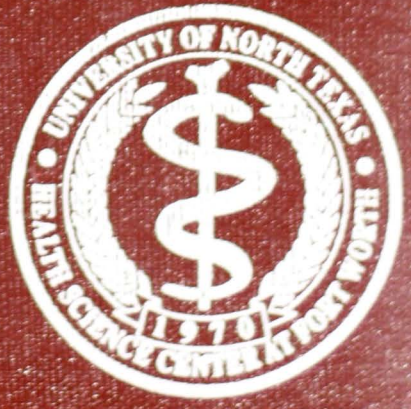
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Approximately 350,000 people experience cardiac arrest in the United States each year, and merely 4-33% of the victims survive to hospital discharge. Cardiac and neurological injuries following resuscitation are the main factors responsible for mortality. Neurodeficit and cognitive dysfunction following recovery from cardiac arrest may persist for up to two years and greatly compromise quality of life in survivors. Loss of effective circulating blood volume during cardiac arrest results in ischemia, energy depletion, ionic imbalance, calcium overload, acidosis and oxidant mediated cytotoxicity. The burst of reactive oxygen species upon reperfusion imposes an oxidant burden resulting in modification of cellular components such as membrane phospholipids and proteins, and the initiation of inflammatory and cell death cascades. This injury is most pronounced in organs with high metabolic demands such as the heart and brain.

Therapies aimed at reducing metabolic impairments such as energy depletion and oxidative stress may mitigate post-resuscitation complications, improve survival and enhance quality of life. Pyruvate, a natural metabolite of the glycolytic pathway, has been shown to enhance post-ischemic energy and antioxidant reserves, and effects improvements in calcium homeostasis and metabolic acidosis. The main purpose of this investigation was to evaluate pyruvate as a corrective metabolic intervention during

cardiopulmonary resuscitation and examine its cardio- and neuroprotective effects following recovery from cardiopulmonary arrest.

To address these objectives a canine model of 5 min cardiopulmonary arrest, open chest cardiac compressions (OCCC) and resuscitation was developed. In the first study intravenous sodium pyruvate or control NaCl was administered during the first 30 min of resuscitation and its effects on cardiac function and metabolites examined through the first 3 h following return of spontaneous circulation. Cardiac arrest resulted in a severe collapse of myocardial phosphocreatine phosphorylation potential and antioxidant redox state. Pyruvate treatment substantially enhanced recovery of energy and antioxidant reserves during early reperfusion. Pyruvate also enhanced contractile performance and carotid blood flow at 15-25 min return of spontaneous circulation (ROSC), and better maintained cardiac function at 3 h ROSC. Thus a latent effect of temporary metabolic correction by intravenous pyruvate therapy during early resuscitation was manifest as improved cardiac function, 3 h after the acute insult.

Oxidative stress during resuscitation can modify membrane lipids and proteins. Inactivation of myocardial enzymes may exacerbate ischemic derangements of myocardial metabolism. To study the impact of cardiac arrest on left ventricular enzymes, beagles were subjected to cardiac arrest and myocardial enzyme activities were measured in snap-frozen left ventricle. Severe depletion of glutathione (GSH) antioxidant redox state occurred during cardiac arrest, which recovered partially following cardiac massage and then completely during early ROSC. Concomitant with oxidant stress,

activities of phosphofructokinase, citrate synthase, aconitase, malate dehydrogenase, creatine kinase, glucose 6-phosphate dehydrogenase and glutathione reductase fell sharply during arrest, and recovered gradually after resuscitation and ROSC, in parallel with GSH redox state. We then tested whether oxidative stress is responsible for the loss of enzyme activity during cardiac arrest. Metabolic (pyruvate) or pharmacological (N-acetylcysteine) antioxidants were infused *iv* for 30 min immediately before cardiac arrest. Antioxidant pretreatments augmented phosphofructokinase, aconitase and malate dehydrogenase activities before arrest, and enhanced these activities, as well as citrate synthase and glucose 6-phosphate dehydrogenase, during arrest. Cardiac arrest thus reversibly inactivates several important myocardial metabolic enzymes, while protection of these enzymes by antioxidants implicates oxidative stress as a principal mechanism of enzyme inactivation.

The third part of this investigation was directed towards addressing the question whether metabolic correction with pyruvate therapy during early ROSC, would extend protection and enhance neurological recovery over an extended period of 3 days following cardiac arrest-resuscitation. Neurological evaluation in the days following recovery from cardiac arrest revealed considerable impairment of function. Activation of matrix metalloproteinases and increased myeloperoxidase activity were also detected in frozen brain tissue. Loss of viable neuronal structure and cell death as indicated by histological evidence and TUNEL were detected 3 days following arrest. Treatment with pyruvate for the first hour of reperfusion prevented neurological deficit on days 1 and 2 of recovery, partially mitigated the inflammatory response and prevented neuronal loss.

By preventing early metabolic disturbances during resuscitation and immediate reperfusion, intravenous pyruvate therapy protected the heart and brain from dysfunction and injury. The following figure summarizes these major findings.

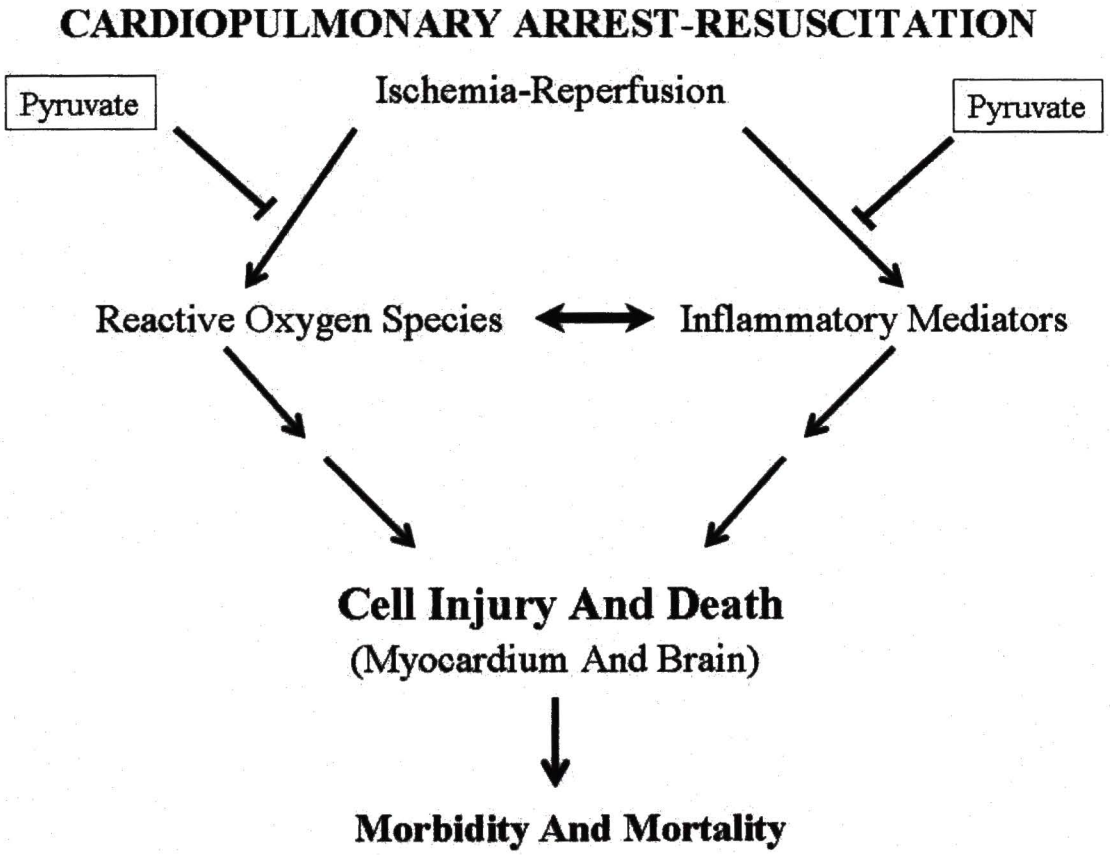

Figure: *Pyruvate mediated metabolic protection following cardiopulmonary arrest-resuscitation.*

PYRUVATE PROTECTION OF MYOCARDIUM AND BRAIN FOLLOWING CARDIOPULMONARY ARREST AND RESUSCITATION

Arti B. Sharma, M.B.B.S

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PYRUVATE PROTECTION OF MYOCARDIUM AND BRAIN FOLLOWING CARDIOPULMONARY ARREST AND RESUSCITATION

DISSERTATION

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LIST OF ABBREVIATIONS

ACO	Aconitase
ANOVA	Analysis of variance
AOP	Mean aortic pressure
ARR	Cardiac arrest
BL	Pre-arrest baseline
CA1	*Cornus ammonis* region 1 of hippocampus
CK	Creatine Kinase
CPR	Cardio-pulmonary resuscitation
Cr	Creatine
CS	Citrate synthase
DAPI	4', 6-diamidino-2-phenylindole
G6PDH	Glucose-6 phosphate dehydrogenase
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GR	Glutathione reductase
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
LDH	Lactate dehydrogenase
LVEDP	Left ventricular end diastolic pressure
MCT	Monocarboxylate transporter
MDH	Malate dehydrogenase

MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NAC	*N*-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NDS	Neurodeficit score
OCCC	Open-chest cardiac compressions
$ONOO^-$	Peroxynitrite
PCr	Phosphocreatine
PFK	Phosphofructokinase
Pi	Inorganic phosphate
ROSC	Return of spontaneous circulation
ROS	Reactive oxygen species
TUNEL	Terminal deoxynucleotidyltransferase mediated dUTP-biotin nick-end labeling

Chapter I

INTRODUCTION

The studies described in the following chapters were conducted to test metabolic intervention with antioxidant and energy substrate sodium pyruvate, in preventing cardiac and neurological damage following cardiopulmonary arrest and resuscitation. It is important to consider that cardiac arrest is a short transient insult resulting in whole body ischemia. Most victims cannot be resuscitated if emergency care is not delivered within the first 5-6 minutes[19] and survival from ventricular fibrillation cardiac arrest decreases 7 to 10% with each minute without defibrillation.[55] Despite technical and therapeutic advances in cardiopulmonary resuscitation (CPR), rates of survival to hospital discharge remain very low.[3,7] The situation does not change with in-hospital arrests, and a mere 30 % of the resuscitated patients survive, primarily due to the limited efficacy of conventional CPR.[1] Outcome of cardiac arrest thus remains poor, not only in terms of life expectancy, but also compromised quality of life of survivors after hospital discharge.[15]

The severe mortality associated with cardiac arrest is largely due to rapid pathophysiological changes that occur within seconds of ischemia in high energy turnover organs, especially the heart and brain. Central nervous system damage causes one-third of the deaths while unresponsive myocardial damage results in another third.[25] Following recovery post-resuscitation syndrome,[71] characterized by multi organ

dysfunction and cardiac insufficiency further contributes to morbidity. The morbidity extends beyond hospital stay as ~ 30% of cardiac arrest survivors exhibit symptoms of remnant neurological dysfunction up to two years following resuscitation.[79, 87] Despite its limited efficacy, cardiopulmonary resuscitation (CPR) remains the basic treatment modality during arrest. A paucity of therapeutic interventions exists which can counter early metabolic disturbances occurring during arrest and immediate reperfusion such as loss of high energy phosphates and oxidative stress that eventually culminate in severe tissue injury.[102, 85]

The object of this investigation was to explore the use of pyruvate, a natural aliphatic carbohydrate with antioxidant and energy-generating properties as a therapeutic intervention for protecting the heart and brain and thereby enhancing CPR and recovery from cardiopulmonary arrest and resuscitation. The studies described in the following chapters support the hypothesis that pyruvate enhances cardiac and neurological recovery from cardiac arrest by providing energetic and antioxidant support for the heart and brain.

Pathological changes during cardiac arrest

Cessation of blood flow during cardiac arrest causes global ischemia and severe reduction in coronary blood flow. Lack of blood flow not only results in failure of oxidative phosphorylation, but also accumulation of metabolic end products such as lactic acid. Oxygen deprivation thus causes rapid depletion of high energy phosphates, i.e. ATP and phosphocreatine, early during ischemia, with accumulation of degradation products of these essential compounds. Initial stimulation of glycolysis is unable to sustain ATP

production as accumulation of metabolic end products leads to an eventual rundown of anaerobic glycolysis. The myocardium is thus highly susceptible to ischemic injury owing to its high basal metabolism and considerable energy requirements. Activation of degradative enzymes compromises cellular integrity and remains unchecked as ATP dependent reparative processes are disabled.[31] Energy depletion thus augments myocardial contractile failure.

Anaerobic metabolism leads to accumulation of lactate, H^+ and K^+ ions further exaggerating existing metabolic acidosis. Disruption in acid-base homeostasis has been shown to decrease myocardial contractility[101] and increase defibrillation threshold,[69] making resuscitation harder to achieve. Rapid accumulation of inorganic phosphate (Pi) resulting from increased ATP degradation and stagnation of blood flow also depresses myocardial contractility during ischemia.[38]

Another consequence of ATP depletion is the failure of ion channel pumps leading to disruption of ionic balance in the cell. Na^+ accumulation hinders effective excitation contraction coupling within the cardiomyocyte, while intracellular compartmentalization of Ca^{2+} is lost in spite of unaltered total cell Ca^{2+}. Failure of the SR Ca^{2+} ATPase to sequester Ca^{2+} raises cytosolic Ca^{2+} markedly. Cytosolic calcium is further raised as calcium is taken up across the cell membrane following reperfusion due to reactivation of Na^+–Ca^{2+} exchange.[11] Increased cytosolic Ca^{2+} not only impedes effective contraction, but also depresses myocardial contractility by activation of calpains, that selectively degrade myofibrils[11] and impair activities of functional proteins such as SERCA2a[31] and

Na^+/K^+-ATPase.[42] Such Ca^{2+} activated proteases also cause activation of xanthine dehydrogenase to xanthine oxidase. The reaction catalyzed by xanthine oxidase produces superoxide oxyradical as byproduct, and thus Ca^{2+} indirectly amplifies ROS formation. Metabolic derangements, energy depletion and decreased sensitivity of contractile proteins to calcium during ischemia lead to contractile failure of the myocardium. A rapid decline in the developed pressure can be observed within minutes of global ischemia.

Ischemia-reperfusion injury following return of spontaneous circulation

Lack of terminal electron acceptor during ischemia produces a backlog of reducing equivalents in the mitochondrial respiratory chain, which contribute to generation of reactive oxygen species (ROS). Upon reperfusion the partially reduced mitochondrial ubisemiquinone radical reacts with oxygen to produce superoxide ($\cdot O_2^-$) and this highly reactive molecule generates other oxidants such as hydrogen peroxide. Another source for myocardial ROS is xanthine dehydrogenase following its conversion to xanthine oxidase by thiol group oxidation and calcium activated proteases. Thus ischemia establishes the environment for ROS burst during reperfusion. ROS generation is further augmented by phagocytic nicotinamide adenine dinucleotide phosphate oxidase generated $\cdot O_2^-$ following re-establishment of perfusion. ROS and increased intracellular Ca^{2+} synergistically mediate reperfusion injury and eventual myocardial dysfunction. ROS can modify protein thiol groups and cause formation of mixed disulfides in proteins via glutathione oxidation and thus, impair mitochondrial respiratory chain activity, inactivate critical enzymes and disable ion pumps. ROS are also responsible for peroxidation of various cell components such as membrane phospholipids and thus

compromise cellular integrity. High mitochondrial matrix [Ca^{2+}] along with ROS can induce mitochondrial transition pore opening, an event that eventually results in apoptotic cell death and irreversible reperfusion injury.[26] ROS generated during ischemia-reperfusion can also act as second messengers in various myocardial signaling processes such as Src family of tyrosine kinases, MAP kinases, small GTP binding proteins, activation of stress related nuclear transcription factors and cytokine cascades. Post-resuscitation syndrome is also characterized by high levels of circulating cytokines, mimicking a systemic inflammatory response.[2] Cardiac arrest and resuscitation induce platelet, neutrophil and endothelial activation and a resultant increase in cytokines such as TNF-α, IL-6, IL-8, IL-10 and IL-1β.[2,32] Circulating activities of these inflammatory markers has been used to even predict the outcome of patients admitted after cardiac arrest-resuscitation.[2] Cytokines in turn amplify ROS production. In transgenic mice overexpressing TNF-α, higher levels of hydroxyl radical and an impaired MnSOD antioxidant capacity were detected in the myocardium.[60] Thus the important mediators of reperfusion injury include calcium overload, reactive oxygen species and inflammation.

Cardiac arrest-resuscitation and myocardium

Global myocardial ischemia during cardiopulmonary arrest and hyperemic reperfusion during ROSC result in myocardial injury that manifests as post-resuscitation syndrome.[46] The decrease in cardiac output and hemodynamic instability observed during recovery is secondary to both systolic and diastolic left ventricular dysfunction.[33] Systolic left ventricular dysfunction manifests as decreased cardiac contractility, with a fall in positive dP/dt, lowering of ejection fraction due to inefficient contractions and a rightward shift in

pressure-volume loop relationship.[33,45,88] Compromised left ventricular diastolic function is also evidenced by a rise in left ventricular end diastolic pressure, decreased negative dP/dt, and echocardiographic abnormalities such as decreased mitral valve deceleration time and increased left ventricular isovolumic relaxation time.

Several pathological changes occurring during cardiac arrest and resuscitation may be responsible for the decline in cardiac function following return of spontaneous (ROSC). Post-resuscitation left ventricular dysfunction observed 2-5 h after ROSC, recovers completely within the next 24-48 h.[45] This reversible form of myocardial dysfunction following brief but severe myocardial ischemia is the hallmark of myocardial stunning.[11] First described by Heyndrickx et al. in 1975,[39] following regional myocardial ischemia, myocardial stunning has also been observed following cardiopulmonary arrest-resuscitation.[33, 45, 80, 90] Factors contributing to the development of post-resuscitation dysfunction include underlying cause of sudden cardiac arrest, response time interval i.e. period of ischemia before CPR is begun, and cumulative energy and repetitions of defibrillatory shocks.[25] Molecular mechanisms underlying loss of myocardial contractility include depletion of available energy sources, acidosis, oxidative stress mediated damage, decreased myofilament Ca^{2+} responsiveness, circulating cytokines and catecholamines, loss of myocardial proteins and enzymes. Myocardial acidosis, which contributes to depressed contractile function[92] can occur without significant alterations in systemic bicarbonate concentrations and continue for an hour following successful resuscitation.

