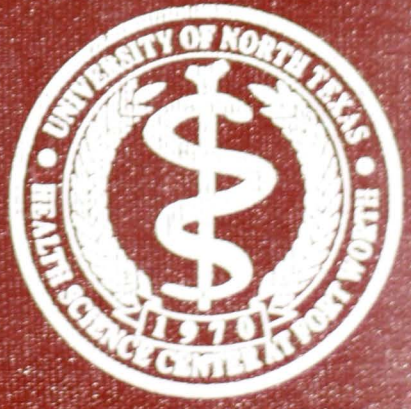
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Ryou, Myoung-Gwi, Novel Cardioprotective and Anti-Inflammatory Mechanisms of Pyruvate-Enhanced Cardioplegia. Doctor of Philosophy (Biomedical Sciences), July 2008, 113 pp, 19 figures, Reference, 176 titles.

Open-heart surgery requires a quiescent surgical field, achieved by cardioplegic arrest of the beating heart. Cardioplegia-induced cardiac arrest, however, imposes global ischemia and reperfusion which depletes myocardial energy reserves and generates free radicals that can damage the heart and induce harmful inflammation. In a recent randomized trial in patients undergoing cardiac surgery on CPB, cardioplegia containing the intermediary metabolite pyruvate, an energy-yielding fuel and antioxidant in myocardium, robustly increased post-surgical cardiac performance. The result of this clinical trial raises an important question: How did the temporary administration of pyruvate-enhanced cardioplegia produce such persistent improvements in cardiac function?

The first portion of this investigation tested the possibility that pyruvate cardioplegia evoked expression of a cardioprotective and anti-inflammatory gene program directed by hypoxia-inducible factor-1 (HIF-1), a transcription factor known to be stabilized and up-regulated by pyruvate. I hypothesized that use of pyruvate-fortified cardioplegia augmented myocardial content of HIF-1α subunit, and activated expression and intracellular signaling by the HIF-1-inducible, cytoprotective hormone erythropoietin (EPO). Also tested were the effects of pyruvate *vs.* conventional glucose cardioplegia on myocardial contents of EPO and its membrane receptor EPO-R, activation (*i.e.*,

phosphorylation) of Erk and Akt, protein kinases implicated in EPO signaling, and myocardial content and activity of the EPO effector, endothelial nitric oxide synthase (eNOS).

In situ swine hearts were arrested for 60 min with cardioplegia containing 188 mM glucose alone (control cardioplegia) or with 24 mM pyruvate (pyruvate-fortified cardioplegia). A sham group was surgically instrumented but not subjected to cardioplegic arrest or CPB. Following 60 min cardioplegic arrest and 30 min reperfusion with cardioplegia free blood, pigs were weaned from the heart-lung machine and recovered for 4 h. At the end of recovery, left ventricular myocardium was excised and fixed with formalin for EPO-R immonohistochemistry. Additional myocardium was snap-frozen and processed for measurements of HIF-1α and EPO mRNA expression, and for immunoblot analysis of contents of HIF-1α, EPO, EPO-R, total and phosphorylated Akt and Erk, and eNOS. Myocardial phosphorylation potential, ATP content and NOS activity were measured by spectrophotometry.

There were no differences in myocardial energy state among the groups, indicating that the energy-enhancing effects of pyruvate subsided after pyruvate cardioplegia cleared from the myocardium. HIF-1α content was increased by 60% 4 h after cardiac arrest with pyruvate *vs.* control cardioplegia, but HIF-1α mRNA abundance was unaltered, indicating pyruvate enhanced HIF-1α content at the post-translational level. Pyruvate cardioplegia effected dramatic increase (*c.* 1000-fold) in EPO mRNA, and increased EPO and EPO-R contents by 58 and 123%, respectively. Pyruvate cardioplegia also increased Akt and Erk phosphorylation, by 38 and 75%, without affecting total

contents of these signaling kinases. Pyruvate cardioplegia also increased myocardial NOS activity by 45% and eNOS content by 81%. Thus, administration of pyruvate-fortified cardioplegia to arrest the heart persistently enhanced the cytoprotective EPO-Akt/Erk-eNOS signaling cascade, at least in part by stabilizing HIF-1.

Anti-inflammatory effects of pyruvate have been demonstrated in various animal models of inflammation. I hypothesized that pyruvate-fortified cardioplegia mitigates inflammation by increasing anti-inflammatory cytokines, reducing ROS, and minimizing neutrophil infiltration. Arterial and coronary sinus plasma was sampled at predetermined points to measure anti-inflammatory cytokines and GSH/GSSG ratio. Left ventricular myocardial contents of acute inflammatory marker C-reactive peptide (CRP), pro-inflammatory enzyme matrix metalloproteinase-3 (MMP3), and the anti-inflammatory factor, tissue inhibitor of metalloproteinase-2 (TIMP2) were examined by immunoblot. CRP was decreased by 74% in myocardium arrested with pyruvate-fortified *vs.* control cardioplegia. Circulating IL-10 sharply increased during CPB, then subsided during recovery, while IL-6 plateaued at 2-4 h recovery. Pyruvate cardioplegia intensified and prolonged the increase in IL-10, and caused myocardium to release IL-6 into coronary effluent. Pyruvate suppressed neutrophil infiltration and maintained the structural integrity of the myocardium. This effect may be due to a sharp increase in myocardial TIMP2 content, which would suppress MMP3 degradation of the extracellular matrix.

I conclude that pyruvate-fortified cardioplegia 1) activates cytoprotective EPO-signaling pathway by enhancing HIF-1α content, and 2) suppresses inflammation by increasing anti-inflammatory cytokines and glutathione redox state, and by suppressing

MMP activity, thereby preventing neutrophil invasion of the myocardial parenchyma. These findings support novel cardioprotective mechanisms afforded by pyruvate-enriched cardiolegia during CPB. The cytoprotective and anti-inflammatory mechanisms may share a common pathway mediated by HIF-1 and EPO.

NOVEL CARDIOPROTECTIVE AND

ANTI-INFLAMMATORY MECHANISMS

OF PYRUVATE-ENHANCED CARDIOPLEGIA.

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NOVEL CARDIOPROTECTIVE AND ANTI-INFLAMMATORY MECHANISMS OF PYRUVATE-ENHANCED CARDIOPLEGIA.

DISSERTATION

Presented to the Graduate Council of the
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For the Degree of

DOCTOR OF PHILOSOPHY

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ABBREVIATIONS

CPB	Cardiopulmonary bypass
CABG	Coronary artery bypass grafting
I/R	Ischemia/reperfusion
$\cdot O_2^-$	Superoxide radical
H_2O_2	Hydrogen peroxide
OH·	Hydroxyl radicals
$ONOO^-$	Peroxynitrite
ROS	Reactive oxygen species
NF-κB	Nuclear factor-κB
TNF	Tumor necrosis factor
IL	Interleukin
GSH	Glutathione
GSSG	Glutathione disulfide
HIF-1	Hypoxia inducible factor-1
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
PFK	Phosphofructokinase
COX2	Cyclooxygenase 2
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase

MMP Matrix metalloproteinase

TIMP Tissue inhibitor of metalloperoxidase-2

TACE TNF converting enzyme

VHL von Hippel-Lindau tumor suppressor protein

ODD Oxygen dependent degradation domain

HRE Hypoxia response element

α-KG α-ketolutarate

CRP C-reactive protein

ELISA Enzyme linked immuno sorbent assay

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INTRODUCTION

Cardiopulmonary bypass (CPB) is applied in various cardiac surgeries, including correction of congenital heart defects and coronary artery bypass grafting (CABG) [1], and allows the heart to be arrested without compromising blood flow to the body. In the first successful open heart surgery involving CPB, Gibbon in 1953 repaired atrial septal defect in an 18 year-old woman[2]. In the following decades, CPB has become a standard treatment in cardiothoracic surgery, and is used in approximately 350,000 surgeries per year in the United States. Nevertheless, CPB is not completely benign, and pathological processes imposing the heart and other organs often occur after surgery involving CPB. [3, 4]

Capillary leakage, acute renal insufficiency, neuropsychiatric dysfunction, a decreased hepatic synthetic capacity, and decreased coagulation, and cardiac ischemia/reperfusion (I/R) injury imposed by application and removal of the aortic cross clamp frequently occur following CPB. [5]

Cardiopulmonary bypass surgery

Delicate cardiac surgeries require that the heart be arrested to provide the surgeon a motionless field. Cardiac arrest is achieved by intracoronary administration of cardioplegia solutions, which contain high concentrations of K^+ to depolarize cells and

interrupt the cyclic depolarization and repolarization of the myocardium that subtends the cardiac cycle. Cardioplegia solution also contains metabolic substrates to help sustain myocardial ATP production during arrest. The heart-lung machine assumes the functions of the heart and lungs during CPB; it withdraws systemic venous blood, oxygenates it and releases CO2, and then delivers the arterialized blood to the aorta under pressure generated by the roller pump.

The extracorporeal circuit through the heart-lung machine contains several components including tubes, roller pump, membrane oxygenator, and cannulas (Figure 1). Tubes made of silicon rubber are used to link CPB components. When the patient's blood passes through this extracorporeal circuit, contact of the blood with the circuit's foreign surfaces triggers an immune reaction, initiated by the a contact protein cascade. Second, a pump is applied to circulate blood through the circuit and patient, and to control blood flow and pressure. Another key component of the heart-lung machine is the membrane oxygenator, which functions like the lungs to oxygenate and clear CO_2 from the blood. Two types of oxygenator, bubble and membrane have been used in CPB. Since the 1980s, the membrane oxygenator has become predominant, and is now the only type of oxygenator used in cardiopulmonary bypass. Fourth, several cannulas are required in CPB surgery. Cannulas are inserted in various locations in the body depending on the type of surgery. A cardioplegia cannula is inserted into the aortic root proximal to the cross-clamp to deliver cardioplegia antegradely to the coronary arteries.

Aside from the extracorporeal circuit, another crucial component of CPB is the cardioplegia solution. In 1955, Melrose administered K^+-enriched saline solution to arrest the heart and to reduce air emboli. Melrose termed the technique " cardioplegia". [6] However, the high KCl concentration caused late phase vascular and myocardial tissue injury. [7, 8] In 1957, crystalloid cardioplegia, which contained much lower concentrations of KCl, was introduced. [9] Lower KCl cardioplegia elicited safer cardiac arrest than prototype. Since then, considerable effort has been directed toward minimizing the potential organ damage in CPB surgery. Now, lactate- or glucose- based cardioplegia solution is widely used in clinical settings. Cardioplegia can be delivered to the myocardium antegradely, via the aortic root and coronary artery, and retrogradely, via the coronary sinus and the coronary veins. Blood cardioplegia (4°C), which has 4 vol of blood and 1vol of crystalloid solution, is used in general CPB.

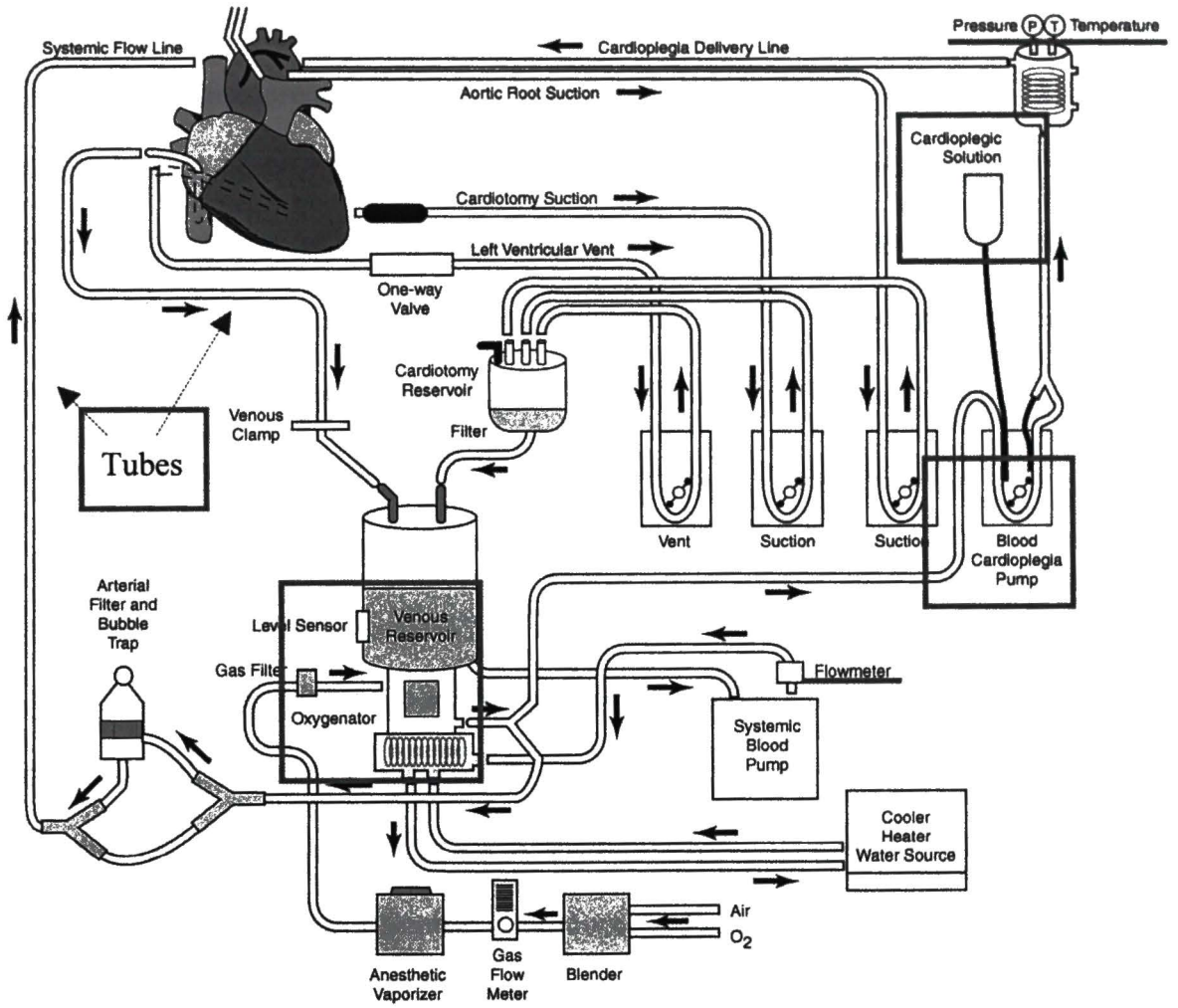

Figure1. *Cardiopulmonary bypass components* Major components are highlighted with red boxes or red underscoring. Arrows indicates direction of flow. Figure from *cardiacsurgery.ctsnetbooks.org.*

Mediators of CPB-induced tissue damage

Despite numerous advances in CPB over the last several decades, the procedure is still thought to contribute to mortality and morbidity of cardiac surgery.[10, 11] CPB stimulates formation of reactive oxygen species, which contribute to initiation and propagation of systemic inflammatory responses. Furthermore, oxidative stress and myocardial ischemia-reperfusion imposed by CPB and cardioplegic arrest activate the pro-apoptotic cysteine aspartic acid-specific protease (caspase) cascade.

1) Reactive oxygen species

Surgical procedures are known to induce oxidative stress. CPB elicits oxyradical formation [12-14], which could contribute to post-bypass myocardial dysfunction. [8, 15, 16] Most free radicals in biological systems are derived from O_2 and NO. The mitochondrial electron transport chain is essential to produce ATP, but reduction of O_2 to H_2O by this process is not 100% efficient. Instead, some partially reduced O_2 leaks from the respiratory chain in the form of superoxide radical ($\cdot O_2^-$). Indeed, as much as 5% of the O_2 consumed in mitochondria is converted to $\cdot O_2^-$ instead of H_2O. Superoxide can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. Neither radical is an especially powerful oxidant, but both can interact with transition metals, such as Fe^{2+} or Cu^+ to generate highly reactive, toxic hydroxyl radicals ($OH\cdot$). Furthermore, nitric oxide (NO), constitutively produced in endothelial cells, can irreversibly combine with $\cdot O_2^-$ in a diffusion limited, biradical condensation to form peroxynitrite ($ONOO^-$). [17]

Reactive oxygen species (ROS) in biological systems can attack a variety of constituent molecules. Proteins, lipids, carbohydrates, and DNA are all potential targets of ROS attack. Polyunsaturated fatty acid residues in lipoproteins have a chemical structure (multiple C=C bonds) that makes them vulnerable targets for free radical oxidation. Furthermore, $ONOO^-$ can inflict lethal cellular injury by initiating a peroxidation cascade,[18] modifying DNA bases,[18] causing DNA single or double strand breaks,[19, 20] depleting cellular thiols,[19] activating matrix metalloproteinases,[21, 22] nitrosating aromatic compounds,[19, 23, 24] and inactivating components of the mitochondrial respiratory chain.[25] Besides, ROS stimulate other pathological phenomena, such as development of arrhythmias[26] and activate apoptotic signaling pathways. [27] Furthermore, ROS are implicated in the mechanism of inflammation associated with cardiopulmonary bypass. In a recent investigation on 20 children who underwent elective heart surgery, oxidative stress occurred immediately after CPB.[28] Oxidative stress provokes phosphorylation of nuclear factor-κB (NF- κB), causing its translocation into the nucleus where it binds to the promoter regions of genes encoding pro-inflammatory proteins.[29] In the pediatric CPB study, pro-inflammatory cytokine release peaked 3-12 h after bypass. [28]

Cardiopulmonary bypass does evoke enhancement of endogenous anti-oxidant defenses. [30] In a clinical investigation, glutathione peroxidase, superoxide dismutase, and total antioxidant capacity increased during CPB and returned to the respective baseline

levels within 24 hours.[30] However, this enhance of increased anti-oxidant defense is not enough to completely neutralize ROS produced during and after CPB.

2) Pro-inflammatory mediators

Inflammation is apowerful physiologic mechanism to defend the body against pathogens. However, inflammation is a central process in CPB-induced tissue damage, and a major source of ROS. The inflammatory cascade, culminating in neutrophil extravasation and tissue invasion, is mediated by the complement cascade, adhesion molecules in membrane of neutrophils and vascular endothelium, and generation of ROS. Generation of humoral inflammatory mediators from invasive immune cells is initiated by activation of contact proteins, including factor XII, factor XI, prekallikrein, and high-molecular-weight kininogen. Blood contact with the extracorporeal circuit activates contact proteins involved in the coagulation cascade, eventually causing the conversion of plasminogen to plasmin. Plasmin not only initiates fibrinolysis but also can trigger the classical complement cascade. [31]

In the immune defense mechanism, complement system is essential to activate cytokine release from circulating immune cells. A series of studies showed that circulating complement precursors are cleaved to their active forms during CPB,[32-39] beginning with C5a. Furthermore, increased terminal complement complex C5b-9 [40] and a decreased number of C5a receptors on neutrophils after CPB indicated that C5a had been generated during CPB. Pediatric clinical studies revealed an increase in C5a at the end of CPB.[36, 37] As described earlier, free radicals are released by activated neutrophils.

