SIRS) (32). The inflammation is severe and is thought to be the cause of many complications following CPB (27). The most common post-surgical complication is respiratory dysfunction, but multiple organs may be damaged by systemic inflammation.

Cardiopulmonary bypass induces an increase in the body's natural antioxidant defenses (34). A recent clinical investigation measured the total antioxidant capacity of the plasma and glutathione peroxidase and superoxide dismutase, which are responsible for the reduction of peroxides and superoxides, respectively. Glutathione peroxidase, superoxide dismutase, and total antioxidant capacity increased significantly during CPB and returned to baseline within 24 hours (34). Plasma alpha-tocopherol, a measure of antioxidant status, gradually increases beginning at 20 min CPB and continuing to increase for 6 hr after CPB (70).

Cardiopulmonary bypass elicits oxyradical formation (14,17,30), which could contribute to post-bypass myocardial dysfunction (13,33,71). The increase in natural antioxidant defenses that occurs during CPB are not sufficient to prevent the detrimental effects of the overwhelming formation of oxyradicals. Fischer *et al.* (18) demonstrated the ability of *N*-acetylcysteine, a pharmacologic antioxidant, to reduce oxidative stress (assessed from 8-isoprostane in coronary sinus blood) and improve post-bypass cardiac performance when intravenously infused in dogs. 8-Isoprostane, a product of lipid peroxidation and measure of oxidative stress (36,58), has been shown to be elevated in blood following CPB in several studies (18,44,66,69). In addition to its vasoconstrictor effects (1), 8-isoprostane has been proposed as a risk marker of coronary heart disease (61). N-acetylcysteine, when given intravenously to patients during and after CABG surgery with CPB, did not prevent postoperative renal dysfunction, medical complications, or mortality (6). The direct effects of the antioxidant on the heart were not studied.

Pyruvate

Pyruvate is a natural aliphatic carbohydrate and metabolic intermediate in mammalian cells. Pyruvate is gaining increased attention as a natural means of protecting the myocardium from damage associated with oxidative stress and ischemia-reperfusion. The physiologic concentration of pyruvate in arterial plasma is between 0.1 and 0.2 m*M* in overnight-fasted pigs (53) and humans (45). While the heart does not rely heavily on pyruvate for normal metabolism due to these submillimolar plasma

concentrations, it is able to respond functionally and metabolically to exogenous pyruvate.

Extracellular pyruvate is cotransported with a proton into the cytoplasm via the electroneutral, sarcolemmal monocarboxylate-proton symporter (23). Transport of cytosolic pyruvate into the mitochondria via the monocarboxylate transporter on the inner mitochondrial membrane (23) serves an essential role for pyruvate's positive inotropic effects (39).

Pyruvate enhances post-ischemic cardiac performance and Ca^{2+} handling.

Adequate ATP synthesis is required to maintain normal cardiac performance. Ischemia reduces oxygen and substrate supply to the heart so that aerobic metabolism and oxidative phosphorylation of ATP are compromised. Depletion of ATP stores reduces available energy supply for critical cellular functions including ion transport and myofilament contraction. Enhancement of post-ischemic myocardial function by pyruvate has been demonstrated in several studies. In a recent investigation (62), pyruvate administration during open-chest cardiac compression and the first 25 min of recovery from cardiopulmonary arrest hastened recovery of phosphorylation potential. Enhancement of ATP phosphorylation potential by pyruvate in this (62) and other investigations (5,29,38,48,73) increases cytosolic Gibbs free energy of ATP hydrolysis (ΔG_{ATP}), the immediate energy source for cellular work. Reductions in ΔG_{ATP} impair calcium handling (sarcoplasmic reticulum Ca^{2+} ATPase) and cross-bridge cycling (actin-myosin ATPase) (25), critical steps in force-generating contraction. Pyruvate has been

shown to increase sarcoplasmic reticular Ca^{2+} uptake and release while increasing both energy state and contractile performance (7,37,39,43,46). Inhibition of the inner mitochondrial membrane pyruvate transporter by α-*cyano*-3-hydroxycinnamate (4) prevented these beneficial effects (39,43). Therefore mitochondrial oxidation of pyruvate likely generates the energy required for enhancement of Ca^{2+} handling and, thus, cardiac contractile performance. In addition, pyruvate reduces ROS formation, which may further explain pyruvate's ability to improve calcium handling and prevent calcium overload upon reperfusion. Furthermore, pyruvate does not increase heart rate (5,51,53), and therefore, does not increase the internal work of the heart.

Pyruvate enhances myocardial antioxidant systems

Oxidative stress has been implicated in the pathogenesis of ischemia-reperfusion induced myocardial dysfunction. Cells contain natural defense mechanisms to neutralize damaging oxyradicals. Enhancements of the cellular antioxidant systems have the potential to reduce oxyradical injury by reducing the associated oxidative stress. The glutathione system can effectively neutralize hydrogen peroxide, peroxynitrite and lipid peroxides. Indeed, this system is the major antioxidant mechanism in mammalian cells. The reducing power of this system is determined by the ratio of reduced glutathione (GSH) to oxidized glutathione disulfide (GSSG). Moreover, changes in GSH/GSSG accurately reflect global changes in all of the intracellular antioxidant redox couples, including the α-tocopherol, ascorbate and thioredoxin systems, because all of these systems are in a state of near equilibrium within the cell (19,26,55,59).

The effect of pyruvate on GSH redox potential was measured in the guinea-pig myocardium challenged with hydrogen peroxide (41). Hearts were perfused with 0.1 mM H_2O_2 for 10 min, followed by 30 min H_2O_2-free washout before 60 min treatment with 5 mM pyruvate or other compounds, and then snap-frozen for measurement of energy and antioxidant metabolites. Cardiac mechanical function progressively declined during and following H_2O_2 exposure before stabilizing at 30% of baseline. H_2O_2 also depleted the myocardial antioxidant reserve, indicated by a decrease in GSH/GSSG. Phosphorylation potential initially was lowered by H_2O_2 but recovered as the cardiac performance fell, due to the decreased energy demands of the H_2O_2 injured, hypocontractile myocardium. Pyruvate treatment restored function to the level seen in H_2O_2-free sham experiments. Pyruvate maintained phosphorylation potential despite this increased energy demand. Pyruvate also enhanced GSH/GSSG. Interestingly, lactate, which is oxidized by the same metabolic pathways as pyruvate but lacks pyruvate's antioxidant properties, did not restore function, phosphorylation potential, nor GSH/GSSG. *N*-Acetylcysteine (NAC), a pharmacologic antioxidant but not an energy yielding fuel, was able to restore GSH/GSSG, but had no salutary effect on contractile performance and failed to maintain phosphorylation potential. Therefore, the unique combination of maintained energy supply in the face of a greater energy demand and augmented GSH/GSSG could be responsible for pyruvate's restoration of contractile function in these oxidatively stressed hearts.

Pyruvate, a powerful antioxidant

Pyruvate has proven effective at reducing organ injury or dysfunction in a variety of models of oxidative stress, including myocardial (2,5,12,15), intestinal (9), or hepatic (63) ischemia-reperfusion injury. Pyruvate protects the myocardium from direct damage by peroxides (42). Figure 3 summarizes the antioxidant mechanisms of pyruvate (40). 1) Pyruvate detoxifies hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) by direct chemical reactions. 2) Pyruvate metabolism increases intracellular citrate (10), which inhibits phosphofructokinase (PFK) (20), causing glucose-6-phosphate to accumulate and thereby provide substrate for the hexose monophosphate shunt, the source of NADPH reducing power to maintain GSH. 3) Conversion of citrate to isocitrate provides substrate for $NADP^+$ dependent isocitrate dehydrogenase, potentially a second source of NADPH. Pyruvate also increases the ATP phosphorylation potential and lowers free cytosolic concentrations of ADP and AMP. This lessens the production of hypoxanthine and xanthine (38), which serve as substrates for ROS formation by xanthine oxidase (64).

Pyruvate's ability to reduce oxidative stress may also play an important role in reducing the inflammatory response to CPB. As previous discussed, ROS are important mediators in the inflammatory response (54). Pyruvate may act as an indirect anti-inflammatory agent by reducing the formation of ROS. In fact, pyruvate inhibits the activation of NF-κB and TNF-α mRNA expression in mice subjected to mesenteric ischemia-reperfusion (68).

Cardioprotection by pyruvate cardioplegia in patients undergoing coronary artery bypass surgery

This novel study (52) investigated the efficacy of pyruvate to protect the heart in patients undergoing cardiac surgery on cardiopulmonary bypass. Patients either received pyruvate (10mM) fortified cardioplegia (n=15) or standard lactate (23.8 mM) based cardioplegia (n=15) (4 vol blood:1 vol crystalloid). The two groups were effectively matched for demographics, pre-surgical cardiac function and markers of ischemic injury. Post-surgical left ventricular stroke work was sharply increased in patients receiving pyruvate cardioplegia compared to those receiving lactate cardioplegia (Figure 4). Pyruvate also lowered coronary sinus Troponin I and creatine phosphokinase-MB release by 67 and 53%, respectively. ß-adrenergic support post-bypass was required for 10 patients in the lactate cardioplegia group but only for 4 patients in the pyruvate cardioplegia group (χ^2: P=0.067). Therefore, pyruvate both reduced myocardial injury and improved post-surgical cardiac function. The benefits of the temporary administration of pyruvate on the arrested myocardium lasted 12 h after cardioplegic arrest. These favorable results are the stimulus for the proposed investigation.

Summary

Cardiopulmonary bypass has come a long way since the humble beginnings of Gibbons. CPB is now standard practice for common surgeries such as CABG, valve replacements, and correction of congenital heart defects. While necessary, CPB has unfortunate and unintended consequences: SIRS, increased oxidative distress, and multi-organ dysfunction that all contribute to post-CPB morbidity and mortality.

A natural metabolite, pyruvate exerts a powerful anti-oxidant and energy yielding benefits in a variety of metabolically stressful situations in the myocardium. A preliminary clinical trial found that pyruvate-fortified cardioplegia reduces myocardial injury and improves post-surgical cardiac performance. The study which follows seeks to identify the mechanisms of pyruvate's salutary effects in the setting of cardioplegic arrest.

Specific aims

The first aim of this investigation was to determine the ability of lactate- and pyruvate-fortified cardioplegia solutions to dampen oxyradical release and bolster endogenous antioxidant defenses during cardioplegic arrest. The experiments were designed to determine the ability of lactate-based and pyruvate-fortified cardioplegias to alter myocardial energetics during cardioplegic arrest. *In situ* adult swine hearts were arrested with one of three cardioplegia solutions containing 188 mM glucose alone (control) or with either 23.8 mM lactate or 23.8 mM pyruvate added for 60 min and reperfused with cardioplegia-free blood for 3 min. 8-Isoprostane, an indicator of oxidative stress, was measured in plasma samples taken before, at 20 min intervals during, and at 1 and 3 minutes after cardioplegic arrest. Glutathione redox potential (GSH/GSSG) and phosphorylation potential were measured in myocardial tissue samples taken at 45 min arrest and 3 min reperfusion. The study was designed to test the hypothesis that pyruvate, more substantially than lactate, reduces oxidative stress associated with cardioplegic arrest and preserves glutathione antioxidant defenses in the

circulation and myocardial tissue during cardioplegic arrest. The study also tested the hypothesis that pyruvate, and to a lesser extent lactate, improves phosphocreatine phosphorylation potential. Pyruvate cardioplegia reduced oxidative stress during arrest and that antecedent lactate and pyruvate cardioplegia prevented lipid peroxidation during the first 3 min of reperfusion. Pyruvate cardioplegia bolstered the endogenous antioxidant state during arrest, while none of the solutions had an effect on GSH/GSSG during reperfusion. Finally, phosphorylation potential tended to increase in all 3 groups during arrest but fell in the control and lactate groups during reperfusion. In contrast, pyruvate cardioplegia maintained phosphorylation potential during reperfusion. Therefore, pyruvate-fortified cardioplegia reduces oxidative stress and preserves myocardial energy state in a large animal model of cardioplegic arrest.

The second aim of this investigation was to determine the effects of cardioplegic arrest and reperfusion on oxidant-sensitive metabolic enzymes, and whether pyruvate, administered during reperfusion rather than in cardioplegia, could reduce oxidative stress, protect or reactivate oxidant-sensitive enzymes, and thereby bolster the energy state of the heart. *In situ* swine hearts were arrested with control cardioplegia solution for 60 min as in specific aim 1. Hearts were then reperfused with cardioplegia-free blood with or without 12 mM pyruvate for 3 min. Tissue metabolites and enzyme activities were measured in left ventricular myocardium snap frozen at 45 min arrest or after 3 min reperfusion. Cardioplegic arrest and reperfusion inactivated several key metabolic enzymes. Pyruvate administration during reperfusion reduced oxidative stress and increased antioxidant redox state. Pyruvate protected the mitochondrial enzymes creatine

kinase and aconitase from inactivation but had no salutary effect on oxidant-inactivated cytosolic enzymes. Pyruvate also increased myocardial energy state when compared to control. Thus, pyruvate administration during reperfusion following cardioplegic arrest prevents mitochondrial enzyme inactivation while reducing oxidative stress and bolstering myocardial energy state.

Significance

This investigation, for the first time, identified favorable effects of pyruvate on oxidative stress, myocardial energy state, and enzyme activity when administered during cardioplegic arrest or reperfusion. Pyruvate may serve as a natural therapeutic for patients undergoing cardiopulmonary bypass with cardioplegic arrest. More research must be done to determine the long term benefits of pyruvate therapy on the heart in this setting.

REFERENCES

1. **Banerjee M, Kang KH, Morrow JD, Roberts LJ and Newman JH.** Effects of a novel prostaglandin, 8-epi-PGF2 alpha, in rabbit lung in situ. *Am J Physiol Heart Circ Physiol* 1992; 263:H660-663.

2. **Bassenge E, Sommer O, Schwemmer M and Bunger R.** Antioxidant pyruvate inhibits cardiac formation of reactive oxygen species through changes in redox state. *Am J Physiol Heart Circ Physiol* 2000; 279:H2431-2438.

3. **Braimbridge MV, Chayen J, Bitensky L, Hearse DJ, Jynge P and Cankovic-Darracott S.** Cold Cardioplegia or continuous coronary perfusion? *J Thorac Cardiovasc Surg* 1977; 74:900-906.

4. **Bunger R and Mallet RT.** Mitochondrial pyruvate transport in working guinea-pig heart. Work-related vs. carrier-mediated control of pyruvate oxidation. *Biochim Biophys Acta* 1993; 1151:223-236.

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

6. **Burns KE, Chu MW, Novick RJ, Fox SA, Gallo K, Martin CM, Stitt LW, Heidenheim AP, Myers ML and Moist L.** Perioperative N-acetylcysteine to prevent renal dysfunction in high-risk patients undergoing CABG surgery: a randomized controlled trial. *JAMA* 2005; 294:342-350.

7. **Chen W, London R, Murphy E and Steenbergen C.** Regulation of the Ca^{2+} gradient across the sarcoplasmic reticulum in perfused rabbit heart. A 19F nuclear magnetic resonance study. *Circ Res* 1998; 83:898-907.

8. **Christen S, Finckh B, Lykkesfeldt J, Gessler P, Frese-Schaper M, Nielsen P, Schmid ER and Schmitt B.** Oxidative stress precedes peak systemic inflammatory response in pediatric patients undergoing cardiopulmonary bypass operation. *Free Radic Biol Med* 2005; 38:1323-1332.

9. **Cicalese L, Lee K, Schraut W, Watkins S, Borle A and Stanko R.** Pyruvate prevents ischemia-reperfusion mucosal injury of rat small intestine. *Am J Surg* 1999; 171:97–100.

10. **Comte B, Vincent G, Bouchard B, Jette M, Cordeau S and Rosiers CD.** A ^{13}C mass isotopomer study of anaplerotic pyruvate carboxylation in perfused rat hearts. *J Biol Chem* 1997; 272:26125-26131.

11. **Cook SA and Poole-Wilson PA.** Cardiac myocyte apoptosis. *Eur Heart J* 1999; 20:1619-1629.

12. **Crestanello JA, Lingle DM, Millili J and Whitman GJ.** Pyruvate improves myocardial tolerance to reperfusion injury by acting as an antioxidant: a chemiluminescence study. *Surgery* 1998; 124:92-99.

13. **Curello S, Ceconi C, Gluili Fd, Panzali AF, Milanesi B, Calarco M, Pardini A, Marzollo P, Alfieri O, Messineo F and Ferrari R.** Oxidative stress during

reperfusion of human hearts: Potential sources of oxygen free radicals. *Cardiovasc Res* 1995; 29:118-125.

14. **Davies S, Duffy J, Wickens D, Underwood S, Hilla A, Alladine F, Feneck R, Dormandy T and Walesby R.** Time-course of free radical activity during coronary artery operations with cardiopulmonary bypass. *J Thorac Cadiovasc Surg* 1993; 105:979-987.

15. **DeBoer LW, Bekx PA, Han L and Steinke L.** Pyruvate enhances recovery of rat hearts after ischemia and reperfusion by preventing free radical generation. *Am J Physiol Heart Circ Physiol* 1993; 265:H1571-576.

16. **Droge W.** Free radicals in the physiological control of cell function. *Physiol Rev* 2002; 82:47-95.

17. **Ferrari R, Ottavio A, Curello S, Ceconi C, Cargnoni A, Marsollo P, Pardini A, Caradonna E and Visioli O.** Occurrence of oxidative stress during reperfusion of the human heart. *Circulation* 1990; 81:201-211.

18. **Fischer UM, Cox CS Jr, Allen SJ, Stewart RH, Mehlhorn U and Laine GA.** The antioxidant N-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest. *J Thorac Cardiovasc Surg* 2003;126:1483-1488.

19. **Freisleben HJ and Packer L.** Free-radical scavenging activities, interactions and recycling of antioxidants. *Biochem Soc Trans* 1993; 21:325-330.

20. **Garland PB, Randle PJ and Newsholme EA.** Citrate as an intermediary in the inhibition of phosphofructokinase in the rat heart muscle by fatty acids, ketone bodies, pyruvate, diabetes, and starvation. *Nature* 1963; 200:169-170.

21. **Gay WA and Ebert PA.** Functional metabolic, and morphologic effects of potassium-induced cardioplegia. *Surgery* 1973; 74:284-290.

22. **Gibbon JH Jr.** The development of the heart-lung apparatus. *Rev Surg* 1970; 27:231-244.

23. **Halestrap AP.** The mitochondrial pyruvate carrier: kinetics and specificities for substrates and inhibitors. *Biochem J* 1975; 148:85-96.

24. **Hearse DJ, Stewart DA and Chain EB.** Recovery from cardiac bypass and elective cardiac arrest. *Circ Res* 1974; 35:448-457.

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

26. **Kehrer JP and Lund LG.** Cellular reducing equivalents and oxidative stress. *Free Radic Biol Med* 1994; 17:65-75.

27. **Kirklin JK.** The postperfusion syndrome: inflammation and the damaging effects of cardiopulmonary bypass. In: Tinker JD, ed. Cardiopulmonary bypass: current concepts and controversies. Philadelphia: Saunders, 1989:131-146.

28. **Kusama Y, Bernier M and Hearse DJ.** Exacerbation of reperfusion arrhythmias by sudden oxidant stress. *Circ Res* 1990; 67:481-489.

29. **Lasley RD, Bunger R, Zhou Z and Mentzer RM Jr.** Metabolically based treatment of stunned myocardium. *J Card Surg* 1994; 9:469-473.

30. **Lazzarino G, Raatikainen P, Nuutinen M, Nissinen J, Tavazzi B, Di Pierro D, Giardina B and Peuhkurinen K.** Myocardial release of malondialdehyde and purine compounds during coronary bypass surgery. *Circulation* 1994; 90:291-297.

31. **Lebedev AV, Levitsky DO, Loginov VA and Smirnov VN.** The effect of primary products of lipid peroxidation on the transmembrane transport of calcium ions. *J Mol Cell Cardiol* 1982; 14:99-103.

32. **Levy JH and Tanaka KA.** Inflammatory response to cardiopulmonary bypass. *Ann Thorac Surg* 2003; 75:715-720.

33. **Li XY, McCay P, Zughaib M, Jeroudi M, Triana JF and Bolli R.** Demonstration of free radical generation in the stunned myocardium in the conscious dog and identification of major differences between conscious and open-chest dog. *J Clin Invest* 1992; 92:1025-1041.

34. **Luyten CR, van Overveld FJ, De Backer LA, Sadowska AM, Rodrigus IE, De Hert SG and De Backer WA.** Antioxidant defense during cardiopulmonary bypass surgery. *Eur J Cardiothorac Surg* 2005; 27:611-616.

35. **MacFarland JA, Thomas LB, Gilbert JW, and Morrow AG.** Myocardial necrosis following elective cardiac arrest induced with potassium citrate. *J Thor Cardiovasc Surg* 1960; 40:200-208.

36. **Mak S and Newton GE.** The oxidative stress hypothesis of congestive heart failure: radical thoughts. *Chest* 2001; 120:2035-2046.

37. **Mallet RT and Bunger R.** Energetic modulation of cardiac inotropism and sarcoplasmic reticular Ca^{2+} uptake. *Biochim Biophys Acta* 1994; 1224:22-32.

38. **Mallet RT and Bunger R.** Metabolic protection of post-ischemic phosphorylation potential and ventricular performance. *Adv Exp Med Biol* 1993; 346:233-241.

39. **Mallet RT and Sun J.** Mitochondrial metabolism of pyruvate is required for its enhancement of cardiac function and energetics. *Cardiovasc Res* 1999; 42:149-161.