Myocardial stress activates cytokines such as IL-6 and tumor necrosis factor alpha (TNF-α) which are not constitutively expressed in the myocardium. Although required to maintain homeostasis in the face of an insult, an exaggerated response leads to functional impairment and cell loss. Cytokine mediated decrease in contractility[51, 74] may serve as an adaptive response during situations of energy supply:demand imbalance such as ischemia. Myocardial stunning associated with cardiac surgery is accompanied by acute phase reaction and an enhanced inflammatory response.[84]

One of the primary mediators of inflammation TNF- α can trigger receptor mediated cell death by binding to surface receptors TNF-R1 and TNF-R2. In a porcine model of cardiac arrest-resuscitation, 7 min of global ischemia produced a 3 fold increase in TNF-α, sustained throughout 90 min ROSC.[72] TNF- α increase was also observed in patients resuscitated following out of hospital cardiac arrest, and peaked at 6 h reperfusion.[43] Myocardial ischemia reperfusion can trigger cell death pathways and add to existing cardiac dysfunction. Thus treatments that reduce oxidative stress and myocardial inflammatory response during ischemia and reperfusion as a result of cardiac arrest and resuscitation can potentially attenuate subsequent myocardial dysfunction and failure.

Cardiac arrest-resuscitation and brain pathology

Although the brain constitutes only 2% of body weight it receives 20 % of the resting cardiac output. A high energy turnover organ, the brain needs a constant supply of oxygen and glucose and is therefore heavily dependent on its blood supply. Autoregulation of cerebral blood flow and regional blood flow in the brain are

mechanisms that protect the brain against loss of blood supply. During severe ischemia autoregulation fails, and neurons are irreversibly injured within 6-8 min. It has been shown that global cerebral ischemia with cardiac arrest results in loss of selective neuronal subpopulations that are more susceptible to ischemic injury.[86] Since cardiopulmonary arrest and resuscitation results in whole body ischemia-reperfusion metabolic and inflammatory response of other organs also influences the outcome.

Cessation of blood supply and consequently O_2 and glucose delivery leads to exhaustion of cerebral glucose supply within 2-4 min[82] and collapse of energy state within 4-5 min.[23,95] Cardiopulmonary resuscitation generating cerebral perfusion pressures (CPP) greater than 60 mmHg can restore cerebral ATP and intracellular pH.[23] Even though ATP is restored by a lower CPP, ~30 mmHg, neuronal pH does not recover.[24] Conditions causing low perfusion pressures may thus impede restoration of normal cerebral pH, i.e. the metabolic disturbances continue even though some parameters such as ATP are deceptively normal. Situations impeding the generation of adequate CPP can thus hamper the restoration of normal cerebral H^+ ion concentration. A fall in pH hastens neuronal injury and death and therefore acidosis following cardiopulmonary arrest and resuscitation can exacerbate neurological injury.

Failure of ion pumps due to energy depletion causes ionic imbalance, neuronal and interstitial swelling[86] and increased Ca^{2+} uptake by neurons.[4] Increased Ca^{2+} promotes loss of cell membrane integrity, increases in arachidonic acid breakdown products and increased cell death signaling and neuronal loss.[95]

In the brain, moreover, certain regions continue to be ischemic even after establishment of ROSC and normal systemic pressures. This low or no-reflow phenomenon observed exacerbates reperfusion injury, and hence global cerebral blood flow is not an adequate measure of local blood supply. Interspersed in regions of low flow are areas receiving normal or hyperemic blood supply.[53, 96] Post-resuscitation cerebral hypoperfusion may be a result of vasoconstriction, decreased red cell elasticity, increased platelet adhesion, endothelial cell swelling, pericapillary edema, and Ca^{2+} influx.[28] This no-reflow phenomenon may persist for up to 24 hours ROSC. Derangements in energy metabolism accompany post-recovery hypoperfusion. Following 6 min of cardiac arrest, Ellef *et al.* [22] were unable to restore ATP in spite of achieving normal cerebral perfusion pressures during CPR. During the first few minutes of reperfusion cerebral oxygen utilization increases beyond prearrest values. One hour into reperfusion energy metabolism and glucose utilization are depressed and remain so for hours depending on the severity of ischemia.[86] This depression in energy generation and utilization is prominent in ischemia susceptible regions such as the hippocampal CA1 subregion.

Inactivation or degradation of metabolic enzymes by reactive oxygen species, such as pyruvate dehydrogenase[65] may also be partially responsible for depressed cerebral metabolism. Oxidant mediated injury can damage lipids, proteins, DNA, RNA and other structural and functional components of neurons. Oxidative stress in the brain, following cardiac arrest and resuscitation is also evident by formation of 8-isoprostane. Plasma 8-isoprostane, an indicator of lipid peroxidation in the brain, increases during the first 30

min ROSC. Improvement in cerebral blood flow by pharmacological intervention lessens the formation of 8-isoprostane.[59]

Reactive oxygen species generated during ischemia and following reperfusion not only depress cerebral energy metabolism but can also trigger cell death signaling and contribute to delayed neuronal death.[29] Oxidants inflict neuronal damage not only by structural modification but also by secondary signaling mechanisms. The mechanisms responsible for ROS mediated cell death may include increased p53 expression, release of mitochondrial cytochrome c, promotion of mitochondrial transition pore formation and, oxidative modification of mitochondrial membrane lipids. By enhancing inflammation ROS may also trigger receptor mediated cell death signaling and thus result in delayed neuronal injury following transient ischemic insult.[85]

In resuscitated patients a systemic inflammatory reaction is observed. Widespread multiorgan ischemia and injury during cardiac arrest, accompanied by oxidative burst during reperfusion trigger 'sepsis' like syndrome in the post-resuscitation phase.[2] Increased systemic IL-8 and polymorphonuclear elastase have been observed in patients following resuscitation.[70] Dogs subjected to cardiac arrest-resuscitation and treated with anti-inflammatory ibuprofen exhibited better survival rates and lower neurological deficit at 24 h ROSC.[54] Microglial activation following post-ischemic insult results in increased production of nitric oxide, oxyradicals and cytokines. Reactive oxygen species also trigger activation of matrix metalloproteinases.[44] Cerebral edema following post-ischemic recovery is associated with injury to the blood brain barrier and activation of basement

membrane degrading MMPs.[97] Activation of MMPs results in subsequent neuronal loss in the hippocampal CA1 area.[58, 78] Increase in myeloperoxidase activity 3 days following transient forebrain ischemia indicates infiltration of brain parenchyma by activated polymorphonuclear leukocytes and macrophages.[94] Inflammatory response in the brain with increased cytokine signalling, MMP activation, and inflammatory cell infiltration mediate delayed neuronal death and ischemic brain damage.[13, 58, 94]

Reactive oxygen species, increased cytosolic Ca^{2+} and inflammation activate proteases which initiate cell death pathways in the brain. Krajewska *et al.*[48] demonstrated that 10 min cardiac arrest in dogs induces proteolytic processing of several procaspases. A time dependent activation of caspases 3, 6, 8 and 10 is detectable in neurons as well as microglia in the ischemia-sensitive regions of the brain. They also demonstrated an increase in proapototic Bcl-2 family proteins. Moreover, these cell death signals were detectable as early as 30 min and continued for up to 3 days of ROSC. Neuronal loss has been documented 3-30 days following cardiac arrest-resuscitation by several investigators.[13, 41, 59, 98]

Cardiopulmonary resuscitation thus presents a complex clinical scenario with multiple metabolic disturbances that initiate and augment one other. The main pathology underlying cardiac arrest is the loss of blood supply, consequent depletion of energy, and oxidative stress during arrest and following reperfusion. Therapies that enhance recovery of energy sources, and counter antioxidant stress could be protective in both the heart and brain. Several treatment modalities have been tried which aim at reducing acute and

delayed myocardial and neurological damage due to cardiac arrest. These include enhancement of perfusion, reducing calcium accumulation, prevention of acidosis, administration of antioxidants, blocking inflammatory signaling and oxidative stress, and inhibiting cell death triggers such as caspase-3 activation and mitochondrial transition pore formation. The following paragraphs describe the multiple effects of pyruvate in preventing energy depletion, oxidative stress, acidosis, inflammation and cell death following ischemia-reperfusion injury in the heart and brain.

Metabolic protection by pyruvate following ischemia reperfusion in the heart and brain.

A natural aliphatic monocarboxylate and metabolic fuel, pyruvate is protective in conditions of ischemia-reperfusion, documented as early as 1980 by Mochizuki et al.[66] A product of glycolysis, pyruvate protects both the myocardium[10, 16] and the brain.[46, 57] At physiological concentration (0.1-0.2mM), plasma pyruvate is not an important myocardial metabolic fuel, unlike fatty acids and glucose. However, when given at supraphysiological concentrations pyruvate is preferentially transported into the myocardium over other monocarboxylates, by the sarcolemmal monocarboxylate transporter (MCT).[62] Pyruvate is rapidly metabolized and enhances myocardial mechanical function.[61] Pyruvate-fortified cardioplegic solution, tremendously improved left ventricular recovery and decreased the release of myofibril proteolysis products troponin -I and creatine kinase (MB), in patients undergoing cardiopulmonary bypass.[100] Pyruvate also enhances contractile performance in isolated perfused and in vivo

myocardium following acute insults, particularly in stunned or ischemically injured myocardium.[6, 16, 63]

In surgically arrested myocardium pyruvate-fortified cardioplegia bolstered phosphocreatine phosphorylation potential, a measure of myocardial energy reserves.[47] By providing readily available substrate for oxidative phosphorylation, augmenting TCA cycle intermediates and stimulating glycolysis, pyruvate increases ATP formation. [62] Increased availability of ATP for actin-myosin crossbridge cycling may enhance effective myofibril contraction. By increasing Gibb's free energy of ATP hydrolysis, available for ion pumps, pyruvate also enhances ion transport and calcium homeostasis. Pyruvate's inotropic effect has been related to enhancement of Ca^{2+} cycling efficiency of sarcoplasmic reticular (SR) Ca^{2+} ATPase. [37, 61, 64] Pyruvate by enhancing SR calcium uptake also allows better diastolic relaxation and greater filling, thus improves cardiac contraction. In collaboration with reactive oxygen species, Ca^{2+} mediates myocardial stunning following ischemia reperfusion injury.[11] As described above increased cytosolic Ca^{2+} results in several pathological changes during ischemia reperfusion, such as ROS formation, activation of proteases and cell death signaling. By improving Ca^{2+} sequestration, pyruvate may also decrease its untoward effects.

Pyruvate directly neutralizes oxidants such as H_2O_2 and organic peroxides, hydroxyl radical ($OH^{\cdot}$) and peroxynitrite ($OONO^-$). Pyruvate can also indirectly enhance antioxidant redox state by promoting NADPH formation which in turn increases GSH/GSSG. In addition, pyruvate may shift the lactate dehydrogenase equilibrium

towards NADH oxidation and by depriving superoxide generating NADH oxidase of its substrate pyruvate can indirectly reduce oxidative stress.[63] Antioxidant state enhancement by pyruvate has been shown to decrease myocardial injury and improve function following ischemia-reperfusion.[18, 21, 47]

Pyruvate also exhibits anti-inflammatory properties. The pyruvate derivative ethyl pyruvate interferes with pro-inflammatory transcription factor, NF-kappa B signaling and lipopolysaccharide mediated IL-6 release in cultured murine macrophage-like RAW 264.7 cells.[36] Ethyl pyruvate attenuates reperfusion injury during hemorrhagic shock and also suppresses pro-inflammatory gene expression.[27] Ethyl pyruvate is eventually metabolized to pyruvate; however ethanol formation occurs during this process. Acidosis promotes cardiomyocyte cell death and thereby can worsen ventricular dysfunction.[52] Exogenous pyruvate is also an alkalotic agent and corrects systemic metabolic and lactic acidosis.[103] Exogenous pyruvate can consume intracellular protons via several mechanisms: 1) its conversion to lactate via lactate dehydrogenase LDH, 2) by reacting with H^+ ion, pyruvate gets oxidized to CO_2 and H_2O via the TCA cycle, 3) activation of pyruvate dehydrogenase complex, 4) increase in citrate formation and resultant increase in TCA cycle flux and consumption of protons, and 5) pyruvate utilization for gluconeogenesis consumes an additional 2 protons. Exogenous pyruvate administration can thus counter acidosis during cardiac arrest resuscitation which is marked by severe acidosis. Depletion of cellular energy state below critical level can trigger apoptotic processes. By preventing this critical drop pyruvate may also reduce cell death following reperfusion. In a model of hemorrhagic shock, pyruvate administration prevented lipid

peroxidation and PARP cleavage in the porcine brain and liver.[68] Following cardioplegic arrest pyruvate improves functional recovery and dampens cell death in rat hearts.[21] Pyruvate by mediating several metabolic improvements may thus diminish post-ischemic injury and enhance cardiac recovery.

Pyruvate protection in the brain

A normal plasma pyruvate concentration of 0.1- 0.2 mM in fasting dogs can be raised to supraphysiological 5 mM by intravenous pyruvate infusion. Exogenous pyruvate is readily taken up by the brain and quickly equilibrates following intravenous infusion.[35] In the central nervous system, pyruvate is co-transported with a single proton in a bidirectional, concentration dependent manner. This transport will therefore be enhanced in conditions of acidosis such as post-ischemic recovery. Pyruvate enhances energy reserves in the brain following hemorrhagic shock in pigs.[61]

Even though pyruvate acts as an antioxidant, delayed pyruvate treatment, 3h after oxidative insult with H_2O_2 also prevented significant neuronal loss.[20] Thus pyruvate may have prevented secondary cell death signaling. Pyruvate has been shown to reduce inflammatory response and ROS formation in the brain.[81, 99] Activated microglia can produce NO, oxyradicals and cytokines[34] and, are thus instrumental in post-ischemic inflammatory response. Ethyl pyruvate treatment in cultured microglia prevents lipopolysaccharide induced inflammatory activation and nuclear factor-κB activation.[46] Intraperitoneal pyruvate administered 1 h post-reperfusion following 12 min ischemia

suppresses hippocampal neuronal death 72 h later. This protection was related to pyruvate's ability to prevent zinc toxicity following ischemia.[57]

Above mentioned studies indicate that intravenous pyruvate therapy following cardiopulmonary arrest and resuscitation may improve metabolic state by bolstering energetics and antioxidant redox state, enhancing calcium homeostasis, reducing inflammation and prevent cell death, and thus enhance cardiopulmonary resuscitation.

SPECIFIC AIMS

Quality of cardiopulmonary resuscitation (CPR) delivered by well-trained hospital staff is often unable to meet the published recommendations, even during in-hospital arrests. Reperfusion adds to ischemic insult and complicates myocardial and neurological injury. Consequently, cardiac arrest results in low survival rates and high morbidity. Lack of blood flow during arrest results in ischemia and consequently severe metabolic derangements due to depletion of ATP and other high energy phosphates, oxidative stress, acidosis and calcium overload. Additionally, oxyradicals modify cell components such as membrane phospholipids and proteins, and initiate pro-inflammatory and apoptotic signaling. Interventions that can reduce ischemic energy depletion and oxidative stress may prevent post-resuscitation complications and improve survival. Exogenous pyruvate administration reinforces post-ischemic energy reserves and antioxidant redox state; pyruvate also improves calcium homeostasis and corrects metabolic acidosis. The global objective of this project was to examine pyruvate mediated protection of the heart and brain during and following CPR and cardioversion. Specifically the investigation aimed 1) to test the effects of intravenous pyruvate in preventing oxidative stress and energy loss in the myocardium during cardiac arrest-resuscitation, and whether, by preventing such metabolic disturbances, pyruvate therapy would enhance electromechanical recovery and cardiac function during 3 h recovery of spontaneous circulation (ROSC), 2) to test whether cardiac arrest-resuscitation alters myocardial metabolic enzyme activities and whether oxidant stress is responsible for these changes, 3) to determine if immediate metabolic intervention with pyruvate during

early recovery prevents the delayed central neural sequelae of cardiac arrest such as neurological dysfunction, inflammation and neuronal death. To investigate these hypotheses, the following specific aims were addressed:

Specific Aim I: To test the hypothesis that intravenous pyruvate during recovery from cardiac arrest conserves myocardial antioxidant and energy reserves, and enhances post-ischemic electrocardiographic recovery and cardiac mechanical function.

Specific Aim II: To test the hypothesis that myocardial metabolic enzymes are inactivated by oxidative stress during cardiac arrest-resuscitation. The hypothesis predicts that intravenous infusion of antioxidants, pyruvate (metabolic) and N-acetyl cysteine (pharmacological) would prevent such inactivation thus implicating oxidative stress as a mechanism of enzyme inactivation.

Specific Aim III: To test the hypothesis that intravenous pyruvate therapy during the first 60 min of recovery from cardiac arrest would enhance neurological recovery, decrease inflammation and prevent delayed neuronal cell death 3 days following acute insult.

By accomplishing these specific aims this investigation sought to evaluate the natural antioxidant pyruvate as a novel treatment modality to effect improvements in CPR, resuscitability and prevention of post-recovery injury to vital organs. Information resulting from this investigation would provide the empirical foundation to support clinical development of pyruvate therapy as a means of protecting vital organs and supporting recovery of patients from cardiac arrest.

EXPERIMENTAL DESIGN

To investigate specific aims I and II acute surgeries were performed and dogs underwent 5 min cardiac arrest, 5 min open chest cardiac massage and defibrillation, and then recovered for up to 3h following return of spontaneous circulation (ROSC). In the third study surgical procedures were performed under sterile conditions and the period of recovery was extended to three days. A concise description of the experimental design and the rationale behind it for each of the aims is given below.

Intravenous pyruvate mediated enhancement of myocardial electromechanical recovery from cardiopulmonary arrest-resuscitation.

Despite recent improvements in the quality of resuscitative efforts and the promptness of emergency response teams, CPR is much less effective than physiological cardiopulmonary function for delivering O_2 and nutrients to the brain, heart and other internal organs. Consequently, cardiac arrest results in high mortality.[22,40] Loss of ATP production during ischemia compromises several energy dependent processes required to maintain cell integrity and function. Cytotoxic oxidants produced due to buildup of electrons within mitochondrial respiratory chain components inflict further injury on the energy depleted cells. A natural fuel and powerful antioxidant, pyruvate could mitigate the harmful effects of energy depletion and oxidative stress in arrested and reperfused myocardium. To study the effect of intravenous pyruvate therapy on myocardial function following cardiac arrest-resuscitation, beagles were subjected to 5 min cardiopulmonary

arrest and 5 min open chest cardiac compressions (OCCC), followed by defibrillation with internal paddles. An arrest interval of 5 min with 5 min of low flow OCCC ensured a high success rate of resuscitation while producing sufficient pathological changes to clearly delineate an effect of pyruvate treatment. An open chest preparation also facilitated 1) delivery of direct cardiac massage, which considerably improves chances of successful resuscitation[8] and permits control of arterial pressures generated during CPR, and 2) precision sampling of myocardium at desired times to allow measurements of labile energy metabolites. A total of 64 beagles (33 males/31 females) were randomly assigned to non-arrested sham controls or cardiopulmonary arrest-resuscitation with intravenous pyruvate or NaCl as iso-osmotic vehicle control. At 5 min arrest a 2 M solution of either pyruvate or NaCl was administered via peripheral vein and the infusion (0.125 $mmol \cdot kg^{-1} \cdot min^{-1}$) continued for 25 min ROSC. In pilot studies it was determined that pyruvate administered at the rate of 0.125 $mmol \cdot kg^{-1} \cdot min^{-1}$ achieved therapeutically effective plasma concentrations. Return of spontaneous circulation following defibrillation was defined by mean aortic blood pressures of $\geq$ 60 mmHg and sinus cardiac rhythm. Arterial plasma was collected at various times during ROSC, flash frozen and then analyzed for carbohydrate concentrations (pyruvate, lactate and glucose). Partial pressures of O_2 and CO_2 and pH of arterial blood were maintained within physiological limits during ROSC. Myocardial metabolites [adenosine triphosphate (ATP), phosphocreatine (PCr), creatine (Cr), inorganic phosphate (P_i), lactate, pyruvate, glutathione (GSH) and glutathione disulfide (GSSG)], were measured in left ventricular myocardium clamp frozen at the end of ROSC.