Hydroxyl radical (•OH) directly cleaves C5 into its activated form.[41] Additionally, activated factor XII and plasmin promote further generation of C3a and C5a.[31, 42, 43] C3a and C5a stimulate monocytes to transcribe mRNA encoding proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin 1 (IL-1), and interleukin 6 (IL-6).[38, 44, 45] When high concentrations of C3a and C5a are present, TNF, IL-1, and IL-6 are released from monocytes.[38, 46] Additionally, binding of C5a to its receptors on neutrophils and monocytes activates those cells and directs them into the inflammatory process.[46] Finally, the extent of complement activation during CPB is closely associated with the duration of CPB and with the age of the patient.[33]

Cytokines are proteins or glycoproteins that mediate inflammatory signals to stimulate immune system. Cytokines are produced by and released from their cells of origin, and then they exert endocrine functions, similar to hormons.[47] Examples of cytokines are TNF, interleukin, interferons, and several growth factors.[48]

There are two types of tumor necrosis factors, TNF-α and TNF-β. TNF-α, also known as cachectin, is produced by monocytes and mononuclear phagocytes. Cachectin is so named because it can elicit hemorrhagic necrosis of tumors and because it plays a pivotal role in causing cachexia of chronic diseases.[49] The rate of TNF-α increase after pro-inflammatory stimulus is the fastest among cytokines, implicating it as an initiator of inflammation. Plasma TNF-α concentration peaks in bimodal fashion 2 h and 18 - 24 h after CPB.[3] TNF-α stimulates release of other humoral inflammatory factors, including

IL-1, IL-6, leukotrienes, and platelet activating factor.[49] Furthermore, TNF-α stimulates neutrophil degranulation and adherence to endothelial cells.[49] On the other hand, TNF-β, produced by lymphocytes, stimulates neutrophil phagocytosis.[49]

Interleukins enhance communication between white blood cells, and orchestrate many aspects of the inflammatory response.[50] IL-6 is produced by various cell types, including monocytes, macrophages, lymphocytes, fibroblasts, keratinocytes, endothelial cells, and muscle cells in response to their stimulation by TNF-α, endotoxin, and IL-1.[51-53] IL-6 plays numerous roles in biological systems. IL-6 showed antiviral activity and enhancement of antibody production by the activated B-cell.[54, 55] Distinctive functions of IL-6, dependent on the cell or tissues of origin, have been reported. IL-6 produced from muscle has an anti-inflammatory function, which in part, increases IL-10, IL-1 receptor antagonist, and cortisol [56], a documented anti-inflammatory hormone.[57]

Cellular inflammatory adhesion molecules also play important roles in the inflammatory response. The interaction between neutrophils and endothelial cells occurs after neutrophils are activated by pro-inflammatory cytokines. This adherence of neutrophil and endothelial cells requires a reduction of vascular shear forces and the expression of specific surface adhesion molecules by endothelial cells and neutrophils.[58-60] These adhesion molecules include selectins expressed on both endothelial cells and neutrophils, integrin expressed only on white blood cells, and immunoglobulin

superfamily expressed only on endothelial cell. Ligand binding to specific receptors is required to facilitate neutrophil adhesion to endothelium.

3) Apoptotic mediators

The three groups of factors contributing to CPB-induced tissue damage, including ROS and pro-inflammatory cytokines, also are capable of inducing mechanisms leading to programmed cell death, *i.e.* apoptosis. Myocardial ischemia is a leading cause of cardiac apoptosis. Cardioplegia induced cardiac arrest imposes global ischemia on the heart. In rat heart, increased apoptotic indexes were reported from as early as 3 h to as long as 1 month after CPB.[61] Two distinct apoptosis mechanisms have been identified, cytochrome-C dependent apoptotic pathway and death receptor mediated apoptotic pathway. The death receptor pathway only plays a secondary role in the initiation of cardiac apoptosis. The release of cytochrome-C from the mitochondrial intermembrane space initiates the main apoptotic cascade. Activation of caspase cascade is well documented in animal models and human cases of heart failure.[62, 63] Downstream caspases (caspase-3,-6,-7) are activated several hours before morphological changes. The mitochondrial apoptosis pathway is regulated by anti-apoptotic Bcl-2 and pro-apoptotic Bax.[64] The Bcl-2/Bax ratio profoundly influences cardiac apoptosis rate.[65] Also, the protein kinases, Erk and Akt, have been implicated in anti-apoptotic signaling. [66-68]

Pyruvate: powerful cardioprotection against CPB-induced tissue damage.

Pyruvate, a natural aliphatic carbohydrate and intermediary metabolite in mammalian cells, is well recognized as a powerful anti-oxidant and endogenous energy provider. Recently, anti-inflammatory functions of pyruvate [69] and its derivative, ethyl pyruvate[70, 71] have been demonstrated. Furthermore, pyruvate has been found to stabilize hypoxia inducible factor-1α (HIF-1α) in human cancer cells under non-hypoxic environments.[72] HIF-1 activates expression and synthesis of anti-apoptotic and anti-inflammatory mediators including erythropoietin (EPO). In the last part of this introduction, the possible role of pyruvate enhancement of HIF-1 in pyruvate-fortified cardioplegia-induced protection against CPB caused tissue damage is discussed.

1) Pyruvate, a powerful anti-oxidant

Pyruvate effectively reduces organ damage from myocardial, [73-76] intestinal,[77] or hepatic[78] ischemia-reperfusion (I/R) injury. I/R produces a massive amount of ROS during ischemia and especially when O_2 is reintroduced into the tissue at the beginning of reperfusion. Pyruvate's antioxidant actions are protective against I/R injury. ROS generated from electron transport chain, xanthine oxidase and/or other sources cause cellular and/or organ damage via alteration of cellular proteins and changes in lipid bilayer integrity. Pyruvate detoxifies hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) by direct chemical reactions.[79, 80] In the cellular metabolism, pyruvate inhibits phosphofructokinase (PFK) by increasing intracellular citrate concentration.[81] This

inhibition of PFK causes accumulation of glucose-6-phosphate which, in turn, provides the substrate for hexose monophosphate shunt, which generates NADPH reducing power to maintain GSH. GSH/GSSG exists in near equilibrium with other soluble and intramembranous antioxidant redox couples.[82-84] Therefore, GSH/GSSG ratio serves as an index of the redox state of the cellular antioxidant system. Pyruvate also increases the ATP phosphorylation potential and lowers free cytosolic ADP and AMP. By decreasing AMP, pyruvate reduces the production of hypoxanthine and xanthine, which are substrates for ROS formation by xanthine oxidase. Accordingly, pyruvate added to cardioplegia solution could dampen ROS-induced alterations of cellular proteins and membrane phospholipids.

2) Pyruvate, an anti-inflammatory factor

Inflammation is a complex, multi-factorial immune response in the body. Reactive oxygen species (ROS) are important indicators and mediators of inflammation, [29] so that pyruvate treatment could minimize inflammatory response in CPB by neutralizing excessive ROS. In fact, hepatic NF-κB and TNF-α mRNA expression in mice was effectively inhibited by pyruvate in I/R.[85] Pyruvate's anti-oxidant function alone, however, cannot fully explain pyruvate's anti-inflammatory capabilities, because other ROS scavengers, including N-acetylcysteine [86, 87], pyrollidine dithiocarbamate [88], and dimethyl sulfoxide [88], could not block the activation of pro-inflammatory transcription factor, NF-κB. NF-κB activates more than 200 genes including those encoding pro-inflammatory cytokines, TNF, IL-6, and IL8, and the inflammatory enzyme

cyclooxygenase (COX)-2. Ethyl pyruvate treatment decreased the expression of pro-inflammatory transcripts of inducible nitric oxide synthase (iNOS), COX 2, and IL-6.[71] Therefore, inhibition of NF-κB activation could be a pivotal mechanism of pyruvate's anti-inflammatory capabilities. Coagulation factors and tissue factor are closely related to the systemic inflammation. Incubation of human monocyte-like cells with ethyl pyruvate suppressed secretion of pro-inflammatory mediators in response to LPS, and also the expression of pro-coagulant proteins and tissue factor was inhibited.[70]

Matrix metalloproteinases (MMP) are pivotal to development of inflammation.[89] MMP regulate physical barriers, modulate inflammatory mediators such as cytokine and chemokine, and establish chemokine gradients in inflamed tissues which serve to attract immune cells to the site of infection or injury.[90] MMPs have TNF-cleaving activity.[91, 92] TNF is expressed on the T-cell and macrophages as a membrane bound protein (26kD) and it is activated by cleavage to a 17kD soluble cytokine by TNF converting enzyme (TACE). MMP-1, -2, -3, -9, -12, 14, -15, and -17 have TACE activity.[91, 92] Likewise, IL-1β, another potent pro-inflammatory cytokine, is activated by MMPs.[93] Recently, Sharma et al. reported that pyruvate treatment in a canine model of cardiopulomonary arrest and resuscitation model inhibited MMP activity.[94]

Collectively, These reports suggest that pyruvate in cardioplegia solution might mitigate inflammatory responses to CPB surgery by dampening ROS generation and inhibiting MMP activity.

3) Pyruvate, an anti-apoptotic mediator

Cardiomyocyte apoptosis may contribute to cardiac insufficiency that impairs recovery from CPB. ROS and inflammation are co-conspirators in the development of apoptosis. Pyruvate's anti-apoptotic functions are not as widely known as its energy-yielding, anti-oxidant, and anti-inflammatory functions. In addition, Lu et al. demonstrated yet another effect of pyruvate, its inhibition of the O_2-dependent degradation of hypoxia inducible factor-1α (HIF-1α) (Figure 2). HIF-1 has two subunits, α and β, which are constitutively expressed in mammalian cells. HIF-1 activity is controlled primarily by adjusting the rate of HIF-1α degradation. Under non-hypoxic environment, key proline residues in HIF-1α are hydroxylated in a reaction catalyzed by prolyl hydroxylase. Oxygen, α-ketoglutarate, and iron are required for prolyl hydroxylase activity. Prolyl hydroxylation facilitates the binding of von Hippel-Lindau tumor suppressor protein (VHL) to the O_2 dependent degradation domain (ODD) on HIF-1α.[95-98] Binding of VHL to ODD targets HIF-1α for poly-ubiquitylation. Ubiquitinylated HIF-1α undergoes proteosomal degradation.

Pyruvate inhibits prolyl hydroxylase activity by competing with its substrate, α-ketoglutarate for access to the enzyme's catalytic core. Without proteosomal degradation, HIF-1α translocates into the nucleus, where it heterodimerizes with HIF-1β subunits,[99] forming the active transcription factor. HIF-1 binds hypoxia response element (HRE) domains and initiates transcription of erythropoietin (EPO) and other hypoxia-response genes. Hypoxia inducible factor-1 regulates more than 100 genes [100] encoding proteins

that mediate adaptive responses to hypoxia, including erythropoiesis,[101] angiogenesis,[102] modulation of vascular tone,[103] anti-apoptosis,[104] anti-inflammation, and energy metabolism.[105, 106] The list of HIF-1-response genes continues to grow. EPO expression is regulated by HIF-1. EPO, classically known as erythropoietic cytokine, recently has been reported to exert cardio-[107-109] and neuro-protective function,[110] in part by inhibiting apoptosis of neuron and cardiomyocytes. The anti-apoptotic effect of EPO is mediated by increased phosphorylation of PI3K/Akt [111]and MAPK/Erk.[109] EPO's binding to EPO-R initiates EPO signaling pathway. Pyruvate in cardioplegia solution protects the myocardium from apoptosis by stabilizing HIF-1α in the face of hyperoxic surgical conditions. Thus, it is conceivable that pyruvate could enhance expression of EPO and activate EPO's anti-apoptotic signaling mechanisms.

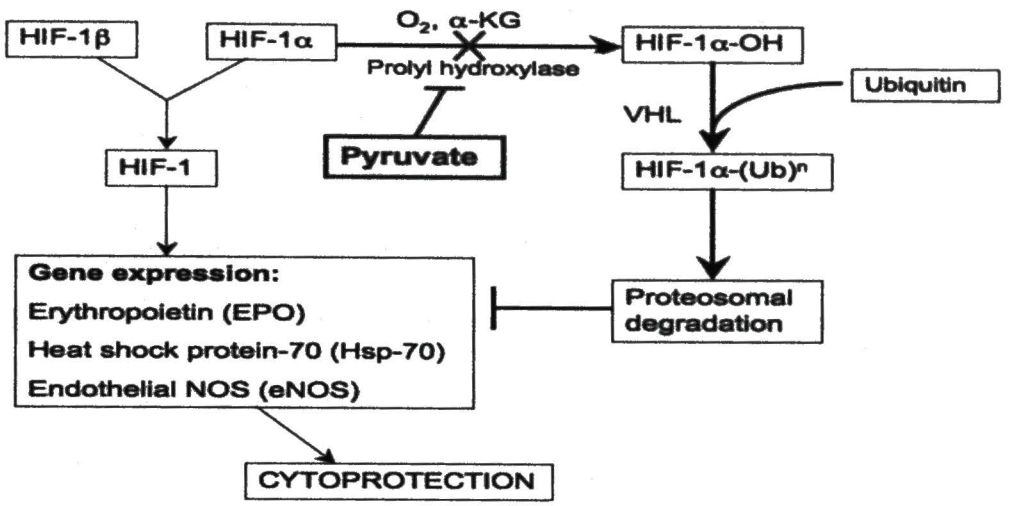

Figure 2. *By blocking HIF-1α degradation, pyruvate enhances HIF-1-activated gene expression.* HIF-1α subunits are degraded under non-hypoxic conditions. HIF-1α is directed into the degradation pathway by prolyl hydroxylation. Pyruvate interferes with the prolyl hydroxylase reactions, thereby stabilizing HIF-1α and provoking expression of HIF-1-responsive genes. HIF-1: Hypoxia inducible factor-1; HIF-1α and 1β: HIF-1α and β subunts; HIF-1α-OH: hydroxylated HIF-1α; α-KG: α-ketoglutarate.

Summary

Cardiopulmonary bypass is a mainstay of most open-heart surgeries, such as CABG, valve replacement, and correction of congenital heart defects. Nevertheless, undesirable consequences, such as oxidative stress and multi-organ dysfunction, have been reported during and after CPB.

Pyruvate, a natural metabolic intermediate, is a powerful anti-oxidant and endogenous energy provider in myocardium. Due to its anti-oxidant effect alone or in combination with its other favorable effects, pyruvate minimizes inflammatory responses and acts as a non-hypoxic HIF-1α stabilizer. Activated HIF-1 stimulates the synthesis of EPO, an anti-inflammatory, anti-apoptotic, cytoprotective cytokine. The studies described herein were conducted to define the mechanisms of pyruvate's cytoprotection via activation of EPO signaling pathway, and anti-inflammatory effects in a domestic swine model of CPB.

Specific aims

The first objective of this investigation is to compare the ability of conventional glucose based versus pyruvate-fortified cardioplegia solutions to maintain HIF-1α in the face of hyperoxic surgical conditions, and, subsequently, to activate cytoprotective EPO signaling pathways 4 h after CPB. Specific Aim 1 tested the hypothesis that administration of pyruvate-fortified cardioplegia during CPB augmented myocardial HIF-1α content, enhanced expression and content of EPO and EPO-R, and activated the kinases that mediate EPO signaling. *In situ* adult swine hearts were arrested for 60 min with 4:1 blood:crystalloid cardioplegia. The crystalloid component contained 188 mM glucose ± 24 mM pyruvate. After 30 min cardiac reperfusion with cardioplegia-free blood, the pigs were weaned from CPB. Left ventricular myocardium was sampled at 4 h recovery for immunoblot measurements of HIF-1α, EPO, EPO receptor (EPO-R), the

EPO signaling kinases Akt and Erk, and endothelial nitric oxide synthase (eNOS), a downstream effector of EPO signaling.

The second goal of this investigation was to delineate the anti-inflammatory effect of pyruvate-fortified cardioplegia. *In situ* swine heart were arrested as described in specific aim 1. Arterial and coronary sinus plasma were sampled in predetermined time points to measure anti-inflammatory cytokines and glutathione redox state. At 4 h of recovery, myocardium was collected and extracted for immunoblot analysis of C-reactive protein (CRP), metalloperoxidase-3 (MMP3), tissue inhibitor of metalloperoxidase-2 (TIMP2), and to measure nitrotyrosine concentration by ELISA. LV myocardium was also formalin fixed to monitor neutrophil infiltration. Specific Aim 2 tested the hypothesis that use of pyruvate-enriched cardioplegia mitigates inflammatory response during CPB by suppressing ROS and neutrophil invasion to tissue, and by stimulating anti-inflammatory cytokine production.

Significance

This investigation, for the first time, demonstrated that pyruvate–enriched cardioplegia activates the anti-apoptotic EPO signaling pathway. Although, previous studies have demonstrated the presence of HIF-1 and EPO-receptors, in myocardium, until now the expression of EPO mRNA and endogenous formation of EPO protein in the heart has not been reported. These novel results lead us to propose that pyruvate may

serve as a natural therapeutic for patients undergoing CPB with cardioplegia arrest. More research must be done to determine the involvement of other HIF isoforms, e.g. HIF-2, and the exact origination of EPO mRNA in heart, cardiac myocyte or endothelial cells in coronary vasculature.