40. **Mallet RT and Sun J.** Antioxidant properties of myocardial fuels. *Mol Cell Biochem* 2003; 253:103-111.

41. **Mallet RT, Squires JE, Bhatia S and Sun J.** Pyruvate restores contractile function and antioxidant defenses of hydrogen peroxide-challenged myocardium. *J Mol Cell Cardiol* 2002; 34:1173-1184.

42. **Marcengill MB, Puri S, Puri SK, Mohanram A, Leonova E, Raymond RM and Watts JA.** Antioxidant effects of pyruvate in isolated rat hearts. *J Mol Cell Cardiol* 1995; 27:2059-2067.

43. **Martin BJ, Valdivia HH, Bunger R, Lasley RD and Mentzer RM Jr.** Pyruvate augments calcium transients and cell shortening in rat ventricular myocytes. *Am J Physiol* 1998; 274:H8-17.

44. **Mehlhorn U, Krahwinkel A, Geissler HJ, LaRosee K, Fischer UM, Klass O, Suedkamp M, Hekmat K, Tossios P and Bloch W.** Nitrotyrosine and 8-isoprostane formation indicate free radical-mediated injury in hearts of patients subjected to cardioplegia. *J Thorac Cardiovasc Surg* 2003; 125:178-183.

45. **Meierhenrich R, Jedicke H, Voigt A and Lange H**. The effect of erythropoietin on lactate, pyruvate and excess lactate under physical exercise in dialysis patients. *Clin Nephrol* 1996; 45:90-97.

46. **Mellors LJ, Kotsanas G and Wendt IR.** Effects of pyruvate on intracellular Ca^{2+} regulation in cardiac myocytes from normal and diabetic rats. *Clin Exp Pharmacol Physiol* 1999; 26:889-897.

47. **Melrose DG, Dreyer B, Bentall HH, and Baker JBE.** Elective cardiac arrest. *Lancet* 1955; ii:21-22.

48. **Mentzer RM Jr, Van Wylen DG, Sodhi J, Weiss RJ, Lasley RD, Willis J, Bunger R, Habil and Flint LM.** Effect of pyruvate on regional ventricular function in normal and stunned myocardium. *Ann Surg* 1989; 209:629-633.

49. **Morales AR, Fine G, and Taber RE.** Cardiac surgery and myocardial necrosis. *Arch Pathol* 1967; 83:71-79.

50. **Ng CS, Wan S, Yim AP and Arifi AA.** Pulmonary dysfunction after cardiac surgery. *Chest* 2002; 121:1269-1277.

51. **Ochiai K, Zhang J, Gong G, Zhang Y, Liu J, Ye Y, Wu X, Liu H, Murakami Y, Bache RJ, Ugurbil K and From AH**. Effects of augmented delivery of pyruvate on

myocardial high-energy phosphate metabolism at high workstate. *Am J Physiol Heart Circ Physiol* 2004; 281:H1823-1832.

52. **Olivencia-Yurvati AH, Blair JL, Baig M and Mallet RT.** Pyruvate-Enhanced cardioprotection during surgery with cardiopulmonary bypass. *J Cardiothorac Vasc Anesth* 2003; 17:715-720.

53. **Panchal AR, Comte B, Dudar B, Roth B, Chandler M, Des Rosiers C, Brunengraber H and Stanley WC.** Acute hibernation decreases myocardial pyruvate carboxylation and citrate release. *Am J Physiol Heart Circ Physiol* 2001; 281:H1613-1620.

54. **Paparella D, Yau TM and Young E.** Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. *Eur J Cardiothorac Surg* 2002; 21:232-244.

55. **Pascoe GA and Reed DJ.** Cell calcium, vitamin E, and the thiol redox system in cytotoxicity. *Free Radic Biol Med* 1989; 6:209-224.

56. **Romaine-Davis A.** *John Gibbon and his heart-lung machine.* Philadelphia: University of Pennsylvania Press, 1991:33.

57. **Salerno TA and Ricci M.** *Myocardial Protection.* Elmsford: Blackwell Publishing, Inc., 2004.

58. **Sakamoto H, Corcoran TB, Laffey JG and Shorten GD.** Isoprostanes--markers of ischaemia reperfusion injury. *Eur J Anaesthesiol* 2002; 19:550-559.

59. **Schafer FQ and Buettner GR.** Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001; 30:1191-1212.

60. **Schmitz C, Weinreich S, Schneider R, Schneider D, Speth I, Schulze-Rauschenbach C, Pohl C and Welz A.** Off-Pump versus on-pump coronary artery bypass: can OPCAB reduce neurologic injury? *Heart Surg Forum* 2003; 6:127-130.

61. **Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brummer J, Gutzki FM, Berger J, Frolich JC and Boger RH.** Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study. *Circulation* 2004; 109:843-848.

62. **Sharma, AB, EM Knott, J Bi, RR Martinez, J Sun and RT Mallet.** Pyruvate improves cardiac electromechanical and metabolic recovery from cardiopulmonary arrest and resuscitation. *Resuscitation* 2005; 66:71-81.

63. **Sileri P, Schena S, Morini S, Rastellini C, Pham S, Benedetti E and Cicalese L.** Pyruvate inhibits hepatic ischemia-reperfusion injury in rats. *Transplantation* 2001; 72:27–30.

64. **Tenney SM.** A theoretical analysis of the relationship between venous blood and mean tissue oxygen pressures. *Respir Physiol* 1974; 20:283-296.

65. **Todd GJ and Tyers GFO**. Potassium induced arrest of the heart: effect of low potassium concentrations. *Surg Forum* 1975; 26:255-256.

66. **Tossios P, Bloch W, Huebner A, Raji MR, Dodos F, Klass O, Suedkamp M, Kasper SM, Hellmich M and Mehlhorn U.** N-acetylcysteine prevents reactive oxygen species-mediated myocardial stress in patients undergoing cardiac surgery. *J Thorac Cardiovasc Surg* 2003; 126:1513-1520.

67. **Tyers GFO, Todd GJ, Neibauer IM, Manley NJ, and Waldenhausen JA**. The mechanism of myocardial damage following potassium citrate (Melrose) cardioplegia. *Surgery* 1975; 78:45-53.

68. **Uchiyama T, Delude RL and Fink MP.** Dose-dependent effects of ethyl pyruvate in mice subjected to mesenteric ischemia and reperfusion. *Intensive Care Med* 2003; 29:2050-2058.

69. **Ulus AT, Aksoyek A, Ozkan M, Katircioglu SF and Basu S.** Cardiopulmonary bypass as a cause of free radical-induced oxidative stress and enhanced blood-borne isoprostanes in humans. *Free Radic Biol Med* 2003; 34:911-917.

70. **Ulus AT, Aksoyek A, Ozkan M, Katircioglu SF, Vessby B, and Basu S.** Oxidative stress and changes in alpha- and gamma-tocopherol levels during coronary artery bypass grafting. *Ann N Y Acad Sci* 2004; 1031:352-356.

71. **Vergely C, Maupoil V, Benderitter M and Rochette L.** Influence of the severity of myocardial ischemia on the intensity of ascorbyl free radical release and on postischemic recovery during reperfusion. *Free Rad Biol Med* 1998; 24:470-479.

72. **Willman VL, Cooper T, Zafiracopoulos P, and Hanlon CR.** Depression of ventricular function following elective cardiac arrest with potassium citrate. *Surgery* 1959; 46:792-796.

73. **Zhou Z, Lasley RD, Hegge JO, Bunger R and Mentzer RM Jr.** Myocardial stunning: a therapeutic conundrum. *J Thorac Cardiovasc Surg* 1995; 110:1391-1401.

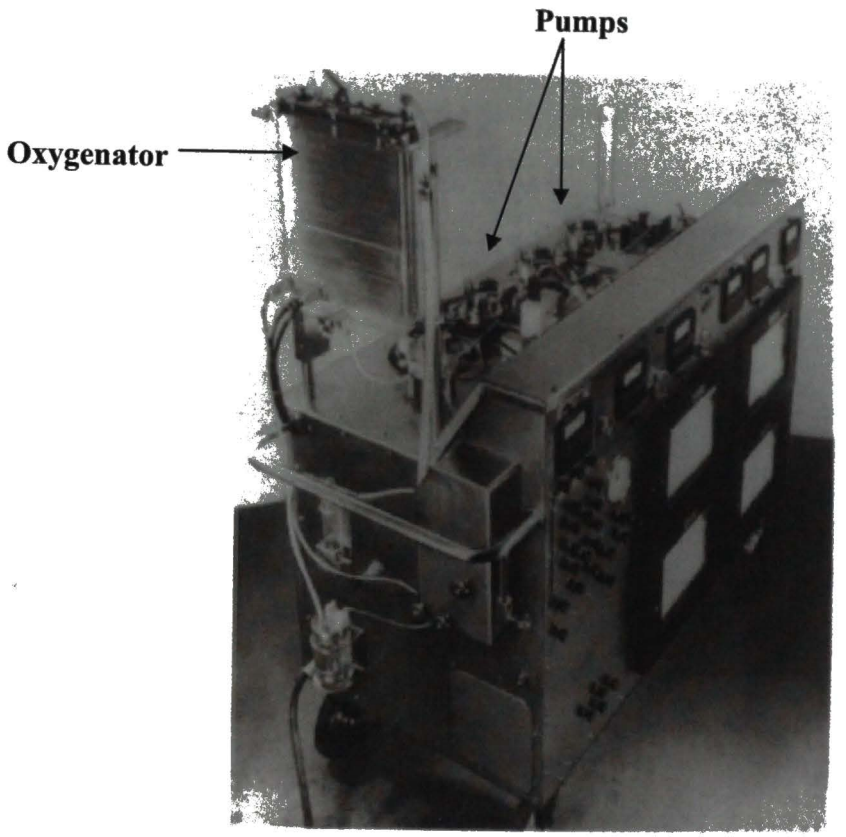

Figure 1. Model II. Used by Gibbon for the first successful coronary artery bypass procedure. Figure from reference 56.

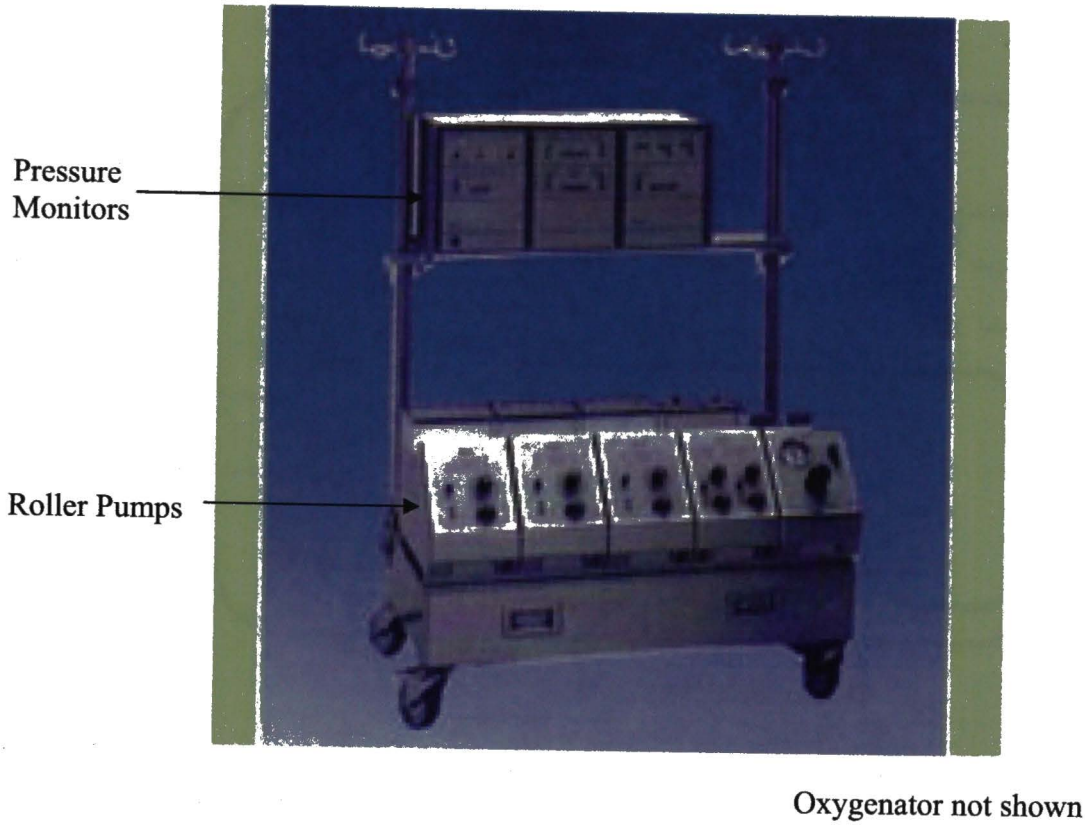

Figure 2. *Modern-day cardiopulmonary bypass machine.*

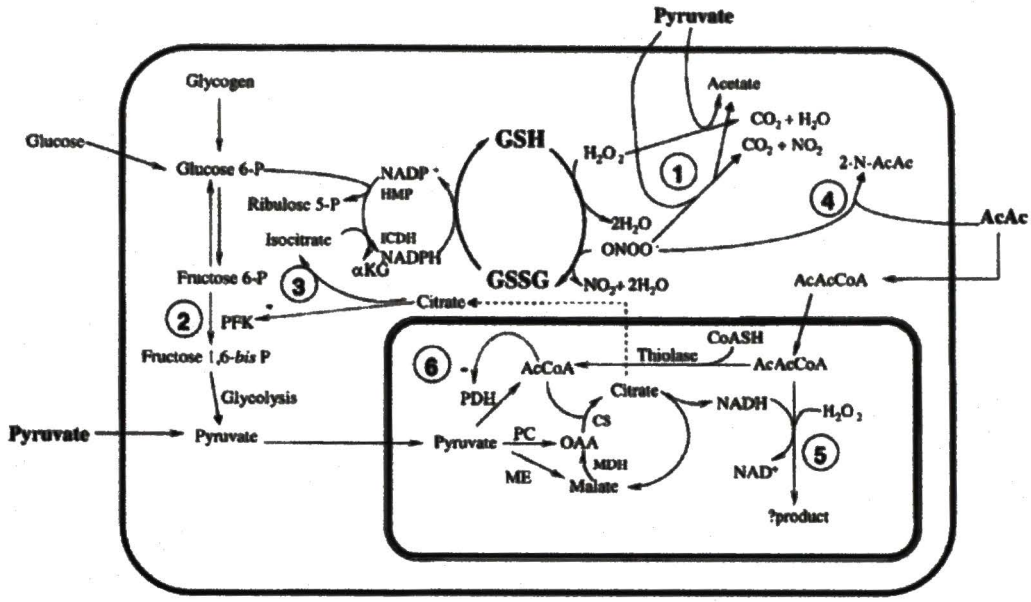

Figure 3. *Antioxidant mechanisms of pyruvate and acetoacetate.* Pyruvate and acetoacetate (AcAc) could relieve oxidant demands on the myocardial GSH system and maintain its redox potential by several mechanisms. (1) Pyruvate detoxifies peroxides and peroxynitrite in direct decarboxylation reactions. Pyruvate carboxylation by pyruvate carboxylase (PC) and/or malic enzyme (ME) increases mitochondrial citrate formation; citrate effluxes to the cytosol where it increases NADPH formation by (2) suppressing phosphofructokinase (PFK) activity and diverting glucose 6-phosphate into the hexose monophosphate pathway (HMP), or (3) by generating isocitrate substrate for $NADP^+$ dependent isocitrate dehydrogenase (ICDH). The diagram also depicts antioxidant mechanisms of the ketone, acetoacetate (AcAc). (4) Acetoacetate combines with peroxynitrate to generate a nontoxic nitrosylated product. (5) Acetoacetate activation yields acetoacetyl CoA (AcAcCoA), which detoxifies hydrogen peroxide in the presence of NADH forming an unknown product. (6) Oxidation of AcAcCoA to acetyl CoA (AcCoA) suppresses pyruvate dehydrogenase (PDH), diverting endogenous pyruvate into citrate-generating carboxylation. αKG: α-ketoglutarate. Figure from reference 40.

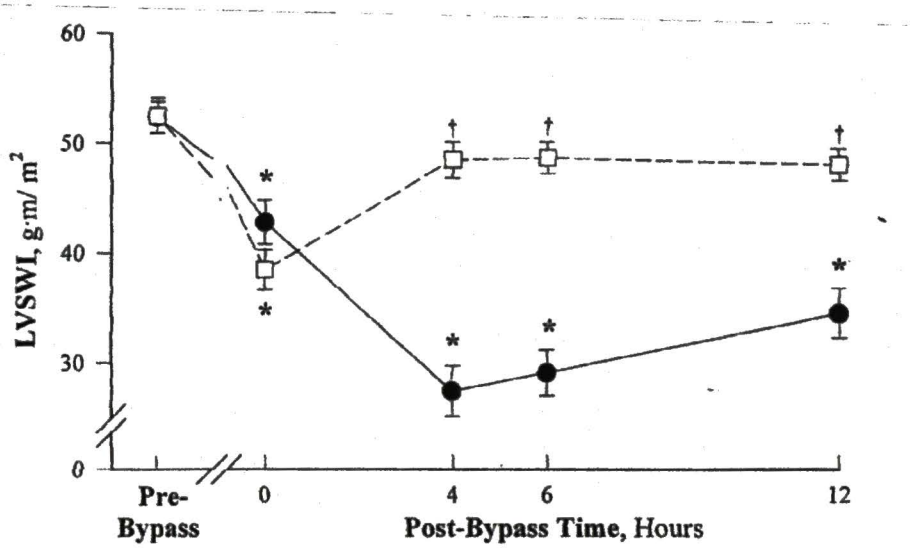

Figure 4. *Impact of pyruvate-fortified cardioplegia on left ventricular contractile performance following cardiopulmonary bypass surgery.* Left ventricular stroke work index (LVSWI) was measured at pre-bypass baseline and at 0-12 h post-bypass in hearts arrested with lactate- (closed circles) and pyruvate-fortified (open squares) cardioplegia solution during bypass. Values are means ± SEM; n = 15 per group. $^{*}P < 0.05$ *vs.* prebypass; $^{\dagger}P < 0.05$ *vs.* lactate cardioplegia group. Figure from reference 52.

CHAPTER II

Pyruvate-fortified cardioplegia suppresses oxidative stress and enhances phosphorylation potential of arrested myocardium

E. Marty Knott[1], Myoung-Gwi Ryou[1], Jie Sun[1], Abraham Heymann[1], Arti B. Sharma[1], Yu Lei[1], Mirza Baig[3], Robert T. Mallet[1], Albert H. Olivencia-Yurvati[2]

Departments of Integrative Physiology[1] and Surgery[2],
University of North Texas Health Science Center, Fort Worth, TX
Department of Pharmacy[3], Plaza Medical Center of Fort Worth, Fort Worth, TX

American Journal of Physiology Heart and Circulatory Physiology
September 2005; 289(3):H1123-30

ABSTRACT

Cardioplegic arrest for bypass surgery imposes global ischemia on the myocardium which generates oxyradicals and depletes myocardial high energy phosphates. The glycolytic metabolite pyruvate, but not its reduced congener lactate, increases phosphorylation potential and detoxifies oxyradicals in ischemic and post-ischemic myocardium. This study tested the hypothesis that pyruvate mitigates oxidative stress and preserves energy state in cardioplegically arrested myocardium. *In situ* swine hearts were arrested for 60 min with a 4:1 mixture of blood and crystalloid cardioplegia solution containing 188 mM glucose alone (control) or with additional 23.8 mM lactate or 23.8 mM pyruvate, then reperfused for 3 min with cardioplegia-free blood. Glutathione (GSH), glutathione disulfide (GSSG), and energy metabolites (phosphocreatine (PCr), creatine (Cr), inorganic phosphate (P_i)) were measured in myocardium snap frozen at 45 min arrest and 3 min reperfusion to determine antioxidant GSH redox state (GSH/GSSG) and PCr phosphorylation potential ([PCr]/([Cr][P_i])). Coronary sinus 8-isoprostane indexed oxidative stress. Pyruvate cardioplegia lowered 8-isoprostane release approximately 40% during arrest *vs.* control and lactate cardioplegia. Lactate and pyruvate cardioplegia dampened ($P < 0.05$ *vs.* control) the surge of 8-isoprostane release following reperfusion. Pyruvate doubled GSH/GSSG *vs.* lactate cardioplegia during arrest, but GSH/GSSG fell in all 3 groups following reperfusion. Myocardial [PCr]/([Cr][P_i]) was maintained in all 3 groups during arrest. Pyruvate cardioplegia doubled [PCr]/([Cr][P_i]) *vs.* control and lactate cardioplegia following reperfusion. Pyruvate cardioplegia mitigates oxidative stress during cardioplegic arrest and enhances myocardial energy state upon reperfusion.

INTRODUCTION

Despite recent advances in minimally invasive surgical interventions, cardiopulmonary bypass (CPB) remains the mainstay method for coronary revascularization (39). Moreover, CPB is essential for a variety of cardiac surgical procedures including valve replacement and correction of congenital heart defects. CPB allows the heart to be arrested without compromising blood flow to the body. However, prolonged arrest of the heart with potassium-based cardioplegia solutions interrupts coronary flow which may ultimately inflict myocardial ischemic injury. CPB elicits reactive oxygen species (ROS) formation (10, 11, 20), which could contribute to post-bypass myocardial dysfunction (9, 22, 46). ROS injure cardiomyocytes by chemically modifying and inactivating enzymes and by oxidizing phospholipids, increasing membrane permeability and impairing ion transport (21). ROS and reactive nitrogen intermediates are central to the pathogenesis of ischemic heart disease, myocardial infarction and stunning, and cardiac failure (4, 25). They have also been shown to induce arrhythmias (19) and activate apoptotic signaling pathways (8). Accordingly, intravenous administration of the pharmacological antioxidant *N*-acetylcysteine reduced myocardial lipid peroxidation and improved post-bypass cardiac performance in dogs (12).