Oxidative stress inactivates myocardial metabolic enzymes during cardiac arrest-resuscitation.

Generation of oxidants following myocardial ischemia-reperfusion modifies myocardial proteins, [77] that may include metabolically important enzymes. Inactivation, degradation and loss of such proteins may in turn hamper recovery of various pathways required for repair and maintenance of cell functions, such as glycolysis, mitochondrial electron transport, ATP synthesis and transport and repair of structural damage. In the first study it was obvious that cardiac arrest resulted in severe oxidative stress in the myocardium and depleted cardiac reducing power, the glutathione redox state (GSH/GSSH). This study therefore examined the effect of cardiopulmonary arrest-resuscitation on myocardial metabolic enzymes. Dogs were subjected to 5 min cardiopulmonary arrest and 5 min resuscitation, and then defibrillated to restore spontaneous circulation. Clamp-frozen left ventricular biopsies were taken at various times up to 180 min ROSC. Enzyme activities and glutathione redox state were measured with colorimetric assays. As anticipated, depletion of antioxidant state paralleled fall in enzyme activities, and both recovered gradually after reperfusion. To elucidate whether oxidant mediated injury resulted in enzyme inactivation, antioxidant pretreatments (pyruvate or N-acetyl cysteine) were infused intravenously. Maximal oxidative stress and enzyme inactivation occurred during arrest. Accordingly, these treatments were administered immediately before cardiopulmonary arrest, so they would be present in the myocardium at the onset of cardiac arrest.

Pyruvate prevents neurological injury during 3 days recovery from cardiopulmonary arrest and resuscitation.

A mere 3-10% of patients successfully resuscitated from cardiac arrest are able to return to their previous lifestyles.[95] Neurological injury during transient ischemic episodes manifests as usually unrecognized delayed neuronal loss that can continue up to a month following acute insult.[57, 98] Oxidative stress and inflammation following transient cerebral ischemia contribute to delayed neuronal loss.[59, 13] To examine whether early metabolic correction by pyruvate enhanced neurological recovery from arrest, mongrel dogs were subjected to cardiopulmonary arrest-resuscitation protocol under sterile surgical conditions. The animals were then allowed to recover over next 3 days. Intravenous pyruvate or equimolar NaCl was infused throughout 5 min OCCC and the first 55 min of ROSC. Kristo *et al.* found that pyruvate treatment was more effective in preventing post-ischemic damage when administered for a 60 min period, instead of a shorter duration.[50] Cerebral metabolism and oxygen consumption following cardiopulmonary arrest and resuscitation returns to normal within the first hour of recovery.[73] Also, the results from the first study indicated that most metabolic disturbances recover within 1 h ROSC. This study therefore examined neuroprotection following 60 min of pyruvate infusion. Arterial plasma 8-isoprostane was monitored as an index of systemic oxidative stress. To measure inflammation matrix metalloproteinase (MMP) and myeloperoxidase activities were assayed using fluorogenic MMP assay and a colorimetric assay, respectively, in frozen samples of the ischemia-susceptible hippocampal CA1 subregion. Neuronal death in the CA1 area was examined by fluorometric assay of activated caspase-3, histological quantification and TUNEL assay on day 3 of ROSC.

METHODS

This section describes the considerations behind choosing certain techniques and analytical procedures used in these studies. A detailed description of the experimental design and methods for each study are given in the chapters that follow. To induce cardiac arrest a 9 V direct current shock was administered on the epicardium, to initiate ventricular fibrillation. The most common pathology underlying sudden cardiac arrest, ventricular fibrillation[76] depletes cardiac energy resources rapidly due to uncoordinated cardiomyocyte contraction and deranged cation homeostasis. Electrocardiogram was used to confirm induction of ventricular fibrillation and the return to normal sinus rhythm following defibrillation.

Pyruvate has been shown to boost cardiac function when administered < 30 min after reperfusion,[56, 89] and other antioxidants given immediately before or at reperfusion also mitigate reperfusion injury.[12] Therefore the ability of pyruvate to enhance resuscitation and cardiac contractile recovery was tested by administering pyruvate during CPR and the first 25 min of ROSC. Adenosine triphosphate (ATP), the immediate energy source for muscle contraction, is rapidly degraded during ischemic conditions. However, measurement of ATP concentration alone does not provide sufficient information about the cellular energy state. Instead it is the Gibbs free energy of ATP hydrolysis that determines whether sufficient energy reserves are available for effective muscle contraction. Therefore myocardial phosphocreatine phosphorylation potential $[PCr]/\{[Cr][P_i]\}$, an indicator of Gibbs free energy of ATP hydrolysis was measured to

define the energy state of the heart during cardiopulmonary arrest-resuscitation.[91] Vehicle control for sodium pyruvate infusion was 2M NaCl administered at the same rate as pyruvate, i.e., 0.125 $mmol \cdot kg^{-1} \cdot min^{-1}$. It has been observed that hypertonic fluids are protective during resuscitation.[14, 49] By using 2 M NaCl as control, the protective effects of pyruvate could be attributed to its enhancement of myocardial metabolism and not its hypertonicity per se.

To determine the effect of cardiac arrest-resuscitation on key myocardial metabolic enzymes, enzyme activities were measured at 37° C using spectrophotometric methods.[9] Left ventricular myocardium was snap frozen with liquid N_2-precooled Wollenberger tongs, in order to arrest myocardial metabolism. Enzyme activities were measured in protein extracts of myocardium. Activities of myocardial enzymes were expressed as units per mg protein, where one unit equals 1 μmol substrate converted to product per min. All groups were measured in the same assay sequence to minimize effects of differences in buffer preparation. Enhancements in enzyme activities in arrested hearts could have resulted from artifactual effects of residual antioxidants NAC and pyruvate in the extracts, which may have protected the enzymes during extraction. To exclude this possibility untreated myocardium was extracted in the absence and presence of added antioxidants, and enzyme activities remeasured. Adding the antioxidants did not affect the measured enzyme activities. The glutathione redox state (GSH:GSSG) remains in a state of near equilibrium with other intracellular antioxidant systems.[30] Therefore the glutathione redox state was used as a global index of the collective status of myocardial antioxidant reserves.

Lastly, we examined pyruvate's ability to prevent delayed injury to the brain following cardiopulmonary arrest-resuscitation. Several investigators have considered 72 h ROSC as the optimal time point to examine neuronal loss in areas susceptible to injury following transient ischemia.[5, 57, 98] Neuronal loss and inflammation was therefore examined in the hippocampal CA1 subregion 3 days after cardiopulmonary arrest-resuscitation. Pyruvate or NaCl control were infused iv (0.125 $mmol \cdot kg^{-1} \cdot min^{-1}$) throughout 5 min OCCC and the first 55 min following cardioversion. Sham dogs underwent similar surgical procedures as the two arrested groups but weren't subjected to cardiac arrest protocol, nor did they receive intravenous infusions of pyruvate or NaCl. The sham group controlled for stress and inflammatory response to invasive surgical procedures and general anesthesia. The sham preparation also allowed control for procedural artifacts in histological appearance of neurons. Lastly, fluorogenic assays were used to detect activated caspase-3 and matrix metalloproteinase activities. By expressing activities per mg protein, potential differences due to a variable response in protein synthesis or efficiency of extraction were nullified.

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Chapter II

PYRUVATE IMPROVES CARDIAC ELECTROMECHANICAL AND METABOLIC RECOVERY FROM CARDIOPULMONARY ARREST AND RESUSCITATION

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Abstract

Severe depletion of myocardial energy and antioxidant resources during cardiac arrest culminates in electromechanical dysfunction following recovery of spontaneous circulation (ROSC). A metabolic fuel and natural antioxidant, pyruvate augments myocardial energy and antioxidant redox states in parallel with its enhancement of contractile performance of stunned and oxidant-challenged hearts. This study tested whether pyruvate improves post-arrest cardiac function and metabolism. Beagles were subjected to 5 min cardiac arrest and 5 min open-chest cardiac compression (OCCC: 80 compressions min^{-1}; aortic pressure 60–70 mmHg), then epicardial dc countershocks (5–10 J) were applied to restore sinus rhythm. Pyruvate was infused i.v. throughout OCCC and the first 25 min ROSC to a steady-state arterial concentration of 3.6 ± 0.2 mM. Control experiments received NaCl infusions. Phosphocreatine phosphorylation potential (PCr) and glutathione/glutathione disulfide ratio (GSH/GSSG), measured in snap-frozen left ventricle, indexed energy and antioxidant redox states, respectively. In control experiments, left ventricular pressure development, dP/dt and carotid flow initially recovered upon defibrillation, but then fell 40–50% by 3 h ROSC. ST segment displacement in lead II ECG persisted throughout ROSC. PCr collapsed and GSH/GSSG fell 61% during arrest. Both variables recovered partially during OCCC and completely during ROSC. Pyruvate temporarily increased PCr and GSH/GSSG during OCCC and the first 25 min ROSC and enhanced pressure development, dP/dt and carotid flow at 15–25 min ROSC. Contractile function stabilized and ECG normalized at 2–3 h ROSC, despite post-infusion pyruvate clearance and waning of its metabolic benefits. In

conclusion, intravenous pyruvate therapy increases energy reserves and antioxidant defenses of resuscitated myocardium. These temporary metabolic improvements support post-arrest recovery of cardiac electromechanical performance.

Keywords: Cardiac arrest; Electrocardiography; Free radical; Metabolism; Open-chest cardiac compression (OCCC); Stunning; Myocardial

Abbreviations: AOP, mean aortic pressure; ARR, cardiac arrest; BL, pre-arrest baseline; CPR, cardiopulmonary resuscitation; Cr, creatine; dP/dtmax, dP/dtmin, maximum and minimum rates of left ventricular pressure change; GSH, glutathione; GSSG, glutathione disulfide; LVEDP, left ventricular end-diastolic pressure; OCCC, open-chest cardiac compression; PCr, phosphocreatine; PCr, phosphocreatine phosphorylation potential (i.e. [PCr]/{[Cr][Pi]}); Pi, intracellular inorganic phosphate; ROSC, recovery of spontaneous circulation.

Introduction

Despite recent progress in delivery of emergency medical care, cardiac arrest remains the leading cause of death in the United States and Western Europe. Only a minority of victims survive to hospital discharge, [1] even when arrest occurs in the hospital. [2] Cardiopulmonary resuscitation (CPR) remains the only available intervention to sustain the victim until cardioversion; however, the systemic arterial pressures generated by CPR are inadequate to prevent ischemic deterioration of underperfused organs, including the heart. Cardiac injury during arrest and resuscitation culminates in post-arrest cardiac insufficiency, the 'post-resuscitation syndrome' [3] characterized by low cardiac output, hemodynamic instability and myocardial stunning [3] and [4].

Ischemic tissue injury is caused by ATP depletion, which compromises energy dependent processes that maintain cellular function and integrity and reactive oxygen species (ROS) that attack and disable cellular proteins. Even brief periods of cardiac arrest threaten energy reserves and compromise function and viability of cardiomyocytes. Although interventions that preserve cellular energy resources and minimize ROS formation could interrupt the pathogenesis of cardiac injury during CPR, such interventions remain elusive.

Pyruvate, a natural aliphatic monocarboxylate and product of glycolysis, has been found to be protective against ischemic and oxidant-induced injury of the myocardium [5], [6], [7] and [8]. A readily oxidized metabolic fuel, pyruvate bolsters cytosolic energy state,

thereby providing energy to maintain cellular processes, including Ca^{2+} transport [9] and [10] in the face of metabolic challenges. Pyruvate also functions as an antioxidant [11] and [12]. Recent studies in this laboratory demonstrated that pyruvate increased glutathione (GSH) and NADPH redox potentials in stunned [13] and H2O2-challenged [14] guinea-pig hearts. Pyruvate also has proven effective in, in situ hearts of large mammals. For example, pyruvate restored contractile function of stunned canine [15] and porcine [8] myocardium and decreased infarction following prolonged coronary occlusions in pigs [16].

This study tested the ability of pyruvate to improve post-arrest mechanical function, preserve energy resources and bolster antioxidant defenses in, in situ canine hearts. Pyruvate was infused systemically throughout open-chest cardiac compression (OCCC) and the first 25 min of post-defibrillation recovery. Pyruvate treatment increased myocardial energy state during OCCC and glutathione redox state following defibrillation. Moreover, pyruvate hastened electrocardiographic recovery, increased left ventricular contractility during the early recovery period and lessened later declines in cephalic blood flow. These results suggest that pyruvate, by providing crucial metabolic energy and antioxidant support, could protect the myocardium during CPR, and thus, facilitate post-arrest cardiac recovery.

Materials and methods

Surgical preparation, instrumentation and electrocardiography

Animal experimentation was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication 85–23, revised 1996). Adult beagles (8–16 kg; 33 males, 31 females) were fasted overnight, then randomly assigned to the pyruvate, NaCl vehicle or sham control groups described below. Dogs were sedated with morphine sulfate (3 mg kg^{-1} s.c.) and anesthetized with α-chloralose (100 mg kg^{-1} i.v.; Sigma, St. Louis, MO). Supplemental α-chloralose was administered as needed to maintain anesthesia. The dogs were intubated and mechanically ventilated (Harvard Apparatus Respirator) with room air enriched with supplemental oxygen. Vinyl cannulae were inserted into the femoral arteries and advanced into the abdominal aorta for measurement of blood pressure and blood sampling. Arterial pH, PO2 and PCO2 were kept within their respective physiological limits (7.35–7.45, 95–105 and 35–45 mmHg) by administering $NaHCO^3$ *iv* and by adjusting tidal volume and ventilatory frequency. Femoral veins were cannulated for administration of sodium pyruvate or NaCl and supplemental anesthetic. Core temperature was monitored with a rectal thermometer and maintained at 36–37 °C with heating pads. A Transonic 2SB flow probe was placed around the left common carotid artery for measurement of cephalic blood flow.

Standard limb lead II electrocardiogram was continuously monitored. ST segment displacement, an early measure of cardiac ischemic injury [17] and [18], was determined

from the vertical deflection of the ST segment relative to the TP segment. ST displacement was expressed as a fraction of the vertical QRS deflection to control for between-experiment differences in signal amplitude.

The heart was exposed via a left lateral thoracotomy through the fifth intercostal space and suspended in a pericardial cradle. A Millar Instruments model SPR-5243F pressure transducer was inserted into the left atrial appendage and advanced into the left ventricle to monitor intraventricular pressure, the rate of pressure change (dP/dt) and heart rate. A vinyl cannula was placed in the right atrium and its distal end connected to a Statham pressure transducer to monitor right atrial pressure and to administer intracardiac medications during the arrest/resuscitation protocol. Positive end-expiratory pressure of 2 cm H2O was applied following thoracotomy to prevent atelectasis. Hemodynamic and electrocardiographic data were acquired with IOX-Base-8 and IOX-Cardio-8 software (EMKA Technologies, Falls Church, VA).

Cardiac arrest and cardiopulmonary resuscitation protocol

Baseline measurements were taken after post-surgical stabilization. Ventricular fibrillation arrest was initiated by applying 9 V current to the left ventricular epicardium. Mechanical ventilation was interrupted at the onset of cardiac arrest. At 4.5 min arrest, a 1 mg bolus of epinephrine (adrenaline) was injected into the right atrium. Internal CPR was continuously administered between 5 and 10 min arrest by OCCC (80 compressions min−1) to establish and maintain a mean aortic pressure of approximately 60 mmHg. The dogs were mechanically ventilated at 12 cycles min−1 during CPR. At 10 min arrest, a 5

J dc countershock was delivered to the epicardium with internal paddles (Burdick dc-190 defibrillator). In the event that the first countershock failed to achieve cardioversion, intervening OCCC was performed during the 30 s interval before the next attempt. Up to four 5 J countershocks, followed by up to three 10 J countershocks were administered, with intervening OCCC. One dog could not be defibrillated and was excluded from further analysis. Recovery of spontaneous circulation (ROSC) was confirmed by spontaneous cardiac depolarization and mean aortic pressure ≥60 mmHg. NaHCO3 (10 mEq) was injected into the right atrium at 30 s ROSC. In the event of ventricular tachycardia, lidocaine (6 mg) was administered via the right atrium to help restore sinus rhythm. Following defibrillation, ventilatory frequency was set initially at 20 cycles min−1 to reduce post-arrest hypercapnia, then gradually decreased after hypercapnia abated.

Sodium pyruvate was infused continuously into the right femoral vein from the start of OCCC to 25 min ROSC. The infusion rate (0.125 $mmol \cdot kg^{-1} \cdot min^{-1}$) achieved a steady state pyruvate concentration of 3.5 mM in the systemic arterial blood (Fig. 1A). Untreated dogs received equimolar NaCl 'vehicle' infusion during the same period. Sham control dogs were surgically prepared, instrumented and monitored for the same duration as the other groups, but were not subjected to the cardiac arrest protocol and did not receive pyruvate or NaCl infusion.

Analytical procedures

Partial pressures of O2 and CO2 and pH of arterial blood samples were measured in an Instrumentation Laboratory Synthesis 30 blood gas analyzer and Instrumentation Laboratory model 682 Co-oximeter. Arterial glucose and lactate concentrations were measured in a Yellow Springs Instruments model 2300-l lactate analyzer. For pyruvate measurements, plasma was obtained by centrifugation of arterial blood, then flash frozen in liquid N2. The plasma samples were thawed and extracted with 1 volume of 0.6 N HClO4 to precipitate proteins. Pyruvate in plasma extract was assayed colorimetrically [19] in a Shimadzu Instruments model UV-1601 dual wavelength UV–vis spectrophotometer (337 nm measuring wavelength, 417 nm reference wavelength, ε = 5.65 $M^{-1}cm^{-1}$).

At the conclusion of the experiment, flash-frozen biopsies of the anterior left ventricular wall were obtained with liquid N_2-precooled Wollenberger tongs. Myocardial metabolites {ATP, phosphocreatinine (PCr), creatine (Cr), inorganic phosphate (Pi), lactate, pyruvate, glutathione and glutathione disulfide (GSSG)}, were extracted and assayed by standard techniques [20] and [21]. Myocardial energy state was assessed from PCr phosphorylation potential, i.e. [PCr]/{[Cr][Pi]} [20]. GSH/GSSG ratio was taken as a measure of GSH redox state [13] and [14].