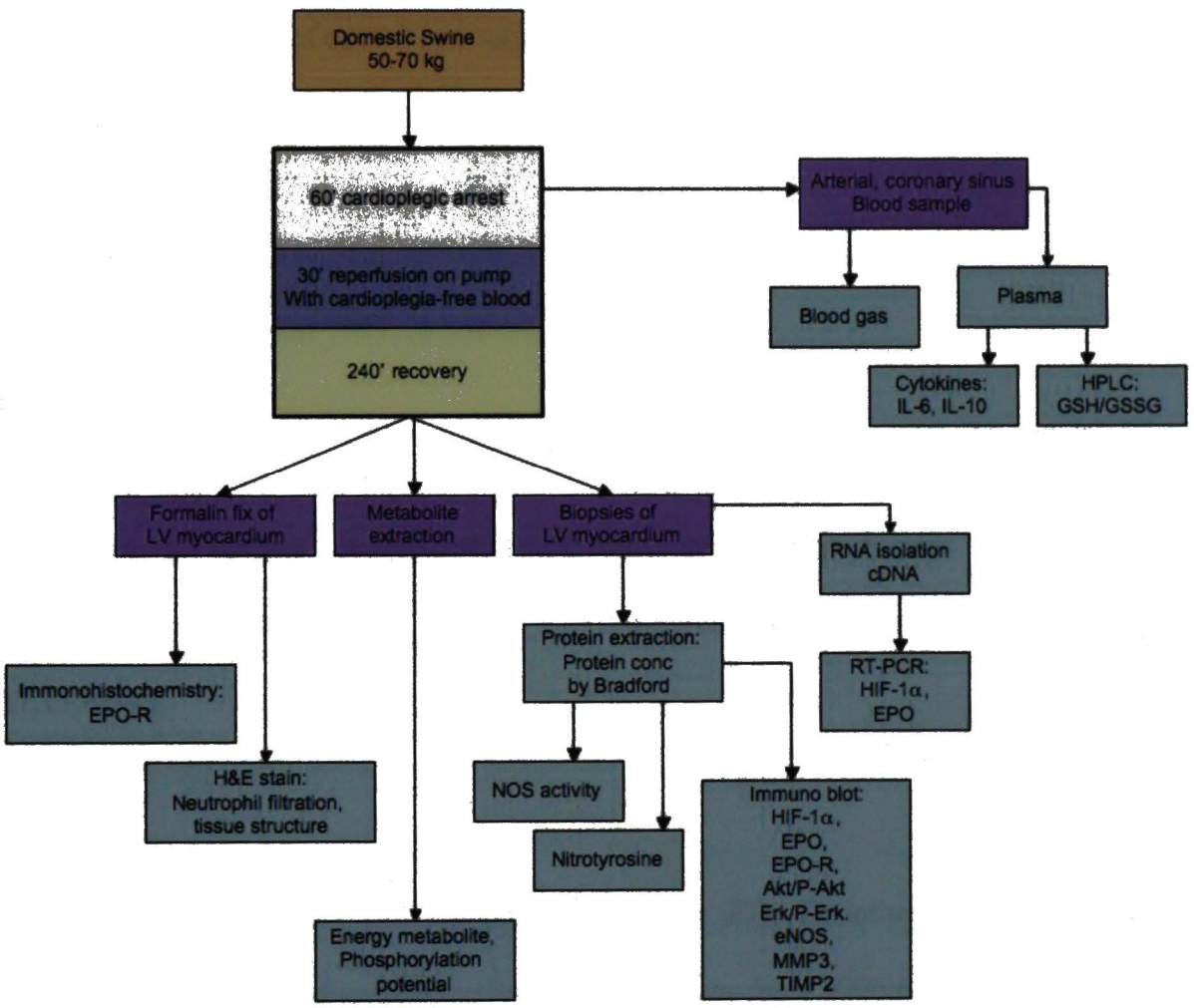

Figure 3. *Experimental Design.* Flow diagram shows main experimental protocols, and target variables as address the specific aims.

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CHAPTER II

Pyruvate-fortified cardioplegia evokes myocardial erythropoietin signaling in swine undergoing cardiopulmonary bypass

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ABSTRACT

Background: Administration of pyruvate-fortified cardioplegia to patients undergoing cardiopulmonary bypass (CPB) enhanced post-surgical recovery of cardiac function. Pyruvate reportedly suppresses degradation of the α subunit of hypoxia-inducible factor-1 (HIF-1), an activator of the gene encoding the cardioprotective cytokine erythropoietin (EPO). This study tested the hypothesis that pyruvate-enriched cardioplegia evoked novel myocardial EPO expression and mobilized EPO signaling mechanisms. **Methods:** Hearts of domestic pigs maintained on CPB were arrested for 60 min with 4:1 blood:crystalloid cardioplegia. The crystalloid component contained 188 mM glucose ± 24 mM pyruvate. After 30 min cardiac reperfusion with cardioplegia-free blood, the pigs were weaned from CPB. Left ventricular myocardium was sampled at 4 h recovery for immunoblot measurements of HIF-1α, EPO, EPO receptor (EPO-R), the EPO signaling kinases Akt and Erk, and endothelial nitric oxide synthase (eNOS), a downstream effector of EPO signaling. **Results:** Pyruvate-fortified cardioplegia induced myocardial EPO mRNA expression, and increased HIF-1α, EPO and EPO-R protein contents by 60, 58 and 123%, respectively ($P<0.05$) *vs.* control cardioplegia. Pyruvate cardioplegia also enhanced Akt and Erk phosphorylation, NOS activity and eNOS content by 38, 76, 45 and 81%, *vs.* respective values in control cardioplegia-treated myocardium ($P<0.05$). **Conclusions:** Pyruvate-fortified cardioplegia induced myocardial EPO expression and activated key elements of EPO's signaling cascades. By stabilizing HIF-1α, pyruvate-fortified cardioplegia may evoke sustained activation of EPO-induced cardioprotective signaling.

INTRODUCTION

Cardioplegia-induced cardiac arrest, a mainstay of cardiac surgery, imposes myocardial ischemia by interrupting coronary blood flow [1, 2]. In a small, randomized trial in patients undergoing coronary artery revascularization on cardiopulmonary bypass (CPB), the use of pyruvate-fortified *vs.* conventional cardioplegia enhanced post-surgical recovery of cardiac function and shortened hospitalization [3]. In a swine CPB model, cardioplegic arrest and reperfusion provoked oxidative stress which inactivated myocardial enzymes and produced cardiac edema [4, 5], but pyruvate cardioplegia mitigated these detrimental effects and increased myocardial energy state. However, pyruvate is rapidly cleared [6], so its acute metabolic effects likely were not responsible for the persistent post-CPB enhancement of cardiac performance in the clinical trial [3].

The heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1) activates an extensive gene program encoding proteins that mediate erythropoiesis, angiogenesis, ATP production, and antioxidant defense [7-9]. HIF-1's α and β subunits are constitutively expressed in cardiomyocytes, but HIF-1α is rapidly degraded under normoxic conditions, which limits HIF-1-induction of gene expression. HIF-1α is targeted for proteosomal degradation by Fe^{2+}-, O_2- and α-ketoglutarate-dependent hydroxylation of two proline residues, catalyzed by prolyl hydroxylase [7-9]. Pyruvate can interfere with this mechanism by displacing α-ketoglutarate from prolyl hydroxylase's catalytic core [10]. By stabilizing HIF-1α, pyruvate may augment HIF-1-induced expression of cardioprotective genes.

The HIF-1-induced hematopoietic cytokine erythropoietin (EPO) has been found to protect myocardium from ischemic injury [11-13]. Interaction of EPO with its membrane receptor (EPO-R) initiates signaling cascades, mediated by Akt and extracellular signal-regulated kinase (Erk), which suppress mitochondrial permeability transition pore opening [14, 15], an activator of apoptotic cell death. Although myocardium expresses EPO-R [11, 16, 17], reports of myocardial EPO expression are sparse and equivocal.

This study tested the hypothesis that administration of pyruvate-fortified cardioplegia during CPB augmented myocardial HIF-1α content, enhanced expression and content of EPO and EPO-R, and activated the kinases that mediate EPO signaling. This potentially cardioprotective mechanism was examined in porcine myocardium 4 h after CPB, when cardioplegia had cleared from the myocardium and pyruvate's acute metabolic effects had subsided.

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-Four domestic swine of either sex, weighing 50-70 kg, were assigned randomly to three groups of 8 animals. The CPB groups received crystalloid cardioplegia containing 188 mM glucose alone (control group) or with 24 mM pyruvate

(pyruvate group). Sham animals were surgically prepared, but not subjected to cardiac arrest or CPB.

Surgical preparation. After overnight fast, pigs were sedated with ketamine (10 mg/kg *im*) and xylazine (1 mg/kg *im*), anesthetized with propofol (2 mg/kg *iv*), and then mechanically ventilated with 0.5-2% isoflurane supplemented with O_2 to maintain a surgical plane of anesthesia. During CPB, anesthesia was maintained by continuous infusion of propofol, 0.2 mg kg^{-1} min^{-1} *iv*.

The femoral artery and vein were cannulated for sampling blood and infusing medications, respectively. The arterial cannula was connected to a pressure transducer (model 1290C, Hewlett-Packard) to monitor blood pressure. The heart was exposed by median sternotomy and supported in a pericardial cradle. Catheters were inserted into the coronary sinus to sample coronary venous blood, and into the inferior vena cava via the right atrium to withdraw blood for the extracorporeal CPB circuit. The abdominal aorta was cannulated to deliver oxygenated blood from the heart-lung machine. A cannula was inserted into the aortic root for antegrade cardioplegia infusion. Crystalloid cardioplegia (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. Pyruvate cardioplegia was prepared by equimolar substitution of 24 mM sodium pyruvate for NaCl. Crystalloid solutions were combined with 4 vol whole blood before infusion.

Experimental protocol. The pig was connected to the heart-lung machine following heparin administration (300 U/kg *iv*). After aortic cross-clamp, cardiac arrest was induced by antegrade infusion of 1200 ml cardioplegia. Additional cardioplegia (400 ml) was infused at 20 and 40 min arrest. After 60 min arrest, the heart was reperfused for 30 min with cardioplegia-free blood, and then the pig was separated from bypass and allowed to recover off-pump for 4 h. Arterial PO_2, PCO_2, pH, and HCO_3^- and K^+ concentrations were measured in an Instrumentation Laboratory model 1730 blood gas analyzer. $NaHCO_3$ and K^+ were administered *iv* to correct acidemia and hypokalemia. The α-adrenergic vasoconstrictor phenylephrine was infused *iv* as needed to maintain mean arterial pressure at 60-70 mm Hg.

Plasma erythropoietin. Coronary sinus and systemic arterial plasma was obtained by sedimenting formed elements. Plasma EPO concentrations were measured by enzyme linked immunosorbent assay (Biochain, Hayward, CA). Immune complexes detected at 450 nm were quantified by comparison with a recombinant EPO standard curve. Myocardial EPO uptake or release was assessed from coronary sinus-arterial EPO concentration differences.

Myocardial biopsies. At 4 h recovery, myocardium was sampled for measurements of proteins, mRNA and metabolites. The left ventricular anterior wall was snap-frozen *in situ* with liquid N_2-precooled tongs [18]. Additional myocardium was excised and fixed in alcoholic formalin solution for immunohistochemical detection of EPO-R.

Myocardial energy state. Myocardial pyruvate and energy metabolites including ATP, phosphocreatine (PCr), creatine (Cr), and inorganic phosphate (Pi) were extracted and colorimetrically assayed as previously described [18]. PCr phosphorylation potential, *i.e.* [PCr]/([Cr][Pi]), and ATP content provided measures of myocardial energy state [18].

Analyses of myocardial proteins. Myocardial proteins were extracted [5, 19] for immunoblot analyses of HIF-1α, EPO, EPO-R, total and phosphorylated Erk and Akt, and endothelial nitric oxide synthase (eNOS). Total protein concentrations in extracts were measured by the Bradford method [20]. Proteins (50 μg/lane) were separated by electrophoresis (100V for 90 min) on 10% SDS-PAGE gels, and then electrophoretically transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies for 2 h at room temperature, washed in TTBS and then exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h. Immune complexes were detected by enhanced chemiluminescence (Thermo-Fisher, Rockford, IL) of H_2O_2. Primary antibodies were mouse monoclonal anti-HIF-1α (Abcam, Cambridge, MA), anti-EPO (R&D Systems, Minneapolis, MN), anti-(P-^{473}S)-Akt and anti-(P-^{202}T, P-^{204}Y)-Erk (Cell Signaling, Danvers, MA), and rabbit polyclonal anti-EPO-R (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt and anti-eNOS (Stressgen, Ann Arbor, MI), and anti-Erk (Abcam). Anti-mouse and anti-rabbit secondary antibodies were from Kirkegaard and Perry (Gaithersburg, MD). Each membrane was stripped and re-probed with anti-actin antibody (Stressgen, Victoria, BC) to detect actin as loading control. Band densities were measured in an AlphaEase FC 4.0 densitometer (AlphaInnotech, San Leandro, CA), and then normalized to actin band

densities. NOS activity was measured with a Griess reaction-based kit (Oxis International, Portland, OR) as recently described [21].

Immunohistochemistry. Myocardium was fixed and processed for immunohistochemistry of EPO-R as previously described [22]. Ovalbumin was used to block non-specific binding. Slides were incubated with primary EPO-R antibody (Santa Cruz) or, for control slides, IgG. After overnight incubation, slides were incubated with donkey anti-rabbit immunoglobulin (Invitrogen, Carlsbad, CA) and mounted. EPO-R was detected using fluorescence microscopy and images were recorded using an Olympus digital camera.

Real time RT-PCR measurements of mRNA. Real time reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess myocardial abundances of HIF-1α, EPO and actin mRNA. Total RNA was isolated using Trizol™ Regent (Invitrogen). cDNA was synthesized from total RNA with Taqman reverse transcriptase (Applied Biosystems, Foster City, CA). HIF-1α, EPO, and actin cDNA were amplified in a Smart cycler II (Cepheid™, Sunnyvale, CA) by using Applied Biosystems SYBR Green Master Mix Kit with specific primers for HIF-1α (forward: 5'-GCCAGAACCTCCTGTAACCA-3'; reverse: 5'-CCTTTTCCTGCTCTGTTTGG-3'), EPO (forward: 5'-CCAAAGCAGGAGGAATTCAG-3'; reverse: 5'-GCTGTTGTGGGAGTCTCCAT-3') and α-actin (forward: 5'-TCATCACCATCGGCAACG-3'; reverse: 5'-TTCCTGATGTCCACGTCGC-3'). Abundances of amplified genes were assessed by analysis of cycle threshold.

Statistical analysis. Values are means ± SEM. Between-group comparisons of mean values were accomplished by one-way ANOVA. When ANOVA detected statistical significance, Tukey's *post hoc* test was applied to identify the specific differences. P values < 0.05 were taken to indicate statistically significant differences.

RESULTS

Myocardial pyruvate content and energy state. Recent studies in this model demonstrated that pyruvate-enriched cardioplegia acutely increased myocardial pyruvate and ATP contents and phosphocreatine phosphorylation potential, *i.e.* $[PCr]/([Cr][P_i])$ [4,5]. At 4 h recovery, pyruvate content was similar in the 3 groups (Figure 1A) indicating complete clearance of the pyruvate cardioplegia. Similarly, pyruvate enhancement of ATP content (Figure 1B) and phosphorylation potential (Figure 1C) subsided by 4 h post-CPB.

Hypoxia inducible factor-1α content. Myocardial HIF-1α mRNA abundances at 4 h recovery were similar in the control cardioplegia, pyruvate cardioplegia and sham groups (Figure 2A, B). In contrast, HIF-1α protein content was increased by 60-70% ($P<0.05$) in the pyruvate cardioplegia group *vs.* the other two groups (Figure 3). Thus, 60 min exposure to pyruvate-fortified cardioplegia during CPB augmented myocardial HIF-1α content 4 h later without altering abundance of the corresponding mRNA.

Pyruvate-induced expression and release of erythropoietin. EPO mRNA was nearly undetectable in sham and control cardioplegia-treated left ventricular myocardium, but was strikingly increased ($P<0.001$) 4 h after arrest with pyruvate-fortified cardioplegia (Figure 2A). Indeed, EPO mRNA content was increased ~ 1000-fold in pyruvate-treated *vs.* control and sham myocardium (Figure 2C). EPO protein content was increased by 58% 4 h after treatment with pyruvate *vs.* control cardioplegia (Figure 4).

The changes in EPO expression and content were associated with altered myocardial EPO uptake/release. At pre-CPB baseline, EPO concentrations were similar in arterial and coronary sinus plasma, indicating essentially no cardiac EPO release before arrest. EPO concentration in coronary sinus plasma progressively increased following pyruvate-enhanced CPB (Figure 5A), but declined after control CPB (Figure 5A). Coronary sinus-arterial EPO concentration difference increased in the pyruvate group throughout recovery, but fell to negative values in the control group (Figure 5B). Thus, augmented myocardial EPO content following pyruvate cardioplegia paralleled net EPO release into the coronary circulation, in contrast to myocardial EPO uptake following control cardioplegia treatment.

Myocardial erythropoietin receptors. Immunohistochemistry revealed robust EPO-receptor (EPO-R) densities in cardiomyocyte and coronary endothelial membranes of non-CPB sham myocardium (Figure 6A.1). EPO-R were severely depleted at 4 h recovery following CPB with control cardioplegia (Figure 6A.2). In contrast, pyruvate-fortified cardioplegia prevented EPO-R depletion (Figure 6A.3,4). Myocardial EPO-R content was

analyzed by immunoblot (Figure 6B, C). In accord with the immunohistochemistry results, EPO-R content fell 35% in control cardioplegia treated *vs.* sham myocardium, but increased appreciably following pyruvate cardioplegia treatment (Figure 6C). Thus, CPB with control cardioplegia depleted myocardial EPO-R, but pyruvate-fortified cardioplegia stabilized and even increased EPO-R content 4 h later.

Total and phosphorylated Akt and Erk. Myocardial contents of the EPO signaling kinases, Akt (Figure 7) and Erk (Figure 8) were similar in the 3 groups. Pyruvate-fortified cardioplegia increased Akt phosphorylation by 38% *vs.* control cardioplegia (Figure 7C). The effects of pyruvate cardioplegia on Erk phosphorylation were even more striking (Figure 8C). Thus, Erk phosphorylation 4 h after pyruvate-enhanced CPB was increased by 126 and 76% above respective values in sham and control cardioplegia-treated myocardium. These results indicate that pyruvate administration during CPB enhanced subsequent activation of Akt and Erk.

Nitric oxide synthase. Phosphorylated Erk and Akt activate endothelial NOS (eNOS), which produces cytoprotective NO. NOS activity and eNOS content fell in parallel, by 35 and 32%, respectively, in control cardioplegia-arrested *vs.* sham myocardium (Figure 9). Pyruvate-fortified cardioplegia maintained NOS activity and eNOS content at the respective sham values. Thus, pyruvate cardioplegia-induced enhancements of EPO, EPO-R, P-Erk and P-Akt were associated with preservation of myocardial eNOS.