Enhancements of the cellular antioxidant systems have the potential to mitigate injury during CPB by reducing the associated oxidative stress. The glutathione (GSH) system can effectively neutralize hydrogen peroxide, lipid peroxides and peroxynitrite. Indeed, this system is the major antioxidant mechanism in mammalian cells (38). The

reducing power of the GSH system is determined by the ratio of GSH to oxidized glutathione disulfide (GSSG). Moreover, changes in GSH/GSSG accurately reflect global changes in all of the intracellular antioxidant redox couples, including the α-tocopherol, ascorbate and thioredoxin systems, because these other antioxidants are in a state of near equilibrium with GSH/GSSG (13, 17, 35, 38).

A natural carbohydrate and glycolytic product, pyruvate acts as both an antioxidant and energy-yielding fuel in myocardium. An α-keto carboxylate, pyruvate can reduce peroxides (7) and convert peroxynitrite to NO_2^- (44). These non-enzymatic reactions decompose pyruvate to CO_2 and acetate. Secondly, anaplerotic flux of pyruvate into the TCA cycle increases citrate content (26); citrate supports metabolic pathways that generate NADPH, the source of reducing power for glutathione reductase (26). Indeed, exogenous pyruvate was found to increase GSH/GSSG in post-ischemic stunned (41) and hydrogen peroxide injured (25) guinea pig myocardium. We recently demonstrated that pyruvate-fortified cardioplegia ameliorates myocardial injury and improves post-surgical recovery of cardiac function in patients undergoing cardiopulmonary bypass, allowing significant reduction in post-surgical hospital stay (33).

The purpose of this investigation was to examine possible metabolic mechanisms for these improvements, with special attention focused on myocardial energy and antioxidant redox states. *In situ* adult swine hearts were arrested with blood cardioplegia solutions in which the crystalloid components contained 188 mM glucose alone (control)

or with additional 23.8 mM lactate or 23.8 mM pyruvate. Oxidative stress was assessed by measuring 8-isoprostane concentration in coronary sinus blood. Left ventricular myocardial biopsies were obtained during arrest and at 3 min reperfusion for measurement of glutathione redox state and energy metabolites. These experiments demonstrated that pyruvate-fortified cardioplegia dampens oxidative stress and bolsters endogenous antioxidant defenses. Additionally, administration of pyruvate-fortified cardioplegia during arrest enhanced myocardial energetic recovery upon reperfusion. These results extend the previously established benefits of pyruvate on the heart to a large animal model of cardioplegic arrest.

METHODS

Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, and was conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (NIH 85-23, revised 1996). Fifty-five adult domestic swine of either sex weighing 45-60 kg were randomly assigned to one of four experimental groups.

Surgical procedures

After an overnight fast, pigs were pre-medicated with ketamine (10 mg/kg, im) and xylazine (1 mg/kg, im). Following sedation, anesthesia was induced with propofol (2 mg/kg, iv) and maintained by mechanical ventilation with 1-3% isoflurane supplemented with O_2 and propofol (1 mg/kg/hr, iv). Cannulae were placed in femoral arteries to monitor arterial pressure (Hewlett-Packard model 1290C pressure transducer) and sample

arterial blood. Plasma Lyte A (Baxter Healthcare Corp, Deerfield, IL) was administered via a femoral vein to help maintain adequate blood volume. After median sternotomy, a pericardial cradle was fashioned for exposure and support of the heart. A catheter was placed through the right atrial appendage and advanced into the coronary sinus to sample coronary sinus blood (Figure 1).

Cardioplegic arrest

After instrumentation, each pig was administered 300 U/kg of heparin iv. 1.2 l of arterial blood was then withdrawn at a rate of 30 – 50 ml/min from a femoral artery using a roller pump (3M Health Care, Ann Arbor, MI). The blood was collected in a flask, stirred and maintained at 37°C. This blood volume was sufficient to perform cardioplegia arrest and to reperfuse the heart with cardioplegia-free blood for 3 min. The hearts did not fully recover mechanical function within 3 min reperfusion, so post-arrest cardiac performance was not monitored in this investigation. Figure 1 depicts the instrumentation of the heart. Drainage catheters were placed in each ventricular cavity via apical stab wounds and secured with purse-string sutures. A cannula (1.5 mm I.D.) for delivery of blood cardioplegia was placed in the ascending aorta and secured with a purse-string suture. Clamps were placed on the aorta distal to the cannula and on the superior and inferior vena cavae to effectively isolate the heart. Initial induction of cardiac arrest was accomplished with antegrade infusion, via the aortic cannula, of a minimum of 400 ml cold (4°C) blood cardioplegia (4 vol blood:1 vol crystalloid solution). Arrest was maintained by infusing 100-150 ml cardioplegia into the aortic root at 20 min intervals. Following 60 min arrest, the heart was reperfused for 3 min at 100-

140 ml/min with cardioplegia-free blood via the aortic cannula. Perfusion pressure (Hewlett-Packard model 1290C pressure transducer) and flow rates (Transonic Flowprobe, Transonic Systems Inc.) were monitored during delivery of cardioplegia and cardioplegia-free blood. Perfusion pressure was maintained between 65 – 75 mm Hg.

Cardioplegia solutions

All cardioplegia solutions were prepared aseptically the morning of the experiment. Control cardioplegia group contained 0.9% NaCl as the main diluent. The lactate cardioplegia was prepared using Ringer's lactate solution. The pyruvate cardioplegia group was administered a pyruvate-fortified Ringer's-based cardioplegia solution, prepared by diluting sodium pyruvate powder (Sigma, St. Louis, MO) in sterile 0.9% NaCl. Pyruvate and lactate concentrations were 23.8 mM in the respective cardioplegia solutions. Electrolytes and other constituents were added to each solution to match the composition of the control cardioplegia. NaCl was added to control cardioplegia to equal the molar amounts of sodium lactate and sodium pyruvate added to the lactate and pyruvate cardioplegia solutions. All 3 crystalloid cardioplegia solutions contained 104 mM NaCl, 135 mM $NaHCO_3$, 91 mM KCl, 6 mM $CaCl_2$, 188 mM glucose, 68 U/L insulin, and 676 mg/l lidocaine. Final pH was 7.6. Crystalloid solutions were combined with 4 vol blood before administration. Dilution of crystalloid in plasma lowered the pyruvate and lactate concentrations to 6-7 mM, well within the optimally cardioprotective pyruvate concentration range of 5-10 mM defined in isolated hearts (5).

Arterial fuels, blood gases and plasma 8-isoprostane

Blood samples were collected before initiation of cardioplegic arrest, at initial arrest, at 20 and 40 min arrest, and at 1 and 3 min reperfusion. Arterial blood samples were obtained from the femoral artery before arrest and from the aortic perfusion line carrying blood cardioplegia during arrest or whole blood during reperfusion. Coronary venous blood was sampled from the coronary sinus. Blood gases, pH, and HCO_3^- were measured in an Instrumentation Laboratory model 1730 blood gas analyzer. Pyruvate concentrations in plasma extracts were measured by colorimetric assays (49) in a Shimadzu Instruments model UV-1601 spectrophotometer. Glucose and lactate concentrations were measured in a Yellow Springs Instruments model 2300 L-lactate analyzer. Glucose and lactate concentrations reported by this analyzer have been verified by spectrophotometry. 8-Isoprostane was measured in coronary sinus plasma using a competitive immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Plasma 8-isoprostane concentrations were normalized for whole blood hemoglobin concentration and expressed as percent of pre-arrest baseline.

Myocardial metabolites, antioxidant redox state and cytosolic energy state

Snap frozen biopsies (*c.* 5 g) of the left ventricular apex were taken *in situ* by compressing tissue in Wollenberger tongs precooled in liquid N_2 (15). Biopsies were stored at -80° C until metabolite extraction. Three experimental series were performed. In the first series, myocardium was sampled at 45 min arrest (8 experiments per group). In the second series, myocardium was biopsied at 3 min reperfusion in 7 control, 10 lactate and 8 pyruvate cardioplegia experiments. The third series was a sham control protocol (n = 6) in which myocardium was sampled 1 hr 45 min after sternotomy but

without cardiac instrumentation or cardioplegic arrest. Experiments were terminated after biopsy.

Myocardial metabolites were extracted (27, 41, 42) and assayed spectrophotometrically (3). Phosphocreatine phosphorylation potential, i.e. [PCr]/([Cr][P_i]) provided a measure of myocardial energy state (2, 45). Intracellular P_i was determined as previously reported (25). Glutathione (GSH) and glutathione disulfide (GSSG) in myocardial extracts were measured in a Shimadzu Instruments model LC-10AT high performance liquid chromatography system equipped with a fluorescence detector (29). The ratio of GSH/GSSG was taken as a measure of the antioxidant redox potential of the myocardium.

Statistical analysis

Data are reported as means ± SEM. Arterial blood gases, pH, and HCO_3^- as well as plasma metabolite and 8-isoprostane data at different time points within each group were compared by repeated measures analysis of variance (ANOVA). Between-group comparisons of these variables at selected time points and myocardial metabolite values were accomplished by factorial ANOVA. When ANOVA detected statistical significance, *post hoc* multiple comparisons versus sham values were made using Dunn's method, and other between-group comparisons were performed using Student-Newman-Kuels multiple comparison test. Student's t-tests were performed for within-group comparisons of data collected at 3 min reperfusion *vs.* 45 min arrest. P values < 0.05 were considered statistically significant.

RESULTS

Arterial acid-base chemistry and hematocrit

Arterial blood gases, pH, and HCO_3^- are reported in Figure 2. Infusion of HCO_3^- in crystalloid cardioplegia controlled arterial pH (Figure 2A) and increased arterial PCO_2 via the carbonic anhydrase equilibrium (Figure 2B), in parallel with increased HCO_3^- concentration (Figure 2C). Arterial O_2 saturation (Figure 2D) was maintained at approximately 97% in all 3 cardioplegia groups. Hematocrit (Figure 2E) fell during arrest due to fluid administration and the addition of the crystalloid cardioplegia component; accordingly, O_2 content (Figure 2F) also decreased during arrest and reperfusion.

Arterial and tissue carbohydrates

Hearts were arrested with one of three cardioplegia solutions, glucose alone (control) or glucose with additional 23.8 mM lactate or pyruvate. Inclusion of pyruvate in crystalloid cardioplegia produced a plasma pyruvate concentration of about 7 mM in blood cardioplegia (Figure 3A). Plasma pyruvate concentration did not increase in the control or lactate cardioplegia groups during arrest. Similarly, plasma lactate concentration increased to 6 mM in the lactate cardioplegia group, but was only slightly elevated in the control and pyruvate cardioplegia groups (Figure 3B). Arterial glucose concentration during arrest averaged about 40 mM in each of the three cardioplegia groups (Figure 3C).

Myocardial uptake and release of carbohydrate substrates are reported in Figure 4. Baseline rates are not reported since total coronary flow was not measured before arrest. Myocardial pyruvate uptake (Figure 4A) was between 0.4 and 0.5 mmol/min in the pyruvate group during cardioplegic arrest. There was no net pyruvate uptake in the pyruvate cardioplegia group during cardioplegia-free reperfusion, nor in the control and lactate cardioplegia groups at any time. Lactate was released from the heart throughout the protocol in the control and pyruvate cardioplegia groups, but a brief period of net lactate uptake was seen during early arrest in the lactate group (Figure 4B). Glucose (Figure 4C) was taken up during arrest in all 3 groups. Upon reperfusion, net glucose release occurred as glucose-enriched cardioplegia was washed out of the organ by cardioplegia-free arterial blood.

Pyruvate (Figure 5A), lactate (Figure 5B), and citrate (Figure 5C) contents were measured in arrested and reperfused myocardium and compared to respective contents in non-arrested sham hearts. Pyruvate content in the sham group was 0.32 ± 0.11 μmol/g dry. Pyruvate content in the control and lactate groups did not change during arrest or reperfusion. During arrest, pyruvate content increased fivefold in the pyruvate cardioplegia group (1.50 ± 0.39 μmol/g dry), but was no longer significantly elevated after reperfusion (0.80 ± 0.17 μmol/g dry) due to pyruvate clearance by washout and metabolism. Myocardial lactate content doubled in the control cardioplegia group and increased fivefold in the lactate and pyruvate groups during arrest. Although lactate

content did not change following reperfusion of the lactate and pyruvate groups, it increased threefold in the control group to a level similar to that in the other groups.

Pyruvate carboxylase (36, 37) and malic enzyme (40) carboxylate pyruvate to form the TCA cycle intermediates oxaloacetate and malate, respectively, culminating in the formation of citrate. Citrate content increased 2.5-fold in the control and lactate groups during arrest and remained elevated following reperfusion. Pyruvate cardioplegia produced an even greater increase in citrate content during arrest and reperfusion *vs.* control and lactate cardioplegia.

Coronary sinus 8-isoprostane

To assess oxidative stress during cardioplegic arrest and reperfusion, the lipid peroxidation product, 8-isoprostane (30, 43), was measured in coronary sinus plasma. 8-isoprostane concentrations were normalized to hemoglobin concentration of coronary sinus blood to control for hemodilution, and reported as percentages of pre-arrest baseline (Figure 6). Pyruvate cardioplegia suppressed 8-isoprostane release in the arrested and reperfused heart, relative to control cardioplegia. This effect was most striking during reperfusion, when 8-isoprostane increased six-fold in the control cardioplegia group. Unexpectedly, lactate cardioplegia also dampened oxidative stress compared with control, but only during reperfusion.

Glutathione antioxidant system

Glutathione (GSH) is the major intracellular antioxidant in the heart. The ratio of GSH to oxidized glutathione disulfide (GSSG), *i.e.* GSH/GSSG, serves as an index of the global redox state of the myocardium's antioxidant defenses (38). Accordingly, GSH, GSSG (Figure 7A) and GSH/GSSG (Figure 7B) were determined in arrested and reperfused myocardium and compared to respective values in non-arrested sham hearts. During cardioplegic arrest GSH content was maintained in all three groups. While not statistically significant, GSSG tended to fall in the control and pyruvate groups, thus raising the GSH redox state. Reintroduction of cardioplegia-free arterial blood to the myocardium upon reperfusion induced oxidative stress: GSH content fell by approximately 25%, and GSSG increased in all three cardioplegia groups. Thus, GSH redox state was depressed following reperfusion.

Energy metabolites and phosphorylation potential

The impact of cardioplegic arrest and reperfusion on myocardial energy state was examined by measuring creatine kinase reactants and phosphorylation potential. ATP was maintained near the sham control value during arrest and reperfusion in all 3 groups (Table). Phosphocreatine (PCr) and creatine (Cr) contents did not differ appreciably among the cardioplegia groups.

Phosphocreatine phosphorylation potential ($[PCr]/([Cr][P_i])$), an index of cytosolic energy state according to the creatine kinase equilibrium (45), permitted assessment of the effects of cardioplegic arrest and reperfusion on myocardial energy reserves. Phosphorylation potential in all three groups tended to increase by 45 min

arrest (Figure 8A) despite interruption of coronary flow. Upon reperfusion, phosphorylation potential returned to pre-arrest baseline in the control and lactate cardioplegia groups. Pyruvate cardioplegia maintained the higher phosphorylation potential even after reperfusion at a level (275 ± 38 M^{-1}) roughly double that of the control (148 ± 32 M^{-1}), lactate (116 ± 16 M^{-1}) and non-arrested sham (109 ± 8 M^{-1}) groups. This enhancement of phosphorylation potential was due in large part to decreased intracellular P_i concentration in the pyruvate cardioplegia group (Figure 8B).

The wet/dry ratio (Table) monitors myocardial water content and tissue edema. The ratio did not change during arrest, but increased in all 3 groups after 3 min reperfusion. Thus, reperfusion of cardioplegically arrested myocardium appeared to produce some moderate edema, irrespective of the fuel composition of the cardioplegia.

DISCUSSION

A recent clinical trial (33) demonstrated marked, sustained enhancement of postsurgical cardiac function in patients administered pyruvate- *vs.* lactate-fortified cardioplegia during CPB. This investigation aimed to delineate the mechanisms of pyruvate protection of the cardioplegically arrested myocardium. Pyruvate cardioplegia produced a plasma pyruvate concentration of approximately 7 mM, well within the optimally cardioprotective range of 5-10 mM pyruvate defined in isolated perfused heart preparations (5). Myocardial energy and antioxidant redox states were measured during cardioplegic arrest and at 3 min reperfusion, a time of intense oxidative stress.

Antioxidant effects of pyruvate

Reactive oxygen species (ROS) are formed as a result of myocardial ischemia and reperfusion in animal models (34) and in humans (9), and have been implicated in post-ischemic myocardial dysfunction (9, 22, 46). In this study, pyruvate cardioplegia minimized myocardial 8-isoprostane release, a measure of lipid peroxidation, during arrest and reperfusion. Pyruvate also increased the glutathione redox state of the heart during arrest. The concentration of 8-isoprostane in systemic arterial plasma of patients increases during CPB (43). 8-Isoprostane concentration also increased in coronary sinus blood 30 min after CPB in dogs (12). In addition, intravenous administration of the pharmacologic antioxidant *N*-acetylcysteine throughout CPB reduced 8-isoprostane, preserved systolic function, and hastened myocardial edema resolution (12). Although cardiac performance could not be studied in this investigation, the established correlation between reduced oxidative stress and improved function could help explain the marked enhancement of post-CPB cardiac function in patients arrested with pyruvate-fortified cardioplegia (33).

Pyruvate cardioplegia, more so than control and lactate cardioplegia, increased myocardial citrate content. Anaplerotic pyruvate carboxylation generates the Krebs cycle intermediates malate and oxaloacetate; condensation of oxaloacetate with acetyl CoA yields citrate (26). Citrate remained elevated in the pyruvate cardioplegia group at 3 min reperfusion even though pyruvate content had decreased by this time. Citrate generates NADPH, the source of reducing power to maintain GSH, through two pathways. First, citrate is converted to isocitrate, generating substrate for $NADP^+$-dependent isocitrate

dehydrogenase (1). Second, as citrate accumulates, it constrains phosphofructokinase activity (14), causing glucose-6-phosphate to accumulate (41) and thereby providing substrate for the NADPH-generating hexose monophosphate shunt.

Lactate does not possess any known antioxidant properties (2), so the mechanism of lactate suppression of post-arrest lipid peroxidation is unclear. Lactate could be rapidly converted to its antioxidant congener pyruvate by lactate dehydrogenase, but neither myocardial pyruvate content nor arterial plasma pyruvate concentrations were increased by lactate *vs.* control cardioplegia. Hypothetically, pyruvate accumulation may have been minimized if pyruvate formed from lactate oxidation were immediately consumed in non-enzymatic detoxification of peroxides and peroxynitrite (26).

Myocardial energy state

Phosphocreatine phosphorylation potential, a measure of myocardial energy state (5, 45), was maintained or even enhanced during arrest, but fell at 3 min reperfusion in the control and lactate cardioplegia groups. Administration of pyruvate cardioplegia during arrest maintained phosphorylation potential during early reperfusion, primarily by reducing intracellular concentration of inorganic phosphate at the latter time point. Enhancement of phosphorylation potential by pyruvate (5, 24, 32, 34, 50) increases cytosolic Gibbs free energy of ATP hydrolysis (ΔG_{ATP}) (27). Reductions in ΔG_{ATP} impair calcium handling (sarcoplasmic reticulum Ca^{2+} ATPase) and cross-bridge cycling (actin-myosin ATPase) (16), critical steps in force-generating contraction. Pyruvate has been shown to increase sarcoplasmic reticular Ca^{2+} uptake and release while increasing

both energy state and contractile performance in isolated perfused hearts (6, 23, 27) and cardiomyocytes (28, 31).

Typically, cardioplegic arrest results in accumulation of NADH (47) since mitochondrial respiration is constrained by a decreased supply of oxygen. Glycolysis, an important source of ATP for sarcoplasmic reticular (SR) Ca^{2+} uptake (48), could become limited by NADH accumulation and concominant decrease in NAD^+ (18). Pyruvate cardioplegia increased myocardial pyruvate and lactate contents five-fold during arrest. Through its conversion to lactate, pyruvate oxidizes NADH to NAD^+, which could relieve glycolytic constraint. In contrast, lactate oxidation generates NADH which could further impair glycolysis during arrest.

Limitations

This novel *in situ* heart preparation permits direct examination of the effects of cardioplegia on the organ. However, effectively isolating the heart from the organism by cross-clamping the great vessels precludes study of the systemic response to the stresses of CPB. In addition, only 1.2 L of arterial blood could be safely withdrawn from the pig without provoking circulatory collapse despite replacement with equal volume of isotonic saline. The volume of blood available after blood cardioplegia administration was only sufficient for 3 min of reperfusion, so post-arrest recovery of cardiac performance could not be studied. Blood from donor animals was not administered to avoid the pro-inflammatory and pro-oxidant effects of allogenic blood.