Statistics

Data are reported as means ± S.E.M. The sample sizes of each group were chosen to detect a difference in maximum dP/dt of 400 mmHg s^{-1} and in GSH/GSSG of 8 at power ≥0.8. With 10 unpaired samples and estimated S.D. of 300 for dP/dt, and 6 unpaired

samples and estimated S.D. of 6 for GSH, the power of the study was estimated at 0.83 for dP/dt and 0.85 for GSH/GSSG at $\alpha = 0.05$. Accordingly, 10 experiments per group were conducted for analysis of hemodynamic function during the full protocol, and 6 experiments per group were conducted for measurement of biochemical variables at the intermediate time points of 5 min arrest, 5 min CPR and 25 min ROSC. Between group comparisons of hemodynamic variables, ST displacement and metabolite contents were accomplished by one-way ANOVA. Repeated-measures ANOVA was used for comparing values at different time points within each group. Student–Newman–Keul's multiple comparison tests were performed when ANOVA detected significant differences. P-values < 0.05 were taken to indicate statistical significance.

Results

Cardiopulmonary resuscitation and defibrillation

Continuous intravenous infusion of pyruvate or NaCl was initiated at 5 min cardiac arrest and maintained throughout CPR and the first 25 min ROSC. Open-chest cardiac compressions at 80 compressions min−1 and mechanical ventilation at 12 cycles min^{-1} were initiated at 5 min arrest. During CPR, aortic pressure (NaCl: 62 ± 3 mmHg; pyruvate: 60 ± 3 mmHg), cardiac output (NaCl: 16.7 ± 4.5 ml min^{-1} kg^{-1}; pyruvate: 12.0 ± 2.8 ml $min^{-1}kg^{-1}$) and carotid blood flow (NaCl: 2.0 ± 0.2 ml min^{-1} kg^{-1}; pyruvate: 1.6 ± 0.2 ml min^{-1} kg^{-1}) were similar in the two treatment groups (P = NS). Beginning at 5 min CPR, epicardial dc countershocks were applied at 30 s intervals with intervening OCCC until sustained defibrillation and ROSC were attained. Cumulative electrical energies required to achieve ROSC were 10.6 ± 3.1 J in NaCl-infused controls and 10.3 ± 1.4 J in pyruvate-treated dogs (P = NS).

Arterial carbohydrates and acid–base chemistry

Arterial plasma concentrations of pyruvate, lactate and glucose during the cardiac arrest/resuscitation protocol are presented in Fig. 1. Plasma carbohydrate concentrations were stable throughout the sham control experiments. In the NaCl-infused dogs, plasma lactate concentration increased approximately five-fold and glucose concentration doubled during CPR and 0–15 min ROSC. Arterial pyruvate concentration increased from 0.1 to 0.5 mM over the same period, roughly in parallel with lactate. Carbohydrate concentrations gradually returned to baseline values by 2 h ROSC.

Intravenous pyruvate infusion increased arterial pyruvate concentration from pre-arrest baseline of 0.22 ± 0.02 mM to 15.4 ± 2.8 mM at 3 min CPR (Fig. 1A). Pyruvate concentration fell after defibrillation despite continuous infusion, then stabilized at 3.6 ± 0.2 mM. Its concentration gradually returned to baseline after infusion was discontinued at 25 min ROSC. Pyruvate administration intensified plasma lactate accumulation during CPR and ROSC (Fig. 1B). Even after pyruvate was discontinued, arterial lactate and glucose concentrations exceeded those of the NaCl-infused dogs.

In the NaCl experiments, systemic arterial pH (Fig. 2B) and bicarbonate concentration (Fig. 2C) fell sharply during CPR and the first 5 min ROSC, then gradually returned to sham control levels by 40 min ROSC. Similar acidemia occurred in the pyruvate group at 5 min ROSC, but afterwards, moderate alkalemia and bicarbonate accumulation ensued. This alkalemia and base excess persisted for 2 h. Accordingly, the amount of supplemental bicarbonate administered to the pyruvate dogs (0.45 ± 0.15 mEq kg^{-1}) was only 17% of that given to the NaCl dogs (2.8 ± 0.3 mEq kg^{-1}). Moderate plasma Na+ accumulation occurred in both cardiac arrest groups (Fig. 2D).

Left ventricular contractile function

Left ventricular mechanical performance was assessed from heart rate × developed pressure product, left common carotid flow (a measure of cranio-cephalic blood flow) and peak positive and negative dP/dt. Rate × pressure product gradually increased in the sham experiments due to a modest increase in heart rate (Fig. 3A). In both arrested

groups, rate × pressure product exceeded baseline at 5 min ROSC. This variable then declined sharply in the NaCl experiments, but fell more gradually and was better maintained in the pyruvate-treated experiments.

The maximum value of the first derivative of left ventricular pressure, dP/dtmax, served as an index of myocardial contractility, and the minimum dP/dt value during isovolumetric relaxation, dP/dtmin, provided a measure of lusitropic function. Baseline dP/dt values were similar in the three groups, and both dP/dtmax and dP/dtmin were stable throughout the sham control experiments (Fig. 3B). dP/dtmax was somewhat elevated above baseline in both arrested groups at 5 min ROSC. Afterward, dP/dtmax of NaCl experiments quickly fell and remained below pre-arrest baseline and sham control values for the duration of the experiment. dP/dtmax fell more gradually in the pyruvate group, but by 60–90 min ROSC dP/dtmax and dP/dtmin were similar in the NaCl and pyruvate groups. Later, dP/dtmax and to a lesser extent dP/dtmin partially recovered in the pyruvate group, but not in the NaCl experiments. Thus, temporary pyruvate support during CPR and early ROSC minimized the later impairments of left ventricular dP/dt and carotid blood flow despite the decline in plasma pyruvate to near baseline concentrations.

Carotid blood flow was stable throughout the sham control protocol (Fig. 3C). In NaCl-treated, arrested dogs, carotid blood flow was impaired immediately after defibrillation but nearly recovered to pre-arrest baseline by 40 min ROSC. Flow then declined over the next 2 h ROSC before stabilizing at 50% of baseline. Pyruvate treatment facilitated rapid,

robust recovery of carotid flow. After pyruvate infusion, carotid flow temporarily fell to that of the NaCl experiments, but was better maintained at 150–180 min ROSC.

Left ventricular preload, i.e. end-diastolic pressure and afterload, i.e. mean aortic pressure, are shown in Fig. 3D. End-diastolic pressure did not differ among the groups at any time. Although a tendency toward increased aortic pressure in the pyruvate versus NaCl experiments was noted at 90–180 min ROSC, possibly due to pyruvate stabilization of cardiac performance, these differences were not statistically significant. Thus, the effects of treatment on left ventricular rate × pressure product and dP/dt were not due to differences in loading conditions.

Electrocardiography

Post-arrest electrocardiographic dysfunction was assessed from the vertical displacement of the ST segment from the TP segment, scaled to the height of the QRS deflection, in limb lead II electrocardiogram. Both the NaCl and pyruvate experiments displayed marked ST segment displacement at 25 min ROSC, just before the infusions were discontinued (Fig. 4), but subsequent electrocardiographic recovery differed in the two groups. In the NaCl group, appreciable ST displacement persisted for 3 h ROSC. In contrast, ST displacement completely resolved by 2 h ROSC in the pyruvate group.

Tissue pyruvate and lactate

Profound ischemia during cardiac arrest increased myocardial lactate content several-fold (Fig. 5). In the NaCl group, the progressive decline in tissue lactate during OCCC and

ROSC mirrored the declining plasma lactate concentration (Fig. 1B). In the pyruvate group, tissue pyruvate content was highest during CPR, then fell 60% by 25 min ROSC despite continued infusion. By 180 min ROSC, pyruvate had almost completely cleared from the myocardium. Lactate content of the pyruvate group exceeded that of the NaCl group at 25 min ROSC, then fell with the decline in tissue pyruvate content.

Energy metabolites and phosphorylation potential

Adenosine triphosphate is the immediate energy source for myocardial contractile performance and cardiac electrophysiological function, both of which recovered more completely in pyruvate- than NaCl-treated dogs. Accordingly, the impacts of cardiac arrest, CPR and pyruvate therapy on high-energy phosphate metabolites and phosphocreatine phosphorylation potential [PCr]/{[Cr][Pi]}, a measure of Gibbs free energy of ATP hydrolysis [22] were determined in left ventricular myocardium. ATP content fell by 40% during 5 min cardiac arrest (Fig. 6A), but declined no further during CPR and slowly recovered during ROSC in both treatment groups. Phosphocreatine content changed even more than ATP during the protocol (Fig. 6B), as expected [23].

Phosphocreatine and creatine varied reciprocally; thus, creatine accumulated during arrest and returned to baseline as phosphocreatine recovered during OCCC and ROSC. Pyruvate tended to increase phosphocreatine and lower creatine content during OCCC, but had no appreciable impact on either compound after ROSC. Phosphocreatine phosphorylation potential collapsed during cardiac arrest (Fig. 6C). The 5 min CPR did produce a modest, albeit partial recovery of phosphorylation potential despite continued

arrest. Phosphorylation potential recovered completely by 25 min ROSC. During CPR, pyruvate administration doubled phosphorylation potential ($P < 0.05$ versus NaCl). Although pyruvate infusion was maintained until 25 min ROSC, its enhancement of phosphorylation potential was no longer statistically significant ($P \sim 0.1$). At 3 h ROSC, phosphorylation potential was nearly identical in the two groups.

Glutathione redox state

Myocardial antioxidant defenses were assessed from the redox state of glutathione, the most abundant low molecular weight antioxidant in cardiomyocytes and a central component of the cell's antioxidant defenses [24]. The GSH/GSSG redox state fell by over 60% within 5 min cardiac arrest (Fig. 7); thus, this brief period of arrest imposed oxidant stress on the myocardium, despite the interruption of O2 delivery. GSH redox state partially recovered by 5 min CPR, and pyruvate administration tended to increase it even further. GSH/GSSG recovered almost completely by 25 min ROSC. Pyruvate increased GSH/GSSG by an additional 60% at this time. The enhanced GSH redox state waned only slightly post-pyruvate. Thus, the greatest enhancement of GSH redox state by pyruvate occurred after defibrillation, and this effect proved more persistent than pyruvate enhancement of phosphorylation potential.

Discussion

Depletion of myocardial energy reserves and antioxidant defenses during cardiac arrest culminates in protracted contractile dysfunction, the 'post-resuscitation syndrome', after sinus rhythm is restored. This study sought to determine whether systemic administration of pyruvate during resuscitation and the early recovery period could mitigate subsequent cardiac impairment in open-chest dogs. After 5 min cardiac arrest, OCCC was administered for 5 min; then, direct epicardial countershocks were delivered to restore spontaneous cardiac rhythm. Cardiac function temporarily recovered following defibrillation, but later weakened as indicated by appreciable declines in carotid blood flow and left ventricular dP/dt. Electrophysiological dysfunction was manifested by marked ST segment displacement following defibrillation, which only partially resolved over the next 3 h.

Intravenous pyruvate infusion, initiated during cardiac massage and sustained for the first 25 min of post-arrest recovery, increased left ventricular contractility (i.e. dP/dtmax), lusitropy (dP/dtmin) and carotid blood flow, albeit modestly. After pyruvate infusion was discontinued, the decline in cardiac function to that of NaCl controls was in parallel with the fall in arterial pyruvate concentration. However, the later declines in dP/dtmax, dP/dtmin and carotid flow were less severe in the pyruvate experiments. Pyruvate did not prevent ST segment displacement during early recovery, but effected complete normalization of electrocardiographic function by 2–3 h ROSC. Thus, temporary pyruvate administration during CPR and early recovery ameliorated cardiac mechanical

impairment and hastened electrophysiological recovery even after pyruvate cleared from the circulation.

Phosphorylation potential, glutathione and pyruvate-enhanced contractile function

Pyruvate has repeatedly been found to increase phosphocreatine phosphorylation potential in isolated, perfused heart preparations [10] and [25], but has less impact on phosphorylation potential in in vivo canine heart [27] and [28]. Pyruvate enhancement of myocardial energy state could be modulated by energy demand, especially in vivo. During OCCC, when arrested hearts did not perform external work, and thus, had decreased energy demands, pyruvate doubled phosphorylation potential versus the NaCl-infused controls. In the first 25 min ROSC, pyruvate treatment increased myocardial contractile state and, thus, energy demand, which may have constrained pyruvate enhancement of phosphorylation potential during recovery. A similar phenomenon was reported by Ochiai et al. in in situ canine myocardium [27] in that study, pyruvate, administered i.v. to a systemic concentration of 3.5 ± 0.4 mM, did not independently increase myocardial PCr/ATP ratio (a 31P NMR-detectable measure of phosphorylation potential), but did partially maintain PCr/ATP ratios in the face of adrenergic stimulation. Nevertheless, the improved contractile function at 15–25 min ROSC in the present study could not be due solely to increased energy state.

The glutathione redox couple functions in near-equilibrium with other intracellular antioxidants, including the ascorbate/dihydroascorbate system of the cytosol and the lipid-soluble Vitamin E and lipoic acid systems of cellular membranes [24] and [29].

Accordingly, GSH redox state provides an integrated measure of the global redox potential of all the cellular antioxidant systems. GSH/GSSG fell sharply, by 61%, during cardiac arrest, but partially recovered during OCCC. A further decline in GSH/GSSG might be expected during OCCC, if its partial restoration of coronary flow and myocardial O_2 delivery had produced a burst of oxyradical production [30]. Instead, the partial recovery of GSH/GSSG during OCCC and its full recovery during ROSC suggest that 5 min arrest in this preparation did not provoke a robust oxyradical burst following reperfusion.

Pyruvate treatment increased GSH redox state following ROSC. Pyruvate's α-keto-carboxylate structure and unique metabolism make it a powerful antioxidant in myocardium and other tissues [12]. We previously demonstrated that pyruvate potentiates β-adrenergic inotropism in stunned guinea-pig myocardium [7]. Pyruvate's antioxidant properties, manifest as increased GSH/GSSG, appeared to effect this potentiation [13], possibly by restoring β-adrenergic signaling mechanisms impaired by oxyradical attack [31]. Recent investigations in rabbit [32] and human [33] myocardium have confirmed pyruvate's interaction with catecholamines. It seems likely that pyruvate potentiated the inotropic response to endogenous catecholamines released during cardiac arrest [34] and [35] to boost cardiac performance during ROSC.

Increased GSH redox state may have contributed to pyruvate enhancement of electrocardiographic recovery. The sarcolemmal Na^+, K^+ ATPase and sarcoplasmic reticular Ca^{2+} ATPase are two prime targets of oxyradical attack [36], [37] and [38].

Impairment of these ion pumps disrupts transmembrane cation electrochemical gradients, producing electrocardiographic dysfunction. ST segment displacement persisted throughout ROSC in the NaCl group, but resolved by 2 h in the pyruvate experiments. It is possible that the pyruvate-enhanced GSH redox state either prevented or reversed oxyradical modifications of ion pumps.

Plasma pyruvate concentrations

During CPR, intravenous pyruvate administration produced systemic arterial pyruvate concentrations of 15.4 ± 2.8 mM, far above the steady-state concentrations achieved following defibrillation. This initial spike in arterial pyuvate likely resulted from adding exogenous pyruvate to the limited circulating volume generated by OCCC. During ROSC, arterial pyruvate concentrations stabilized at approximately 3.5 mM at the same infusion rate. These concentrations approach the optimum concentration range (5–10 mM) for pyruvate enhancement of contractile performance in stunned isolated guinea-pig hearts. Arterial pyruvate concentrations gradually subsided post-infusion.

Arterial pyruvate concentration increased noticeably in the NaCl controls, especially during the first 60 min ROSC. Increased pyruvate paralleled lactate concentration, which plateaued at 5–15 min ROSC and likely resulted from lactate oxidation by circulating lactate dehydrogenase. Although well below levels achieved by intravenous administration, these pyruvate concentrations may have been sufficient to produce some enhancement of contractile function and metabolism. Conversely, pyruvate infusion exacerbated arterial lactate accumulation. At high concentrations, lactate can suppress

myocardial function [40] and may have constrained the improvements in cardiac function and metabolism produced by pyruvate administration.

Pyruvate and post-resuscitation acid–base balance

Pyruvate infusion produced marked alkalinization of systemic arterial blood following defibrillation, accompanied by HCO^- accumulation. These effects slowly waned after pyruvate infusion was discontinued. At least three mechanisms may have contributed to this phenomenon: (1) with a pKa of 2.5, pyruvate is a base and H^+ acceptor at plasma pH; (2) some of the infused pyruvate was reduced to lactate by the H^+ consuming lactate dehydrogenase reaction, which intensified systemic lactate accumulation during ROSC; (3) pyruvate and lactate enter cells via a plasma membrane monocarboxylate:H^+ co-transport mechanism [41], which removes H^+ from the extracellular fluid. Decreased H^+ concentration in the blood would increase $HCO3^-$ concentration via carbonic anhydrase. Interestingly, arterial pH and $HCO3^-$ concentration remained elevated long after pyruvate infusion, which lowered the $HCO3^-$ requirement to maintain pH. A recent investigation in pigs [16] reported a similar $HCO3^-$ accumulation during i.v. pyruvate infusion, although it did not reach statistical significance in that study.

Limitations

This study was conducted in an open-chest, anesthetized canine preparation to facilitate tissue sampling and to standardize aortic pressures at 60–70 mmHg during cardiac massage. Important differences between this model and closed-chest cardiac arrest must be acknowledged. For example, the dogs were maintained under a surgical plane of

anesthesia, the coronary perfusion pressures produced by OCCC in this study exceed those generated by closed-chest pre-cordial compressions, and defibrillatory countershocks were delivered directly to the heart. Pre-intervention arrest and CPR were limited to 5 min each to ensure high rates of cardioversion and ROSC in this investigation. These phases are typically longer in clinical settings, particularly in cardiac arrests outside of hospital. Therefore, it is pre-mature to extrapolate the results of this study to closed-chest cardiac arrest.

Intravenous pyruvate administration produced a moderate, sustained increase in plasma sodium. Higher pyruvate concentrations or more prolonged infusions may have proven even more efficacious, but would have incurred a greater sodium burden.

Pyruvate was the only intervention tested in this study. Compared with other metabolic fuels and antioxidants, pyruvate was uniquely able to simultaneously increase mechanical performance, phosphorylation potential and GSH redox state of isolated hearts [7], [13] and [14]. Whether other metabolic substrates or pharmacological antioxidants can improve post-arrest recovery of in situ myocardium remains to be tested.