COMMENT

This study tested the hypothesis that administration of pyruvate-enriched cardioplegia to arrest the heart during CPB induced myocardial expression and activation of erythropoietin and its signaling mechanisms 4 h later. Compared to conventional glucose-fortified cardioplegia, pyruvate-enriched glucose cardioplegia increased 1. left ventricular myocardial contents of HIF-1α, EPO mRNA and protein, and EPO receptors; 2. phosphorylation (*i.e.*, activation) of Erk and Akt, protein kinases implicated in EPO-induced cardioprotection [13, 23, 24]; and 3. content and activity of the Erk/Akt effector, eNOS. Thus, pyruvate-fortified cardioplegia evoked sustained enhancement of key components of EPO's cardioprotective signaling mechanism.

Pyruvate cardioplegia and erythropoietin signaling. Cardioplegia-induced cardiac arrest affords a quiescent field for cardiac surgical procedures, and slows myocardial energy consumption. Nevertheless, interruption of coronary blood flow during CPB imposes ischemic stress on the heart which often causes functional cardiodepression for several h post-bypass [25, 26]. In a randomized phase I clinical trial, administration of novel, pyruvate-fortified cardioplegia afforded robust, persistent improvements in post-bypass cardiac performance *vs.* conventional cardioplegia, hastening hospital discharge [3].

This study sought to decipher mechanisms that may contribute to the sustained improvements in cardiac function after pyruvate-enhanced CPB. Attention was focused on signaling cascades initiated by HIF-1 induction of EPO and related genes. Although the

previously reported [4, 5] increases of myocardial ATP content and phosphorylation potential afforded by pyruvate-enriched cardioplegia had subsided by 4 h recovery, major elements of the EPO cascade were enhanced. EPO mRNA expression, virtually undetectable in sham and control myocardium, was robust in pyruvate-treated myocardium. Indeed, EPO mRNA may be expressed only when HIF-1 is stabilized. Pyruvate-enhanced EPO mRNA expression paralleled increased myocardial EPO content and EPO release into the coronary effluent. Sham and control cardioplegia-treated myocardium also contained appreciable amounts of EPO despite the near-absence of EPO mRNA. Circulating EPO likely contributed to myocardial EPO content in these groups. Conversely, EPO release throughout recovery may have limited the increase in myocardial EPO following pyruvate treatment.

Immunohistochemistry revealed endowment of porcine myocardium with EPO receptors, as previously shown in rodent hearts [11, 16, 17]. EPO-R were depleted in myocardium arrested with control cardioplegia, but sharply increased in myocardium receiving pyruvate-fortified cardioplegia. Pyruvate treatment also provoked phosphorylation of EPO's downstream kinases, especially Erk, and augmented content of eNOS, which Erk and Akt stimulate to produce cardioprotective nitric oxide [14, 15]. Thus, pyruvate-fortified cardioplegia enhanced the EPO signaling cascade in left ventricular myocardium, an effect possibly initiated by pyruvate stabilization of HIF-1α.

Pyruvate stabilization of HIF-1α. HIF-1 orchestrates gene expression and synthesis of an array of cytoprotective proteins, including EPO, EPO-R and eNOS [27]. HIF-1 transcriptional activity is regulated by the dynamic balance of constitutive expression *vs.* degradation of its α subunit. In the presence of O_2, Fe^{2+} and α-ketoglutarate, hydroxylation of two proline residues (^{402}P, ^{564}P) by prolyl hydroxylase [28] targets HIF-1α for proteosomal degradation, thereby suppressing HIF-1-dependent gene expression [8, 9].

Pyruvate cardioplegia increased HIF-1α content but not its mRNA, in accord with the concept that pyruvate enhances HIF-1α content by preventing its degradation, not by increasing its synthesis. Pyruvate and its carboxylation product oxaloacetate directly suppress HIF-1α degradation by competing with the essential cofactor α-ketoglutarate for access to prolyl hydroxylase's catalytic core [10, 29]. In addition, a recent study in rat cortical neurons revealed strong correlations between HIF-1α content and glutathione redox state, *i.e.* GSH/GSSG [30]. The authors proposed that increased GSH/GSSG could suppress activation of proteosomes by reactive oxygen species and, thus, preserve HIF-1α [30]. Pyruvate-fortified cardioplegia increased myocardial GSH/GSSG in the porcine CPB model [4]. Collectively, inhibition of prolyl hydroxylase and proteosomes by pyruvate and its metabolic products, including oxaloacetate and GSH, could suppress HIF-1α degradation and, thus, augment HIF-1-induction of EPO, EPO-R and eNOS.

Erythropoietin signaling and cardioprotection. Classically a hematopoietic cytokine, EPO recently has been found to powerfully protect myocardium from ischemia-reperfusion-

injury [11, 12]. EPO occupation of its receptor mobilizes two parallel signaling cascades culminating in phosphorylation of Erk and Akt [14, 15]. These kinases stimulate eNOS to produce nitric oxide, which activates mitochondrial ATP-sensitive K^+ ($mitoK_{ATP}$) channels. Although pyruvate's impact on $mitoK_{ATP}$ channels is unknown, Kerr *et al.* [31] showed that pyruvate suppressed mitochondrial permeability transition in post-ischemic rat myocardium, a protective effect potentially mediated by $mitoK_{ATP}$ channels [32].

Limitations. Definitive proof linking the EPO-Erk/Akt-eNOS mechanism to pyruvate-induced cardioprotection requires pharmacological, siRNA and/or transgenic strategies, but the size and complexity of the porcine CPB model preclude such approaches. Myocardium was analyzed at 4 h recovery to study HIF-1-induced gene expression and protein synthesis. It is unlikely that this time point coincided with maximum pyruvate-evoked gene expression, protein content or kinase activation. Studies of metabolic stress and pyruvate treatment in isolated cardiomyocytes would permit manipulation of signaling pathways and analysis of time-courses of mRNA expression, protein synthesis and phosphorylation. Such studies are natural extensions of the current investigation.

The impact of pyruvate-enhanced cardioplegia on cardiac mechanical function was not assessed in this study. Phenylephrine was infused to maintain systemic arterial pressure at 60-70 mm Hg, ensuring adequate myocardial perfusion to avoid confounding effects of ischemia. Systemic arterial pressures at 4 h reperfusion were 65 ± 4 and 72 ± 6 mm Hg in the control and pyruvate groups ($P > 0.2$). However, the phenylephrine dosage required to

maintain arterial pressure in the pyruvate group, 1.0 ± 0.3 mg kg^{-1}, was only 45% that of the control cardioplegia group, 2.2 ± 0.5 mg kg^{-1} ($P < 0.05$), indirect evidence of improved cardiac function in the pyruvate cardioplegia group.

Implications. This study reveals a novel cardioprotective mechanism of pyruvate, distinct from and more persistent than its enhancements of myocardial energy and antioxidant redox states [4-6]. For the first time, a metabolic substrate is found capable of inducing erythropoietin formation within the myocardium and activating EPO signaling. This novel cardioprotective mechanism can be mobilized during CPB by administering pyruvate-enriched crystalloid cardioplegia.

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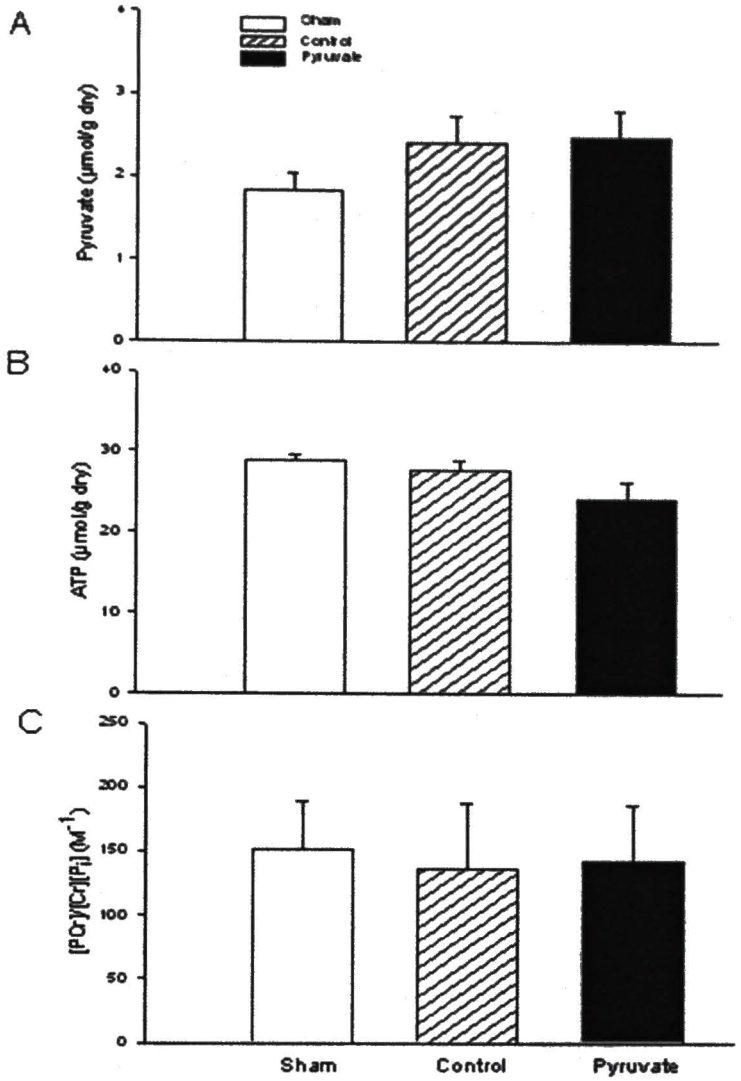

Figure 1. *Myocardial pyruvate content and energy state.* In this figure and in Figures 2-4 and 6-9, variables were measured in left ventricular myocardium sampled 4 h after arrest with control (hatched bars) or pyruvate-fortified (filled bars) cardioplegia, or in non-arrested sham experiments (open bars). Values in all figures are means ± SEM from the same 8 experiments/group. Panels A, B: pyruvate and ATP contents; panel C: phosphocreatine (PCr) phosphorylation potential. Cr: creatine; P_i: inorganic phosphate. No statistically significant differences were detected.

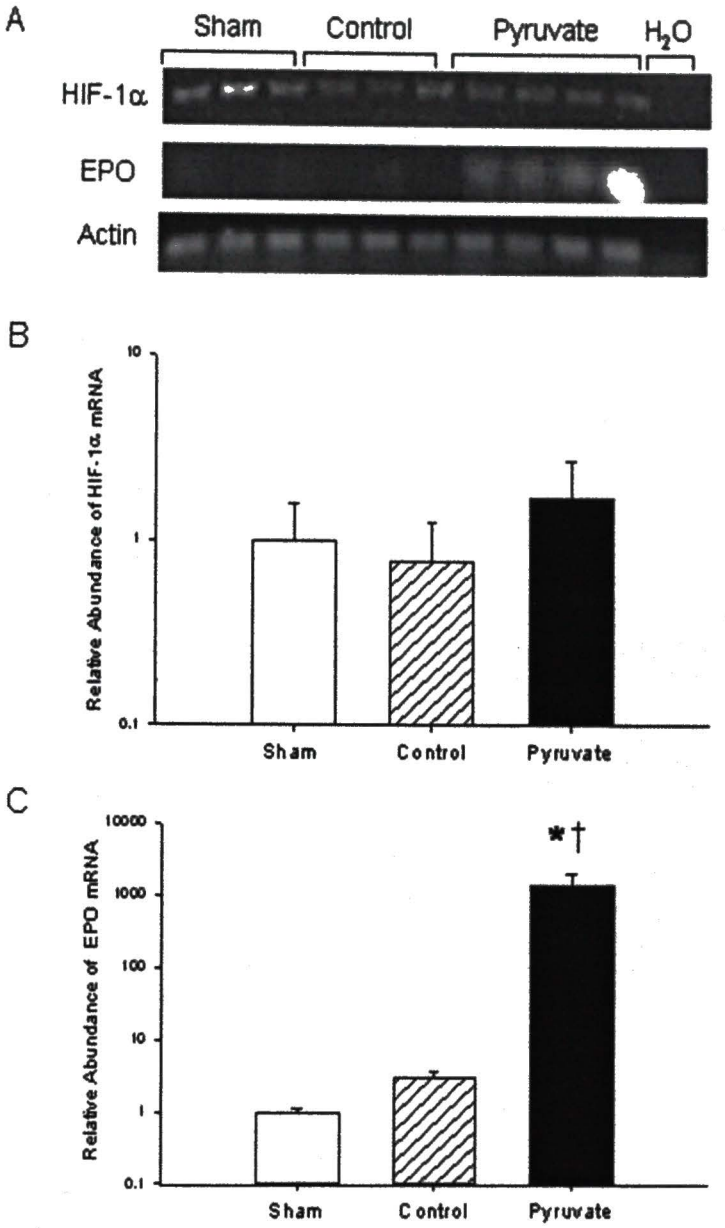

Figure 2. *HIF-1α and EPO mRNA expression in left ventricular myocardium.* Panel A: ethidium bromide-stained reverse transcriptase-PCR gel. Panels B, C: HIF-1α (panel B) and EPO (panel C) mRNA abundances, normalized to α-actin mRNA and presented relative to sham values. In this figure and in Figures 3, 4 and 6-9, mean sham value = 1.0. Values are plotted on logarithmic scale. *$P < 0.001$ *vs.* control; †$P < 0.001$ *vs.* sham.

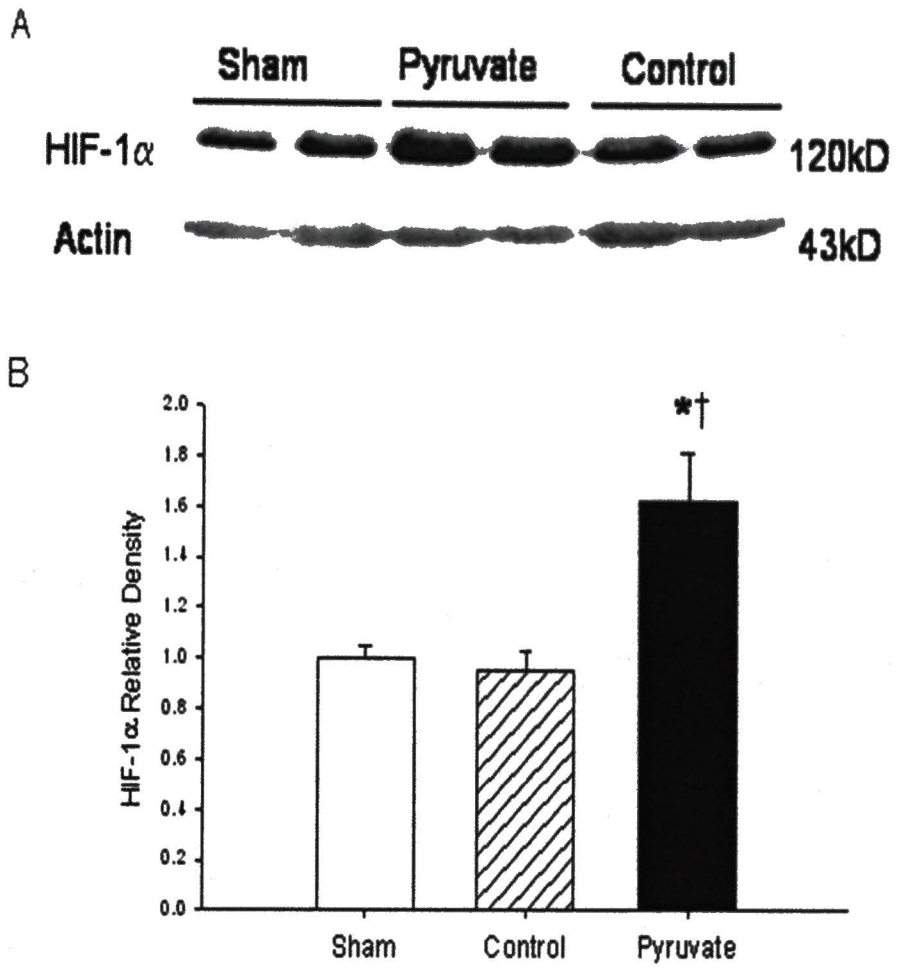

Figure 3. *Left ventricular myocardial HIF-1α content.* Panel A: typical immunoblot of HIF-1α and actin loading control. Panel B: relative HIF-1α contents. Immunoblot band densities in this and the following figures are normalized to actin as a loading control, measured on the same membranes. *$P < 0.001$ *vs.* control; †$P < 0.001$ *vs.* sham.

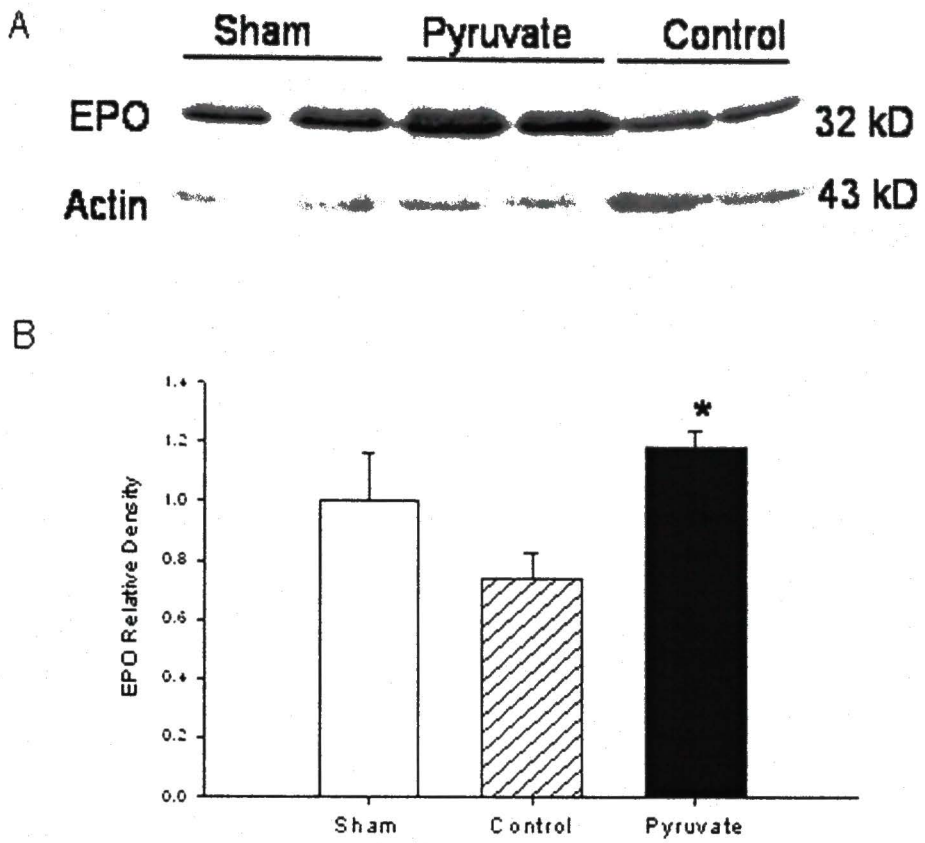

Figure 4. *Erythropoietin (EPO) content.* Panel A: typical immunoblots of HIF-1α and actin. Panel B: relative HIF-1α contents. *$P < 0.05$ *vs.* control.