Summary and conclusions

Compared to control and lactate-fortified cardioplegia solutions, pyruvate cardioplegia significantly reduced oxidative stress in the heart during cardioplegic arrest. Moreover, antecedent administration of pyruvate or lactate cardioplegia during arrest suppressed the reperfusion burst of lipid peroxidation. A metabolic antioxidant, pyruvate enhanced GSH/GSSG antioxidant redox state during arrest. GSH/GSSG fell upon reperfusion in all 3 cardioplegia groups. Phosphorylation potential was maintained or even enhanced during arrest in all 3 groups, but fell after reperfusion in the control and lactate cardioplegia groups. Antecedent pyruvate cardioplegia bolstered myocardial phosphorylation potential even after reperfusion.

This investigation demonstrated for the first time that pyruvate-fortified cardioplegia reduces oxidative stress and preserves myocardial energy state in a large animal model of cardioplegic arrest. Further study of the beneficial effects of pyruvate on the heart during cardiopulmonary bypass is warranted.

ACKNOWLEDGEMENTS

The outstanding technical assistance of Rudolfo Martinez (Edward Via Virginia College of Osteopathic Medicine), Arthur G. Williams, Jr. (University of North Texas Health Science Center), and Amar Joumma (Plaza Medical Center of Fort Worth) is gratefully acknowledged.

GRANTS

This study was supported by grants from the Osteopathic Heritage Foundation (02-18-522) and the National Heart, Lung and Blood Institute (HL071684).

REFERENCES

1. **Andres A, Satrustegui J and Machado A.** Development of NADPH-producing pathways in rat heart. *Biochem J* 186: 799-803, 1980.

2. **Bassenge E, Sommer O, Schwemmer M and Bünger R.** Antioxidant pyruvate inhibits cardiac formation of reactive oxygen species through changes in redox state. *Am J Physiol Heart Circ Physiol* 279: H2431-2438, 2000.

3. **Bergmeyer HU.** *Methods of Enzymatic Analysis.* New York: Academic Press, 1983.

4. **Bolli R.** Causative role of oxyradicals in myocardial stunning: a proven hypothesis. *Basic Res Cardiol* 93: 156-162, 1998.

5. **Bünger R, Mallet RT and Hartman DA.** Pyruvate-enhanced phosphorylation potential and inotropism in normoxic and postischemic isolated working heart. *Eur J Biochem* 180: 221-233, 1989.

6. **Chen W, London R, Murphy E and Steenbergen C.** Regulation of the Ca^{2+} gradient across the sarcoplasmic reticulum in perfused rabbit heart. *Circ Res* 83: 898-907, 1998.

7. **Constantopoulos G and Barranger JA.** Nonenzymatic decarboxylation of pyruvate. *Anal Biochem* 139: 353-358, 1984.

8. **Cook SA and Poole-Wilson PA.** Cardiac myocyte apoptosis. *Eur Heart J* 20: 1619-1629, 1999.

9. **Curello S, Ceconi C, de Giuli F, Panzali AF, Milanesi B, Calarco M, Pardini A, Marzollo P, Alfieri O and Messineo F.** Oxidative stress during reperfusion of human hearts: potential sources of oxygen free radicals. *Cardiovasc Res* 29: 118-125, 1995.

10. **Davies S, Duffy J, Wickens D, Underwood S, Hilla A, Alladine F, Feneck R, Dormandy T and Walesby R.** Time-course of free radical activity during coronary artery operations with cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 105: 979-987, 1993.

11. **Ferrari R, Alfieri O, Curello S, Ceconi C, Cargnoni A, Marzollo P, Pardini A, Caradonna E and Visioli O.** Occurrence of oxidative stress during reperfusion of the human heart. *Circulation* 81: 201-211, 1990.

12. **Fischer UM, Cox CS,Jr, Allen SJ, Stewart RH, Mehlhorn U and Laine GA.** The antioxidant *N*-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest. *J Thorac Cardiovasc Surg* 126: 1483-1488, 2003.

13. **Freisleben HJ and Packer L.** Free-radical scavenging activities, interactions and recycling of antioxidants. *Biochem Soc Trans* 21: 325-330, 1993.

14. **Garland PB, Randle PJ and Newsholme EA.** Citrate as an intermediary in the inhibition of phosphofructokinase in rate heart muscle by fatty acids, ketone bodies, pyruvate, diabetes, and starvation. *Nature* 200: 169-170, 1963.

15. **Itoya M, Mallet RT, Gao ZP, Williams AG, Jr., and Downey HF.** Stability of high-energy phosphates in right ventricle: myocardial energetics during right coronary hypotension. *Am J Physiol Heart Circ Phsiol* 271: H320-328, 1996.

16. **Kammermeier H.** High energy phosphate of the myocardium: concentration versus free energy change. *Basic Res Cardiol* 82 Suppl 2: 31-36, 1987.

17. **Kehrer JP and Lund LG.** Cellular reducing equivalents and oxidative stress. *Free Radic Biol Med* 17: 65-75, 1994.

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

19. **Kusama Y, Bernier M and Hearse DJ.** Exacerbation of reperfusion arrhythmias by sudden oxidant stress. *Circ Res* 67: 481-489, 1990.

20. **Lazzarino G, Raatikainen P, Nuutinen M, Nissinen J, Tavazzi B, Di Pierro D, Giardina B and Peuhkurinen K.** Myocardial release of malondialdehyde and

purine compounds during coronary bypass surgery. *Circulation* 90: 291-297, 1994.

21. **Lebedev AV, Levitsky DO, Loginov VA and Smirnov VN.** The effect of primary products of lipid peroxidation on the transmembrane transport of calcium ions. *J Mol Cell Cardiol* 14 Suppl 3: 99-103, 1982.

22. **Li XY, McCay PB, Zughaib M, Jeroudi MO, Triana JF and Bolli R.** Demonstration of free radical generation in the "stunned" myocardium in the conscious dog and identification of major differences between conscious and open-chest dogs. *J Clin Invest* 92: 1025-1041, 1993.

23. **Mallet RT and Bünger R.** Energetic modulation of cardiac inotropism and sarcoplasmic reticular Ca^{2+} uptake. *Biochim Biophys Acta* 1224: 22-32, 1994.

24. **Mallet RT and Bünger R.** Metabolic protection of post-ischemic phosphorylation potential and ventricular performance. *Adv Exp Med Biol* 346: 233-241, 1993.

25. **Mallet RT, Squires JE, Bhatia S and Sun J.** Pyruvate restores contractile function and antioxidant defenses of hydrogen peroxide-challenged myocardium. *J Mol Cell Cardiol* 34: 1173-1184, 2002.

26. **Mallet RT and Sun J.** Antioxidant properties of myocardial fuels. *Mol Cell Biochem* 253: 103-111, 2003.

27. **Mallet RT and Sun J.** Mitochondrial metabolism of pyruvate is required for its enhancement of cardiac function and energetics. *Cardiovasc Res* 42: 149-161, 1999.

28. **Martin BJ, Valdivia HH, Bünger R, Lasley RD and Mentzer RM, Jr.** Pyruvate augments calcium transients and cell shortening in rat ventricular myocytes. *Am J Physiol Heart Circ Physiol* 274: H8-17, 1998.

29. **Medved I, Brown MJ, Bjorksten AR, Leppik JA, Sostaric S and McKenna MJ.** *N*-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. *J Appl Physiol* 94: 1572-1582, 2003.

30. **Mehlhorn U, Krahwinkel A, Geissler HJ, LaRosee K, Fischer UM, Klass O, Suedkamp M, Hekmat K, Tossios P and Bloch W.** Nitrotyrosine and 8-isoprostane formation indicate free radical-mediated injury in hearts of patients subjected to cardioplegia. *J Thorac Cardiovasc Surg* 125: 178-183, 2003.

31. **Mellors LJ, Kotsanas G and Wendt IR.** Effects of pyruvate on intracellular Ca^{2+} regulation in cardiac myocytes from normal and diabetic rats. *Clin Exp Pharmacol Physiol* 26: 889-897, 1999.

32. **Mentzer RM, Jr, Van Wylen DG, Sodhi J, Weiss RJ, Lasley RD, Willis J, Bünger R, and Flint LM.** Effect of pyruvate on regional ventricular function in normal and stunned myocardium. *Ann Surg* 209: 629-633, 1989.

33. **Olivencia-Yurvati AH, Blair JL, Baig M and Mallet RT.** Pyruvate-enhanced cardioprotection during surgery with cardiopulmonary bypass. *J Cardiothorac Vasc Anesth* 17: 715-720, 2003.

34. **Opie LH.** Reperfusion injury and its pharmacologic modification. *Circulation* 80: 1049-1062, 1989.

35. **Pascoe GA and Reed DJ.** Cell calcium, vitamin E, and the thiol redox system in cytotoxicity. *Free Radic Biol Med* 6: 209-224, 1989.

36. **Peuhkurinen KJ.** Regulation of the tricarboxylic acid cycle pool size in heart muscle. *J Mol Cell Cardiol* 16: 487-495, 1984.

37. **Peuhkurinen KJ, Nuutinen EM, Pietilainen EP, Hiltunen JK and Hassinen IE.** Role of pyruvate carboxylation in the energy-linked regulation of pool sizes of tricarboxylic acid-cycle intermediates in the myocardium. *Biochem J* 208: 577-581, 1982.

38. **Schafer FQ and Buettner GR.** Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191-1212, 2001.

39. **Schmitz C, Weinreich S, Schneider R, Schneider D, Speth I, Schulze-Rauschenbach C, Pohl C and Welz A.** Off-Pump versus on-pump coronary artery bypass: can OPCAB reduce neurologic injury? *Heart Surg Forum* 6: 127-130, 2003.

40. **Sundqvist KE, Hiltunen JK and Hassinen IE.** Pyruvate carboxylation in the rat heart. Role of biotin-dependent enzymes. *Biochem J* 257: 913-916, 1989.

41. **Tejero-Taldo MI, Caffrey JL, Sun J and Mallet RT.** Antioxidant properties of pyruvate mediate its potentiation of beta-adrenergic inotropism in stunned myocardium. *J Mol Cell Cardiol* 31: 1863-1872, 1999.

42. **Tejero-Taldo MI, Sun J, Caffrey JL and Mallet RT.** Pyruvate potentiates beta-adrenergic inotropism of stunned guinea-pig myocardium. *J Mol Cell Cardiol* 30: 2327-2339, 1998.

43. **Ulus AT, Aksoyek A, Ozkan M, Katircioglu SF and Basu S.** Cardiopulmonary bypass as a cause of free radical-induced oxidative stress and enhanced blood-borne isoprostanes in humans. *Free Radic Biol Med* 34: 911-917, 2003.

44. **Vásquez-Vivar J, Denicola A, Radi R, and Augusto O.** Peroxynitrite-mediated decarboxylation of pyruvate to both carbon dioxide and carbon dioxide radical anion. *Chem Res Toxicol* 10: 786-794, 1997.

45. **Veech RL, Lawson JW, Cornell NW and Krebs HA.** Cytosolic phosphorylation potential. *J Biol Chem* 254: 6538-6547, 1979.

46. **Vergely C, Maupoil V, Benderitter M and Rochette L.** Influence of the severity of myocardial ischemia on the intensity of ascorbyl free radical release and on postischemic recovery during reperfusion. *Free Radic Biol Med* 24: 470-479, 1998.

47. **Wahr JA, Olszanski D, Childs KF, and Bolling SF.** Dichloroacetate enhanced myocardial functional recovery post-ischemia : ATP and NADH recovery. J *Surg Res* 63: 220-224, 1996.

48. **Xu KY, Zweier JL and Becker LC.** Functional coupling between glycolysis and sarcoplasmic reticulum Ca^{2+} transport. *Circ Res* 77: 88-97, 1995.

49. **Yi KD, Downey HF, Bian X, Fu M and Mallet RT.** Dobutamine enhances both contractile function and energy reserves in hypoperfused canine right ventricle. *Am J Physiol Heart Circ Physiol* 279: H2975-2985, 2000.

50. **Zhou Z, Lasley RD, Hegge JO, Bünger R and Mentzer RM, Jr.** Myocardial stunning: a therapeutic conundrum. *J Thorac Cardiovasc Surg* 110: 1391-1400, 1995.

Table. Myocardial Creatine Kinase Reactants

	n	ATP	PCr	Cr	Wet/Dry
Sham	6	27 ± 2	41 ± 2	78 ± 3	4.64 ± 0.25
45' ARREST					
Control	7	26 ± 1	55 ± 2	78 ± 4	4.72 ± 0.05
Lactate	7	25 ± 2	49 ± 4	87 ± 5	4.74 ± 0.05
Pyruvate	7	25 ± 1	51 ± 3	79 ± 9	4.83 ± 0.08
3' REPERFUSION					
Control	7	24 ± 1	52 ± 6	97 ± 6	5.23 ± 0.07 *†
Lactate	10	23 ± 1	45 ± 3	98 ± 5	5.42 ± 0.19 *†
Pyruvate	8	24 ± 1	50 ± 3	90 ± 8	5.55 ± 0.18 *†

ATP, phosphocreatine (PCr), and creatine (Cr) contents (μmol•g dry^{-1}) were measured in left ventricular tissue biopsied at 45 min arrest and 3 min reperfusion. Mass ratios of wet/dry tissue are also listed. Means ± SEM *P < 0.05 *vs.* sham; †P < 0.05 *vs.* arrest.

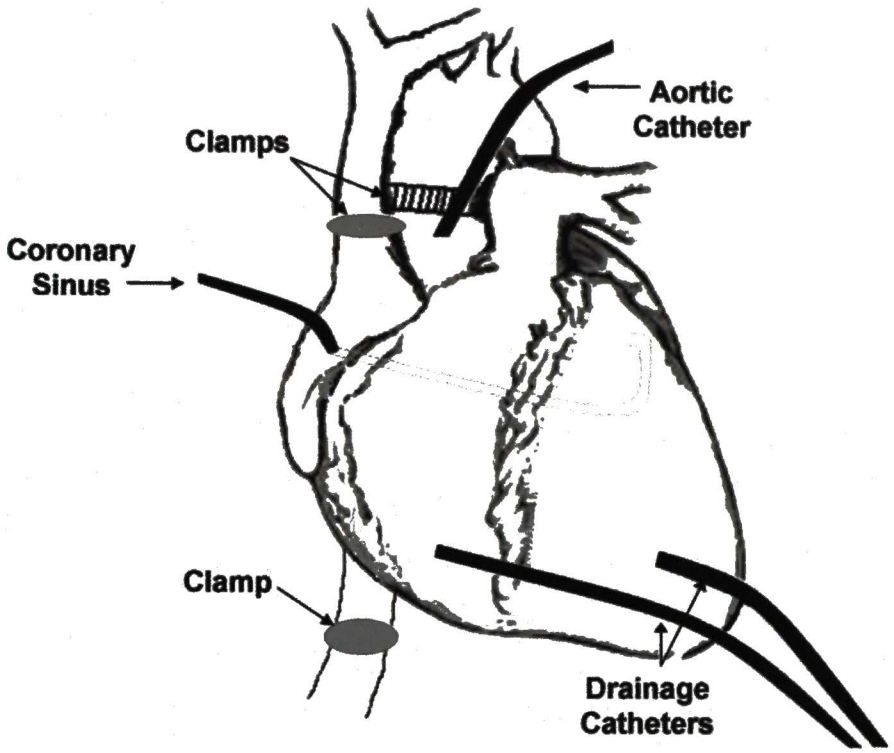

Figure 1. *Instrumentation of the heart.* Cardioplegia is administered via the aortic cannula. Cross-clamping the superior and inferior vena cavae and ascending aorta isolates the heart, facilitating study of the direct effects of different cardioplegic solutions on the organ. Coronary sinus blood is sampled to measure 8-isoprostane. Drainage catheters prevent overfilling of the ventricles.

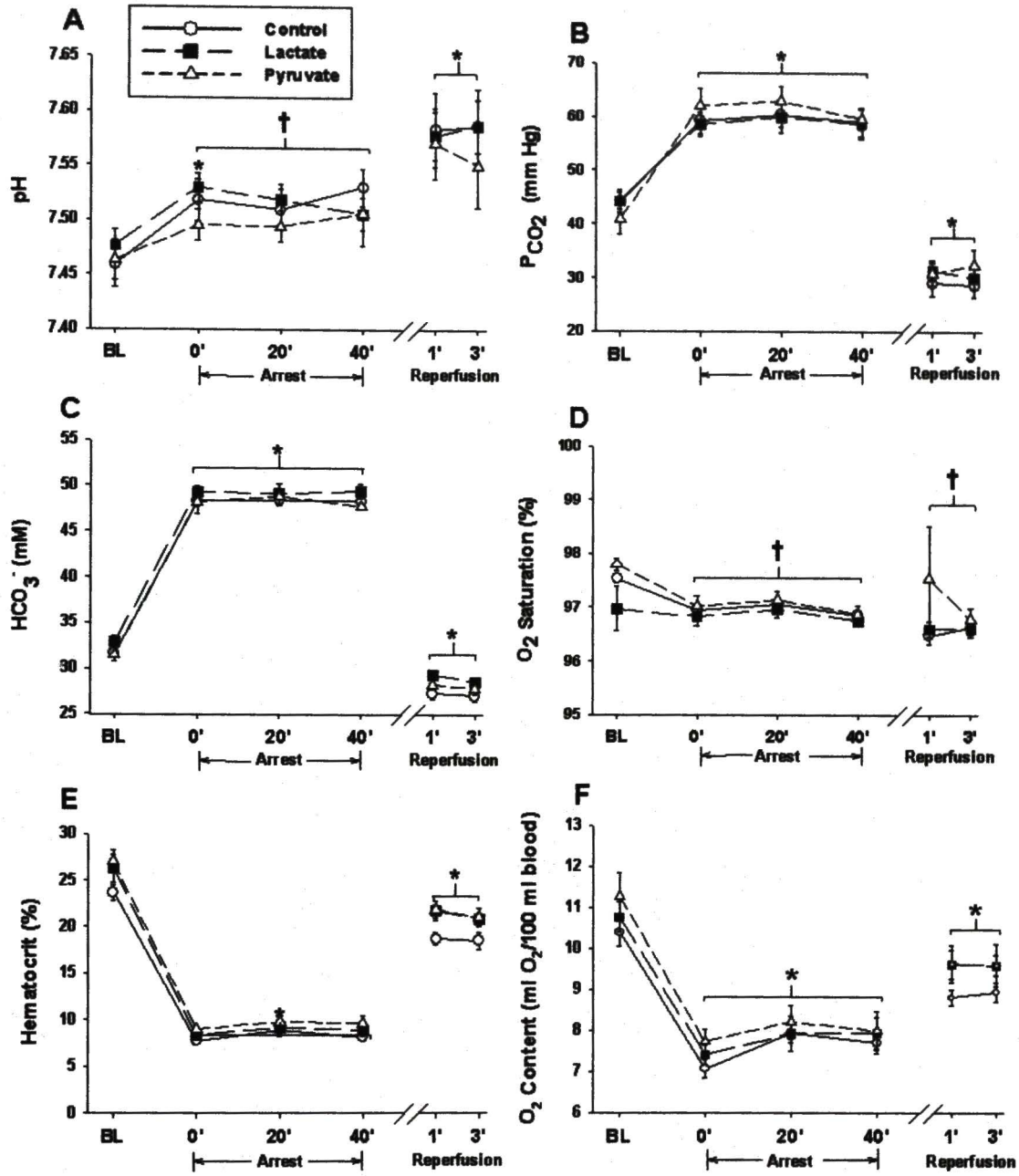

Figure 2. *Arterial blood gases and acid-base chemistry.* pH (Panel A), PCO_2 (Panel B), HCO_3^- (Panel C), O_2 saturation (Panel D), hematocrit (Panel E), and O_2 content (Panel F) were measured at pre-arrest baseline (BL), at 20 min intervals during arrest, and at 1 and 3 min reperfusion. *P < 0.05 *vs.* baseline in all three groups; †P < 0.05 *vs.* baseline, control group only. Values in this and the following figures are means ± SEM.

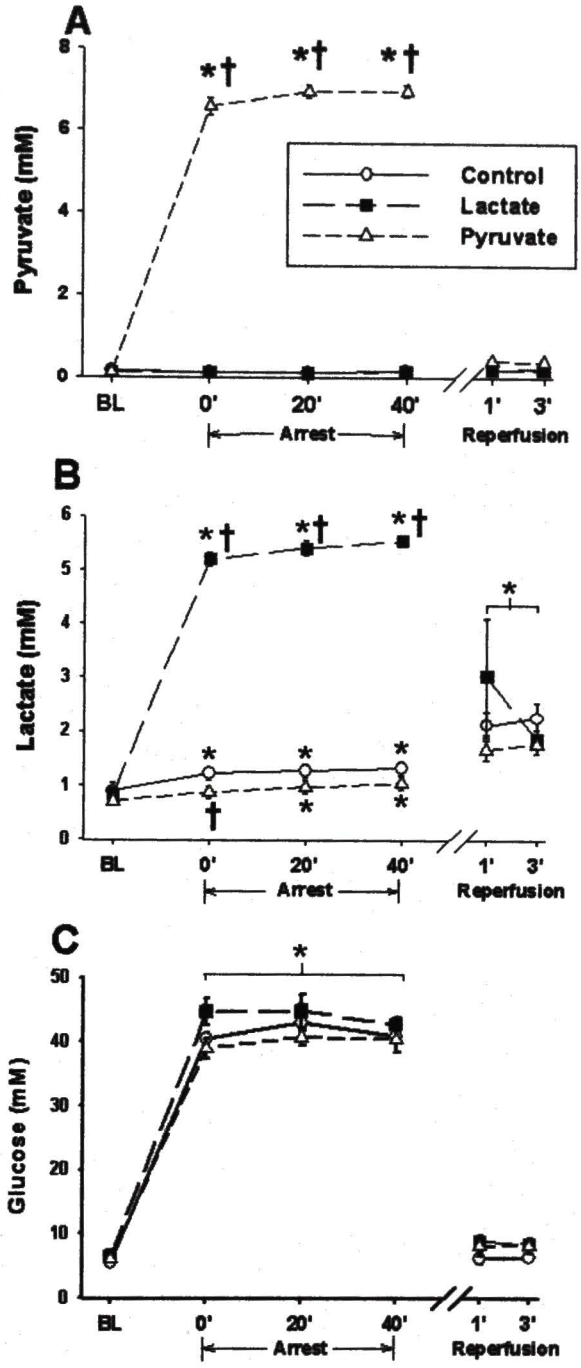

Figure 3. *Arterial plasma carbohydrate concentrations.* Pyruvate (Panel A), lactate (Panel B), and glucose (Panel C) were measured in arterial plasma. *P < 0.05 *vs.* baseline; †P < 0.05 *vs.* control.