Conclusions

This investigation tested the ability of intravenously administered pyruvate to restore myocardial electromechanical function and energy and antioxidant resources during open-chest cardiac compression and defibrillation. The results of this investigation

support the following conclusions: (1) open-chest cardiac compression partially restores myocardial energy and antioxidant redox states; (2) intravenous pyruvate infusion improves post-arrest myocardial electromechanical function; (3) pyruvate bolsters critical energy resources and glutathione redox state in resuscitated myocardium. Thus, temporary pyruvate therapy to improve myocardial energy and antioxidant states in the peri-resuscitation period produces more sustained improvements in left ventricular mechanical and electrocardiographic function, even though its metabolic benefits wane post-infusion.

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Figure legend

Figure. 1. *Plasma carbohydrate concentrations.* Pyruvate (panel A), lactate (panel B) and glucose (panel C) were measured in systemic arterial blood sampled during pre-arrest baseline (BL), at 3 min cardiac arrest (A), at 3 min cardiopulmonary resuscitation (C) and for 180 min following recovery of spontaneous circulation (ROSC). The period of intravenous pyruvate or NaCl infusion is indicated by the bars at the top of each panel. Values are means ± S.E.M. from 11 pyruvate-treated ( ), 11 NaCl-treated ( ) and 10 non-arrested sham control (●) experiments. *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group.

Figure. 2. *Arterial acid–base chemistry and sodium.* PCO2 (panel A), pH (panel B) and bicarbonate (panel C) and sodium (panel D) were measured in systemic arterial blood samples. Data are from the same experiments as Fig. 1. ARR: cardiac arrest; CPR: cardiopulmonary resuscitation; other abbreviations as in Fig. 1. Sham control (●); NaCl group (□), pyruvate group (▲). *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group.

Figure. 3. *Left ventricular contractile function.* Left ventricular mechanical performance was assessed from heart rate times developed pressure, i.e. mean aortic pressure (AOP) minus left ventricular end-diastolic pressure (LVEDP; panel A), the first derivative of left ventricular pressure, dP/dt (panel B) and carotid blood flow (panel C). AOP and LVEDP are presented in panel D. Abbreviations are the same as in Fig. 1 and Fig. 2. *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group.

Figure. 4. *Electrocardiographic dysfunction following cardiac arrest, CPR and defibrillation.* Vertical ST segment displacement from the TP segment was normalized to the height of the QRS complex to correct for between-experiment differences in electrical signal. Open bars: sham controls; hatched bars: NaCl group; filled bars: pyruvate-treated group. Values are from the same experiments as Fig. 1, Fig. 2 and Fig. 3. *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group.

Figure. 5. *Myocardial lactate and pyruvate contents.* Metabolites in this figure and Fig. 6 and Fig. 7 were measured in extracts of snap-frozen myocardium. Values are means ± S.E.M. from 6 to 11 experiments. *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group.

Figure. 6. *Myocardial high-energy phosphates.* ATP (panel A); phosphocreatine (PCr) and creatine (Cr) (panel B) were measured in snap-frozen myocardium. Creatine contents of each group are indicated by the finely hatched bars that overlap the PCr bars. PCr phosphorylation potentials (panel C) were computed from PCr and Cr contents and intracellular Pi concentration, as previously reported [20] and [21]. Abbreviations on the abscissa are the same as in Fig. 5. Panels A and C: *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group. Panel B: *P < 0.05 vs. sham PCr content; ‡P < 0.05 vs. sham Cr content.

Figure. 7. *Glutathione redox state.* Glutathione (GSH) and glutathione disulfide (GSSG) were measured in snap-frozen left ventricular biopsies to determine glutathione redox state, GSH/GSSG. Abbreviations on the abscissa are the same as in Fig. 5 and Fig. 6. *P < 0.05 vs. sham; †P < 0.05 vs. NaCl group.

Figures

Figure 1

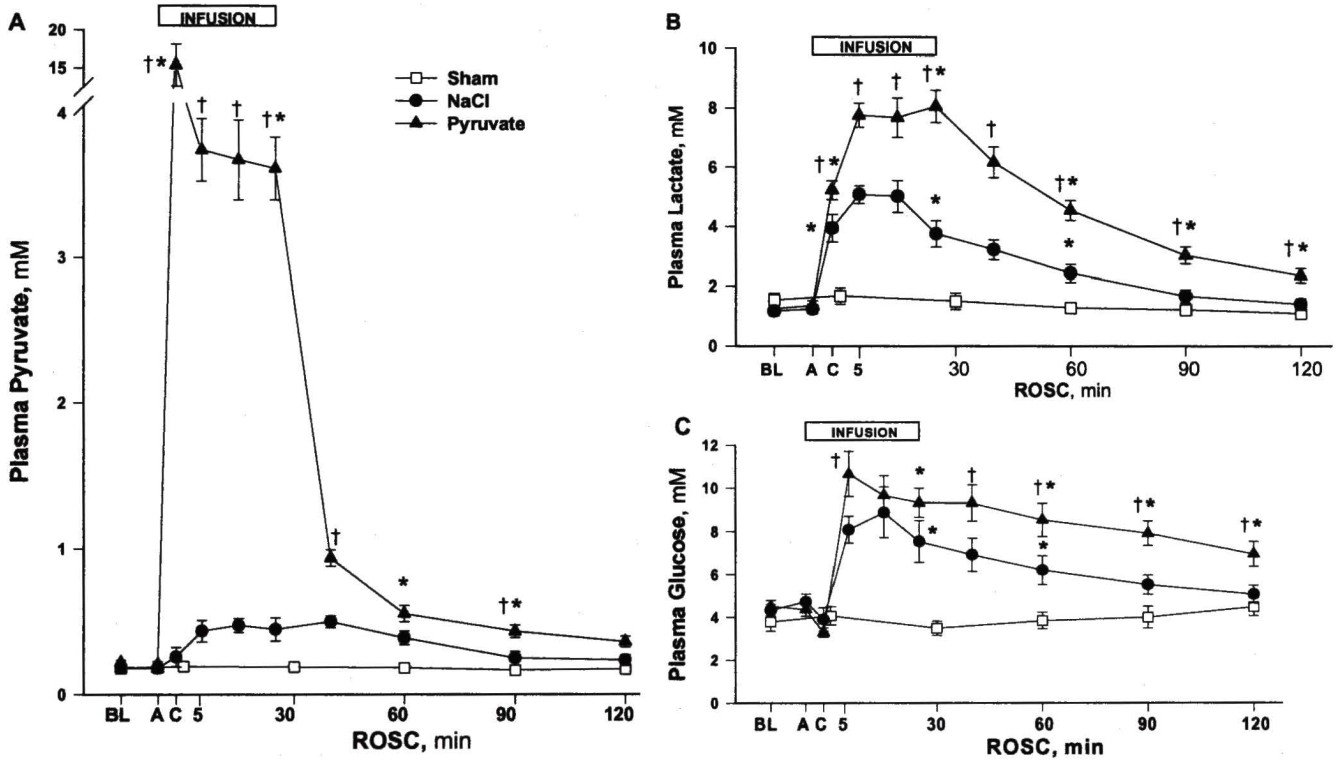


Figure 2.

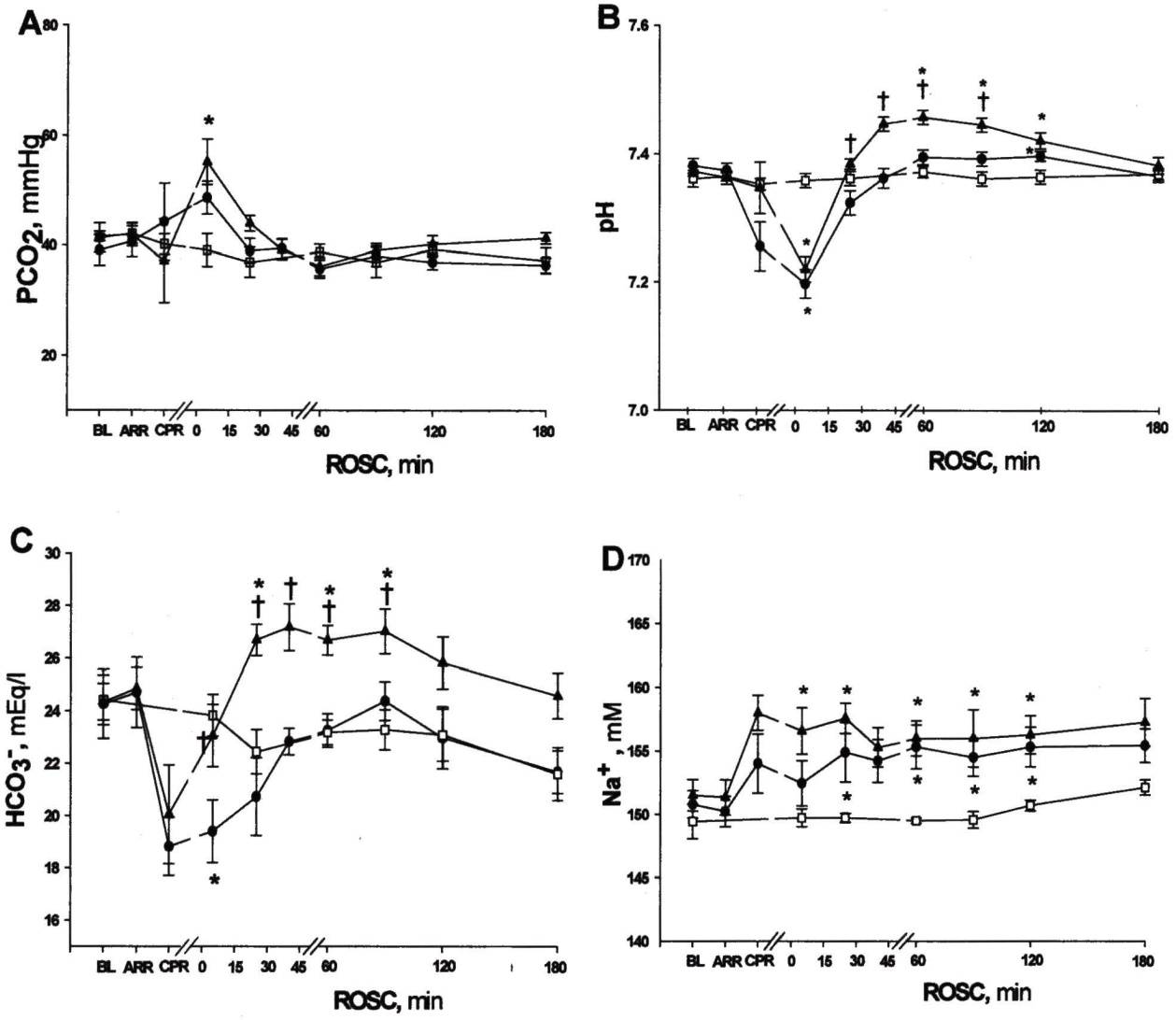

A
PCO_2, mmHg
80
60
40
20
BL ARR CPR 0 15 30 45 60 120 180
ROSC, min
B
pH
7.6
7.4
7.2
7.0
BL ARR CPR 0 15 30 45 60 120 180
ROSC, min
C
HCO_3^-, mEq/l
30
28
26
24
22
20
18
16
BL ARR CPR 0 15 30 45 60 120 180
ROSC, min
D
Na^+, mM
170
165
160
155
150
145
140
BL ARR CPR 0 15 30 45 60 120 180
ROSC, min

Figure 3

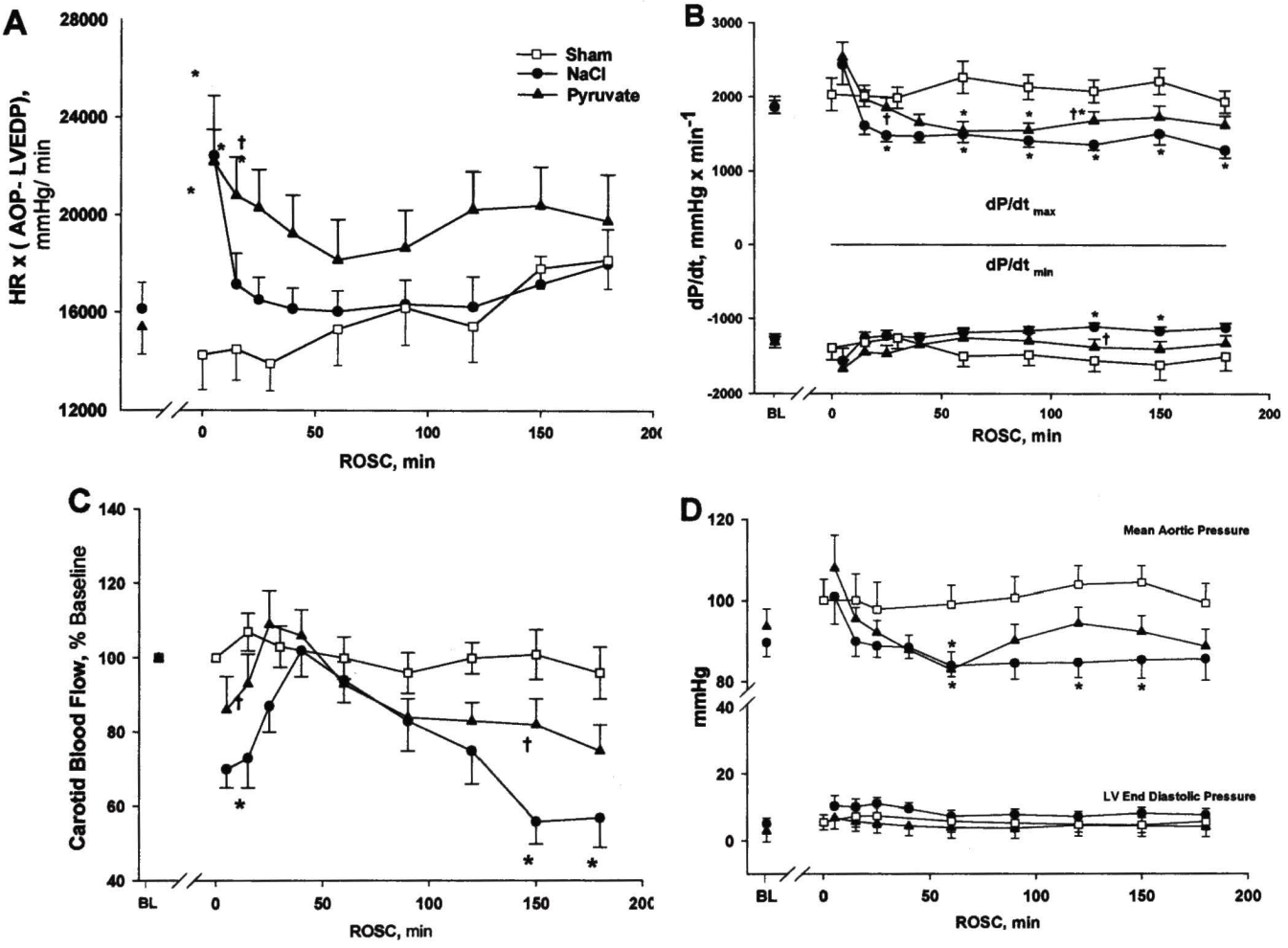

A
HR x (AOP- LVEDP), mmHg/ min
28000
24000
20000
16000
12000
Sham
NaCl
Pyruvate
0
50
100
150
ROSC, min
B
dP/dt, mmHg x min-1
3000
2000
1000
0
-1000
-2000
dP/dtmax
dP/dtmin
BL
ROSC, min
C
Carotid Blood Flow, % Baseline
140
120
100
80
60
40
BL
ROSC, min
D
mmHg
120
100
80
40
20
0
Mean Aortic Pressure
LV End Diastolic Pressure
BL
ROSC, min

Figure 4

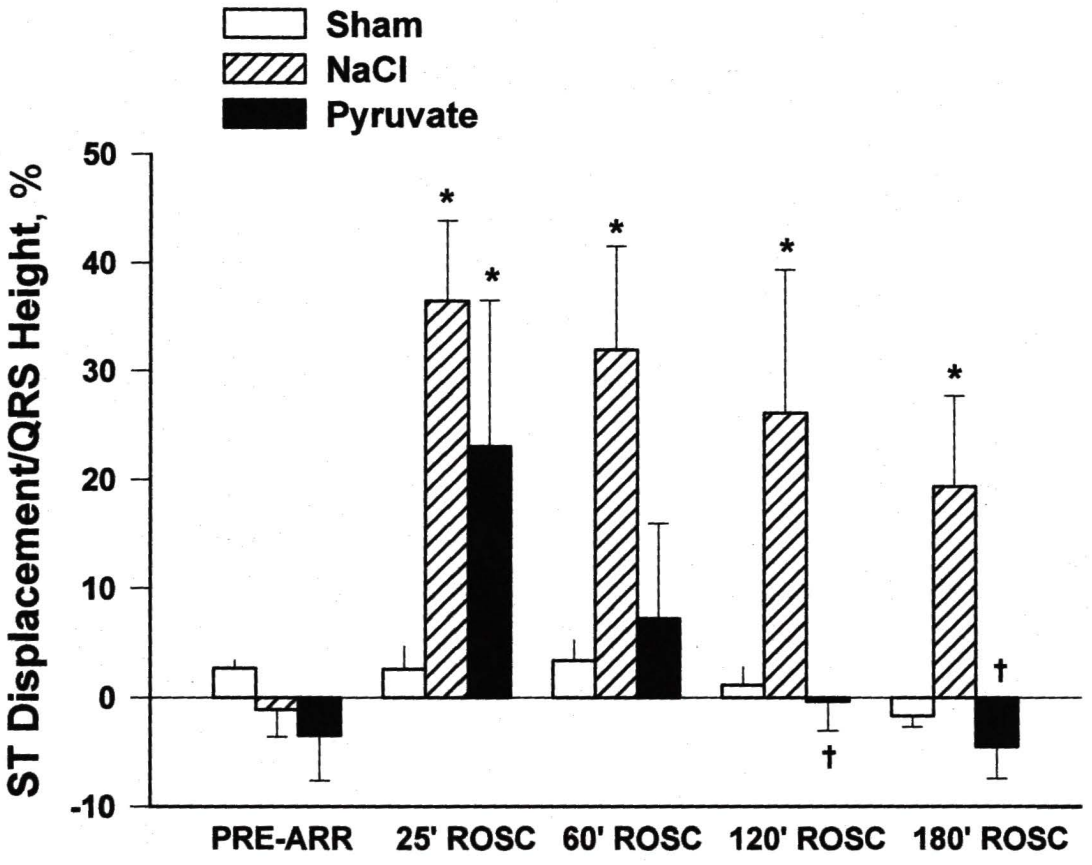

Sham
NaCl
Pyruvate
ST Displacement/QRS Height, %
50
40
30
20
10
0
-10
*
*
*
*
*
†
†
PRE-ARR
25' ROSC
60' ROSC
120' ROSC
180' ROSC