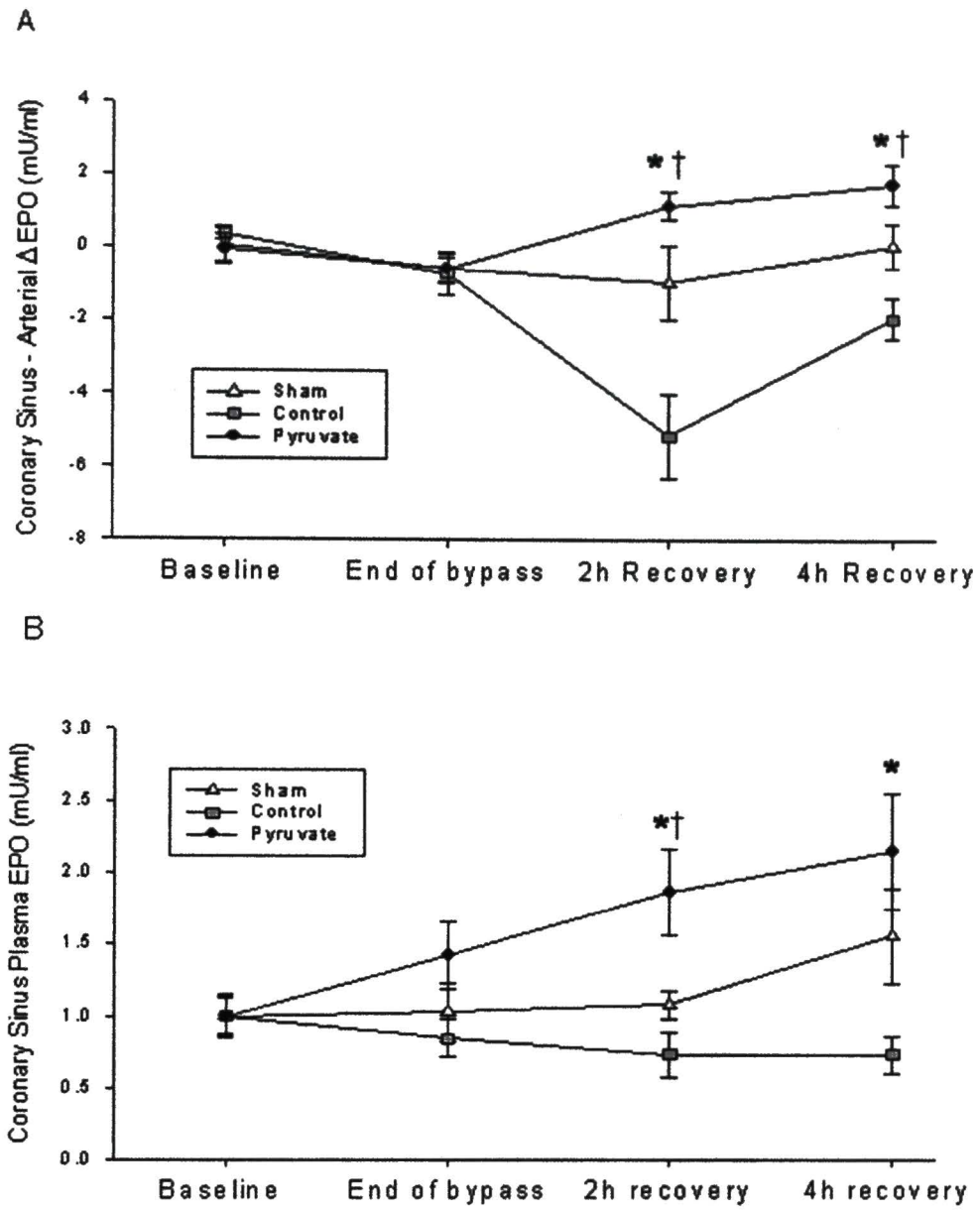

Figure 5. *Myocardial EPO uptake/release.* Panel A: coronary sinus EPO concentrations. Panel B: coronary sinus – systemic arterial EPO concentration differences; negative values indicate net myocardial uptake. *$P < 0.001$ *vs.* control; †$P < 0.001$ *vs.* sham.

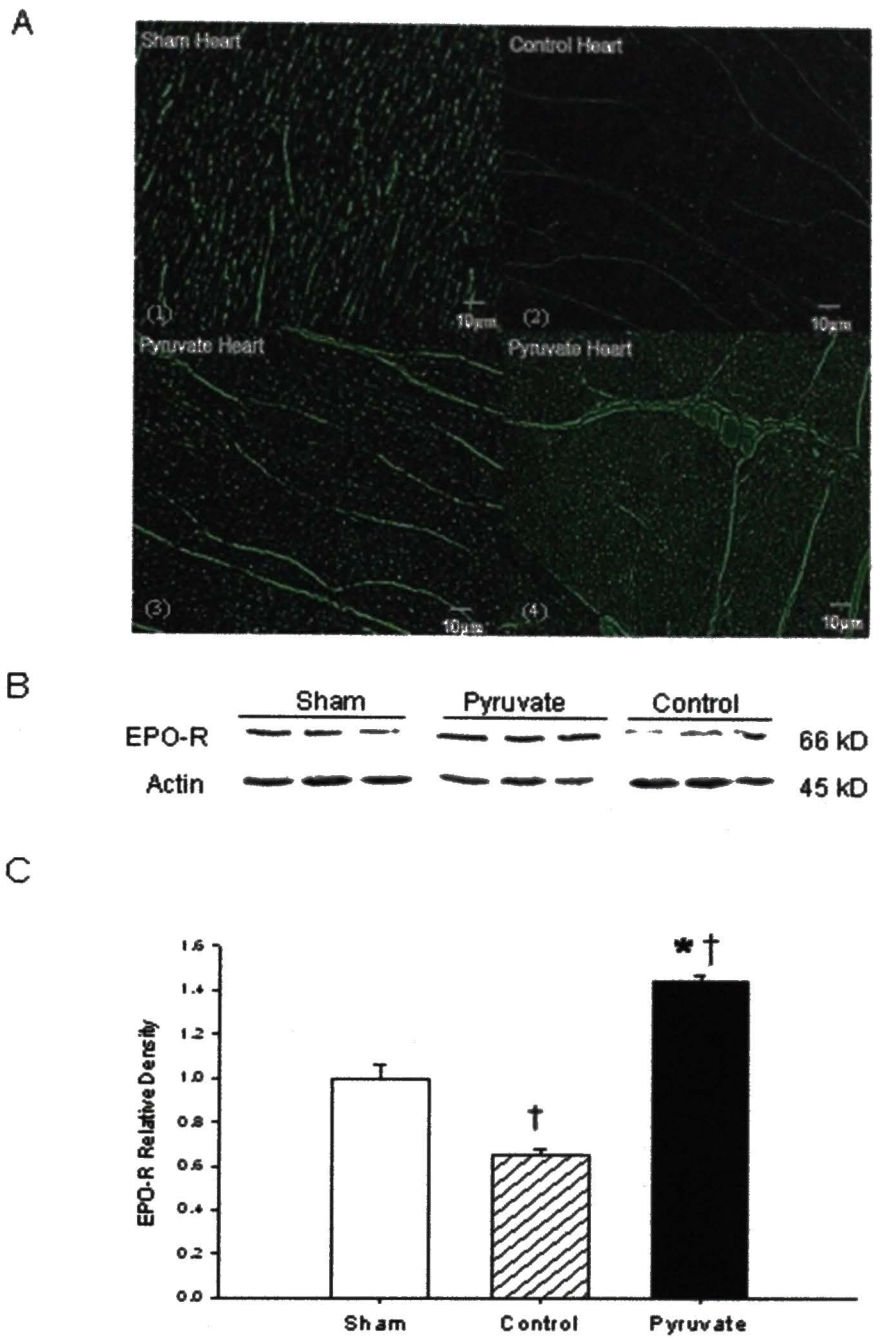

Figure 6. *EPO receptors (EPO-R).* Panel A: Immunohistochemically detected EPO-R (green) in left ventricular myocardium from sham (1), control cardioplegia-treated (2) and pyruvate cardioplegia-treated (3, 4) experiments. Panel B: typical immunoblot of EPO-R and actin. Panel C: EPO-R content. *P < 0.05 *vs.* control.

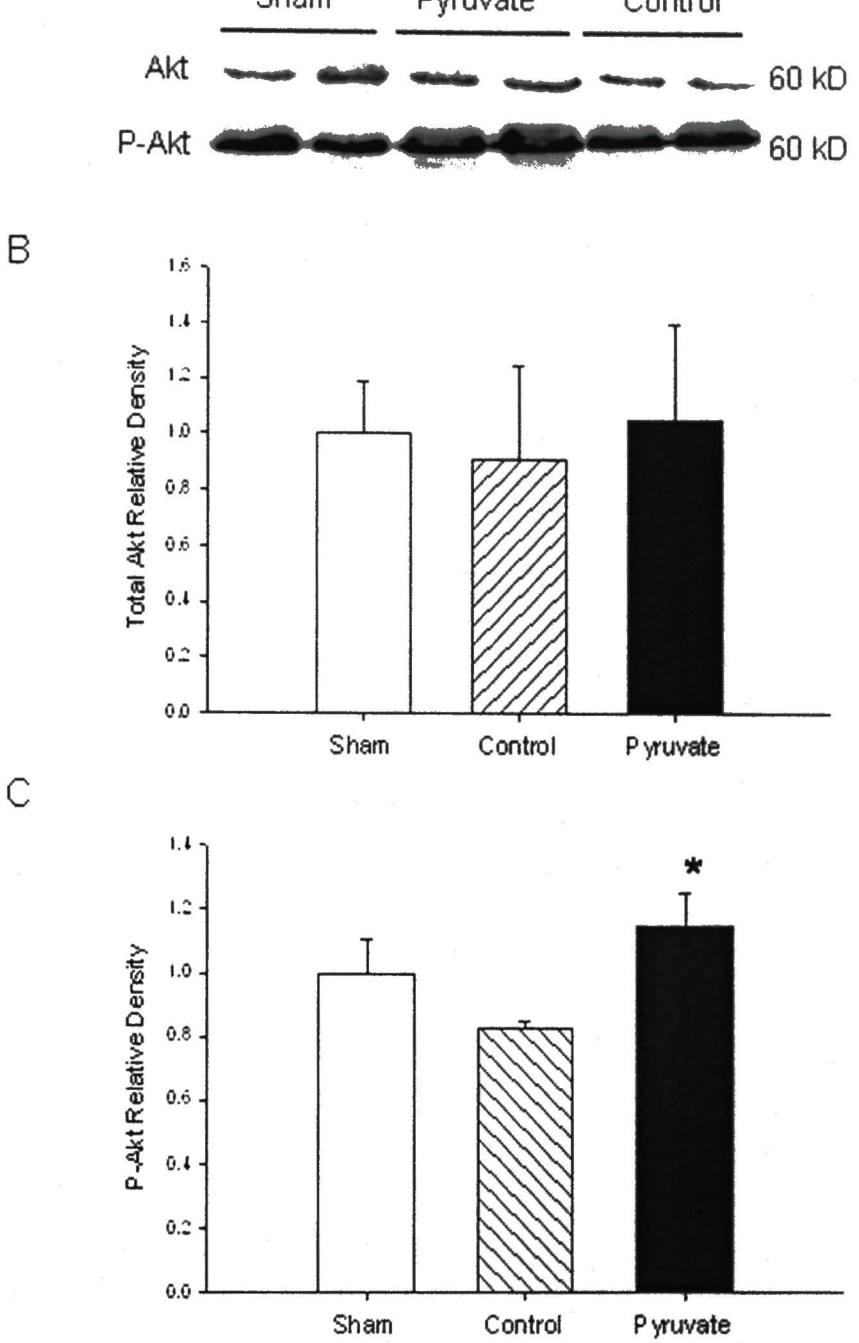

Figure 7. *Akt and phospho-Akt (P-Akt) contents.* Panel A: typical immunoblot of Akt and P-Akt. Panels B, C: total (panel B) and phosphorylated (panel C) Akt contents. *P < 0.05 *vs.* control.

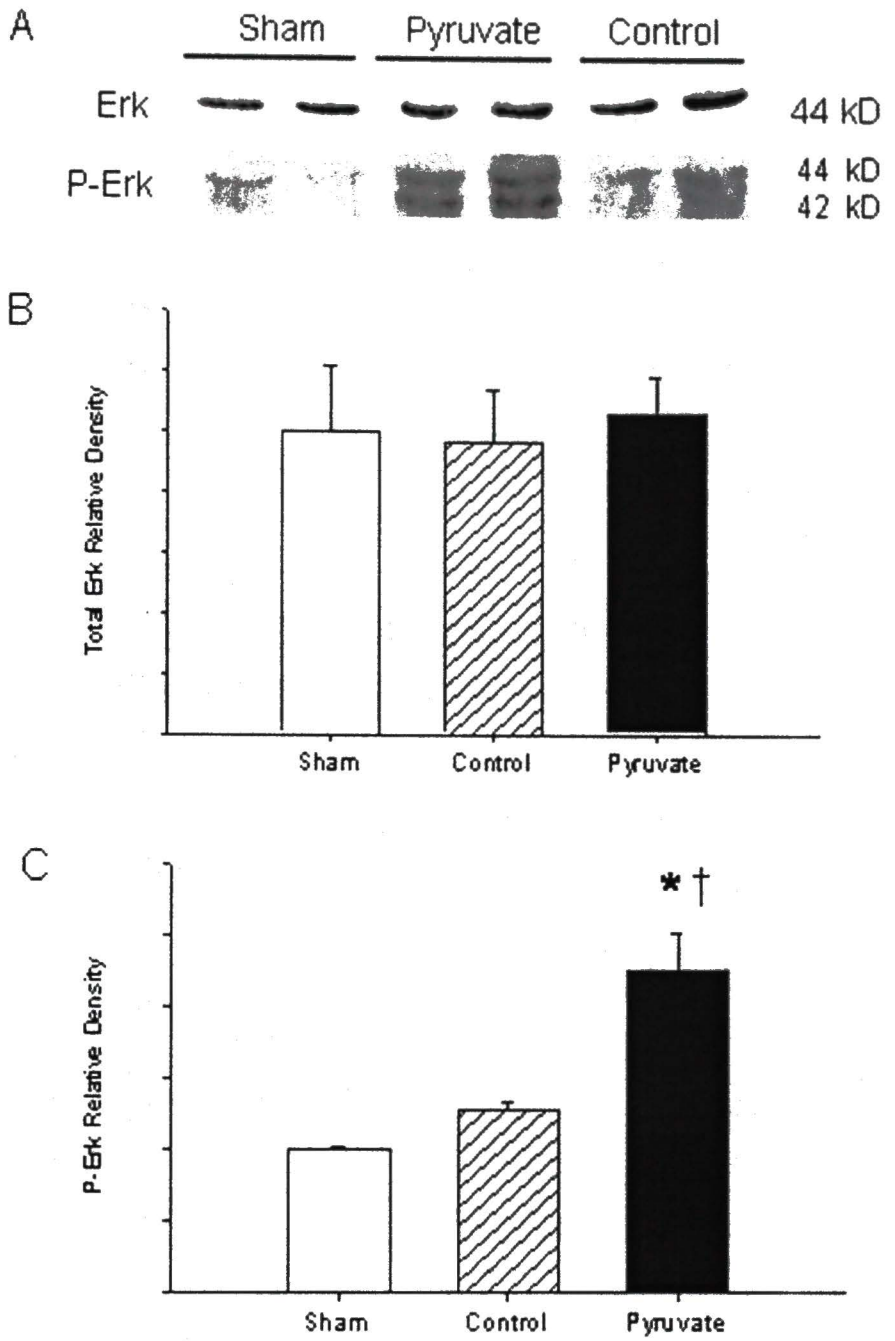

Figure 8. *Erk and phospho-Erk (P-Erk) contents.* Panel A: typical immunoblot of Erk and P-Erk. Panels B, C: total (panel B) and phosphorylated (panel C) Erk contents. *$P < 0.05$ *vs.* control; †$P < 0.05$ *vs.* sham.