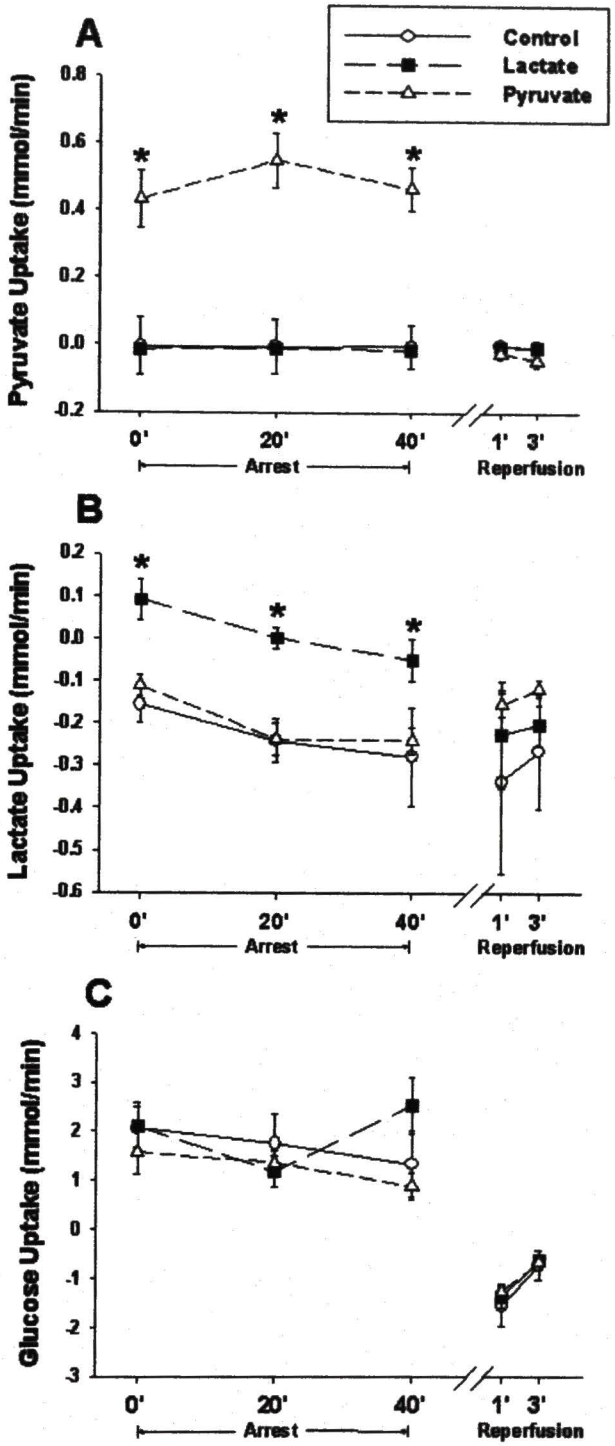

Figure 4. *Carbohydrate uptake.* Pyruvate (Panel A), lactate (Panel B), and glucose (Panel C) uptakes equaled arterial – coronary sinus concentration differences times perfusion flow rate. *$P < 0.05$ *vs.* control.

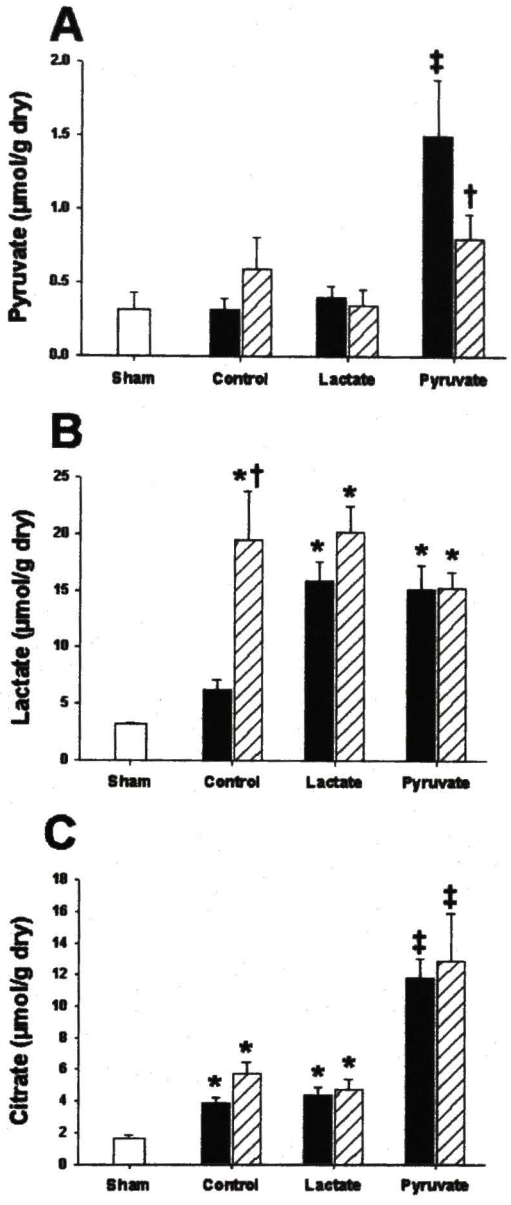

Figure 5. *Myocardial pyruvate, lactate, and citrate contents.* Pyruvate (Panel A), lactate (Panel B), and citrate (Panel C) contents were measured in snap-frozen left ventricular biopsies taken at 45 min arrest and at 3 min reperfusion in control, lactate, and pyruvate cardioplegia groups, and at 105 min post-sternotomy in non-arrested sham hearts. $^{*}P < 0.05$ *vs.* sham; $\dagger P < 0.05$ *vs.* respective values in the same group at 45 min arrest; $\ddagger P < 0.05$ *vs.* sham, control, and lactate.

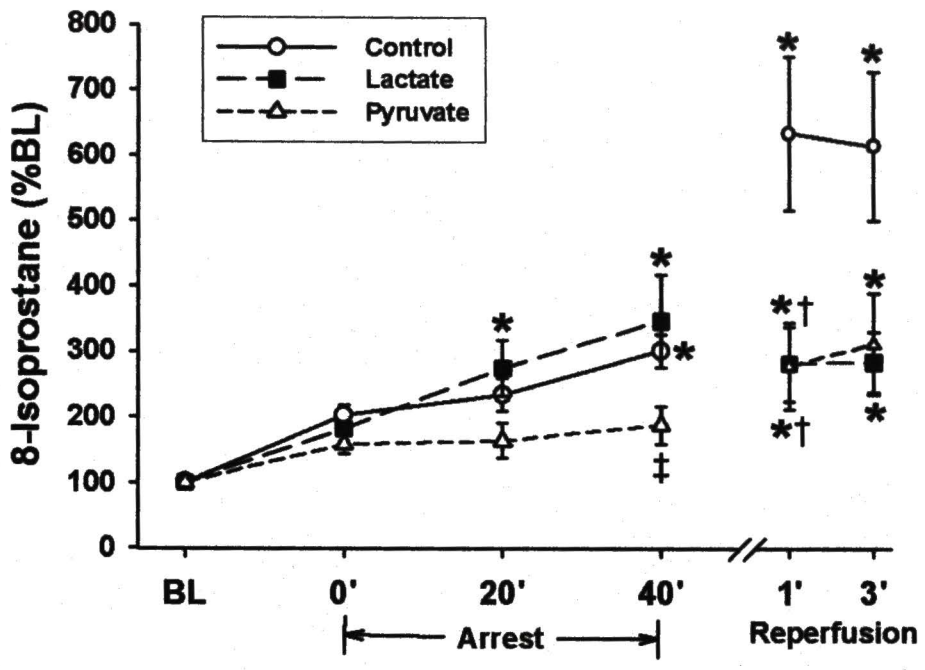

Figure 6. *Coronary sinus 8-isoprostane concentration.* 8-isoprostane was measured in coronary sinus plasma. Measured concentrations were divided by hemoglobin (Hgb) concentration in coronary sinus blood to control for varying degrees of hemodilution, and expressed as percentages of pre-arrest baseline. Actual baseline values were 3.77 ± 0.25, 2.70 ± 0.59, and 5.10 ± 1.75 ng 8-isoprostane/g Hgb in the control, lactate and pyruvate groups, respectively. *P < 0.05 *vs.* baseline; †P < 0.05 *vs.* control; ‡P < 0.05 *vs.* lactate.

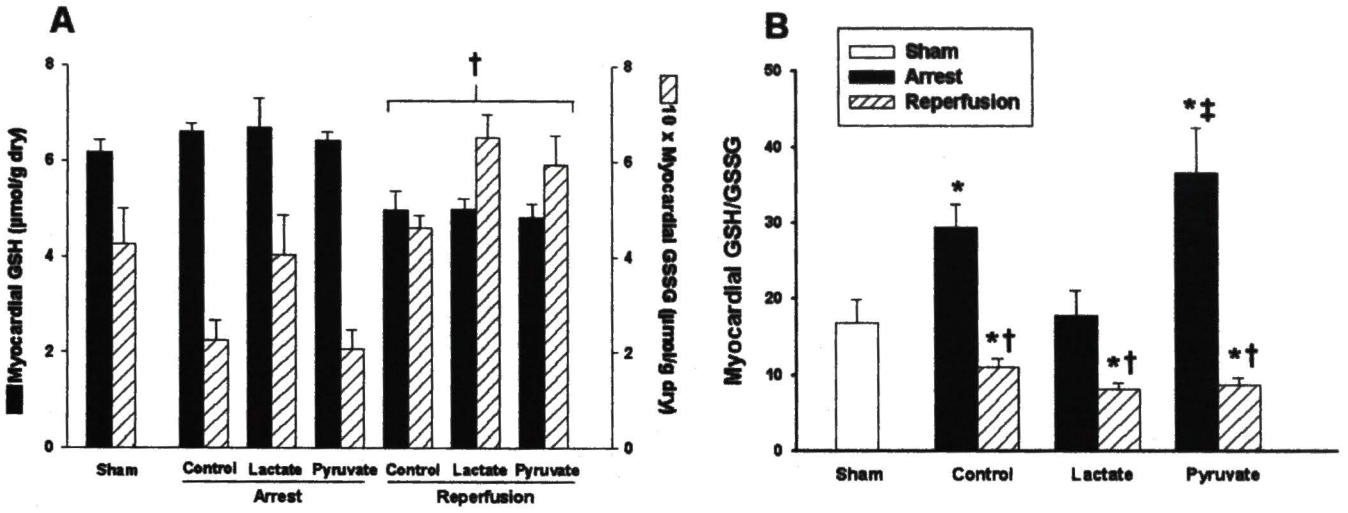

Figure 7. *Myocardial glutathione redox state.* Panel A: Glutathione (GSH: solid bars) and glutathione disulfide (GSSG: hatched bars) contents were measured in left ventricular myocardium at 45 min arrest and 3 min reperfusion in the cardioplegia groups, and at 105 min post-sternotomy in non-arrested sham hearts. GSH and GSSG values are plotted on different scales. Panel B: Glutathione redox state (GSH/GSSG) at 45 min arrest (black bars) and 3 min reperfusion (hatched bars) was computed from GSH and GSSG contents. *$P < 0.05$ *vs.* sham; †$P < 0.05$ *vs.* respective values in the same group at 45 min arrest; ‡$P < 0.05$ *vs.* lactate.

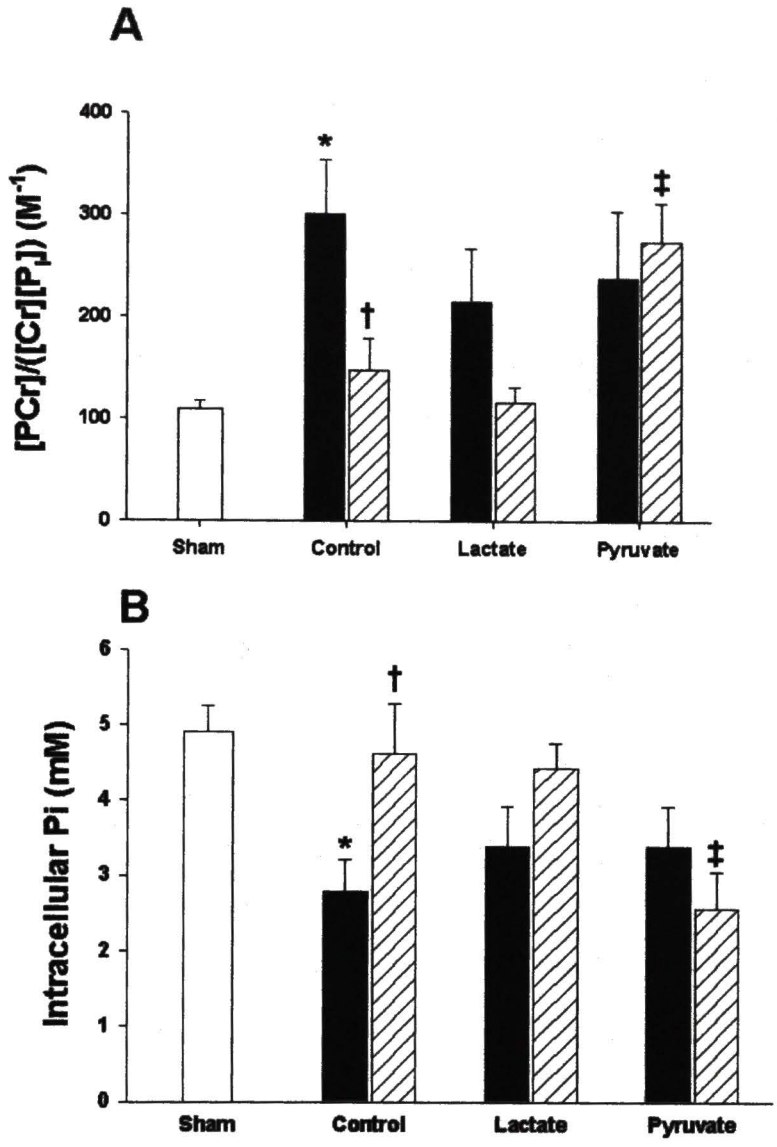

Figure 8. *Myocardial phosphorylation potential and inorganic phosphate.* Panel A: Phosphocreatine phosphorylation potential ([PCr]/([Cr][P_i])) was computed from phosphocreatine (PCr) and creatine (Cr) contents (Table) and intracellular inorganic phosphate (P_i) concentration (Panel B) measured in left ventricular myocardium sampled at 45 min arrest and 3 min reperfusion. *$P < 0.05$ *vs.* sham; †$P < 0.05$ *vs.* respective values in the same group at 45 min arrest; ‡$P < 0.05$ *vs.* sham, control, and lactate.

CHAPTER III

Oxidative Stress Induced Changes in Enzyme Activity

Our first investigation (Chapter 2) revealed that pyruvate-fortified cardioplegia reduced oxidative stress during cardioplegic arrest and reperfusion (9). Previous studies have demonstrated that antioxidant therapy with *N*-acetylcysteine reduces oxidative stress and improves post-arrest myocardial function (5). One possible mechanism for this improvement may be the ability of antioxidant therapy to protect vital metabolic enzymes from inactivation by oxyradicals formed during cardiac surgery. To date, the activities of metabolic enzymes during cardioplegic arrest have not been investigated. In the following investigation, we measured the activity of metabolic enzymes known to be affected by oxidative stress.

Creatine kinase (CK) plays a crucial role in myocardial energy utilization as different CK isoenzymes catalyze the transfer of high energy phosphate from the site of ATP production in the mitochondria to ATP-consuming processes elsewhere in the cardiomyocyte, *e.g.* myofilaments and ion-transporting ATPases. Under conditions of extreme oxidative stress, CK may be inactivated by S-thiolation of a cysteine residue in the catalytic core via reaction with glutathione disulfide (GSSG) (17), peroxynitrite ($ONOO^-$) (10), hydrogen peroxide (H_2O_2) (13,18), and superoxide (12), and by nitration of tyrosine residues by $ONOO^-$ (14).

Aconitase is a citric acid cycle enzyme that converts citrate to isocitrate. The activity of aconitase depends on the redox state of its cubane $[4Fe\text{-}4S]^{2+}$ cluster (16).

Oxyradicals disassemble the cubane cluster and result in inactivation of the enzyme. Aconitase has been shown to be inactivated by nitric oxide (15), H_2O_2 (11), $O_2^{\bullet-}$ and $ONOO^-$ (7). Loss of aconitase activity is commonly used as a biomarker of oxidative damage (2). Inactivation is reversible when oxidative stress is resolved but may be irreversible when oxidative stress is prolonged (2).

During aerobic metabolism, phosphofructokinase (PFK) is the key regulatory enzyme of glycolysis (3). However, during anaerobic glycolysis, the main source of ATP production during cardioplegic arrest, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) becomes the major rate-limiting enzyme (3). Therefore, changes in activity of either PFK or GAPDH could potentially effect ATP production. GAPDH has a reactive cysteine residue at position 149 which has been shown to undergo oxidation with direct H_2O_2 exposure (8). Oxidative stress associated with ischemia reperfusion injury results in S-glutathiolation of GAPDH which is reversed by antioxidant treatment (4). Another glycolytic enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is inactivated by oxidative modification of an active site lysine residue (6). Lactate dehydrogenase (LDH) is not inactivated in isolated guinea-pig hearts during H_2O_2 exposure (11).

A natural carbohydrate and glyocolytic product, pyruvate has both direct and indirect antioxidant properties in myocardium (9). Exogenous pyruvate was found to increase the reducing power of the GSH system in post-ischemic stunned (19) and hydrogen peroxide injured (11) guinea pig myocardium. In our first investigation, pyruvate-fortified cardioplegia increased GSH redox state during cardioplegic arrest but

not during early reperfusion when the burst of oxyradical formation is likely the greatest (9). However, the ability of pyruvate administration during reperfusion to protect the antioxidant defenses and prevent ROS inactivation of key enzymes has not been tested.

REFERENCES

1. **Bulteau AL, Ikeda-Saito M, Szweda LI. Bulteau AL, Ikeda-Saito M and Szweda LI.** Redox-dependent modulation of aconitase activity in intact mitochondria. *Biochemistry* 2003; 42:14846-14855.

2. **Bulteau AL, Lundberg KC, Ikeda-Saito M, Isaya G and Szweda LI.** Reversible redox-dependent modulation of mitochondrial aconitase and proteolytic activity during in vivo cardiac ischemia/reperfusion. *Proc Natl Acad Sci USA* 2005; 102:5987-5991.

3. **Das DK and Maulik N.** Bioenergetics, ischemic contracture and reperfusion injury. *EXS* 1996; 76:155-173.

4. **Eaton P, Wright N, Hearse DJ and Shattock MJ.** Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol* 2002; 34:1549-1560.

5. **Fischer UM, Cox CS,Jr, Allen SJ, Stewart RH, Mehlhorn U and Laine GA.** The antioxidant *N*-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest. *J Thorac Cardiovasc Surg* 2003; 126:1483-1488.

6. **Friguet B, Szweda LI and Stadtman ER.** Susceptibility of glucose-6-phosphate dehydrogenase modified by 4-hydroxy-2-nonenal and metal-catalyzed oxidation to proteolysis by the multicatalytic protease. *Arch Biochem Biophys* 1994; 311:168-173.

7. **Hausladen A and Fridovich I.** Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. *J Biol Chem* 1994; 269:29405-29408.

8. **Janero DR, Hreniuk D and Sharif HM.** Hydroperoxide-induced oxidative stress impairs heart muscle cell carbohydrate metabolism. *Am J Physiol Cell Physiol* 1994; 266:C179-188.

9. **Knott, EM, M-G Ryou, J Sun, A Heymann, AB Sharma, Y Lei, M Baig, RT Mallet and Olivencia-Yurvati AH.** Pyruvate-fortified cardioplegia suppresses oxidative stress and enhances phosphorylation potential of arrested myocardium. *Am J Physiol Heart Circ Physiol* 2005; 289:H1123-1130.

10. **Konorev EA, Hogg N and Kalyanaraman B.** Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett* 1998; 427:171-174.

11. **Mallet RT, Squires JE, Bhatia S and Sun J.** Pyruvate restores contractile function and antioxidant defenses of hydrogen peroxide-challenged myocardium. *J Mol Cell Cardiol* 2002; 34:1173-1184.

12. **McCord JM and Russell WJ.** Inactivation of creatine phosphokinase by superoxide during reperfusion injury. *Basic Life Sci* 1988; 49:869-873.

13. **Mekhfi H, Veksler V, Mateo P, Maupoil V, Rochette L and Ventura-Clapier R.** Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. *Circ Res* 1996; 78:1016-1027.

14. **Mihm MJ and Bauer JA**. Peroxynitrite-induced inhibition and nitration of cardiac myofibrillar creatine kinase. *Biochimie* 2002; 84:1013-1019.