Figure 5

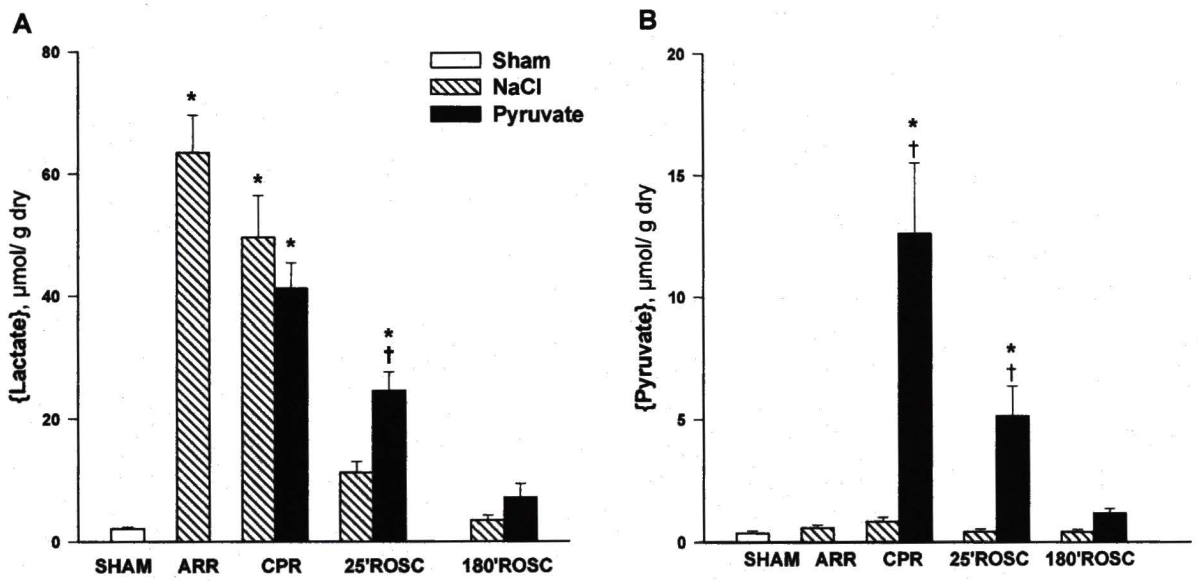

A
B
Sham
NaCl
Pyruvate
{Lactate}, µmol/ g dry
{Pyruvate}, µmol/ g dry
SHAM
ARR
CPR
25'ROSC
180'ROSC
0
20
40
60
80
5
10
15
20
*
†

Figure 6

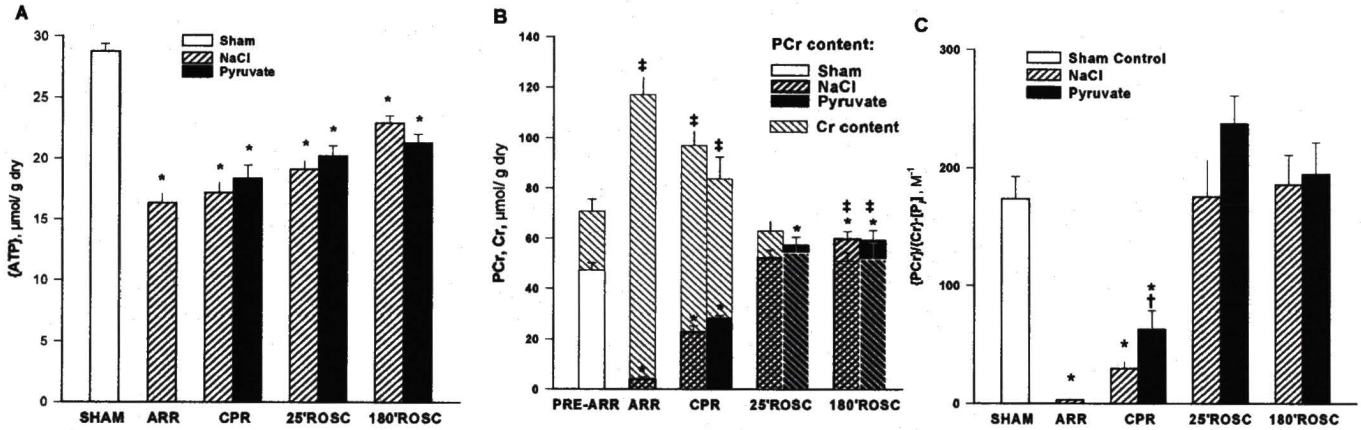
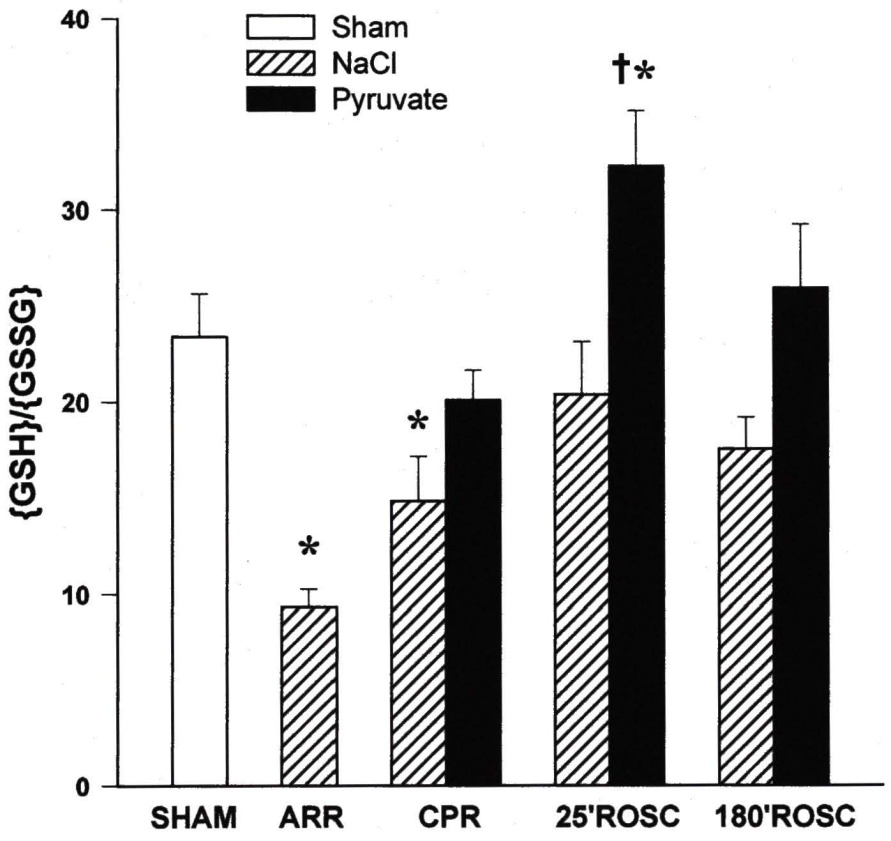

A
Sham
NaCl
Pyruvate
(ATP), µmol/ g dry
SHAM
ARR
CPR
25'ROSC
180'ROSC
B
PCr content:
Sham
NaCl
Pyruvate
Cr content
PCr, Cr, µmol/ g dry
PRE-ARR
ARR
CPR
25'ROSC
180'ROSC
C
Sham Control
NaCl
Pyruvate
SHAM
ARR
CPR
25'ROSC
180'ROSC

Figure 7


Sham
NaCl
Pyruvate
{GSH}/{GSSG}
40
30
20
10
0
*
*
†*
SHAM
ARR
CPR
25'ROSC
180'ROSC

Chapter III

OXIDATIVE STRESS REVERSIBLY INACTIVATES MYOCARDIAL ENZYMES DURING CARDIAC ARREST

Arti B. Sharma, Jie Sun, Linda L. Howard, Arthur G. Williams, Jr., Robert T. Mallet

Abstract

Purpose: Oxidative stress during cardiac arrest may inactivate myocardial enzymes, and thereby exacerbate ischemic derangements of myocardial metabolism. This study examined the impact of cardiac arrest on left ventricular enzymes. **Methods:** Beagles were subjected to 5 min cardiac arrest and 5 min open chest cardiac compressions (OCCC) before epicardial DC countershocks were applied to restore sinus rhythm. Glutathione/glutathione disulfide redox state (GSH/GSSG) and a panel of enzyme activities were measured in snap-frozen left ventricle. To test whether oxidative stress during arrest inactivated the enzymes, metabolic (pyruvate) or pharmacological (*N*-acetylcysteine) antioxidants were infused *iv* for 30 min before arrest. **Results:** During cardiac arrest, activities of phosphofructokinase, citrate synthase, aconitase, malate dehydrogenase, creatine kinase, glucose 6-phosphate dehydrogenase and glutathione reductase fell by 56, 81, 55, 34, 42, 55 and 45%, respectively, coincident with 50% decline in GSH/GSSG. OCCC effected full recovery of glutathione reductase and partial recovery of citrate synthase and aconitase, in parallel with GSH/GSSG. Phosphofructokinase, malate dehydrogenase, creatine kinase and glucose 6-phosphate dehydrogenase recovered only after cardioversion. Antioxidant pretreatments augmented phosphofructokinase, aconitase and malate dehydrogenase activities before arrest, and enhanced these activities, as well as citrate synthase and glucose 6-phosphate dehydrogenase, during arrest. **Conclusions:** Cardiac arrest reversibly inactivates several important myocardial metabolic enzymes. Antioxidant protection of these enzymes implicates oxidative stress as a principal mechanism of enzyme inactivation during arrest.

Key Words: glutathione, pyruvate, *N*-acetylcysteine, phosphofructokinase, creatine kinase, citrate synthase

Abbreviations: ACO: aconitase; ANOVA: analysis of variance; ARR: cardiac arrest; BL: pre-arrest baseline; CK: creatine kinase; Cr: creatine; CS: citrate synthase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulfide; G6PDH: glucose 6-phosphate dehydrogenase; LDH: lactate dehydrogenase; MDH: malate dehydrogenase; NAC: *N*-acetylcysteine; OCCC: open chest cardiac compression; PCr: phosphocreatine; PFK: phosphofructokinase; P_i: inorganic orthophosphate; ROSC: recovery of spontaneous circulation

Introduction

Cardiac arrest imposes severe myocardial ischemia which depletes high energy phosphates. Reactive oxygen species (ROS) generated during ischemia (4, 31) can modify cellular components including phospholipids, structural proteins, ion transporters and enzymes. Inactivation of metabolic enzymes can exacerbate ischemic depletion of myocardial energy and antioxidant reserves.

Several myocardial enzymes are susceptible to modification and inactivation by ROS, including enzymes of glycolysis [phosphofructokinase (13, 21, 24), glyceraldehyde 3-phosphate dehydrogenase (22)], TCA cycle [aconitase (10, 14), malate dehydrogenase (17)], intracellular energy transport [creatine kinase (24, 40)] and antioxidant defense [glutathione reductase (41), glutathione peroxidase (41), glucose 6-phosphate dehydrogenase (26)]. Enzyme modifications may be reversible or permanent, depending on the severity of oxidative stress, culprit oxidant species, and chemical nature of the modification. Cells are armed with enzyme systems that can reverse some protein modifications and restore protein function. These systems include the protein disulfide reductases thioreductase and thioredoxin (2), glutaredoxins (29) catalyzing glutathione-disulfide oxidoreductions, and sulfiredoxin (7) which reduces cysteine-sulphinic acid derivatives formed by oxidation of cysteine residues. Other modifications such as carbonylation are irreversible and divert proteins to ubiquitination and proteosomal degradation (16, 31).

This study examined the impact of cardiac arrest and resuscitation on a panel of metabolic enzymes in left ventricular myocardium. Beagles underwent 5 min cardiac arrest, followed by 5 min open chest cardiac compression, defibrillation, and 3 h recovery. Myocardial enzyme activities were measured in left ventricular biopsies. Several enzymes were partially inactivated during cardiac arrest and recovered after sinus rhythm was restored. The changes in enzyme activities paralleled glutathione redox state, a measure of myocardial antioxidant state. To test whether oxidative stress inactivated the enzymes, antioxidants pyruvate and *N*-acetylcysteine were administered before arrest. The antioxidant pretreatments prevented subsequent collapse of several enzyme activities during cardiac arrest, in some cases by increasing pre-arrest activities.

Methods

All animal experimentation conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 85–23, revised 1996), and was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center. Adult beagles (11.3 ± 0.3 kg; 40 males, 51 females) were randomly assigned to either cardiac arrest-resuscitation or the antioxidant pretreatment groups described below.

Surgical preparation and instrumentation

After overnight fast, dogs were sedated with morphine sulfate (3 mg kg^{-1} *sc*) and anesthetized with α-chloralose (100 mg kg^{-1} *iv*). Supplemental α-chloralose was administered as needed to maintain anesthesia. Surgical preparation and instrumentation were performed as recently described (35). Briefly, the dogs were intubated and mechanically ventilated with room air enriched with supplemental O_2. Vinyl catheters were placed in the abdominal aorta via femoral artery for sampling arterial blood and measuring blood pressure, in a femoral vein for antioxidant infusion, and in the other femoral vein for administration of supplemental anesthetic and $NaHCO_3$. Arterial pH, PO_2, and PCO_2 were maintained within physiological limits by adjusting tidal volume and ventilatory frequency, ventilating with supplemental O_2, and administering $NaHCO_3$. Body temperature was maintained at 36-37°C by use of a circulating water heating pad.

The heart was exposed via left lateral thoracotomy in the 5th intercostal space and suspended in a pericardial cradle. A vinyl cannula was placed in the right atrium to

administer intracardiac medications. Lead II electrocardiography was used to confirm ventricular fibrillation during arrest and sinus rhythm after cardioversion.

Cardiac arrest and cardiopulmonary resuscitation protocol

Cardiopulmonary arrest (35) was induced by applying 9V current to the left ventricular epicardium and interrupting mechanical ventilation. At 4.5 min of arrest, a 1 mg epinephrine bolus was injected into the right atrium. Open chest cardiac compressions (OCCC, 80 compressions min^{-1}) were administered at 5-10 min cardiac arrest to achieve a mean aortic pressure of ~ 60 mmHg. Mechanical ventilation was resumed at 12 cycles min^{-1} during OCCC. Beginning at 10 min arrest, defibrillatory DC countershocks were delivered to the epicardium with internal paddles. Up to four 5J countershocks with intervening OCCC were delivered at 30 s intervals, followed by up to three 10J countershocks, until cardioversion was achieved.

Recovery of spontaneous circulation (ROSC) was defined as mean aortic pressure of $\geq$ 60 mmHg and spontaneous cardiac rhythm. In the event of ventricular tachyarrhythmia lidocaine (6 mg bolus) was injected into the right atrium to restore sinus rhythm. Experiments were terminated by collecting freeze clamp biopsies of the left ventricular anterior wall at pre-arrest baseline, 5 min arrest, 5 min OCCC, 25 min ROSC and 180 min ROSC, using liquid N_2-precooled Wollenberger tongs. Biopsies were stored at -90°C before analyses of metabolites and enzymes.

Antioxidant pretreatments

Following surgical instrumentation and stabilization of cardiac function, the dogs received intravenous infusion of either sodium pyruvate (0.125 mmol kg^{-1} min^{-1}), a natural energy metabolite and antioxidant (27) or *N*-acetyl cysteine (0.06 mmol kg^{-1} min^{-1}), a pharmacologic antioxidant (27). At 30 min infusion the heart was arrested and antioxidant infusion was simultaneously discontinued. Left ventricular myocardium was biopsied at 30 min pre-arrest infusion or at 5 min arrest.

Analytical procedures

Myocardial metabolites were extracted and measured by spectrophotometric assays (24). Arterial plasma was obtained by centrifugation of freshly drawn whole blood and flash frozen in liquid N_2. Glutathione:glutathione disulfide (GSH/GSSG) redox state and *N*-acetylcysteine concentration in plasma were measured by high-performance liquid chromatography with fluorescence detection (24). 8-isoprostane, a product of non-enzymatic oxidative modification of tissue phospholipids, is considered to be a reliable marker of oxidative stress (19). Competitive enzyme immunoassay for 8-isoprostane (Cayman Chemical, Ann Arbor, MI) was performed in arterial plasma, according to the manufacturer's instructions.

Enzymes were extracted from snap frozen left ventricular myocardium (24). Briefly, tissue was pulverized to a fine powder in a precooled mortar under liquid N_2, then homogenized in 6-8 vol ice-cold phosphate buffer. Sulfhydryl reducing agents were omitted from the buffer to avoid spontaneous recovery of enzymes inactivated by cardiac arrest. Following centrifugation (20 min at 4°C and 116,000 g_{max}) the pellet was re-

extracted twice in 4 vol phosphate buffer and the three supernatant fractions combined and stored at -90° C. Enzyme activities were measured at 37° C in a Shimadzu Instruments model UV-1601 dual wavelength UV-vis spectrophotometer according to spectrophotometric methods described in Bergmeyer (6). Activities were determined from the rates of formation or disappearance of NADPH or NADH monitored at 337 nm wavelength ($\varepsilon = 6.24\ mol^{-1} \cdot cm^{-1}$). Extract protein concentration was measured with the Coomassie plus kit (Pierce, Rockford, IL). Enzyme activities were expressed as units per mg protein, where one unit equals 1 μmol substrate converted to product per min. To exclude the possibility that antioxidant enhancements of enzyme activities resulted from residual antioxidant in the snap-frozen myocardium, untreated myocardium was extracted in the absence and presence of added pyruvate and *N*-acetylcysteine (0.8 $\mu mol\ g^{-1}$).

Statistical analyses

Data are reported as means ± S.E.M. Between-group comparisons of myocardial metabolites, GSH/GSSG and enzyme activities were accomplished by single factor analysis of variance (ANOVA). Single factor ANOVA also was used for within-group comparisons of myocardial enzymes and metabolites at different time points. Repeated measures ANOVA was used for within-group comparisons of plasma GSH/GSSG, 8-isoprostane and antioxidant concentrations. When ANOVA detected statistical significance, Student-Newman-Keuls multiple comparison test was applied to identify specific differences among mean values. P values < 0.05 were taken to indicate statistically significant differences. Statistical analyses were performed using SigmaStat version 3.1 software.

Results

Enzyme inactivation during cardiac arrest and recovery during resuscitation and ROSC

The impact of cardiac arrest, cardiopulmonary resuscitation effected by open-chest cardiac compressions (OCCC) and recovery of spontaneous circulation (ROSC) on a panel of key metabolic enzymes was examined in canine left ventricular myocardium. Activity of phosphofructokinase, the principal rate-controlling glycolytic enzyme, fell by 56% during cardiac arrest, remained depressed during OCCC, and then slowly recovered during 3 h ROSC (Figure 1A). Two other glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (Figure 1B) and lactate dehydrogenase (Figure 1C), were not appreciably inactivated during the protocol, although a trend towards lower lactate dehydrogenase activity was detected during arrest, OCCC and at 25 min ROSC. Among TCA cycle enzymes, citrate synthase activity fell the most steeply during cardiac arrest, by 81%, then substantially recovered during OCCC, but no further after ROSC (Figure 2A). Aconitase activity fell 55% during arrest, and then gradually recovered during OCCC and ROSC (Figure 2B). Malate dehydrogenase activity fell 34% during arrest, remained depressed during OCCC, and recovered only after ROSC (Figure 2C). Creatine kinase, which catalyses energy transfer between mitochondria and extramitochondrial ATPases, fell 42% during arrest and did not return to sham control level until 180 min ROSC (Figure 2D).