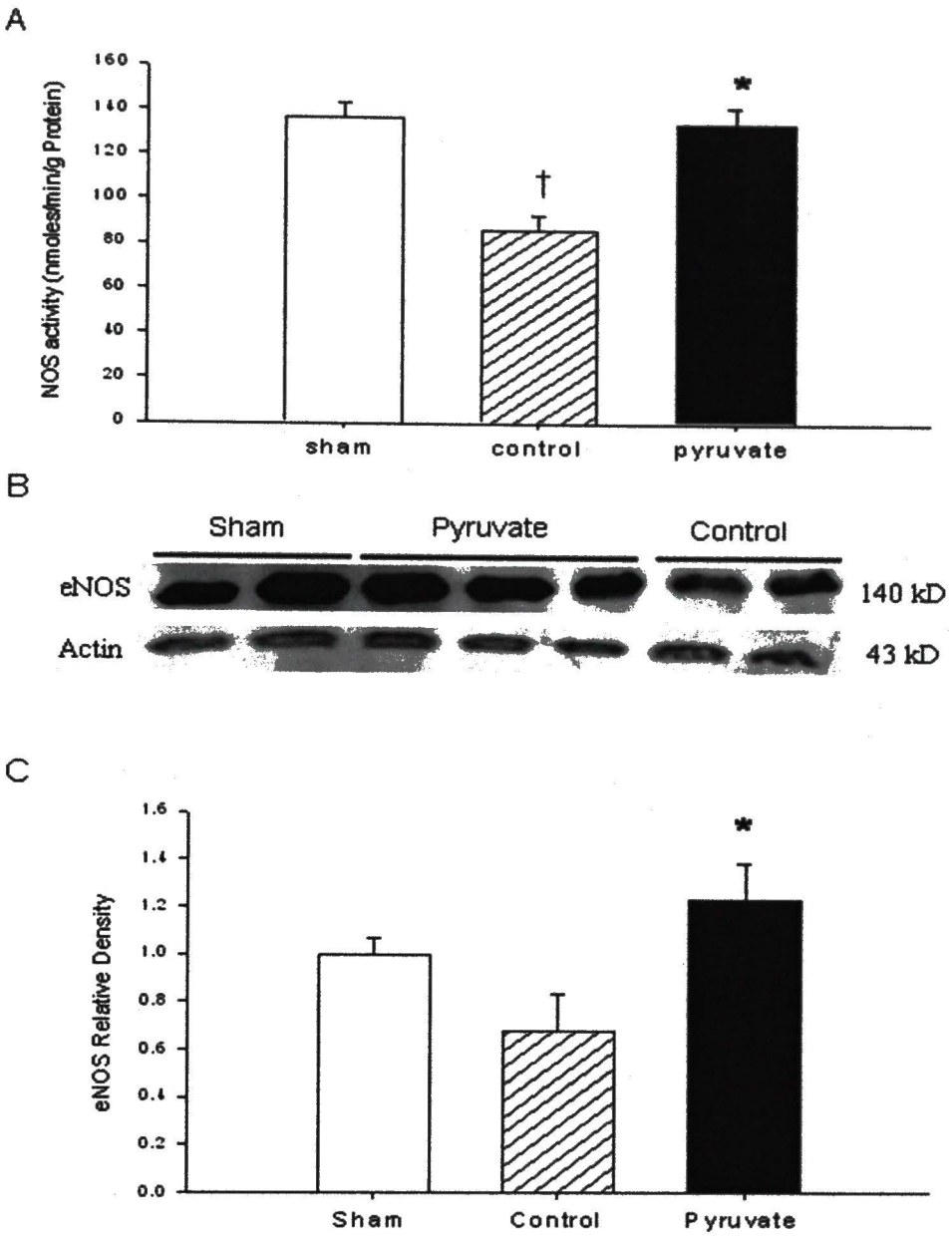

Figure 9. *Nitric oxide synthase (NOS) activity and eNOS content.* NOS activities (panel A) and eNOS contents (panels B, C) were measured in myocardium from the same experiments as in Figures 1-8. Panel B: typical immunoblot of eNOS and actin. *$P < 0.001$ *vs.* control; †$P < 0.001$ *vs.* sham.

CHAPTER III

Pyruvate-enriched cardioplegia suppresses cardiopulmonary bypass-induced inflammation

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ABSTRACT

Background: Cardiopulmonary bypass (CPB) can elicit harmful systemic inflammation. Cardioplegia enriched with pyruvate, an intermediary metabolite and energy-yielding substrate, exerts antioxidant protection in myocardium of pigs subjected to CPB. **Hypothesis:** Pyruvate-fortified cardioplegia mitigates inflammation by increasing anti-inflammatory cytokines, enhancing myocardial antioxidant defenses, and suppressing MMP3 activity. **Methods:** *In situ* swine hearts were arrested for 1 h with 4:1 blood:crystalloid cardioplegia, where the crystalloid component contained 188 mM glucose ± 24 mM pyruvate. Following 30 min reperfusion with cardioplegia-free blood, pigs were weaned from CPB. Cytokines and glutathione redox state were measured in arterial and coronary sinus plasma. At 4 h post-CPB, left ventricular myocardium was sampled for immunoblot analyses of the acute inflammation marker C-reactive peptide (CRP), MMP3 and, tissue inhibitor of metalloproteinase-2 (TIMP2), and to detect neutrophil infiltration. **Results:** Pyruvate cardioplegia produced a sustained increase in glutathione redox state in the coronary sinus plasma. Myocardial CRP content increased over fivefold following CPB with control cardioplegia, but pyruvate cardioplegia prevented this increase. Circulating IL-10 concentrations temporarily increased during and immediately after CPB; pyruvate cardioplegia provoked more sustained and robust enhancement of this anti-inflammatory cytokine. Myocardial IL-6 release gradually increased in both CPB groups during recovery, but more so in the pyruvate group. Pyruvate cardioplegia blunted post-CPB myocardial neutrophil infiltration. **Conclusion:**

Pyruvate-enriched cardioplegia suppresses CPB-induced inflammation during subsequent recovery, an effect potentially mediated by anti-inflammatory cytokines and TIMP-2.

INTRODUCTION

The development and refinement of cardiopulmonary bypass (CPB) technology over the last 50 years has enabled surgeons to correct lethal cardiac structural abnormalities, repair or replace diseased heart valves, and restore coronary circulation to the cardiac muscle. Despite remarkable advances in bypass surgery, the systemic inflammatory response to these highly invasive procedures can injure the heart and other internal organs, causing post-operative morbidity and mortality. A host of factors, including blood contact with the artificial surfaces of the extracorporeal circuit, the use of heparin and protamine to control clotting, hypothermia to slow organ metabolism, anesthesia and surgical trauma, reinfusion of blood collected from the chest cavity and heart, sepsis and endotoxemia due to intestinal mucosal hyperpermeability, and accumulation of microemboli in the blood combine to provoke a massive systemic inflammatory response.[1-8] By damaging the internal organs, including the heart itself, systemic inflammation produces a constellation of post-surgical morbidities, including pulmonary insufficiency and acute respiratory failure,[9, 10] renal insufficiency,[1, 11-13] neurocognitive impairment,[14] cardiodepression[15, 16] and atrial fibrillation.[17, 18] These complications lengthen hospitalization, increase healthcare costs, and can even prove fatal.[19]

Recent studies have revealed anti-inflammatory capabilities of pyruvate and its ethyl derivative. Pyruvate stabilized cardiac function, prevented hepatic cell death and maintained GSH/GSSG during hemorrhagic shock in pigs, [20] and improved survival and

increased arterial pressure in rats subjected to hemorrhagic shock and recovery.[21] Ringers ethyl pyruvate solution suppressed systemic inflammation in mice during resuscitation from hemorrhagic shock.[22] Pyruvate and ethyl pyruvate decreased intestinal mucosal injury and ameliorated mucosal hyperpermeability in mice[23] and rats[24] subjected to mesenteric ischemia/reperfusion. Moreover, ethyl pyruvate suppressed increases in plasma TNF-α and hepatic TNF-α mRNA abundance during intestinal ischemia in mice,[23] dampened the surge in plasma TNF-α and improved survival in a murine sepsis model,[25] and suppressed lipopolysaccharide-induced IL-6 secretion, nitric oxide formation, and expression of IL-6 and iNOS mRNA in cultured murine lymphocytes.[26]

Pyruvate's anti-inflammatory properties raise the possibility that administration of pyruvate-fortified cardioplegia could suppress the inflammatory response to cardiopulmonary bypass. Accordingly, this study tested the hypothesis that pyruvate-fortified cardioplegia minimizes subsequent neutrophil invasion by increasing production of anti-inflammatory cytokines and by increasing myocardial content of tissue inhibitor of matrix metalloproteinase-2. Experiments were conducted in domestic pigs subjected to cardioplegic arrest on cardiopulmonary bypass.

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* (National Institutes of Health publication 85-23, Revised 1996). 24 domestic swine of either sex weight 50-70 kg were assigned randomly to three groups of 8 subjects. Two groups underwent cardiopulmonary bypass, in which the crystalloid component of the cardioplegia contained 188 mM glucose alone (control group) or with 24 mM pyruvate (pyruvate group). A sham group also was studied, in which pigs were surgically prepared but weren't subjected to cardioplegia-induced cardiac arrest, CPB or reperfusion.

Surgical procedure. Pigs were premedicated with ketamine (10 mg/kg *im*) and xylazine (1 mg/kg *im*), anesthetized with propofol (2 mg/kg *iv*), intubated, and mechanically ventilated with 0.5-2% isoflurane supplemented with O_2 to maintain a surgical plane of anesthesia. During CPB, anesthesia was maintained by continuous infusion of propofol, 0.2 mg kg^{-1} min^{-1} *iv*.

The heart was exposed by median sternotomy and supported in a pericardial cradle. Catheters were placed in the femoral artery and coronary sinus for blood sampling, and another catheter was placed in the femoral vein to inject medications. The inferior vena cava was cannulated via the right atrium to withdraw blood for the extracorporeal CPB circuit. The abdominal aorta was cannulated to deliver oxygenated blood from the heart-

lung machine. A cardioplegic line was inserted into the aortic root for antegrade cardioplegia infusion.

Cardioplegic arrest and recovery. Control crystalloid cardioplegia (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. Pyruvate cardioplegia was prepared by equimolar substitution of 24 mM sodium pyruvate for NaCl. The crystalloid solutions were combined with 4 vol whole blood before infusion. After heparin administration (300 U/kg, *iv*), the pig was connected to the heart-lung machine. The aorta was cross-clamped distal to the cardioplegia line. Cardiac arrest was induced by antegrade infusion of 1200 ml of cardioplegia, and maintained by infusing 400 ml of cardioplegia at 20 min intervals. After 60 min of arrest, the heart was reperfused for 30 minutes with cardioplegia-free blood via the aortic cannula, and then the pig was disconnected from the heart-lung machine and recovered for 4 h. $NaHCO_3$ and K^+ were administered *iv* to correct acidemia and hypokalemia, respectively. The α-adrenergic vasoconstrictor phenylephrine was infused *iv* as needed to maintain mean arterial pressure at 60-70 mm Hg throughout recovery.

Blood and tissue sampling. Arterial and coronary sinus plasma samples were collected in a series of pre-determined points to measure blood gas chemistry, glutathione redox state and cytokines. PO_2, PCO_2, pH, and HCO_3^- and K^+ concentrations were measured in an Instrumentation Laboratory model 1730 blood gas analyzer. Plasma was

obtained by sedimentation of formed elements, immediately flash-frozen in liquid N_2, and then stored at -80°C for later analyses of glutathione and cytokines.

After 4 h recovery, left ventricular myocardium was freeze-clamped with Wollenberger tongs precooled in liquid N_2. [27] These samples were stored at -80°C until extraction. Additional myocardium was excised, fixed in formalin, and stained with hematoxylin-eosin to detect neutrophils.

Cytokine measurement. Plasma concentrations of interleukins 6 (IL-6) and 10 (IL-10) were quantified by enzyme-linked immunosorbent assay (ELISA). Commercially available ELISA kits were used for quantification of IL-6 (Biosource, Carlsbad, CA) and IL-10 (R&D Systems, Minneapolis, MN).

Immunoblot analysis. The inflammatory markers, were extracted from frozen myocardiumamined with frozen tissue samples. Frozen myocardium was extracted [28] for immunoblot analyses of C-reactive peptide (CRP), matrix metalloproteinase-3 (MMP3) and tissue inhibitor of matrix metalloproteinases-2 (TIMP2). Total protein concentrations in extracts were determined by the Bradford method. [29] Proteins were separated by electrophoresis (100 V for 2 h) on 10% polyacrylamide gels (20 μg protein/lane), and then were electrophoretically transferred to nitrocellulose membrane. Membranes were incubated in 5% nonfat milk for 2 h to block non-specific binding sites before exposure to antibodies.

Mouse anti-CRP primary antibody was obtained from R&D Systems (Minneapolis, MN). Rabbit anti-MMP3 and TIMP2 antibodies were purchased from Chemicon (Temecula, CA). Anti-mouse and rabbit secondary antibodies were purchased from Kirkegaard & Perry (Gaithersburg, MD). Target bands were visualized with enhanced chemiluminescence (Pierce, Rockford, IL). Each membrane was stripped and re-probed with anti-actin antibody (Stressgen, Victoria, BC) to detect actin as loading control. Band densities were measured in an AlphaEase FC 4.0 densitometer (AlphaInnotech, San Leandro, CA), and then normalized to actin band densities.

Glutathione redox state and nitrotyrosine. Plasma glutathione (GSH) and glutathione disulfide (GSSG) were measured by high performance liquid chromatography. [30] GSH/GSSG concentration ratios were taken as measures of GSH redox state. {[31] Nitrotyrosine (NT), a product of peroxynitrite-induced protein nitrosation, was measured with an ELISA-based kit (Northwest Life Science, Vancouver, WA).

Neutrophil infiltration. Tissue sections (6 μm) were hematocylin-eosin stained for morphological assessment of tissue injury and detection of inflammatory cells. [32] Neutrophils were visualized by light microscopy and images were recorded using an Olympus digital camera.

Statistical analysis. Values are means ± SEM. Mean values among the three experimental groups were compared by single-factor ANOVA. When ANOVA detected

statistical significance, Tukey's *post hoc* test was applied to identify the specific differences. P values < 0.05 were taken to indicate statistically significant differences.

RESULTS

Glutathione redox state in arterial and coronary sinus plasma. The redox state of the central antioxidant glutathione, *i.e.* GSH/GSSG concentration ratio, provides an index of the collective redox state of the antioxidant systems in equilibrium with GSH. [31] Accordingly, GSH/GSSG was measured in arterial (Figure 3A) and coronary sinus (Figure 3B) plasma to indirectly assess the effects of control or pyruvate-enhanced CPB on myocardial antioxidant defenses over the full experimental protocol. Arterial GSH/GSSG was unaltered by control or pyruvate-enhanced CPB, showing a modest upward trend in all 3 groups during the protocol (Figure 3A). In contrast, CPB substantially affected GSH/GSSG in coronary sinus (Figure 3B). In control CPB experiments, GSH/GSSG fell *vs.* respective sham values, especially at 2-4 h recovery. Conversely, GSH/GSSG rose sharply during cardiac arrest with pyruvate-enriched cardioplegia, and continued to increase over the next 2.5 h to a plateau despite reperfusion and washout of the pyruvate cardioplegia. Thus, CPB with control *vs.* pyruvate-enhanced cardioplegia had opposite effects on coronary sinus GSH/GSSG, and these redox effects persisted and even intensified during cardioplegia washout and post-arrest cardiac recovery. Because systemic arterial GSH/GSSG was similar among the

groups, changes in myocardial redox state are likely responsible for the differences in coronary sinus GSH/GSSG.

Myocardial nitrotyrosine. Myocardial nitrotyrosine content, a marker of peroxynitrite, did not differ among the groups, although values in the control group tended to be higher than in the sham or pyruvate-enhanced CPB groups (Figure 4).

Pro- and anti-inflammatory cytokines. Both arterial and coronary sinus plasma showed similar pattern of changes in IL-6 and 10 though out the experimental protocol (Figure 2). There was no significant difference between control and pyruvate groups until 1h of recovery in arterial samples. However, during the late phase of recovery, in the pyruvate group, the IL-6 concentration was significantly elevated *vs.* control group ($P<0.05$). On the other hand, IL-10 concentration was immediately increased after pyruvate-fortified cardioplegia infusion *vs.* control ($P<0.05$). During the recovery period, IL-10 concentration was gradually decreased.

C-reactive peptide. C-reactive protein (CRP) content, a marker of acute inflammation, increased nearly six fold in control CPB *vs.* sham myocardium (Figure 4). In contrast, the use of pyruvate-fortified cardioplegia to arrest the heart during CPB prevented CRP accumulation. Thus, pyruvate-fortified cardioplegia decreased CRP content by 74% *vs.* control ($P<0.001$) (Figure 1), to a value not significantly different from the sham content.

Neutrophil infiltration in left ventricular myocardium. Infiltration of neutrophils into the myocardial parenchyma was detected by histological examination. Invasion of polymorphonucleocytes to LV tissue was observed in histological examination. In control group, more neutrophils were trapped in the LV myocardium as well as coronary blood vessels (Figure 5C). Furthermore, control LV histological structure was more severely damaged as compared to sham and pyruvate groups (Figure 5).

MMP3 and TIMP2. Matrix metalloproteinases degrade the extracellular matrix, permitting neutrophil extravasation and tissue infiltration. The extent of neutrophil filtration is closely related to myocardial MMP activity, which is determined by myocardial contents of MMP and tissue inhibitor of matrix metalloproteinase (TIMP). MMP content did not differ significantly among the 3 groups (Figure 6A). However, sharply increased TIMP2 protein content was detected in the pyruvate group (Figure 6B). The ratio of TIMP to MMP content, a principal determinant of MMP activity, was increased by pyruvate-fortified cardioplegia 2.5-fold *vs.* sham and roughly six fold *vs.* control cardioplegia (Figure 6C). This remarkable enhancement of TIMP2 would inhibit MMP and, thus, suppress degradation of the extracellular matrix and neutrophil invasion of the myocardial parenchyma.

DISCUSSION

Cardiopulmonary bypass initiates a cascade of molecular events (Figure 2) that culminates in activation and extravasation of neutrophils, which infiltrate and damage

target tissues. CPB activates several pro-inflammatory cytokines, most notably interleukins 6 (IL-6) and 8 (IL-8) and tumor necrosis factor (TNF)-α.[23, 27, 33-38] These proteins, produced by vascular endothelium,[27, 38-40] white blood cells[27, 39] and fibroblasts [39] function as chemoattractants to recruit neutrophils to target tissues. [27, 33, 41, 42] Pro-inflammatory cytokines have been implicated in the pathogenesis of post-bypass cardiodepression,[43] lung injury[41] and atrial fibrillation.[44] Neutrophil extravasation and invasion of tissue parenchyma culminates the inflammatory cascade. To penetrate the tissue, neutrophils secrete matrix metalloproteinases (MMPs), which degrade the extracellular matrix and damage cellular proteins.[2] MMPs have been detected in canine myocardium, lung and brain,[45, 46] and porcine lung [9] after cardiopulmonary bypass.

C-reactive protein (CRP), a plasma protein produced by liver, is an acute inflammation marker. Increased CRP in the first several days post-surgery was reported in patients undergoing coronary artery revascularization on-pump.[47] Increased myocardial CRP content in the control cardioplegia group was identical to previous findings in clinical cases. On the other hand, decreased CRP content in the pyruvate group indicated that pyruvate-fortified cardioplegia dampened the acute inflammatory response as compared with conventional cardioplegia.

Circulating concentrations of the anti-inflammatory cytokine IL-10 sharply increased during CPB, and then gradually subsided during post-CPB recovery. Pyruvate cardioplegia augmented circulating IL-10 during CPB and throughout recovery. IL-6

concentrations increased as IL-10 declined, and plateaued at 2-4 h recovery. IL-6 activates a cytoprotective-signaling pathway mediated by phosphatidylinositol-3-kinase (PI3K).[48] Activated PI3K suppressed inflammatory response by suppressing pro-inflammatory mediators contents.