15. **Pellat C, Henry Y and Drapier JC.** IFN-gamma-activated macrophages: detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide and non-heme iron proteins. *Biochem Biophys Res Commun* 1990; 166:119-125.

16. **Ramsay RR, Dreyer JL, Schloss JV, Jackson RH, Coles CJ, Beinert H, Cleland WW and Singer TP.** Relationship of the oxidation state of the iron-sulfur cluster of aconitase to activity and substrate binding. *Biochemistry* 1981; 20:7476-7482.

17. **Reddy S, Jones AD, Cross CE, Wong PS and Van Der Vliet A.** Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue. *Biochem J* 2000; 347:821-827.

18. **Suzuki YJ, Edmondson JD and Ford GD.** Inactivation of rabbit muscle creatine kinase by hydrogen peroxide. *Free Radic Res Commun* 1992; 16:131-136.

19. **Tejero-Taldo MI, Caffrey JL, Sun J and Mallet RT.** Antioxidant properties of pyruvate mediate its potentiation of beta-adrenergic inotropism in stunned myocardium. *J Mol Cell Cardiol* 1999; 31:1863-1872.

CHAPTER IV

Pyruvate Mitigates Oxidative Stress During Reperfusion of Cardioplegia-Arrested Myocardium

E. Marty Knott, B.A.,[1,2] Jie Sun, B.S.,[1] Yu Lei,[1] Myoung-Gwi Ryou, M.S.,[1] Albert H. Olivencia-Yurvati, D.O.,[2] Robert T. Mallet, Ph.D.[1]

Departments of Integrative Physiology[1] and Surgery,[2]

University of North Texas Health Science Center, Fort Worth, TX

Annals of Thoracic Surgery

March 2006; 81: 928-34

ABSTRACT

Background: Cardioplegic arrest and reperfusion of the myocardium imposes oxidative stress that could potentially inactivate metabolic enzymes and compromise energy production. This study determined the impact of cardioplegic arrest and reperfusion on activities of several oxidant-sensitive enzymes, and tested whether pyruvate, a natural metabolic fuel and antioxidant, mitigates oxidant stress, protects enzymes and bolsters myocardial energy state following reperfusion. **Methods:** *In situ* swine hearts were arrested for 60 min with 4:1 blood:crystalloid cardioplegia, and then reperfused for 3 min with cardioplegia-free blood with or without *c.* 12 mM pyruvate. Tissue metabolites and enzyme activities were measured in left ventricular myocardium snap frozen at 45 min arrest and 3 min reperfusion. **Results:** 8-Isoprostane content, a measure of lipid peroxidation, sharply increased upon reperfusion, coincident with a 70% decline in redox state of the intracellular antioxidant glutathione. Aconitase and glucose 6-phosphate dehydrogenase activities fell during arrest; creatine kinase and phosphofructokinase were inactivated upon reperfusion. Pyruvate suppressed 8-isoprostane formation, maintained glutathione redox state, and enhanced phosphocreatine phosphorylation potential, a measure of myocardial energy state, during reperfusion. Pyruvate reactivated creatine kinase and aconitase, which are at least partially mitochondrial enzymes, but did not protect the cytosolic enzymes glucose 6-phosphate dehydrogenase and phosphofructokinase. **Conclusions:** Administration of pyruvate upon reperfusion following cardioplegic arrest mitigates oxidative stress, protects mitochondrial enzymes and increases myocardial energy state. These results support therapeutic application of pyruvate-enhanced reperfusion to prevent cardiac injury following cardioplegic arrest.

INTRODUCTION

Cardioplegic arrest is essential for a variety of surgical procedures including coronary revascularization, valvular repair, and correction of congenital heart defects. Unfortunately, cardioplegic arrest and subsequent reperfusion generate cytotoxic oxygen and nitrogen metabolites, including superoxide [1,2], hydrogen peroxide [1], hydroxyl radical [3,4] and peroxynitrite [5]. These reactive compounds could potentially cause myocardial dysfunction by chemically modifying cellular protein, lipid and nucleic acid components. Several enzymes of intermediary and energy metabolism are among the principal biomolecular targets of reactive oxygen and nitrogen species. Inactivation of these enzymes could impair ATP synthesis and delivery to the contractile machinery.

A natural carbohydrate and metabolic intermediate, pyruvate functions as an antioxidant by virtue of its α-keto-carboxylate chemical structure and its mitochondrial metabolism [6]. The latter favors production of NADPH, the source of reducing power to maintain intracellular antioxidant defenses. Exogenous pyruvate increased the reducing power of the glutathione antioxidant system in post-ischemic stunned [7] and hydrogen peroxide-challenged [8] guinea-pig myocardium. Pyruvate-fortified cardioplegia increased glutathione redox state during cardioplegic arrest but not during the first few min of reperfusion, when oxyradical formation is likely the most intense [9]. On the other hand, whether pyruvate administration during reperfusion after cardioplegic arrest can protect myocardial antioxidant defenses and prevent inactivation of enzymes has not been tested.

This study assessed the impacts of cardioplegic arrest and reperfusion on glycolytic, hexose monophosphate shunt, Krebs cycle and energy shuttling enzymes, and determined whether reperfusion with pyruvate mitigates oxidative stress and protects or restores activities of these enzymes. *In situ* adult swine hearts were cardioplegically arrested for 60 min, then reperfused with cardioplegia-free whole blood with or without *c.* 12 mM pyruvate. Left ventricular myocardial biopsies were taken during arrest and at 3 min reperfusion for measurements of oxidative stress, antioxidant defenses and energy state, and enzyme activities. 8-Isoprostane, a product of lipid peroxidation, accumulated during reperfusion, and glutathione redox state concomitantly fell. Activities of aconitase and glucose 6-phosphate dehydrogenase fell during cardioplegic arrest, whereas creatine kinase and phosphofructokinase were inactivated upon reperfusion. Pyruvate prevented glutathione depletion and 8-isoprostane accumulation, and increased myocardial energy state. Pyruvate selectively reactivated creatine kinase and aconitase, enzymes located at least partially in the mitochondria, suggesting compartmentation of pyruvate metabolism [10,11] may have concentrated its antioxidant actions on this organelle during the first minutes of reperfusion.

MATERIAL AND METHODS

Animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, and was conducted in accordance with the *Guide to the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996). Twenty-seven adult domestic swine of either sex weighing 45-60 kg were randomly assigned to one of 4 experimental groups.

Surgical procedures. The surgical preparation was described recently [9]. In brief, swine were sedated with ketamine + xylazine, anesthetized with *iv* propofol and mechanically ventilated with 1-3% isoflurane supplemented with O_2. Cannulae were placed in femoral arteries to monitor arterial pressure and sample arterial blood. Plasma Lyte A (Baxter, Deerfield, IL) was administered via a femoral vein to maintain blood volume. The heart was exposed by median sternotomy and supported in a pericardial cradle. Coronary venous blood was sampled from a catheter placed in the coronary sinus via the right atrial appendage. The ventricles were drained with catheters inserted through the cardiac apex. A cannula was placed in the ascending aorta to deliver cardioplegia.

Cardioplegic arrest and reperfusion. Each pig was administered 300 U/kg of heparin *iv*. Arterial blood (1.2 l) was then withdrawn at a rate of 30 – 50 ml/min from a femoral artery using a roller pump (3M Health Care, Ann Arbor, MI). The blood was maintained at 37°C and used to prepare cardioplegia and to reperfuse the heart. Clamps were placed on the aorta distal to the cannula and on the superior and inferior vena cavae to effectively isolate the heart. Arrest was achieved with antegrade infusion, via the aortic cannula, of a minimum of 400 ml cold (4°C) blood cardioplegia (4 vol blood:1 vol crystalloid solution) as previously described [9]. Crystalloid cardioplegia solution (pH 7.6) contained 104 mM NaCl, 135 mM $NaHCO_3$, 91 mM KCl, 6 mM $CaCl_2$, 188 mM glucose, 68 U/L insulin, and 676 mg/l lidocaine. Arrest was maintained by infusing 100-150 ml blood cardioplegia into the aortic root at 20 and 40 min. After 60 min arrest, the

heart was reperfused by administering cardioplegia-free blood to the aorta at a rate of approximately 100 ml/min for 3 min. In six pigs, 1 M sodium pyruvate was infused into the aorta at a rate of 0.8 ml/min throughout reperfusion, resulting in a plasma pyruvate concentration of approximately 12 mM. The hearts did not fully recover mechanical function within 3 min, so post-arrest cardiac function was not monitored.

Plasma pyruvate. Pyruvate in aortic and coronary sinus plasma extracts was assayed [12] in a Shimadzu Instruments UV-1601 spectrophotometer. Myocardial pyruvate uptake equaled arterio-venous pyruvate concentration difference multiplied by blood flow rate.

Myocardial metabolites and enzymes. Snap frozen biopsies (*c.* 5 g) of the left ventricular apex were taken *in situ* by compressing tissue in Wollenberger tongs precooled in liquid N_2 [13]. Myocardium was sampled at 45 min arrest (n = 8), or at 3 min reperfusion in absence (n = 7) or presence (n = 6) of pyruvate infusion. In sham controls (n = 6) myocardium was sampled 1 h 45 min after sternotomy but without cardiac instrumentation or arrest. Experiments were terminated after biopsy. Aliquots of frozen myocardium were weighed, dessicated to constant mass, and reweighed to determine tissue water content (ml/g), which equaled 1- (dry mass/wet mass).

Metabolites were extracted from frozen myocardium [12,13] and spectrophotometrically assayed [14]. Phosphocreatine phosphorylation potential provided a measure of myocardial energy state [12,13]. Glutathione (GSH) and

glutathione disulfide (GSSG) were measured in a Shimadzu Instruments LC-10AT high performance liquid chromatography system equipped with a fluorescence detector [9,15]. The GSH/GSSG ratio provided a measure of myocardial antioxidant redox state [16]. Myocardial enzymes were extracted [8,17] and activities measured by spectrophotometry [14]. Extract protein concentration was determined colorimetrically with the Coomassie Plus Kit (Pierce, Rockford, IL), and enzyme activities expressed as U/mg protein.

Myocardial 8-isoprostane. To assess oxidative stress, total (esterified and non-esterified) 8-isoprostane, a product of lipid peroxidation, was measured in myocardium using a competitive immunoassay kit (Cayman Chemical, Ann Arbor, MI). The analyte was extracted from powdered tissue (250 mg) according to the kit instructions, then measured at 405 nm wavelength in a 96 well plate reader (BioTek KCjunior, Winooski, VT).

Statistical analyses. Data are reported as means ± SEM. Factorial analysis of variance (ANOVA) was applied to determine differences in all measured variables. When ANOVA detected statistical significance, *post hoc* between-group comparisons were performed with Student-Newman-Kuels multiple comparison test. Statistical analyses were performed with SigmaStat version 3.1 software. P values < 0.05 were considered statistically significant.

RESULTS

Aortic infusion of pyruvate during reperfusion resulted in a plasma pyruvate concentration of 11.6 ± 0.5 mM being delivered to the heart. Net myocardial uptake of pyruvate was 0.7 ± 0.1 mmol/min at 3 min reperfusion.

Myocardial 8-isoprostane and glutathione redox state. Left ventricular myocardial content of 8-isoprostane, a product of lipid peroxidation and indicator of oxidative stress, did not change during arrest but doubled within 3 min reperfusion (Figure 1). Pyruvate administration during reperfusion prevented the increase in 8-isoprostane. Thus, reperfusion engendered oxidative stress in myocardium, but pyruvate prevented lipid peroxidation due to this oxidant burst.

The ratio of glutathione/glutathione disulfide content, GSH/GSSG, provides a global measure of the redox state of the myocardium's antioxidant defenses [16]. GSH/GSSG increased during arrest, but fell sharply upon reperfusion, coincident with oxidative stress and lipid peroxidation (Figure 1). Pyruvate administration during reperfusion prevented the decline in GSH/GSSG.

Myocardial water content. Myocardial water content was measured to assess edema formation. Water content did not change during cardioplegic arrest, but noticeably increased during reperfusion, from 78.9 ± 0.2 to 81.1 ± 0.1 ml/100 g of myocardium (Figure 2). Pyruvate treatment prevented the increase in myocardial water content during reperfusion.

Myocardial pyruvate and derivatives. Cytosolic lactate dehydrogenase converts pyruvate to its reduced congener, lactate. In mitochondria, pyruvate carboxylation generates Krebs cycle intermediates, leading to increased citrate content [18]. Pyruvate and its derivatives were measured in left ventricular myocardium of arrested, reperfused and sham control hearts (Figure 3). Pyruvate content did not increase in the control group during arrest or reperfusion, but sharply increased as expected during pyruvate administration. Lactate content increased twofold during arrest and another threefold during pyruvate-free reperfusion. Pyruvate treatment prevented lactate accumulation during reperfusion. Citrate content increased during arrest and remained elevated during reperfusion in the control group. Pyruvate administration further increased citrate content.

Myocardial enzymes. Activities of glycolytic (phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase), hexose monophosphate shunt (glucose 6-phosphate dehydrogenase), Krebs cycle (aconitase) and energy shuttling (creatine kinase) enzymes were measured in arrested, reperfused and sham control myocardium. Creatine kinase activity (Figure 4A) did not change during arrest but fell 43% within 3 min of reperfusion. Exogenous pyruvate attenuated creatine kinase inactivation. Like creatine kinase, phosphofructokinase was unaltered during arrest but fell upon reperfusion (Figure 4B). However, pyruvate did not protect phosphofructokinase activity during reperfusion. Aconitase activity fell 40% during arrest and, unlike the other enzymes, partially recovered during reperfusion (Figure 4C).

Pyruvate markedly increased aconitase activity, to a level even 30% higher than in sham myocardium. Glucose 6-phosphate dehydrogenase was inactivated by 26% during arrest, but was unaffected by reperfusion ± pyruvate (Figure 4D). Activities of glyceraldehyde 3-phosphate dehydrogenase (Figure 4E) and lactate dehydrogenase (Figure 4F) were unaltered by arrest, reperfusion or pyruvate.

Myocardial energy state. Left ventricular myocardial ATP content tended to fall during arrest and declined even further upon reperfusion (Figure 5). Pyruvate treatment during reperfusion restored ATP content to the level observed in non-arrested sham controls. To assess the effects of cardioplegic arrest and reperfusion and the impact of pyruvate on myocardial energy state, phosphocreatine phosphorylation potential was computed from intracellular concentrations of phosphocreatine, creatine and inorganic phosphate. Phosphorylation potential nearly tripled during arrest but fell to near sham values within 3 min reperfusion (Figure 5). Pyruvate prevented the fall in phosphorylation potential during reperfusion.

COMMENT

This investigation aimed to determine if the natural antioxidant pyruvate could prevent inactivation of essential metabolic enzymes during the intense oxidative stress produced by reperfusion of cardioplegically arrested myocardium. Enzyme activities, oxidative stress and tissue edema, antioxidant redox state, and phosphorylation potential were measured in left ventricular myocardium sampled during arrest and at 3 min reperfusion with and without pyruvate infusion. Recently [9] pyruvate-fortified

cardioplegia was found to increase GSH/GSSG during arrest, but this antioxidant action quickly subsided during pyruvate-free reperfusion and failed to prevent GSH depletion and myocardial edema. Accordingly, this study investigated whether pyruvate administration exclusively during reperfusion following arrest with pyruvate-free cardioplegia could prevent lipid peroxidation, tissue edema and GSH depletion, and protect enzymes known to be targets of oxidants.

Pyruvate metabolism. Pyruvate infusion into the aorta delivered a plasma pyruvate concentration of 11.6 mM to the heart. This treatment increased pyruvate and citrate contents in the myocardium at 3 min reperfusion, indicating rapid uptake and mitochondrial metabolism of pyruvate. On the other hand, pyruvate actually prevented lactate accumulation, even though pyruvate is the direct precursor of lactate via lactate dehydrogenase. Radioisotope and stable isotope studies in isolated hearts demonstrated substantial subcellular compartmentation of pyruvate metabolism. Thus, exogenous pyruvate was mainly metabolized in the mitochondria, and only gradually converted to lactate [11,19]; indeed, intracellular lactate was not in isotopic equilibrium with exogenous pyruvate [10,19]. Moreover, pyruvate carboxylation in the mitochondria generates the Krebs cycle intermediates malate and oxaloacetate, leading to increased citrate content [18]. An allosteric inhibitor of phosphofructokinase [20], citrate suppresses glycolysis, the principal metabolic source of lactate, and thereby may prevent lactate accumulation. Moreover, pyruvate carboxylation increases the capacity of the Krebs cycle to oxidize acetyl CoA, which would favor pyruvate oxidation over lactate formation.

Antioxidant effects of pyruvate. Reactive oxygen and nitrogen species formed during reperfusion may contribute to myocardial injury following cardioplegic arrest [1-5]. The metabolic antioxidant pyruvate [9] and a pharmacological antioxidant, *N*-acetylcysteine [21], suppressed myocardial 8-isoprostane release during cardioplegic arrest. In this study, oxidative stress was minimal during arrest, but substantial stress accompanied reperfusion with whole blood. Reperfusion with pyruvate-enhanced blood prevented myocardial lipid peroxidation and rapid decline of GSH/GSSG. Thus, pyruvate mitigated reperfusion-induced oxidative stress. Pyruvate functions as an antioxidant by directly detoxifying peroxynitrite [22] and hydrogen peroxide [23] or, through its metabolism to citrate, increasing the supply of NADPH to maintain GSH/GSSG [6,7].

Enzyme activities. Mitochondrial and cytosolic creatine kinase isoenzymes exchange phosphate groups between ATP and phosphocreatine to efficiently transfer high energy phosphate from mitochondria to extramitochondrial ATP-consuming processes. During periods of oxidative stress, creatine kinase may be inactivated by *S*-thiolation or *S*-nitrosation of its catalytic cysteine residue via reaction with glutathione disulfide [24], peroxynitrite [25], hydrogen peroxide [26,27], or superoxide [28], or by nitration of tyrosine residues by peroxynitrite [29]. This investigation for the first time determined the impact of reperfusion on this important enzyme following cardioplegic arrest. Creatine kinase was unaltered during arrest but inactivated upon reperfusion. Pyruvate may protect creatine kinase by maintaining a high glutathione redox state during

reperfusion. Indeed, creatine kinase, inactivated by S-thiolation of its catalytic cysteine, can be directly dethiolated and reactivated by GSH [24,30].

Aconitase, a Krebs cycle enzyme that converts citrate to isocitrate, is another target of oxidants, including nitric oxide [31], hydrogen peroxide [8], superoxide [32] and peroxynitrite [32]. Indeed, loss of aconitase activity is commonly used as a biomarker of oxidative damage [33]. Aconitase activity depends on the redox state of its cubane [4Fe-4S]$^{2+}$ cluster [34]. Oxidants disassemble the cubane cluster and inactivate the enzyme. Inactivation is reversible if oxidative stress is quickly resolved but may become irreversible when oxidative stress is prolonged [35]. This investigation demonstrated inactivation of aconitase during cardioplegic arrest, partial recovery after reperfusion, and robust reactivation of the enzyme by pyruvate. Current evidence suggests that citrate is required for reinsertion of iron into the cubane cluster reversing the posttranslational modifications responsible for aconitase inactivation [35]. Thus, pyruvate enhancement of myocardial citrate content may have produced a salutary effect on aconitase.

Glucose 6-phosphate dehydrogenase, a cytosolic enzyme that catalyzes the initial, rate-controlling reaction of the hexose monophosphate shunt, is inactivated by oxidative modification of a lysine residue in its catalytic core [36]. Activity of this enzyme fell 26% during arrest but no further upon reperfusion, and was unresponsive to pyruvate. The allosterically regulated glycolytic enzyme phosphofructokinase was inactivated only upon reperfusion, yet it, too was unprotected by pyruvate. Neither glyceraldehyde 3-

phosphate dehydrogenase, a known oxidant target [37,38], nor lactate dehydrogenase were affected by arrest, reperfusion, or pyruvate treatment.

The two enzymes reactivated by pyruvate are at least partially mitochondrial (aconitase, creatine kinase mitochondrial isoenzyme), while cytosolic enzymes phosphofructokinase and glucose 6-phosphate dehydrogenase did not respond to pyruvate treatment. It is plausible that pyruvate exerts its antioxidant effects initially in the mitochondria due to its metabolic compartmentation. Citrate accumulation indicates that pyruvate was taken up and metabolized by the mitochondria, yet its conversion to lactate, a cytosolic process, did not occur within 3 min reperfusion.

Myocardial energy state. Oxidant- and pyruvate-induced changes in enzyme activities may affect myocardial energy production. Phosphocreatine phosphorylation potential, a measure of myocardial energy state [39], increased during arrest, but fell at 3 min reperfusion [9]. Pyruvate administration during early reperfusion maintained phosphorylation potential and ATP content. Enhancement of phosphorylation potential by pyruvate [40] increases Gibbs free energy of ATP hydrolysis which defines the amount of energy available for ATP-dependent cardiac performance. In addition to providing readily oxidized fuel for the myocardium [40], pyruvate may have enhanced phosphorylation potential by preventing creatine kinase inactivation during reperfusion. However, it must be noted that despite inactivation of creatine kinase, phosphorylation potential during pyruvate-free reperfusion was similar to that of non-arrested sham hearts.