Cardiac arrest and resuscitation also affected enzyme components of the glutathione antioxidant system. Activities of glucose 6-phosphate dehydrogenase, a major source of NADPH, and glutathione reductase, which uses NADPH reducing power to regenerate

glutathione (GSH) from glutathione disulfide (GSSG), fell by 55 and 45%, respectively, during cardiac arrest (Figure 3). Glutathione reductase quickly recovered during OCCC, but glucose 6-phosphate dehydrogenase only recovered after ROSC. Glutathione peroxidase harnesses GSH reducing power to detoxify H_2O_2 and lipid peroxides. Although cardiac arrest tended to inactivate glutathione peroxidase, the enzyme's activity sharply increased to twice its baseline value during OCCC. Collectively, these results indicate that cardiac arrest reversibly inactivated several important metabolic enzymes. The enzymes spontaneously recovered, albeit at different rates, during OCCC and ROSC.

Glutathione redox state and arterial 8-isoprostane

Arterial plasma 8-isoprostane, a product of free radical oxidation of arachidonic acid and an index of systemic oxidative stress, increased sharply during OCCC and the first 5 min ROSC, and then gradually subsided (Figure 4A). Glutathione redox state (GSH/GSSG), a measure of myocardial antioxidant reducing power, fell sharply during arrest, then progressively recovered during OCCC and the first 25 min ROSC (Figure 4B).

Antioxidant pretreatments and glutathione redox state

To test the hypothesis that oxidative stress imposed by cardiac arrest inactivated myocardial enzymes, the ability of antioxidant pretreatments to protect enzyme activities during cardiac arrest was examined. The pharmacological antioxidant *N*-acetylcysteine (NAC) or the natural metabolic antioxidant pyruvate was systemically infused for 30 min immediately before cardiac arrest. Arterial plasma pyruvate concentration sharply increased during intravenous pyruvate infusion and within 8 min stabilized at ~4 mM

(Figure 5A). Plasma NAC steadily increased over 30 min infusion to a concentration of 1 ± 0.2 mM (Figure 5B). NAC was undetectable in plasma of pyruvate treated and sham control dogs, and NAC infusion did not alter plasma pyruvate concentration.

N-acetylcysteine pretreatment enhanced glutathione redox state in both plasma (Figure 6A) and myocardium (Figure 6B). Pyruvate pretreatment modestly increased GSH/GSSG in plasma, but not in myocardium. At 5 min arrest, GSH/GSSG in NAC- or pyruvate-pretreated myocardium was 48 and 57% greater, respectively, than that of untreated myocardium (Figure 6B). Thus, antioxidant pretreatments partially preserved myocardial GSH reducing power during cardiac arrest.

Antioxidants protect myocardial enzymes during cardiac arrest.

Antioxidants enhanced pre-arrest activities of the glycolytic enzymes phosphofructokinase and lactate dehydrogenase (Figure 7A, B). During arrest, pyruvate pretreatment enhanced phosphofructokinase activity (Figure 7A) and NAC pretreatment increased lactate dehydrogenase activity (Figure 7B). Glyceraldehyde 3-phosphate dehydrogenase was unaltered by cardiac arrest or by antioxidants (Figure 7C).

Neither pyruvate nor NAC affected citrate synthase activity before arrest, but both pretreatments, especially NAC, attenuated inactivation of this TCA cycle enzyme during cardiac arrest (Figure 8A). Pyruvate but not NAC augmented aconitase activity before arrest, but only NAC pretreatment protected the enzyme during arrest (Figure 8B). Both pretreatments increased malate dehydrogenase activity before and during cardiac arrest

(Figure 8C). Creatine kinase was not affected by antioxidant pretreatments (Figure 8D) and antioxidant enhancements of this enzyme during arrest were not significant. Neither glucose 6-phosphate dehydrogenase, the rate-controlling enzyme of the hexose monophosphate shunt, nor glutathione reductase were affected by antioxidants before cardiac arrest, but NAC pretreatment protected both enzymes during arrest (Figures 9A, B). Both antioxidants markedly increased glutathione peroxidase before arrest, but only NAC pretreatment enhanced the enzyme's activity during arrest (Figure 9C).

Enzyme activities are not increased by residual antioxidants in myocardial homogenate.

Residual NAC or pyruvate in homogenates of antioxidant-treated myocardium could conceivably protect enzymes from inactivation during extraction, which would artifactually increase enzyme activities. To test this possibility, 0.8 $\mu mol \cdot g^{-1}$ pyruvate or NAC, equal to the molar amount of pyruvate in pyruvate-pretreated myocardium, were added to homogenates of untreated pre-arrest myocardium before extracting the enzymes. Excess pyruvate or NAC did not alter activities of any of the antioxidant-responsive enzymes (Figure 10), nor that of glucose 6-phosphate dehydrogenase, which was not affected by antioxidants before arrest. Thus, residual antioxidants did not produce spurious enhancements of myocardial enzymes.

Phosphocreatine phosphorylation potential

Both antioxidant pretreatments enhanced phosphocreatine phosphorylation potential, a measure of myocardial energy state (Figure 11). Phosphorylation potential collapsed during cardiac arrest, despite pre-arrest augmentation by antioxidants.

Discussion

This study examined the impact of cardiac arrest and resuscitation on key enzymes of intermediary, energy and antioxidant metabolism in left ventricular myocardium. Several enzymes were inactivated during cardiac arrest, concomitant with depletion of antioxidant reducing power. The enzyme impairments were reversible; all of the enzymes recovered by 3 h ROSC, albeit at different rates, as did glutathione redox state. To test whether oxidative stress inactivated the enzymes during cardiac arrest, antioxidants pyruvate and *N*-acetylcysteine (NAC) were administered *iv* for 30 min before arrest. Pyruvate and NAC unexpectedly increased pre-arrest activities of phosphofructokinase, lactate dehydrogenase, malate dehydrogenase, and glutathione peroxidase. Pyruvate also enhanced aconitase activity. Despite pre-arrest enhancement by antioxidants, activities of these enzymes fell during cardiac arrest. However, in most cases enzyme activities in antioxidant-pretreated myocardium remained above activities in untreated myocardium at 5 min arrest. These antioxidant effects implicate oxidative stress in the mechanism of reversible enzyme inactivation.

Modulation of enzymes by reactive oxygen metabolites and antioxidants

Enzymes are susceptible to oxidative modification during ischemia-reperfusion (14, 21, 24, 40, 41). Several important enzymes were inactivated during cardiac arrest, but no further during OCCC and ROSC. Citrate synthase and glutathione reductase recovered during OCCC; phosphofructokinase, aconitase, malate dehydrogenase, creatine kinase and glucose 6-phosphate dehydrogenase only recovered after ROSC. The spontaneous recovery of these enzymes indicates that neither protein degradation nor irreversible

oxidative damage contributed to their inactivation during arrest. Instead, reversible modifications, *e.g.* sulfhydryl oxidation or thiolation, may have inactivated the enzymes.

During ischemia, phosphofructokinase is reversibly inactivated by dimerization (13) or by translocation from cytosol to membranes (21). Glyceraldehyde 3-phosphate dehydrogenase is also susceptible to oxidative modification and direct oxidant injury (26, 30). Surprisingly, this enzyme was not inactivated in this canine model of cardiac arrest and resuscitation, nor was it affected by antioxidants. Lactate dehydrogenase activity fell only marginally during arrest and was not appreciably affected by OCCC and ROSC. Similarly, Bogaert *et al.* (8) reported that canine brain lactate dehydrogenase is resistant to inactivation following 10 min cardiac arrest and ROSC. The mechanism of the modest activation of lactate dehydrogenase by antioxidants is unclear.

Aconitase activity fell by 55% during cardiac arrest but recovered following OCCC and ROSC. Oxidative disassembly of its catalytic $[4Fe\text{-}4S]^{2+}$ cluster inactivates aconitase, but reductants such as GSH can restore the enzyme (38). Aconitase also can be reversibly inactivated by oxidation of a sulfhydryl moiety near its active site (10). Both of these mechanisms may respond to treatment with sulfhydryl reagents such as NAC.

Citrate synthase, malate dehydrogenase and creatine kinase are inactivated by oxidative modification and thiolation of critical cysteine residues (17, 28, 32). These enzymes are particularly susceptible to S-glutathiolation, in which GSSG, generated by oxidation of GSH, transfers glutathionyl moieties to cysteine sulfhydryls. This modification can be

reversed and the enzyme reactivated with recovery of GSH redox state. By partially preserving GSH/GSSG, pyruvate and NAC may have stabilized citrate synthase, malate dehydrogenase and creatine kinase during arrest.

Glucose 6-phosphate dehydrogenase (G6PDH), the initial and rate-controlling enzyme of the hexose monophosphate shunt, is also sensitive to oxidative stress (24, 26), and is inactivated by thiol:disulfide exchange (42). After GSH is consumed to detoxify ROS, glutathione reductase regenerates the antioxidant. The sulfhydryl antioxidant NAC protected glutathione reductase during arrest without increasing its pre-arrest activity. On the other hand, pyruvate pretreatment, which was as effective as NAC at preserving GSH/GSSG during arrest, did not protect glutathione reductase. Arguably, inactivation of glutathione reductase may have caused the decline in GSH/GSSG during arrest, not *vice versa*. Glutathione reductase is inactivated by *S*-nitrosoglutathione (3), an endogenous nitric oxide donor and is also susceptible to catecholamine induced oxidative stress and its inactivation is reversed by reductants (33). It therefore seems likely that oxidative stress during arrest resulted in glutathione reductase inactivation.

All of the enzymes that were inactivated during cardiac arrest spontaneously recovered by 3 h ROSC. The reversibility of enzyme inactivation indicates that the enzymes were neither degraded nor released from injured cardiocytes; rather, the enzymes were covalently modified, and reversal of these modifications restored catalytic activity. Our recent study in this model of cardiac arrest and resuscitation (35) demonstrated that left ventricular contractile function temporarily recovered following cardioversion, but then

fell markedly by 2-3 h ROSC. The current findings suggest that this cardio-depression was not caused by metabolic impairment, and that damage to other cellular components may have contributed to the contractile dysfunction.

Unlike the other enzymes, glutathione peroxidase activity increased to well above baseline during OCCC and early ROSC, in accordance with reports of glutathione peroxidase activation in response to oxidant stress and ischemia-reperfusion (1, 12, 23). In murine fibroblasts oxidative stress activated the tyrosine kinases c-Alb and Arg, which in turn phosphorylated and activated glutathione peroxidase (12). Pre-arrest activation of the enzyme by antioxidants may have resulted from increased phosphorylation potential, the major determinant of free energy of ATP hydrolysis (39), the thermodynamic driving force for protein phosphorylation. Pyruvate, an energy generating metabolite, also increased glutathione peroxidase activity during ischemia in isolated rat hearts (15).

Glutathione redox state during cardiac arrest and recovery

Myocardial glutathione redox state (GSH/GSSG) was severely depleted at 5 min arrest, but progressively recovered during OCCC and ROSC, indicating that the major oxidative stress occurred during arrest, not reperfusion. Although oxyradical generation during ischemia is not as well characterized as the reperfusion oxidative burst (9), even during ischemia sufficient O_2 is available in cardiomyocytes to generate reactive oxygen species (5). OCCC effects only partial reperfusion of myocardium. Sequential application of OCCC followed by cardioversion and ROSC may have effected incremental reperfusion that dampened the oxyradical burst. Also, activation of glutathione peroxidase during

OCCC and ROSC, and concomitant recovery of GSH reducing power, may have enhanced detoxification of reactive oxygen metabolites.

Glutathione redox state recovered partially during OCCC, despite persistent inactivation of glucose 6-phosphate dehydrogenase, a major source of NADPH reducing equivalents to regenerate GSH from GSSG. On the other hand, full recovery of glutathione reductase during OCCC may have facilitated partial recovery of GSH redox state, provided sufficient NADPH was available from residual glucose 6-phosphate dehydrogenase or other sources.

N-acetylcysteine pretreatment augmented pre-arrest myocardial GSH/GSSG and partially maintained GSH/GSSG during cardiac arrest. Pyruvate did not increase pre-arrest myocardial GSH/GSSG, but pyruvate, like NAC, attenuated depletion of myocardial GSH/GSSG during arrest. By partially preserving GSH/GSSG, antioxidants may have protected oxidant-sensitive enzymes during cardiac arrest.

Both pyruvate and NAC markedly increased phosphocreatine-phosphorylation potential, a measure of myocardial energy state, before arrest. As an energy yielding metabolic substrate, pyruvate enhances myocardial phosphorylation potential following reperfusion (11, 36), but not during ischemia (11, 24). Although NAC is not an energy-yielding fuel, antioxidants (18, 37) including NAC (25) have been found to enhance myocardial energy state. The mechanisms of antioxidant-enhancement of myocardial energetics are unclear.

Limitations

This study was performed in open chest anesthetized dogs and therefore does not precisely mimic the conditions of closed cardiac arrest and CPR. The open chest preparation facilitated control of aortic pressure during OCCC, and allowed heart tissue to be sampled at specific time points. This study did not identify the reactive oxygen metabolites generated during cardiac arrest, or the specific protein modifications that altered enzyme activities. The possibility that antioxidants may hasten post-arrest recovery of myocardial enzymes and thereby enhance post-arrest energy metabolism and contractile performance remains to be tested. The GSH:GSSG system is only one of several redox systems comprising the myocardial antioxidant defenses. However, GSH/GSSG is abundant and exists in a state of near-equilibrium with these other antioxidants (20, 34), so GSH/GSSG provides an integrated index of the collective status of myocardial antioxidant defenses.

Conclusions

Several important myocardial metabolic enzymes are reversibly inactivated by oxidative stress during cardiac arrest. Cardiopulmonary resuscitation effected partial recovery of some of these enzymes, and all of the enzymes eventually recovered following resumption of spontaneous circulation. Pretreatments with metabolic (pyruvate) and pharmacological (NAC) antioxidants alleviated enzyme inactivation during arrest, in part by enhancing pre-arrest activities. These results implicate oxidative stress as a major contributor to reversible inactivation of myocardial enzymes during cardiac arrest.

Acknowledgements

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Figure Legends

Figure 1. *Myocardial glycolytic enzyme activities during cardiac arrest, OCCC and ROSC.* Activities of phosphofructokinase (PFK: panel A), glyceraldehyde 3-phosphate dehydrogenase (GAPDH: panel B) and lactate dehydrogenase (LDH: panel C) were measured at pre-arrest baseline (BL), 5 min cardiac arrest (ARR), 5 min open chest cardiac compressions (OCCC), and at 25 and 180 min after recovery of spontaneous circulation (ROSC). Solid bar: non-arrested sham time control. Values are means ± SEM from 6-9 experiments at each time point. $^{*}P < 0.05$ *vs.* BL.

Figure 2. *TCA cycle enzymes and creatine kinase.* Activities of citrate synthase (CS: panel A), aconitase (ACO: panel B), malate dehydrogenase (MDH: panel C) and creatine kinase (CK: panel D) were measured at the same times and in the same experiments as in Figure 1. Abbreviations as in Figure 1. $^{*}P < 0.05$ *vs.* BL; $^{\dagger}P < 0.05$ *vs.* ARR.

Figure 3. *Myocardial antioxidant enzymes.* Activities of glucose 6-phosphate dehydrogenase (G6PDH: panel A), glutathione reductase (GR: panel B), and glutathione peroxidase (GPx: panel C) were measured at the same times and in the same experiments as in Figures 1 and 2. $^{*}P < 0.05$ *vs.* BL; $^{\dagger}P < 0.05$ *vs.* ARR.

Figure 4. *Measures of oxidative stress: plasma 8-isoprostane and myocardial glutathione redox state.* Panel A: 8-isoprostane was measured in arterial blood samples at pre-arrest baseline (BL), 3 min cardiac arrest (A), 3 min OCCC, and at several times following ROSC. $^{*}P < 0.05$ *vs.* BL; $^{\dagger}P < 0.05$ *vs.* sham at same time. Panel B:

Glutathione redox state (GSH/GSSG) was measured in left ventricular myocardium at pre-arrest baseline (BL), 5 min cardiac arrest (ARR), 5 min OCCC, and at 25 and 180 min after ROSC. Solid bar: non-arrested sham time control. Values are means ± SEM from the same experiments as in Figures 1-3. *P < 0.05 *vs.* BL; †P < 0.05 *vs.* ARR.

Figure 5. *Arterial plasma pyruvate (panel A) and* N*-acetylcysteine (panel B) during pre-arrest antioxidant infusions.* Antioxidants (circles: *N*-acetylcysteine [NAC]; triangles: pyruvate) were infused from 0 to 30 min. Squares: non-infused time controls. NAC was undetectable in plasma of pyruvate and sham experiments. Values are means ± SEM from 7 pyruvate and 9 NAC experiments. *P < 0.05 *vs.* 0 min.

Figure 6. *Effects of antioxidant treatments on plasma and myocardial glutathione redox states.* Panel A: Plasma GSH/GSSG during pre-arrest antioxidant infusions. Means ± SEM from same experiments as in Figure 5. *P < 0.05 *vs.* 0 min. Panel B: GSH/GSSG in left ventricular myocardium at pre-arrest baseline (solid bars) and 5 min cardiac arrest (hatched bars). In the *N*-acetylcysteine (NAC) and pyruvate groups, pre-arrest values were taken at 30 min treatment. Means ± SEM from 6 sham, 12 pyruvate and 12 NAC experiments. *P < 0.05 *vs.* untreated; †P < 0.05 *vs.* pre-arrest.

Figure 7. *Effects of antioxidant treatments on glycolytic enzymes.* In this figure and in Figures 8 and 9, values were taken at 30 min pyruvate or NAC infusion (pre-arrest) or at 5 min cardiac arrest. Enzyme abbreviations as in Figure 1. Open bars: untreated; solid bars: pyruvate pretreated; hatched bars: NAC pretreated. Means ± SEM from 6

experiments per group. *P < 0.05 *vs.* untreated; † P < 0.05 *vs.* pre-arrest; ‡P < 0.05, NAC *vs.* pyruvate.