In our experimental model, circulating IL-6 was greater following pyruvate-fortified cardioplegia than control. Moreover, there was no net IL-6 release in the control group, but appreciable release of the cytokine at 2-4 h recovery after pyruvate cardioplegia. This unexpected finding suggests a pro-inflammatory action of pyruvate. On the other hand, recent evidence suggests IL-6's role in the inflammatory response depends on its origin. Pro-inflammatory IL-6 is mainly produced by monocytes and macrophages in response to pathogenic stimuli, including oxidative stress. On the other hand, IL-6 generated from skeletal- or cardiac muscle showed anti-inflammatory effect. [49] Furthermore, IL-6 stimulates formation of anti-inflammatory IL-10 in later phase [49] and also activates protein kinases, which contribute to the late phase of cardioprotection. [48] Systemic arterial and coronary sinus IL-6 significantly increased in pyruvate *vs.* control CPB group at 2-4 h of recovery. During this period, there was appreciable net release of IL-6 by the myocardium, a striated muscle. Furthermore, ethyl pyruvate did not provoke IL-6 formation in monocyte and macrophage cell lines.[50]

Reactive oxygen species contribute to the development of inflammation. Degranulation of neutrophils enhances endogenous ROS generation, which can damage

cellular components. A well-documented antioxidant, pyruvate directly scavenges peroxides, hydroxyl radicals and peroxynitrite, and increases the redox state of the principal intracellular antioxidant GSH. [51-53] We recently demonstrated increased myocardial activity and content of endothelial nitric oxide synthase (eNOS), an effector of cardioprotective signaling pathways, following pyruvate-enhanced CPB. The fact that myocardial nitrotyrosine content was no greater and even trended downward in the pyruvate CPB group indicates that increased NOS activity did not increase the myocardial peroxynitrite burden. It is possible that the persistently increased GSH/GSSG may have scavenged any excess peroxynitrite during cardiac recovery from CPB.

Matrix metalloproteinases (MMPs) contribute to various physiological and pathological processes including tissue defense, injury, inflammation and repair. [54] MMPs contribute to the inflammatory processes by degrading and remodeling the extracellular matrix, and by modulating inflammatory mediators such as cytokines and chemokines. The effect of MMPs on chemokines creates chemokine gradients in damaged tissues that regulate the movement of immune cells at the site of infection or injury. The mechanisms whereby MMPs modulate immune cell migration to injured tissue include proteolytic processing of chemokines and chemokine receptors, and release of chemotatic fragments or accessory proteins.[55-57] Indeed, MMP-3-null mice had reduced macrophage-chemoattractant activity.[58] Neutrophil recruitment is an essential procedure in inflammation development. MMP3-null mouse showed decreased neutrophil and CD^{4+} lymphocyte recruitment. [59, 60]

Matrix metalloproteinases are inhibited by peptide regulators, the tissue inhibitors of metalloproteinases (TIMPs).[61] Generally, MMP and TIMP originate in the same cells. Accordingly, increased MMP production causes more release of TIMP to maintain the appropriate balance of MMP and TIMP. Disruption of MMP: TIMP balance can intensify the inflammatory response in some diseases. In the current CPB model, MMP3 protein content did not differ among the groups, but TIMP2 protein content was markedly elevated in pyruvate group. These results support the notion that pyruvate-fortified cardioplegia may suppress pro-inflammatory MMP activity by augmenting myocardial TIMP content. By preventing degradation and remodeling of the extracellular matrix, this TIMP-mediated MMP suppression may limit neutrophil invasion and, thus, inflammation in myocardium arrested with pyruvate-fortified cardioplegia.

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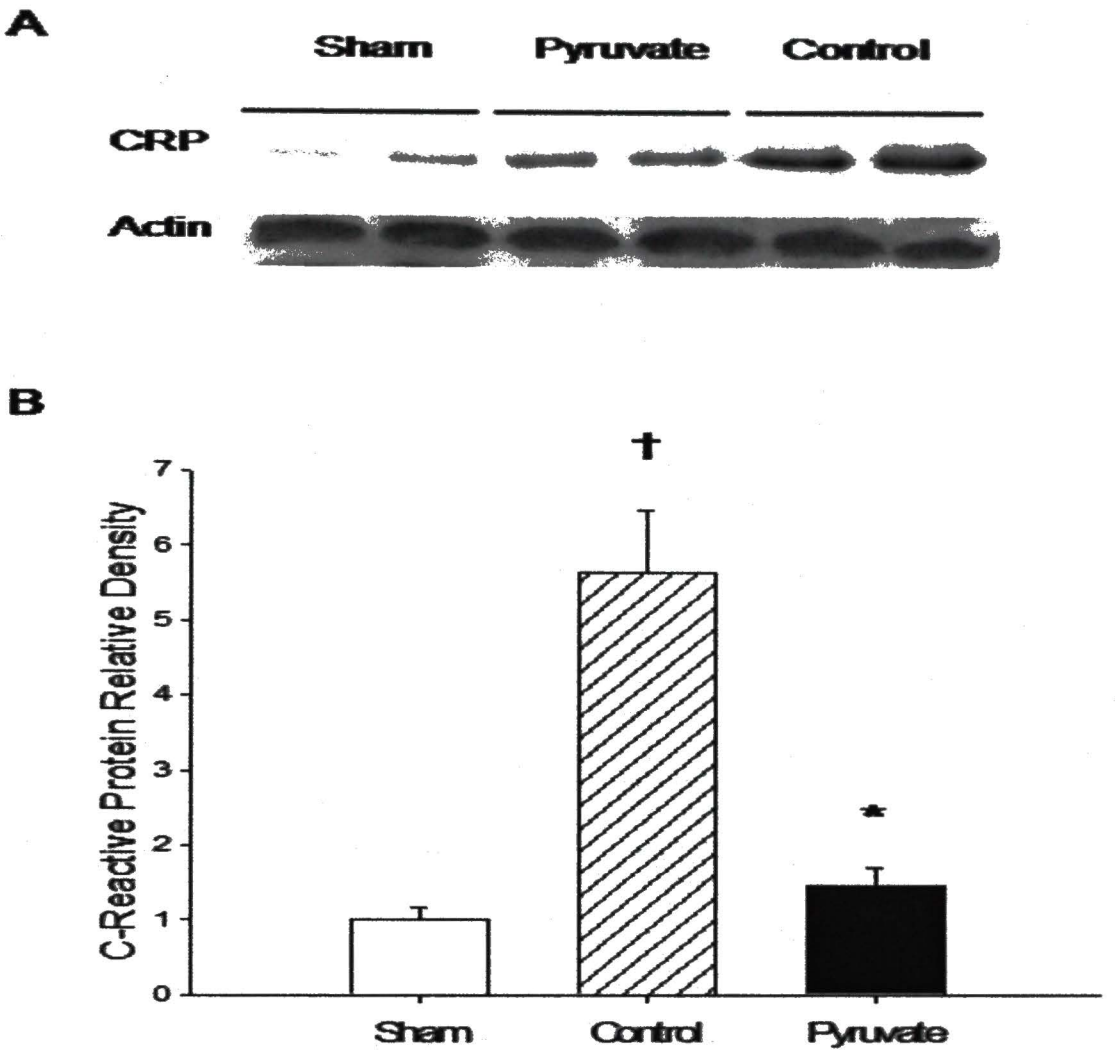

Figure 1. *Left ventricular C-reactive protein content.* Panel A: Immuno blot of C-reactive protein and actin loading control. Panel B: Relative CRP densities of band. Immuno blot band densities in this and the following figures are normalized to actin as a loading control. * $P<0.05$ *vs.* control; † $P<0.05$ *vs.* sham

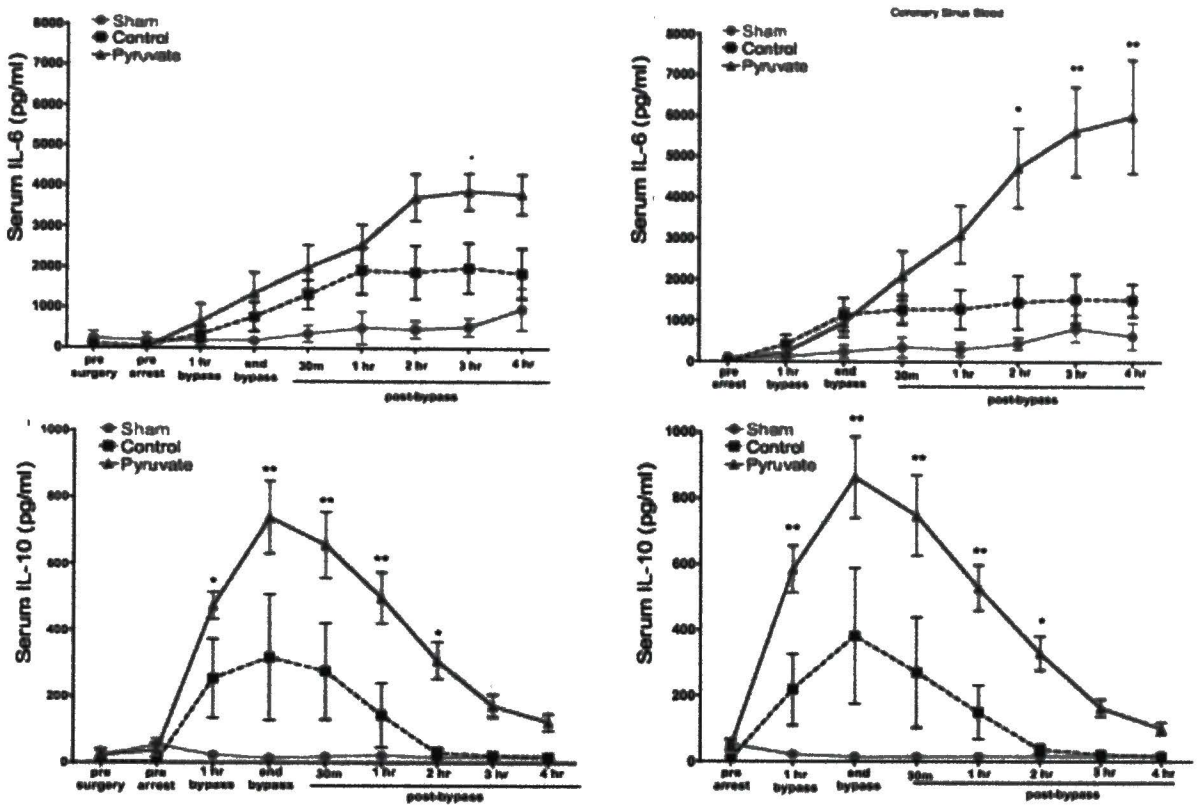

Figure 2. *Arterial and coronary sinus plasma IL-6 and IL-10 concentrations measured before and after CPB.* Data were plotted as mean ± MSE. * P<0.05 *vs.* sham; **P<0.05 *vs.* control and sham

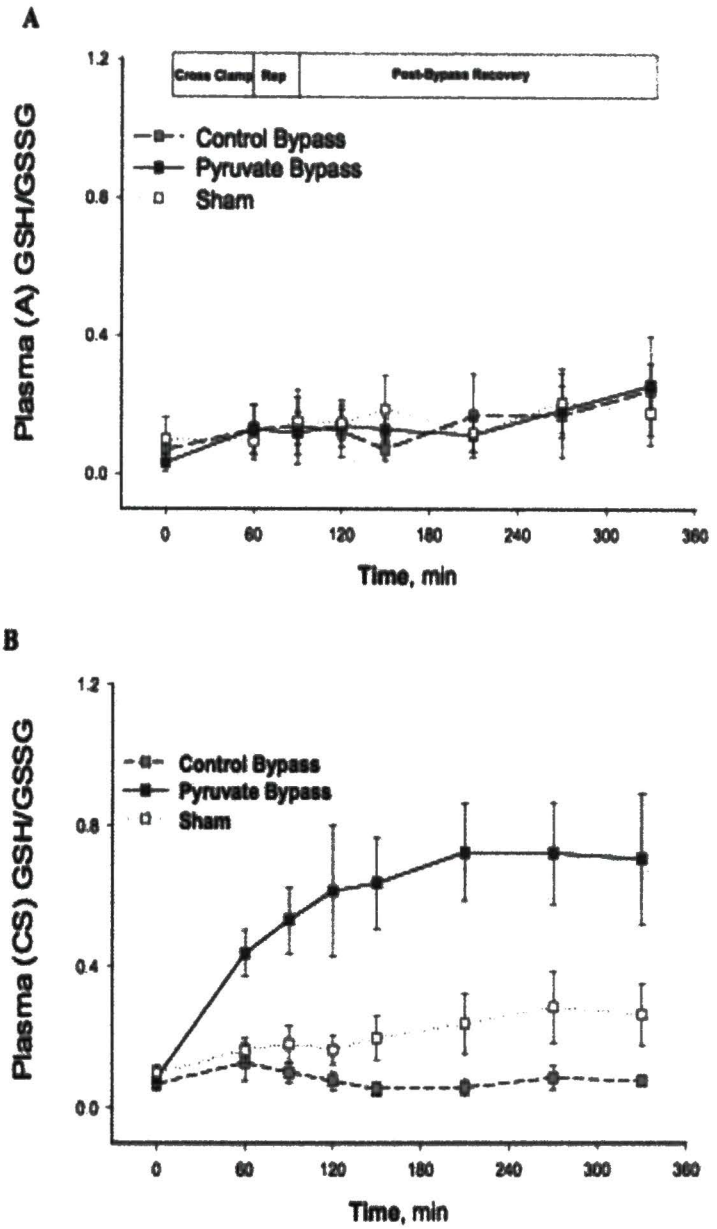

Figure 3. *Arterial and coronary sinus GSH/GSSG redox state.* Data were plotted as mean ± MSE. Panel A displays arterial plasma value. Panel B shows coronary sinus plasma value. After pyruvate treated, pyruvate group shows significant increase in all points *vs.* control and sham. ($P<0.05$)

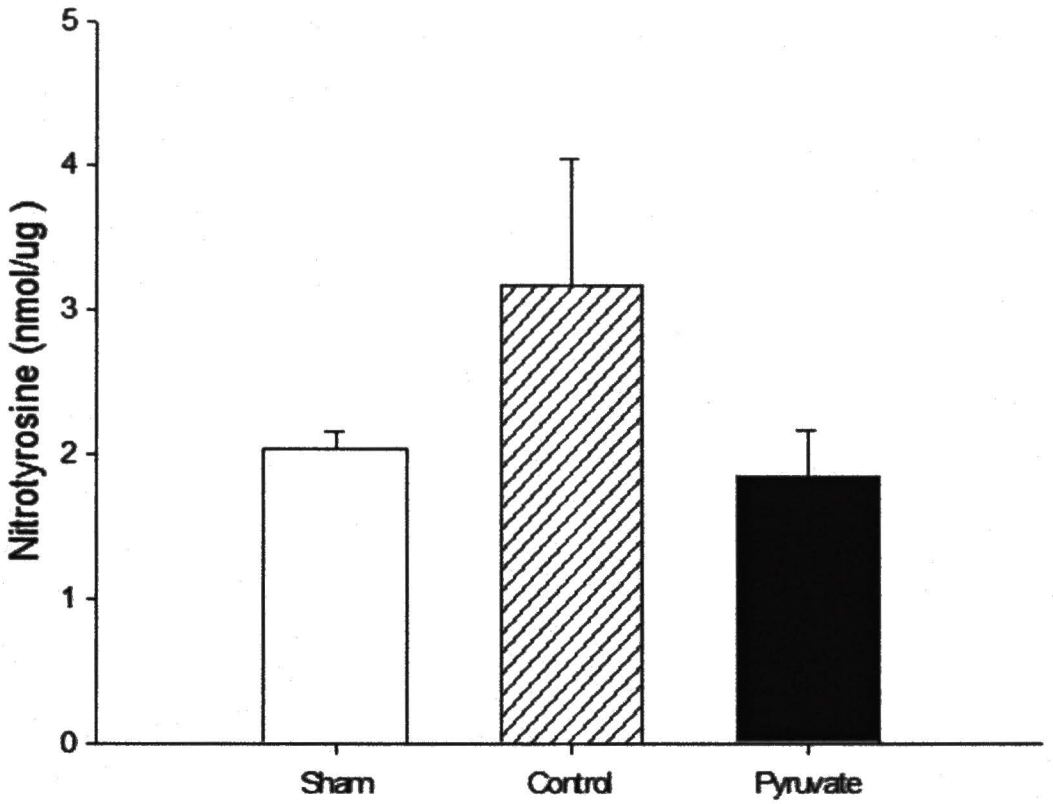

Figure 4. *Nitrotyrosine concentration in left ventricle at the 4 h of recovery from CPB.* Nitrotyrosine concentrations were measured from left ventricular tissue extract. Even though, pyruvate group increased NOS activity, there are no statistical differences between three groups.

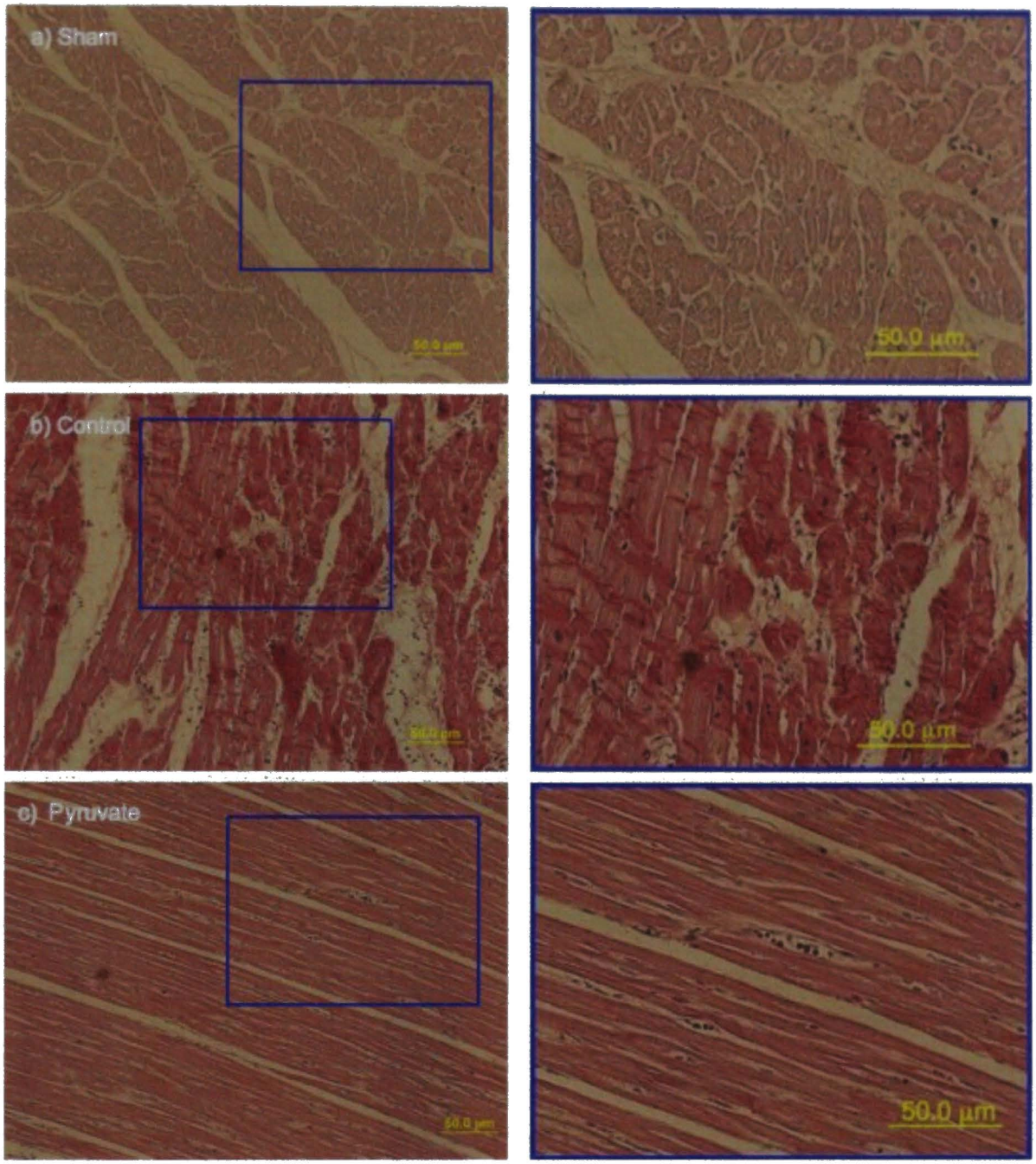

Figure 5. *Histological evidence of neutrophils infiltration in left ventricle.* Tissue slides were prepared by hematocylin-eosin stain. Control slide (panel b) showed high levels of neutrophils infiltration and tissue damage as compared to sham and pyruvate.