Limitations. This *in situ* heart preparation permits direct examination of the effects of cardioplegia and reperfusion on the organ. However, only 1.2 l of arterial blood could be safely withdrawn from the pig without provoking circulatory collapse despite replacement with Plasma Lyte. The volume of whole blood available after blood cardioplegia administration was only sufficient for 3 min of reperfusion, which did not permit complete recovery of mechanical function. Blood from donor animals was not administered to avoid the pro-inflammatory and pro-oxidant effects of allogenic blood. Therefore, it was not possible to determine if pyruvate protection of creatine kinase and aconitase and enhancement of energy state would have improved cardiac mechanical recovery, or prevented arrhythmias or infarction. The optimal pyruvate concentration and duration of treatment remain to be determined.

Summary and conclusions. To our knowledge, this is the first investigation of alterations in myocardial enzyme activities during and immediately following cardioplegic arrest. Creatine kinase activity was maintained during arrest but fell by 3 min reperfusion, while aconitase activity fell during arrest and partially recovered upon reperfusion, despite intense oxidative stress. Pyruvate administration during reperfusion reduced oxidative stress and increased antioxidant state. Pyruvate protected the mitochondrial enzymes creatine kinase and aconitase from inactivation but had no salutary effect on cytosolic enzymes. Pyruvate also increased myocardial energy state. Pyruvate may be a beneficial antioxidant and energy-generating intervention in the setting of cardioplegic arrest.

ACKNOWLEDGEMENTS

This study was supported by grants from the Osteopathic Heritage Foundation (02-18-522) and the National Heart, Lung and Blood Institute (HL071684). This work was completed in partial fulfillment of the requirements for the Ph.D. degree for EMK. The outstanding technical assistance of Arti B. Sharma, Linda Howard and Abraham Heymann is gratefully acknowledged. Mirza Baig, Pharm. D. and Amar Joumma, Department of Pharmacy, Plaza Medical Center of Fort Worth, Fort Worth, TX prepared the cardioplegia solutions.

REFERENCES

1. Prasad K, Chan WP, Bharadwaj B. Superoxide dismutase and catalase in protection of cardiopulmonary bypass-induced cardiac dysfunction and cellular injury. Can J Cardiol 1996;12:1083-1091.

2. Kalawski R, Balinski M, Bugajski P, Wysocki H, Olszewski R, Siminiak T. Stimulation of neutrophil activation during coronary artery bypass grafting: Comparison of crystalloid and blood cardioplegia. Ann Thorac Surg 2001;71:827-831.

3. Menasche P, Grousset C, Gauduel Y, Mouas C, Piwnica A. Prevention of hydroxyl radical formation: A critical concept for improving cardioplegia. Protective effects of deferoxamine. Circulation 1987;76:V180-185.

4. Sellke FW, Shafique T, Ely DL, Weintraub RM. Coronary endothelial injury after cardiopulmonary bypass and ischemic cardioplegia is mediated by oxygen-derived free radicals. Circulation 1993;88:II395-400.

5. Mehlhorn U, Krahwinkel A, Geissler HJ, LaRosee K, Fischer UM, Klass O, Suedkamp M, Hekmat K, Tossios P, Bloch W. Nitrotyrosine and 8-isoprostane formation indicate free radical-mediated injury in hearts of patients subjected to cardioplegia. J Thorac Cardiovasc Surg 2003;125:178-183.

6. Mallet RT and Sun J. Antioxidant properties of myocardial fuels. Mol Cell Biochem 2003; 253:103-111.

7. Tejero-Taldo MI, Caffrey JL, Sun J and Mallet RT. Antioxidant properties of pyruvate mediate its potentiation of beta-adrenergic inotropism in stunned myocardium. J Mol Cell Cardiol 1999; 31:1863-18672.

8. Mallet RT, Squires JE, Bhatia S and Sun J. Pyruvate restores contractile function and antioxidant defenses of hydrogen peroxide-challenged myocardium. J Mol Cell Cardiol 2002; 34:1173-1184.

9. Knott EM, Ryou M-G, Sun J, et al. Pyruvate-fortified cardioplegia suppresses oxidative stress and enhances phosphorylation potential of arrested myocardium. Am J Physiol Heart Circ Physiol, in press.

10. Bünger R. Compartmented pyruvate in perfused working heart. Am J Physiol Heart Circ Physiol 1985;249:H439-49.

11. Lloyd S, Brocks C, Chatham JC. Differential modulation of glucose, lactate, and pyruvate oxidation by insulin and dichloroacetate in the rat heart. Am J Physiol Heart Circ Physiol 2003;285:H163-72.

12. Yi KD, Downey HF, Bian X, Fu M, Mallet RT. Dobutamine enhances both contractile function and energy reserves in hypoperfused canine right ventricle. Am J Physiol Heart Circ Physiol 2000;279:H2975-85.

13. Itoya M, Mallet RT, Gao ZP, Williams AG Jr., Downey HF. Stability of high-energy phosphates in right ventricle: myocardial energetics during right coronary hypotension. Am J Physiol Heart Circ Physiol 1996;271:H320-8.

14. Bergmeyer HU. Methods of Enzymatic Analysis. New York: Academic Press, 1983.

15. Sharma AB, Knott EM, Bi J, Martinez RR, Sun J, Mallet RT. Pyruvate improves electromechanical and metabolic recovery from cardiopulmonary arrest and resuscitation. Resuscitation 2005;66:71-81.

16. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 2001;30:1191-212.

17. Stuewe SR, Gwirtz PA, Agarwal N, Mallet RT. Exercise training enhances glycolytic and oxidative enzymes in canine ventricular myocardium. J Mol Cell Cardiol 2000;32:903-13.

18. Panchal AR, Comte B, Huang H, et al. Partitioning of pyruvate between oxidation and anaplerosis in swine hearts. Am J Physiol Heart Circ Physiol 2000;279:H2390-8.

19. Peuhkurinen KJ, Hassinen IE. Pyruvate carboxylation as an anaplerotic mechanism in the isolated perfused rat heart. Biochem J 1982;202:67-76.

20. Garland PB, Randle PJ, Newsholme EA. Citrate as an intermediary in the inhibition of phosphofructokinase in rat heart muscle by fatty acids, ketone bodies, pyruvate, diabetes, and starvation. Nature 1963;200:169-70.

21. Fischer UM, Cox CS Jr, Allen SJ, Stewart RH, Mehlhorn U, Laine GA. The antioxidant N-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest. J Thorac Cardiovasc Surg 2003;126:1483-8.

22. Vásquez-Vivar J, Denicola A, Radi R, Augusto O. Peroxynitrite-mediated decarboxylation of pyruvate to both carbon dioxide and carbon dioxide radical anion. Chem Res Toxicol 1997;10:786-94.

23. Constantopoulos G, Barranger JA. Nonenzymatic decarboxylation of pyruvate. Anal Biochem 1984;139:353-8.

24. Reddy S, Jones AD, Cross CE, Wong PS, Van Der Vliet A. Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue. Biochem J 2000;347:821-7.

25. Konorev EA, Hogg N, Kalyanaraman B. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett 1998;427:171-4.

26. Mekhfi H, Veksler V, Mateo P, Maupoil V, Rochette L, Ventura-Clapier R. Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. Circ Res 1996;78:1016-27.

27. Suzuki YJ, Edmondson JD, Ford GD. Inactivation of rabbit muscle creatine kinase by hydrogen peroxide. Free Radic Res Commun 1992;16:131-6.

28. McCord JM, Russell WJ. Inactivation of creatine phosphokinase by superoxide during reperfusion injury. Basic Life Sci 1988;49:869-73.

29. Mihm MJ, Bauer JA. Peroxynitrite-induced inhibition and nitration of cardiac myofibrillar creatine kinase. Biochimie 2002;84:1013-9.

30. Konorev EA, Kalyanaraman B, Hogg N. Modification of creatine kinase by S-nitrosothiols: S-Nitrosation vs. S-thiolation. Free Radic Biol Med 2000;28:1671-8.

31. Pellat C, Henry Y, Drapier JC. IFN-gamma-activated macrophages: Detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide and non-heme iron proteins. Biochem Biophys Res Commun 1990;166:119-25.

32. Hausladen A, Fridovich I. Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. J Biol Chem 1994;269:29405-29408.

33. Bulteau AL, Lundberg KC, Ikeda-Saito M, Isaya G, Szweda LI. Reversible redox-dependent modulation of mitochondrial aconitase and proteolytic activity during in vivo cardiac ischemia/reperfusion. Proc Natl Acad Sci USA 2005;102:5987-91.

34. Ramsay RR, Dreyer JL, Schloss JV, et al. Relationship of the oxidation state of the iron-sulfur cluster of aconitase to activity and substrate binding. Biochemistry 1981;20:7476-82.

35. Bulteau AL, Ikeda-Saito M, Szweda LI. Redox-dependent modulation of aconitase activity in intact mitochondria. Biochemistry 2003;42:14846-55.

36. Friguet B, Szweda LI, Stadtman ER. Susceptibility of glucose-6-phosphate dehydrogenase modified by 4-hydroxy-2-nonenal and metal-catalyzed oxidation to proteolysis by the multicatalytic protease. Arch Biochem Biophys 1994;311:168-73.

37. Janero DR, Hreniuk D, Sharif HM. Hydroperoxide-induced oxidative stress impairs heart muscle cell carbohydrate metabolism. Am J Physiol Cell Physiol 1994;266:C179-88.

38. Eaton P, Wright N, Hearse DJ, Shattock MJ. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. J Mol Cell Cardiol 2002;34:1549-60.

39. Veech RL, Lawson JW, Cornell NW, Krebs HA. Cytosolic phosphorylation potential. J Biol Chem 1979;254:6538-47.

40. Mallet RT. Pyruvate: Metabolic protector of cardiac performance. Proc Soc Exp Biol Med 2000;223:136-148.

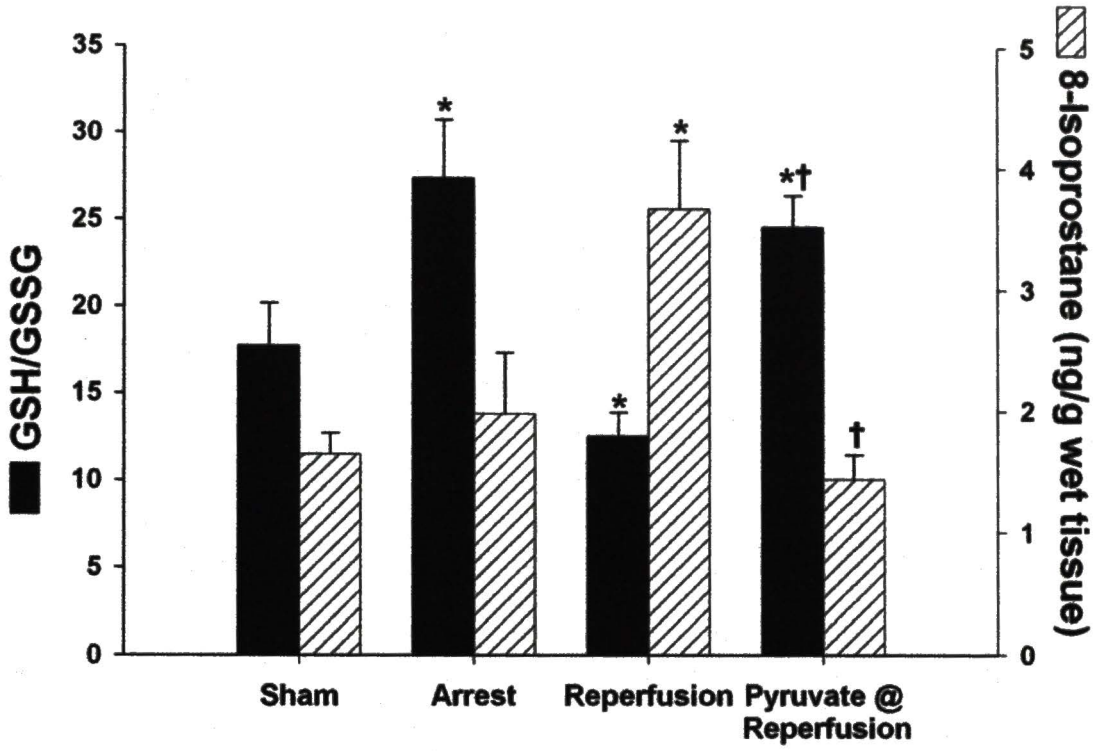

Figure 1. *Antioxidant state and oxidative stress.* Glutathione redox state (GSH/GSSG) and 8-isoprostane content were measured in left ventricular myocardium sampled at 45 min arrest (n = 8), at 3 min reperfusion with (n = 6) and without (n = 7) pyruvate infusion, and at 105 min post-sternotomy in non-arrested sham hearts (n = 6). Values in this and the other figures are means ± SEM. *P < 0.05 *vs.* sham; †P < 0.05 *vs.* reperfusion.

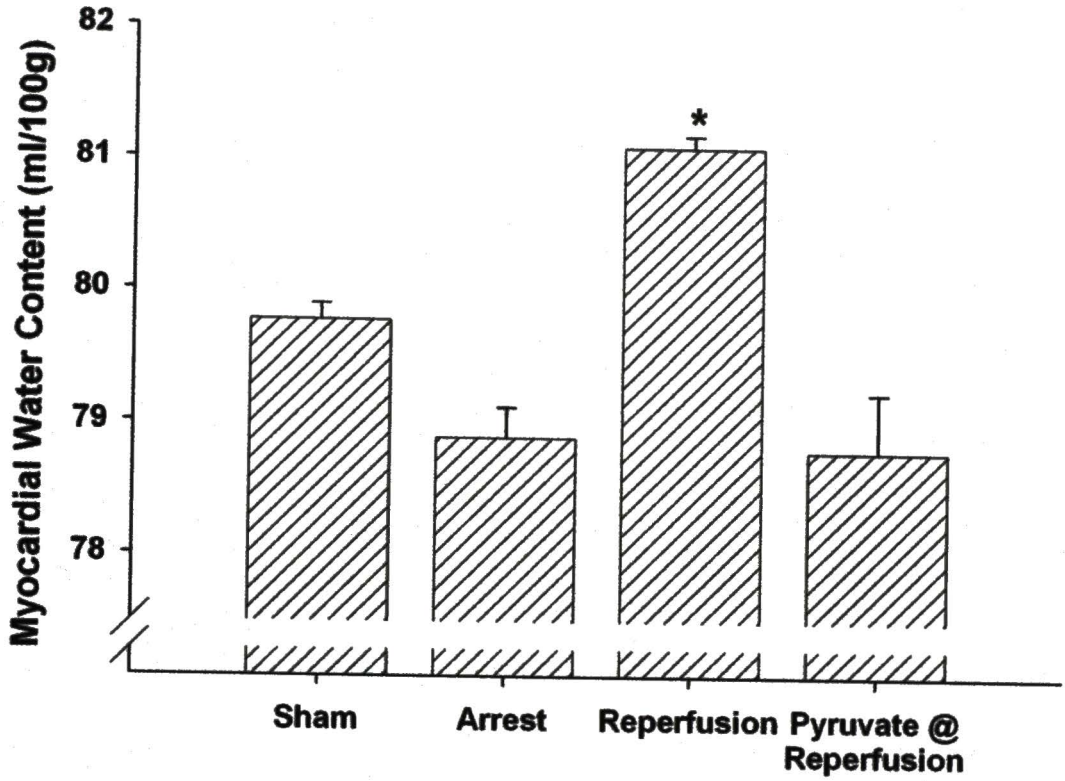

Figure 2. *Myocardial water content.* Tissue water content was determined from masses of fresh and dessicated tissue. $^{*}P < 0.05$ *vs.* all other groups.

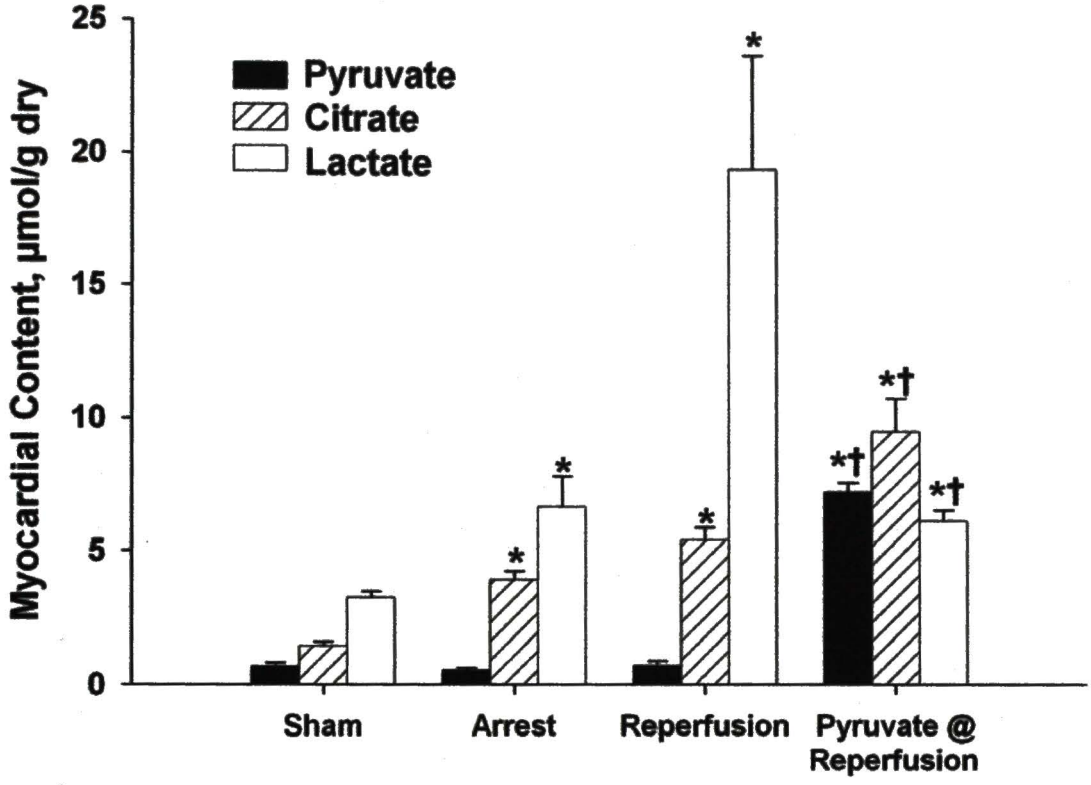

Figure 3. *Myocardial pyruvate and its metabolic derivatives.* Pyruvate (solid bars), lactate (hatched bars) and citrate (open bars) were measured in left ventricular myocardium. *P < 0.05 *vs.* sham; †P < 0.05 *vs.* reperfusion.

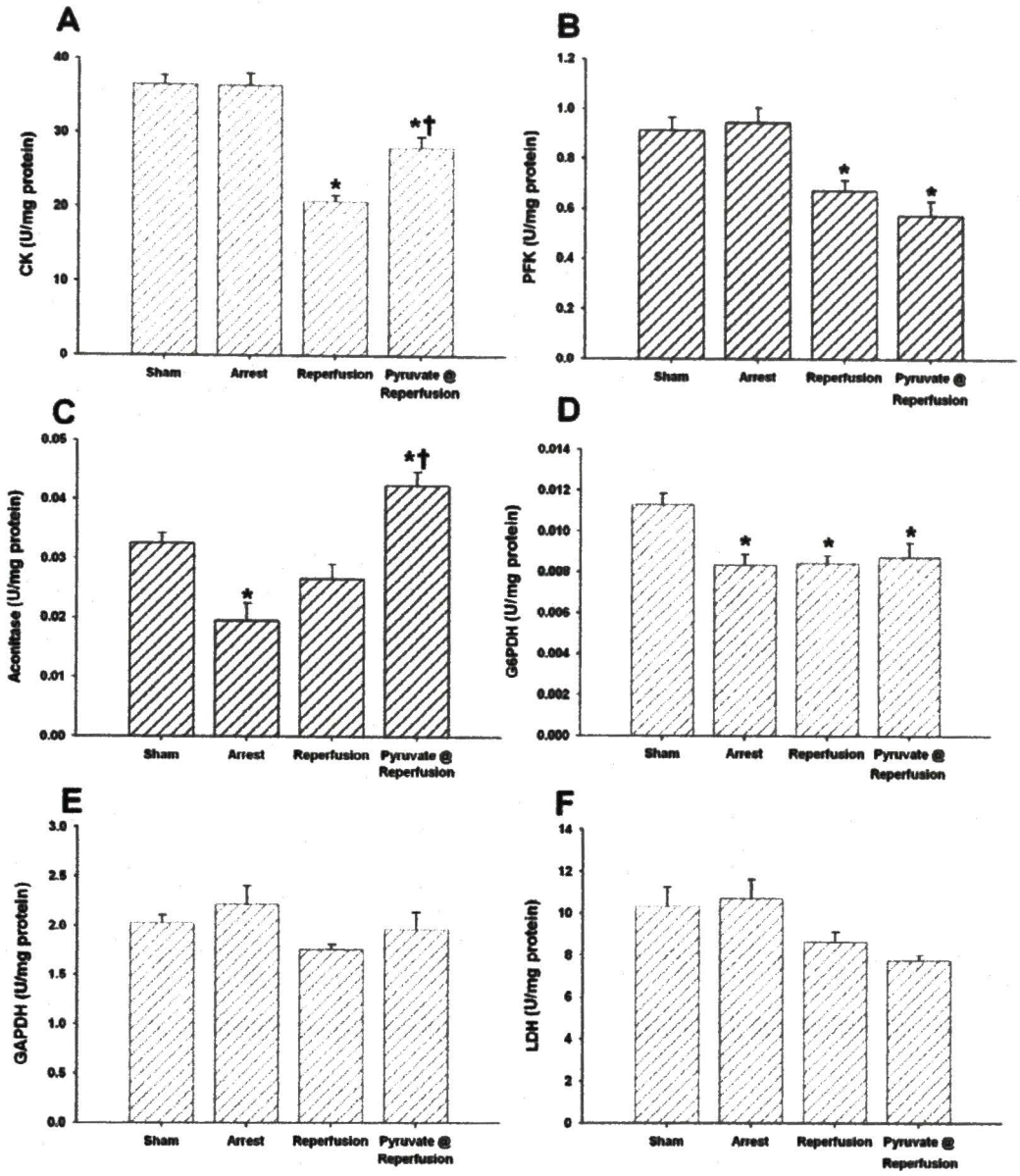

Figure 4. *Myocardial enzymes.* Activities (U/mg protein) of creatine kinase (CK; Panel A), phosphofructokinase (PFK; Panel B), aconitase (Panel C), glucose-6-phosphate dehydrogenase (G6PDH; Panel D), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Panel E), and lactate dehydrogenase (LDH; Panel F) were measured in left ventricular myocardial extracts. $^{*}P < 0.05$ *vs.* sham; †$P < 0.05$ *vs.* reperfusion.

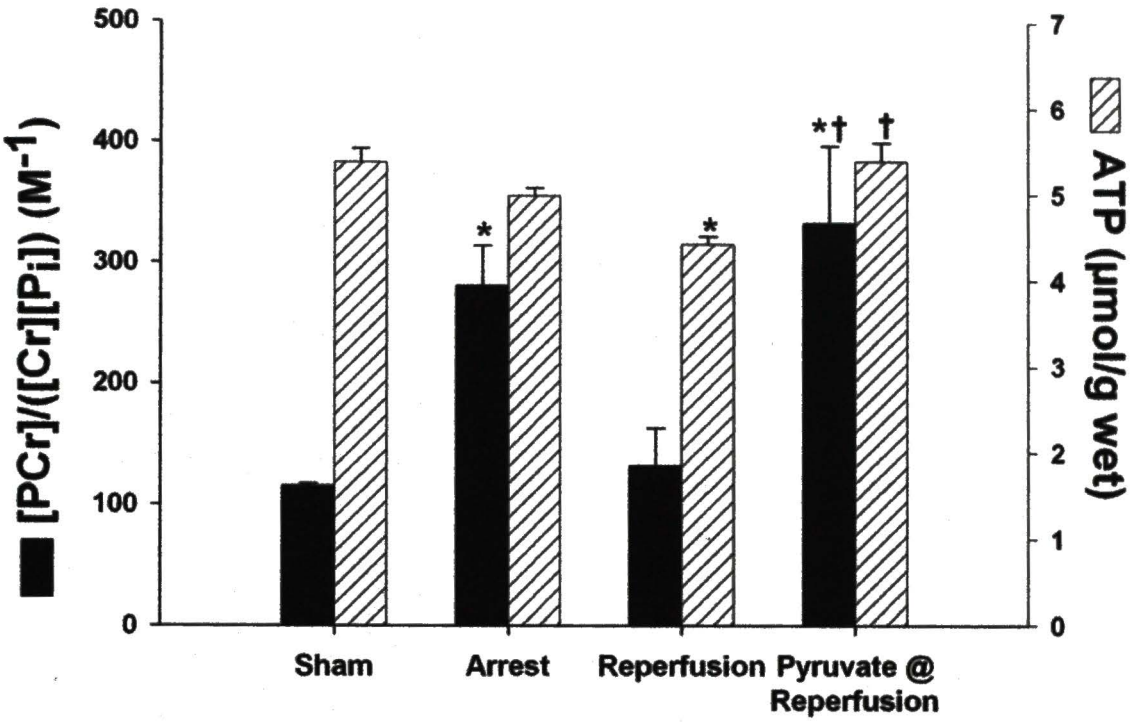

Figure 5. *Myocardial phosphorylation potential and ATP content.* Phosphocreatine phosphorylation potential ([PCr]/([Cr][P_i])) was computed from intracellular concentrations of phosphocreatine (PCr), creatine (Cr) and inorganic phosphate (P_i) measured in left ventricular myocardium. ATP content was measured in the same samples. *$P < 0.05$ *vs.* sham; †$P < 0.05$ *vs.* reperfusion.

CHAPTER V

CONCLUSIONS

The first purpose of this investigation was to determine the effects of the natural metabolite and antioxidant, pyruvate, on oxidative stress and myocardial energy state in the setting of cardioplegic arrest and reperfusion of the *in situ* swine heart. We found that the addition of pyruvate to the crystalloid cardioplegia solution reduced oxidative stress and bolstered myocardial energy state following cardioplegic arrest and reperfusion. Second, we sought to determine the effects of cardioplegic arrest and reperfusion on oxidant-sensitive metabolic enzymes, and whether pyruvate administered during reperfusion had the potential to reduce oxidative stress, protect enzymes, and thereby bolster myocardial energy state. Ischemia-reperfusion associated with cardioplegic arrest inactivated aconitase during arrest and creatine kinase during reperfusion. Pyruvate reduced oxidative stress, increased antioxidant redox state, and protected aconitase and creatine kinase from inactivation. It had no effect on the activity of the cytosolic enzymes studied. From these results, we conclude that:

1. Pyruvate-fortified cardioplegia and administration of pyruvate during early reperfusion increase the antioxidant state of the heart and reduce oxidative stress occurring as a result of cardioplegic arrest and reperfusion.

2. Pyruvate bolsters the myocardial energy state during early reperfusion when administered during cardioplegic arrest or during reperfusion.

3. Cardioplegic arrest and reperfusion inactivates several key metabolic enzymes. Pyruvate administration during reperfusion, the period of most intense oxidative stress, increases the activity of two mitochondrial enzymes during early reperfusion when compared to control.

4. These results suggest that pyruvate-fortified cardioplegia may improve post-CPB mechanical recovery and reduce post-CPB myocardial injury by suppressing oxidative stress, increasing antioxidant state during arrest, protecting enzyme activity, and bolstering myocardial energy state.

CHAPTER VI

PROPOSAL OF FUTURE STUDIES

This investigation, for the first time, provides information concerning the effects of the natural metabolite and antioxidant, pyruvate, on the *in situ* heart subjected to cardioplegic arrest and reperfusion. The studies provide evidence that pyruvate-fortified cardioplegia and pyruvate supplementation during reperfusion may have clinical benefits in patients undergoing cardioplegic arrest for procedures such as on-pump coronary artery bypass grafting and valve replacement. Despite this compelling evidence, more studies remain to be completed in order to further elucidate the mechanisms of cardiac protection by pyruvate. The following experiments are proposed to continue this area of investigation:

1. Determine the effects of pyruvate-fortified cardioplegia on cardiac performance during the first few hours of recovery in a full cardiopulmonary bypass animal model.

2. Determine the effect of pyruvate on the occurrence of post-surgical cardiac arrhythmias such as atrial fibrillation, which are associated with systemic inflammation.

3. Define the optimal cardioprotective concentration of pyruvate in crystalloid cardioplegia.

4. Determine the effects of pyruvate-fortified cardioplegia on oxidative stress and contractile function in a large randomized clinical trial.

Cardiopulmonary bypass induces a systemic inflammatory response

Inflammation is ordinarily a protective response that occurs in response to a potentially harmful event such as infection or surgery. This response is characterized by a complex interaction of humoral and cellular factors that culminates in intracellular pathway activation leading to the release of cytokines, activation of complement and thrombin, and expression of adhesion molecules, among other factors (15). In the setting of CPB, this inflammatory response is due to a combination of multiple factors, including physical contact of blood with the bypass circuit (11), ischemia-reperfusion injury (28), endotoxemia (2), and surgical trauma (23). Despite its many benefits, CPB initiates pro-inflammatory echanisms that culminate in the systemic inflammatory response syndrome (SIRS) (23), characterized by cardiac (5), renal (3), neurologic (26), respiratory (21,30,37), and hepatic (23) dysfunction, as well as bleeding disorders (17).

Neutrophils are activated by interleukin (IL)-8 (39) and suppressed by IL-10 (22). Activated neutrophils express adhesion molecules which cause the cells to adhere to the vascular endothelium (6). These adherent inflammatory cells have the ability to migrate across the endothelium and invade the tissue where they cause damage by producing

reactive oxygen species (19) and by secreting proteases, such as elastase (31). Elastase degrades elastin, a structural component of the extracellular matrix in the lungs, and contributes to post-bypass lung injury (21).

Multiple strategies have been implemented to combat the inflammatory response initiated by CPB. Glucocorticoid therapy has been shown to reduce pro-inflammatory cytokines (14,33) and to increase the anti-inflammatory cytokine, IL-10 (14). Aprotinin, a serine protease, is now commonly used due to its many beneficial effects. Heparin use is universal and has been shown to have anti-inflammatory actions (4) in addition to its intended antithrombotic actions. Reactive oxygen species (ROS) have been shown to activate nuclear factor κB (NF-κB) (34) which regulates the transcription of several inflammatory cytokines. Antioxidants prevent activation of NF-κB (8) and reduce the inflammatory response (41). Victor *et al.* (38) have demonstrated that the pharmacologic antioxidant, N-acetylcysteine reduces cytokine release and NF-κB activation in peritoneal macrophages and lymphocytes from mice. Other treatments such as sodium nitroprusside, inhibition of complement, and monoclonal antibodies have also been investigated (23). Use of heparin coated circuits, application of blood filters, variations in temperature, biventricular bypass and off-pump cardiac surgery have all been used in an attempt to reduce the potentially lethal inflammation caused by CPB. These numerous advances lessen but do not completely prevent CPB-induced inflammation.

Anti-inflammatory effects of pyruvate.

Cardiopulmonary bypass induces a systemic inflammatory response syndrome as discussed above. An ethyl derivative of pyruvate exerts salutary effects on oxidative stress resembling those of sodium pyruvate (32). In addition, ethyl pyruvate was shown to inhibit activation of NF-κB and p38 mitogen activated protein kinase in mice with established lethal sepsis (36). Uchiyama *et al.* (35) have demonstrated that ethyl pyruvate inhibits NF-κB activation and tumor necrosis factor mRNA expression, two components of the inflammatory response, in mice subjected to mesenteric ischemia and reperfusion. The anti-inflammatory actions were associated with decreased intestinal mucosal damage. Yang *et al.* (41) have demonstrated that Ringer ethyl pyruvate solution reduces mRNA expression of the pro-inflammatory cytokine, IL-6, compared to Ringer lactate solution in a murine model of hemorrhagic shock.

Ethanol, a byproduct of ethyl pyruvate breakdown, has been shown to have equivocal effects on NF-κB activation, both inhibiting (10,13) and promoting (25) its activation. In the studies by Uchiyama (35) and Yang (41), ethanol may have contributed to the anti-inflammatory actions of ethyl pyruvate. No studies, to our knowledge, have investigated the potential of sodium pyruvate to reduce inflammatory cytokine expression. It is likely that sodium pyruvate, the proposed cardioplegia fuel, will also exert these anti-inflammatory effects. Pyruvate effectively scavenges reactive oxygen species (7,9,20), which have been shown to activate NF-κB (34). Activation of the NF-κB pathway regulates the expression of several genes involved in the inflammatory response, such as inducible nitric oxide synthase (24), cyclooxygenase-2 (12), tumor necrosis factor (18), and IL-6 (1,15,27,29).

Based on these principles, we hypothesize that pyruvate-fortified cardioplegia may reduce the SIRS associated with CPB. The following specific aims are proposed to address this hypothesis:

1. Determine the ability of pyruvate to suppress the release of inflammatory mediators from isolated human white blood cells.

2. Determine the ability of lactate- and pyruvate-enriched cardioplegia solutions to mitigate the inflammatory response occurring as a result of cardiopulmonary bypass.

REFERENCES

1. **Altavilla D, Saitta A, Guarini S, Galeano M, Squadrito G, Cucinotta D, Santamaria LB, Mazzeo AT, Campo GM, Ferlito M, Minutoli L, Bazzani C, Bertolini A, Caputi AP and Squadrito F.** Oxidative stress causes nuclear factor-kappaB activation in acute hypovolemic hemorrhagic shock. *Free Radic Biol Med* 2001; 30: 1055-1066.

2. **Andersen LW, Landow L, Baek L, Jansen E and Baker S.** Association between gastric intramucosal pH and splanchnic endotoxin, antibody to endotoxin, and tumor

necrosis factor-alpha concentrations in patients undergoing cardiopulmonary bypass. *Crit Care Med* 1993; 21:210-7.

3. **Antunes PE, Prieto D, Ferrao de Oliveira J and Antunes MJ.** Renal dysfunction after myocardial revascularization. *Eur J Cardiothorac Surg* 2004; 25:597-604.

4. **Attanasio M, Gori AM, Giusti B, Pepe G, Comeglio P, Brunelli T, Prisco D, Abbate R, Gensini GF and Neri Serneri GG.** Cytokine gene expression in human LPS- and IFNgamma-stimulated mononuclear cells is inhibited by heparin. *Thromb Haemost* 1998; 79:959-962.

5. **Aybek T, Kahn MF, Dogan S, Abdel-Rahman U, Mierdl S, Kessler P, Wimmer-Greinecker G and Moritz A.** Cardiopulmonary bypass impairs left ventricular function determined by conductance catheter measurement. *Thorac Cardiovasc Surg* 2003; 51:301-305.

6. **Brix-Christensen V, Petersen TK, Ravn HB, Hjortdal VE, Andersen NT and Tonnesen E.** Cardiopulmonary bypass elicits a pro- and anti-inflammatory cytokine response and impaired neutrophil chemotaxis in neonatal pigs. *Acta Anaesthesiol Scand* 2001; 45:407-413.

7. **Bunton, CA.** Oxidation of α-diketones and α-keto-acids by hydrogen peroxide. *Nature* 1949; 163:144.

8. **Christman JW, Lancaster LH and Blackwell TS.** Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. *Intensive Care Med* 1998; 24:1131-1138.

9. **Dobsak P, Courdertot-Masuyer C, Zeller M, Vergely C, Laubriet A, Assem M, Eicher JC, Teyssier JR, Wolf JE and Rochette L.** Antioxidative properties of pyruvate and protection of the ischemic rat heart during cardioplegia. *J Cardiovasc Pharmacol* 1999; 34:651-659.

10. **Fox ES, Cantrell CH and Leingang KA.** Inhibition of the Kupffer cell inflammatory response by acute ethanol: NF-kappa B activation and subsequent cytokine production. *Biochem Biophys Res Comm* 2003; 225:134–140.

11. **Ikuta T, Fujii H, Shibata T, Hattori K, Hirai H, Kumano H and Suehiro S.** A new poly-2-methoxyethylacrylate-coated cardiopulmonary bypass circuit possesses superior platelet preservation and inflammatory suppression efficacy. *Ann Thorac Surg* 2004; 77:1678-1683.

12. **Jobin C, Morteau O, Han DS, and Sartor RB.** Specific NF-κB blockade selectively inhibits tumour necrosis factor-alpha-induced COX-2 but not constitutive COX-1 gene expression in HT-29 cells. *Immunology* 1998; 95:537-543.

13. **Jonsson AS and Palmblad JE.** Effects of ethanol on NF-kappaB activation, production of myeloid growth factors, and adhesive events in human endothelial cells. *J Infect Dis* 2001; 184:761–769.

14. **Kawamura T, Inada K, Nara N, Wakusawa R and Endo S.** Influence of methylprednisolone on cytokine balance during cardiac surgery. *Crit Care Med* 1999; 27:545-548.

15. **Levy JH and Tanaka KA.** Inflammatory response to cardiopulmonary bypass. *Ann Thorac Surg* 2003; 75:715-720.

16. **Livolsi, A, Busuttil V, Imbert V, Abraham RT and Peyron J-F.** Tyrosine phosphorylation-dependent activation of NF-κB: requirement for p56 LCK and ZAP-70 protein tyrosine kinases. *Eur J Biochem* 2001; 268:1508-1515.

17. **Martens S, Matheis G, Wimmer-Greinecker G, Feuerbach J, Jakob A, Mierdl S and Moritz A.** Heparin coating of the extracorporeal circuit combined with leukocyte filtration reduces coagulation activity, blood loss and blood product substitution. *Int J Artif Organs* 2001; 24:484-488.

18. **Meldrum, DR, Shenkar R, Sheridan BC, Cain BS, Abraham E, and Harken AH.** Hemorrhage activates myocardial NF-kappaB and increases TNF-alpha in the heart. *J Mol Cell Cardiol* 1997; 29:2849-2854.

19. **Morse D, Adams D and Maganani B.** Platelets and neutrophil activation during cardiac surgical procedures: impact of cardiopulmonary bypass. *Ann Thorac Surg* 1998; 65:691-695.

20. **Nath KA, Ngo EO, Hebbel RP, Croatt AJ, Zhou B and Nutter LM.** α-Ketoacids scavenge H_2O_2 in vitro and in vivo and reduce menadione-induced DNA injury and cytotoxicity. *Am J Physiol Cell Physiol* 1995; 268:C227-C236.

21. **Ng CS, Wan S, Yim AP and Arifi AA.** Pulmonary dysfunction after cardiac surgery. *Chest* 2002; 121:1269-1277.

22. **Nikolaus S, Bauditz J, Gionchetti P, Witt C, Lochs H and Schreiber S.** Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation. *Gut* 1998; 42:470-476.

23. **Paparella D, Yau TM and Young E.** Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. An update. *Eur J Cardiothorac Surg* 2002; 21:232-244.

24. **Pritts TA, Moon MR, Wang Q, Hungress ES, Salzman AL, Fischer JE and Hasselgren PO.** Activation of NF-kappaB varies in different regions of the gastrointestinal tract during endotoxemia. *Shock* 2000; 14:118-122.

25. **Roman J, Colell A, Blasco C, Caballeria J, Pares A, Rodes J and Fernandez-Checa JC.** Differential role of ethanol and acetaldehyde in the induction of oxidative stress in HEP G2 cells: effect on transcription factors AP-1 and NF-kappaB. *Hepatology* 1999; 30:1473–1480.

26. **Schmitz C, Weinreich S, Schneider R, Schneider D, Speth I, Schulze-Rauschenbach C, Pohl C and Welz A.** Off-Pump versus on-pump coronary artery bypass: can OPCAB reduce neurologic injury? *Heart Surg Forum* 2003; 6:127-130.

27. **Schoonbroodt S, Ferreira V, Best-Belpomme M, Boelaert JR, Legrand-Poels S, Korner M and Piette J.** Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of IκBα in NF-κB activation by oxidative stress. *J Immunol* 2000; 164:4292-4300.

28. **Serraf A, Sellak H, Herve P, Bonnet N, Robotin M, Detruit H, Baudet B, Mazmanian GM and Planche C.** Vascular endothelium viability and function after total cardiopulmonary bypass in neonatal piglets. *Am J Respir Crit Care Med* 1999; 159:544-551.

29. **Shenkar R and Abraham E.** Hemorrhage induces rapid in vivo activation of CREB and NF-kappaB in murine intraparenchymal lung mononuclear cells. *Am J Respir Cell Mol Biol* 1997; 16:145-152.

30. **Shenkman Z, Shir Y, Weiss YG, Bleiberg B and Gross D.** The effects of cardiac surgery on early and late pulmonary functions. *Acta Anaesthesiol Scand* 1997; 41:1193-1199.

31. **Smedly LA, Tonnesen MG, Sandhaus RA, Haslett C, Guthrie LA, Johnston RB Jr, Henson PM and Worthen GS.** Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. *J Clin Invest* 1986; 77:1233-1243.

32. **Tawadrous ZS, Delude RL and Fink MP.** Resuscitation from hemorrhagic shock with Ringer's ethyl pyruvate solution improves survival and ameliorates intestinal mucosal hyperpermeability in rats. *Shock* 2002; 17:473–477.

33. **Teoh KH, Bradley CA, Gauldie J and Burrows H.** Steroid inhibition of cytokine-mediated vasodilation after warm heart surgery. *Circulation* 1995; 92:II347-353.

34. **Thannickal VJ and Fanburg BL.** Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L1005-L1028.

35. **Uchiyama T, Delude RL and Fink MP.** Dose-dependent effects of ethyl pyruvate in mice subjected to mesenteric ischemia and reperfusion. *Intensive Care Med* 2003; 29:2050-2058.

36. **Ulloa L, Ochani M, Yang H, Halperin D, Yang R, Czura CJ, Fink MP and Tracey KJ.** Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. *Proc Natl Acad Sci USA* 2002; 99:12351–12356.

37. **Vargas FS, Uezumi KK, Janete FB, Terra-Filho M, Hueb W, Cukier A and Light RW.** Acute pleuropulmonary complications detected by computed tomography following myocardial revascularization. *Rev Hosp Clin Fac Med Sao Paulo* 2002; 57:135-142.

38. **Victor VM, Rocha M and De la Fuente M.** N-acetylcysteine protects mice from lethal endotoxemia by regulating the redox state of immune cells. *Free Radic Res* 2003; 37:919-929.

39. **von der Hardt K, Schoof E, Kandler MA, Dotsch J and Rascher W.** Aerosolized perfluorocarbon suppresses early pulmonary inflammatory response in a surfactant-depleted piglet model. *Pediatr Res* 2002; 51:177-182.

40. **Williams MA, Cave CM, Quaid G and Solomkin JS.** Chemokine regulation of neutrophil function in surgical inflammation. *Arch Surg* 1999; 134:1360-1366.

41. **Yang R, Gallo DJ, Baust JJ, Uchiyama T, Watkins SK, Delude RLand Fink MP.** Ethyl pyruvate modulates inflammatory gene expression in mice subjected to hemorrhagic shock. *Am J Physiol Gastrointest Liver Physiol* 2002; 283:G212-221.