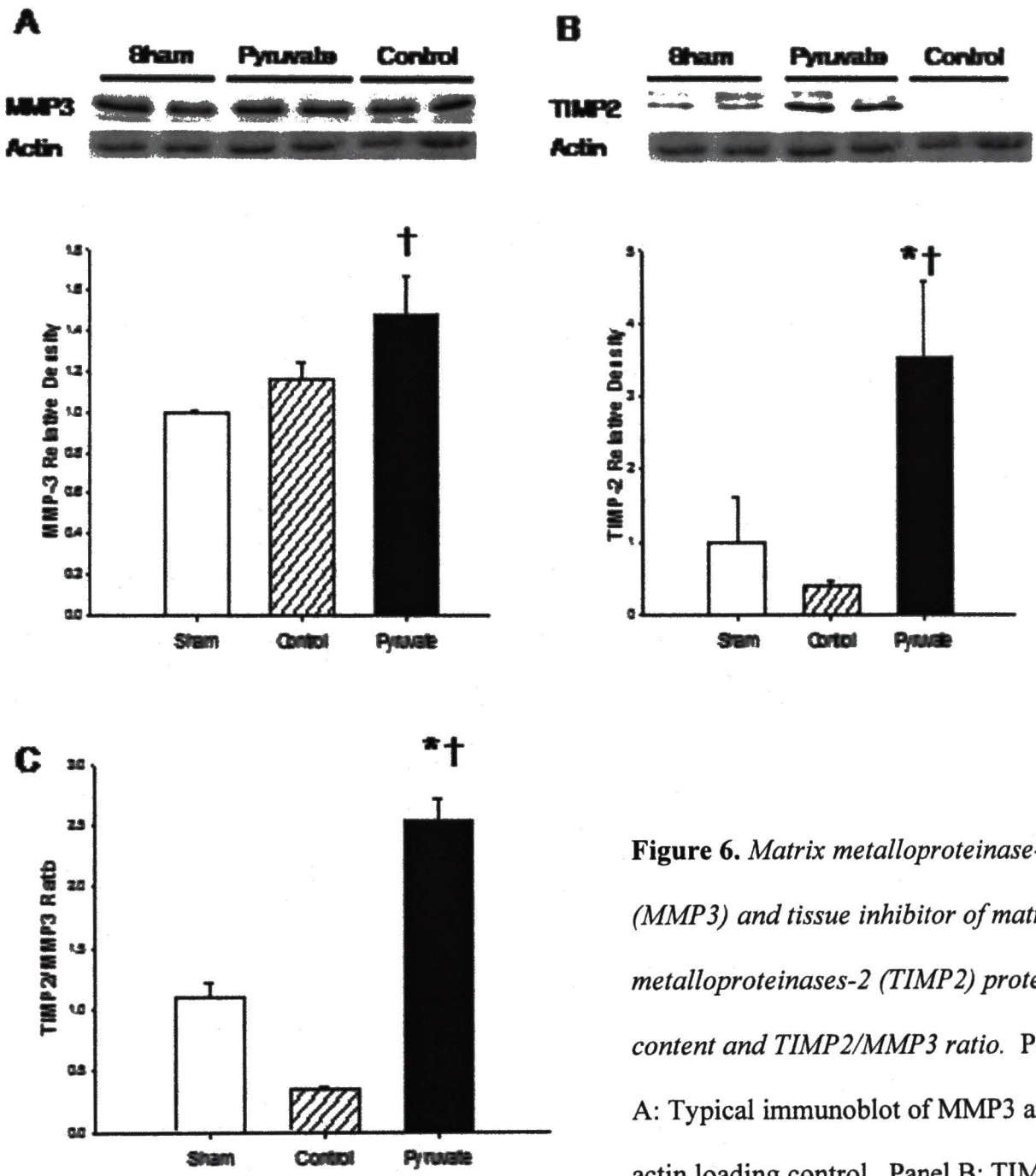

Figure 6. *Matrix metalloproteinase-3 (MMP3) and tissue inhibitor of matrix metalloproteinases-2 (TIMP2) protein content and TIMP2/MMP3 ratio.* Panel A: Typical immunoblot of MMP3 and actin loading control. Panel B: TIMP3 immunoblot and actin loading control. Panel C: TIMP2/ MMP3 Ratio.

* P<0.05 *vs.* Control, † P<0.05 *vs.* Sham

CHAPTER IV

CONCLUSION

This investigation sought to delineate the mechanisms by which pyruvate-fortified cardioplegia protects the myocardium during cardiopulmonary bypass. Specifically examined were the possibilities that pyruvate-enriched cardioplegia activates intracellular signaling mechanisms that protect myocardium from ischemia-reperfusion, and that pyruvate cardioplegia suppresses the inflammatory response to cardiopulmonary bypass. Such actions may subtend the enhancement by pyruvate-fortified cardioplegia of post surgical cardiac recovery in patients undergoing CPB for coronary revascularization [1], and explain how pyruvate-fortified cardioplegia was able to effect persistent enhancement of cardiac performance in these patients several hours after the cardioplegia had cleared from the circulation and myocardium.

The primary goal of this study was to demonstrate cardioprotective mechanism of pyruvate-fortified cardioplegia in swine model of cardiopulmonary bypass. Recent studies in our laboratory demonstrated that pyruvate-fortified cardioplegia reduced oxidative stress and enhanced myocardial antioxidant defenses and energy reserves. These antioxidant and energetic effects could protect the myocardium during the acute oxidative stress imposed by cardioplegic arrest and ischemia-reperfusion, but are unlikely to persist after pyruvate washout. Accordingly, this investigation focused on the possible enhancement by pyruvate-enhanced cardioplegia of cytoprotective proteins and signaling pathways, and pyruvate suppression of systemic inflammation.

We found that myocardial content of α subunit of the oxygen-regulated transcription factor hypoxia inducible factor-1 (HIF-1α) increased following cardiac arrest with pyruvate-enhanced *vs.* conventional control cardioplegia. Increased HIF-1α content was associated with induced expression and synthesis of the anti-apoptotic cytokine, erythropoietin (EPO), and its sarcolemmal receptor, EPO-R. Enhancement of EPO and EPO-R by pyruvate cardioplegia was associated with activation of the cytoprotective signaling kinases Erk and Akt, and increased content and activity of the cardioprotective nitric oxide synthase isoform, eNOS. These signaling elements are known components of EPO's cytoprotective signaling cascade. Second, the hypothetical anti-inflammatory function of pyruvate was examined. The systemic inflammatory reaction to CPB is the central cause of atrial fibrillation, pulmonary and renal insufficiency, neurological impairment and other major postoperative comorbidities. Acute inflammatory marker, C-reactive protein, was significantly lowered in pyruvate group *vs.* control. We also observed that neutrophil filtration was noticeably minimized in pyruvate group as compared to control group. This filtration results were associated with decreased MMP3 activity regulated by increased TIMP2 content. Pyruvate cardioplegia enhanced GSH/GSSG ratio in coronary sinus plasma, an effect that persisted and even intensified during the post-CPB recovery despite washout of the cardioplegia. Due to powerful anti-oxidant effect of pyruvate, in spite of increased NOS activity, nitrotyrosin in pyruvate group was not increased as compared to control group. Also, the anti-inflammatory cytokine, IL-10, and myocardial formation of IL-6, proposed to serve

an anti-inflammatory function when produced within striated muscle, were sharply increased in pyruvate group. These results permit the following conclusion:

1. Pyruvate-fortified cardioplegia increased myocardial content of HIF-1α, the regulated subunit of the transcription factor HIF-1. This pyruvate effect could promote activation of a HIF-1-inducible cardioprotective gene program.

2. Pyruvate-enhanced cardioplegia induced expression and content of the HIF-1-induced cytokine, EPO, in myocardium. This is the first report of myocardial expression and synthesis of this cardioprotective factor.

3. Cardiopulmonary bypass with control cardioplegia depleted myocardial EPO receptor content, but pyruvate cardioplegia preserved EPO receptors.

4. Pyruvate-fortified cardioplegia also increased phosphorylation and, thus, activity of the protein kinases, Erk and Akt, which have been implicated in the signaling mechanisms mediating cardioprotective EPO signaling.

5. Content and activity of endothelial NOS, an effector of the EPO signaling pathway, also were increased by pyruvate-fortified *vs.* control CPB.

6. Pyruvate-fortified cardioplegia increased plasma GSH/GSSG ratio, an effect opposite that of control cardioplegia. This anti-oxidant effect, even though NOS activity in pyruvate group was significantly increased, was closely related with suppressed nitrotyrosin concentration in pyruvate group.
7. The anti-inflammatory cytokine increased sharply in arterial and coronary sinus plasma during and immediately after CPB. Pyruvate-fortified cardioplegia intensified and prolonged the increased in IL-10.

8. Pyruvate-enriched cardioplegia increased myocardial content of TIMP2, the physiological regulator of matrix metalloproteinase-3, an enzyme that degrades the extra cellular matrix, allowing neutrophil infiltration and cardiac remodeling.

9. These results support the hypothesis that pyruvate-fortified cardioplegia improves post-CPB cardiac function by activating EPO signaling, an important anti-apoptotic pathway, and by suppressing the CPB-induced inflammatory response.

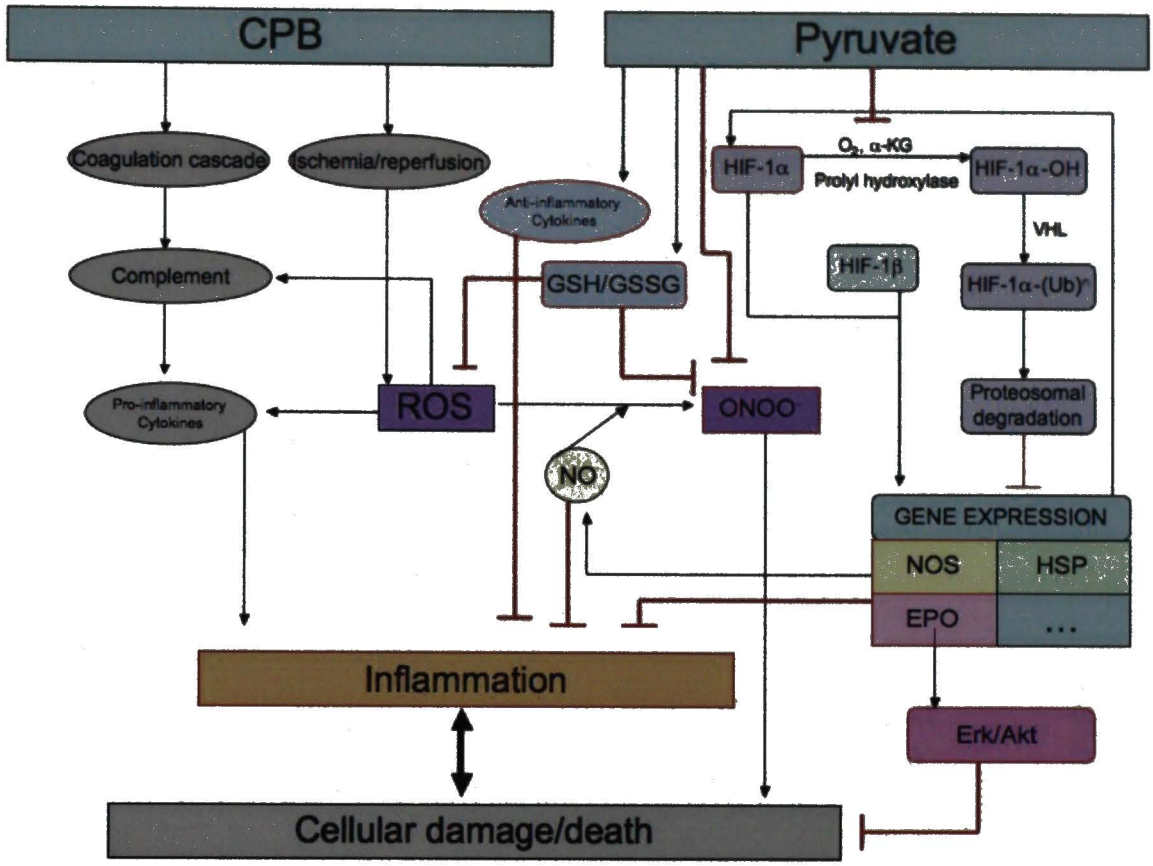

Figure 1. *The mechanism of pyruvate-induced protection against CPB.* Pyruvate's well-appreciated anti-oxidant effect, anti-apoptotic effect by stabilizing HIF-1α, and direct increase in anti-inflammatory cytokines are orchestrated to minimize CPB induced cellular damage. Red-outline of boxes indicates variables we demonstrated in this study. CPB: cardiopulmonary bypass; ROS: reactive oxygen species; NO: nitric oxide; GSH: glutathione; GSSG: Glutathione disulfide; HIF-1α: α subunit of hypoxia inducible factor-1; α-KG: α-ketoglutarate; NOS: nitric oxide synthase; HSP: heat shock protein; EPO: erythropoietin.

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CHAPTER V

PROPOSAL OF FUTUER STUDIES

This investigation, for the first time, demonstrated the involvement of cytoprotective proteins, which are regulated by HIF-1, in pyruvate-fortified cardioplegia induced cardioprotection against CPB. Despite of these convincing evidences, more studies has to be completed in order to further explain the mechanism of cardiac protection by pyruvate in cardioplegia. Even though, the activation of EPO signaling pathway was demonstrated in current studies, actual apoptotic factor was not completely examined. However, anti-apoptotic effect of EPO, previously, has been proposed. The following experiments are proposed to continue this area of investigation:

1. Determine the effects of EPO signaling pathway activation in terms of programmed cell death.
   - Apoptotic markers, including caspases, and Bcl2/Bax ratio, could be examined. Since there is a limitation in antibody availability for swine proteins, RT-PCR for mRNA expression could be the alternative method.

2. Determine further clarification of involvement of EPO in pryruvate-induced cardioprotection.

- EPO-receptor antagonist, recombinant soluble EPO-R, can be used to block the EPO signaling pathway in small rodent model. Also siRNA model can be applied to demonstrate the protective effect of EPO.

3. Define the specific origin of EPO production in the heart; endothelial cell and/or cardiac myocyte.

- In swine experiments, myocyte and endothelial cells in coronary vasculature could not be separated. Using cell lines of human cardiac myocyte and endothelial cells, the source of EPO synthesis could be demonstrated.

The function of HIF-2 in pyruvate-induced protection

The family of HIF-α includes three isoforms: HIF-1α, -2α, and -3α. HIF-3α is an inhibitory PAS domain protein, but HIF -1α and 2α is very similar in structure and functions [1]. HIF-1α and 2α shares most functional domains including bHLH domain [2, 3]. Interestingly, the transgenic mice study, HIF-1α -/-, and HIF-2α -/-, showed various result. HIF-1α -/- mice lead to the lack of VEGF expression and defected angiogenesis in embryonic cell culture [4]. On the other hand, HIF-2α-/- caused defect in fetal catecholamine production [5] or a defect in surfactant deficiency [6]. These results suggested that despite of similar activity on hypoxia response element-linked reporter, HIF-1α and HIF-2α have own specific gene regulatory mechanism.

The stabilization of HIF-1α and -2α is not mainly regulated by the transcription, instead post-translational regulation is the most important part of HIF-1α and -2α regulation. Under normoxic condition, half-life of HIF-1α and 2α is less than 5 min, but under hypoxic conditions, half-life is increased up to 30 min [7]. Even though non-hypoxic HIF-2α stabilizers are not clearly examined, degradation pathway for both HIF-1 and -2 is though prolyl hydroxylation and poly-ubiquitylation [1]. Oxygen dependent degradation (ODD) domain exists in HIF-2α [7, 8]. ODD regulates the HIF-1α and -2α stability by interacting with the von-Hippel-Lindau tumor suppressor gene. Therefore, pyruvate in cardioplegia might stabilize HIF-2α under non-hypoxic conditions. The expression of HIF-2, but not HIF-1, is prominent in highly vascularized organs, such as lung, and endothelium [5, 9]. Thus, HIF-2 shows more organ specific functions.

Based on these characteristics of HIF-1α and -2α, we hypothesized that pyruvate-fortified cardioplegia may maintain both HIF-1α and -2α in heart and brain under non-hypoxic condition. The following specific aims are proposed to address this hypothesis:

1. Determine the ability of pyruvate to stabilize HIF-1α and/or HIF-2α in human endothelial cells and human cardiac myocyte.
2. Compare the content of HIF-1α and -2α in heart and brain in CPB using pyruvate-fortified cardioplegia.
3.

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