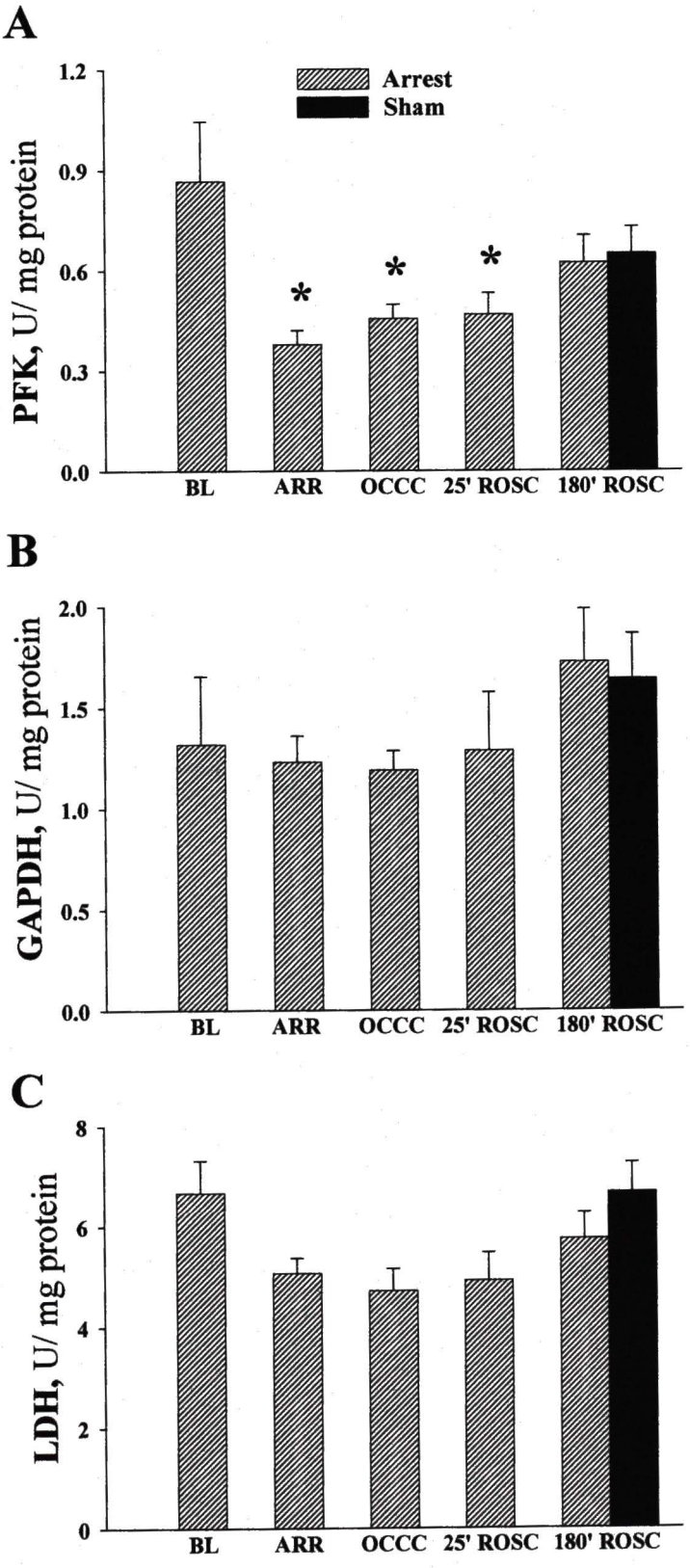
Figure 8. *Effects of antioxidants on TCA cycle enzymes and creatine kinase.* Enzyme abbreviations as in Figure 2. Means ± SEM from the same experiments as in Figure 7. *P < 0.05 *vs.* untreated; † P < 0.05 *vs.* pre-arrest; ‡P < 0.05, NAC *vs.* pyruvate.

Figure 9. *Effects of antioxidants on myocardial antioxidant enzymes.* Enzyme abbreviations as in Figure 3. Values are from same experiments as in Figures 7 and 8. *P < 0.05 *vs.* untreated; † P < 0.05 *vs.* pre-arrest; ‡P < 0.05, NAC *vs.* pyruvate.

Figure 10. *Addition of antioxidants to tissue homogenate did not alter activities of antioxidant-responsive enzymes.* Abbreviations as in Figures 1-3. Means ± SEM for 6 experiments per group. No statistically significant differences between groups were detected.

Figure 11. *Effects of antioxidant pretreatments on myocardial energy state.* Phosphocreatine phosphorylation potential, *i.e.* ({PCr}/{Cr}[P_i]) was measured in left ventricular myocardium at pre-arrest baseline and at 5 min cardiac arrest. Means ± SEM from the same experiments as in Figures 7-9. *P < 0.05 *vs.* untreated; † P < 0.05 *vs.* pre-arrest.

Figure 1.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Arrest
Sham
PFK, U/ mg protein
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0.6
0.3
0.0
*
*
*
BL
ARR
OCCC
25' ROSC
180' ROSC
B
GAPDH, U/ mg protein
2.0
1.5
1.0
0.5
0.0
BL
ARR
OCCC
25' ROSC
180' ROSC
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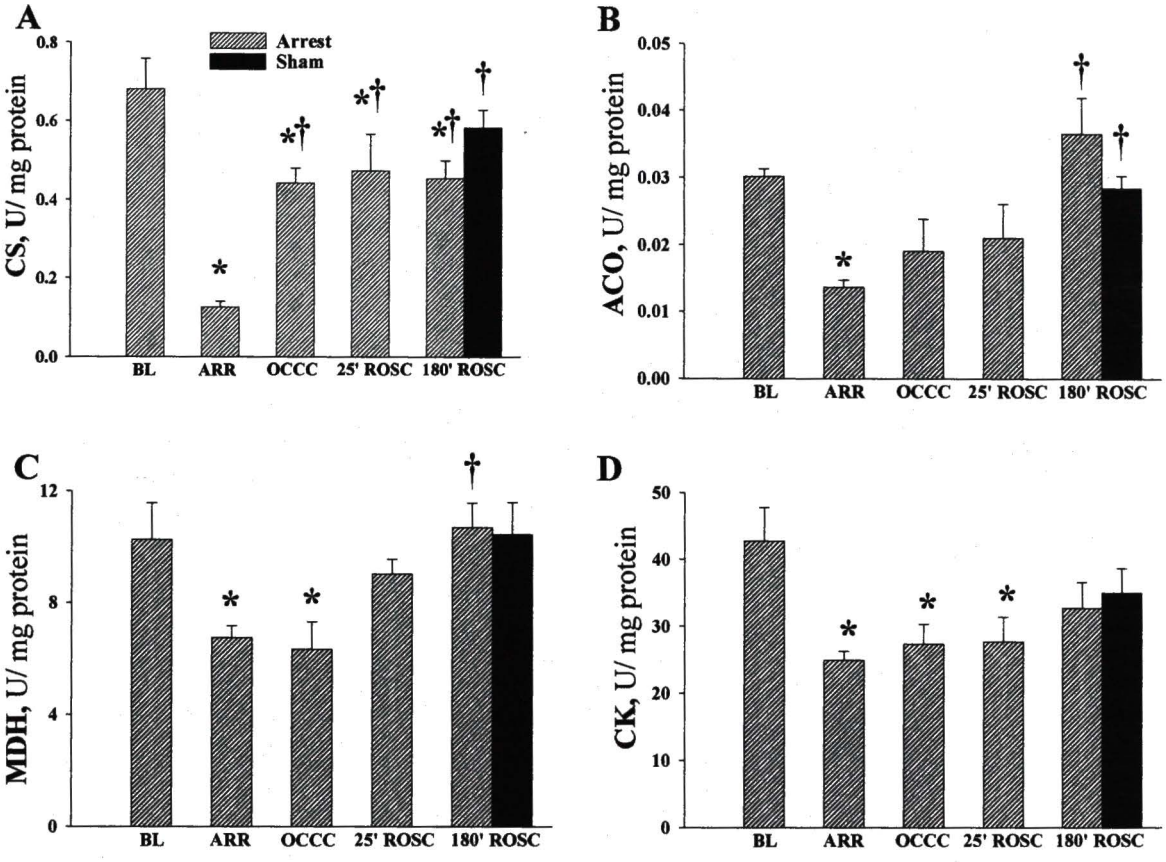
6
4
2
0
BL
ARR
OCCC
25' ROSC
180' ROSC

Figure 2.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ARR
OCCC
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B
ACO, U/ mg protein
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0.01
0.02
0.03
0.04
0.05
BL
ARR
OCCC
25' ROSC
180' ROSC
C
MDH, U/ mg protein
0
4
8
12
BL
ARR
OCCC
25' ROSC
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BL
ARR
OCCC
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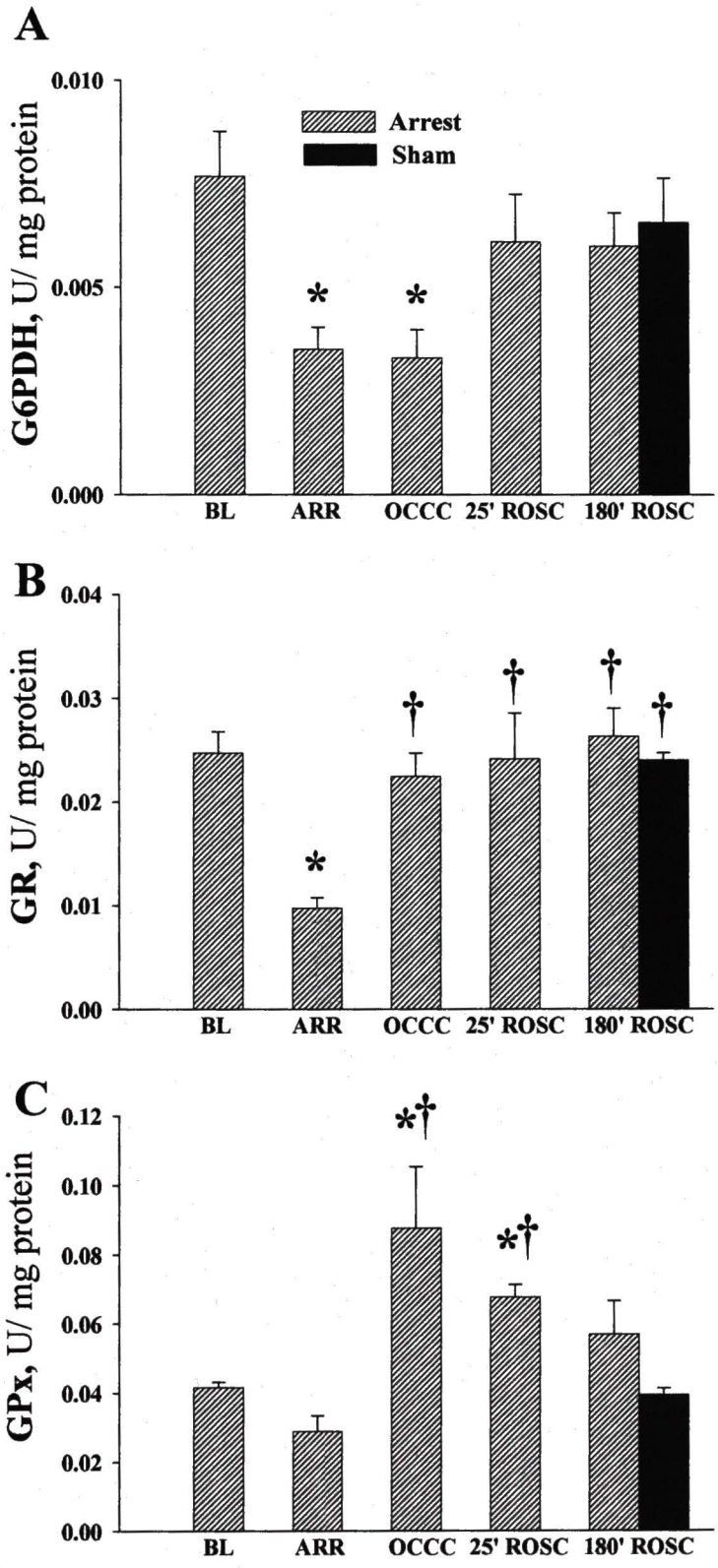
180' ROSC

Figure 3.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G6PDH, U/ mg protein
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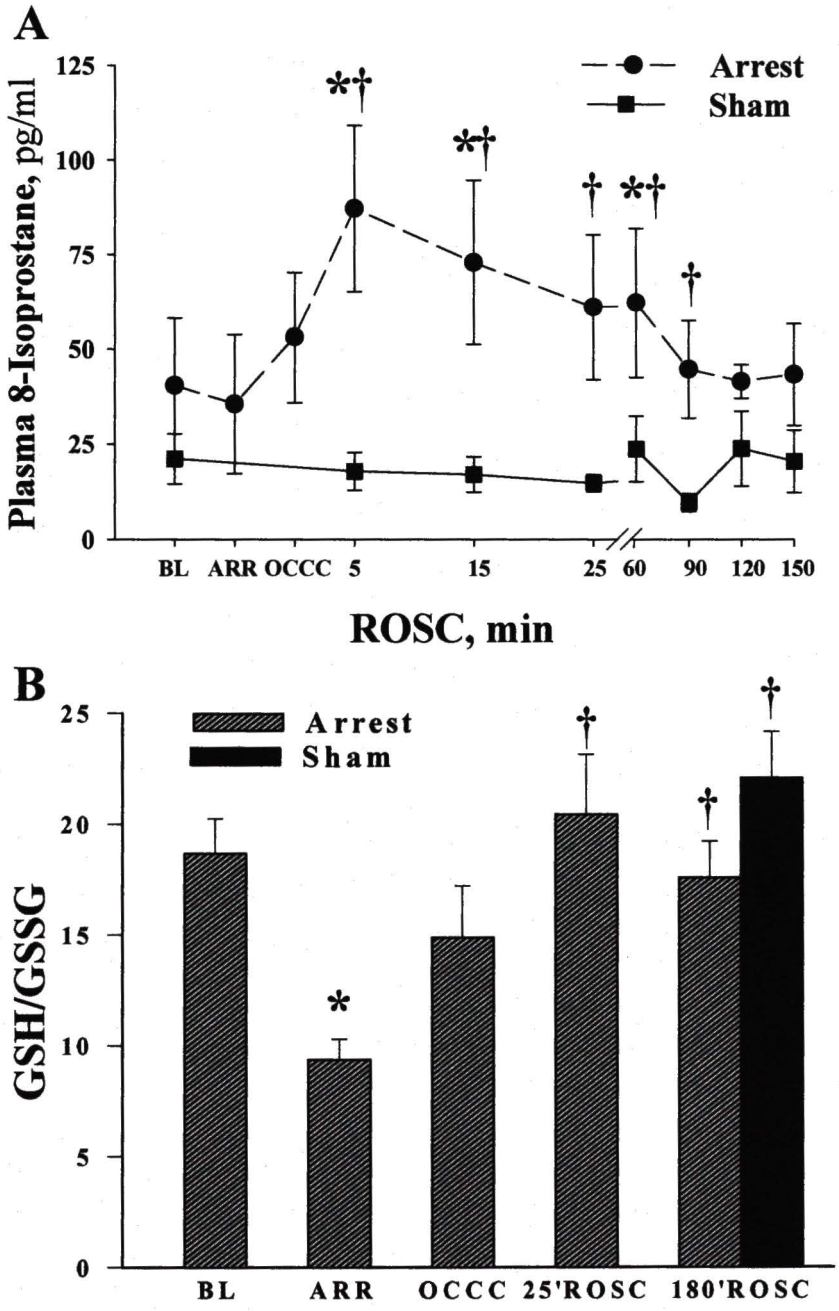
0.005
0.000
Arrest
Sham
*
*
BL
ARR
OCCC
25' ROSC
180' ROSC
B
GR, U/ mg protein
0.04
0.03
0.02
0.01
0.00
*
†
†
†
†
BL
ARR
OCCC
25' ROSC
180' ROSC
C
GPx, U/ mg protein
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*†
*†
BL
ARR
OCCC
25' ROSC
180' ROSC

Figure 4.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Plasma 8-Isoprostane, pg/ml
125
100
75
50
25
0
Arrest
Sham
*†
*†
†
*†
†
BL ARR OCCC 5 15 25 60 90 120 150
ROSC, min
B
GSH/GSSG
25
20
15
10
5
0
Arrest
Sham
*
†
†
†
BL ARR OCCC 25'ROSC 180'ROSC

Figure 5.

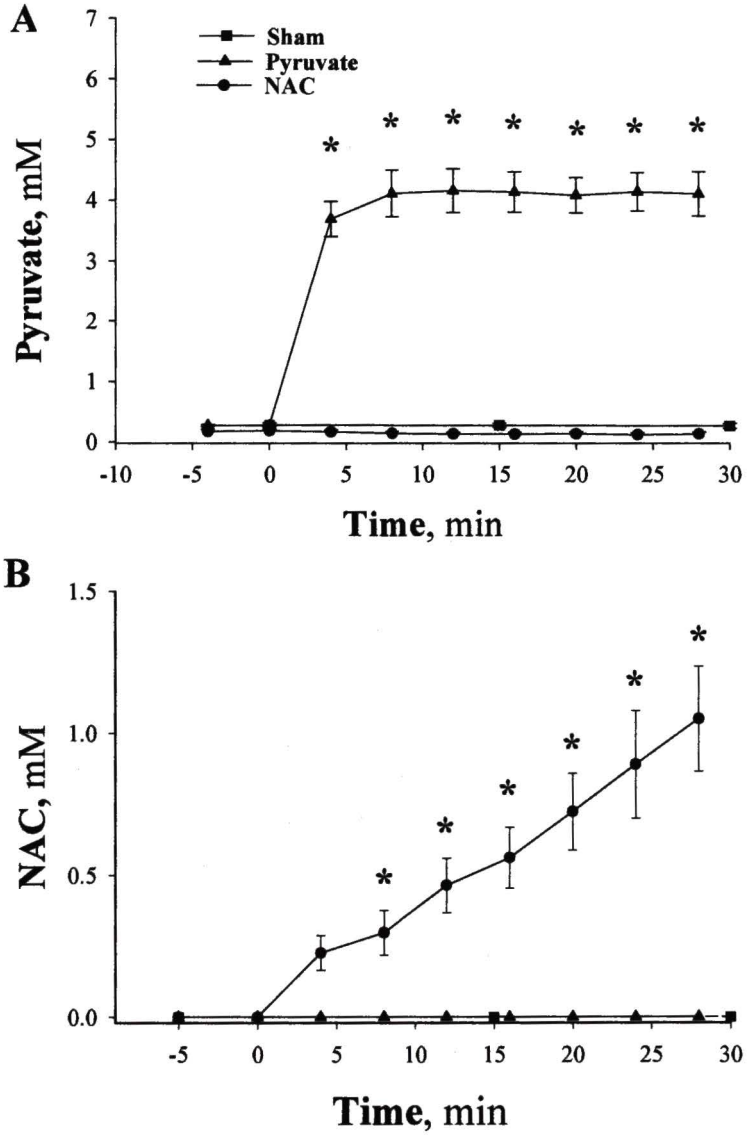


Figure 6.

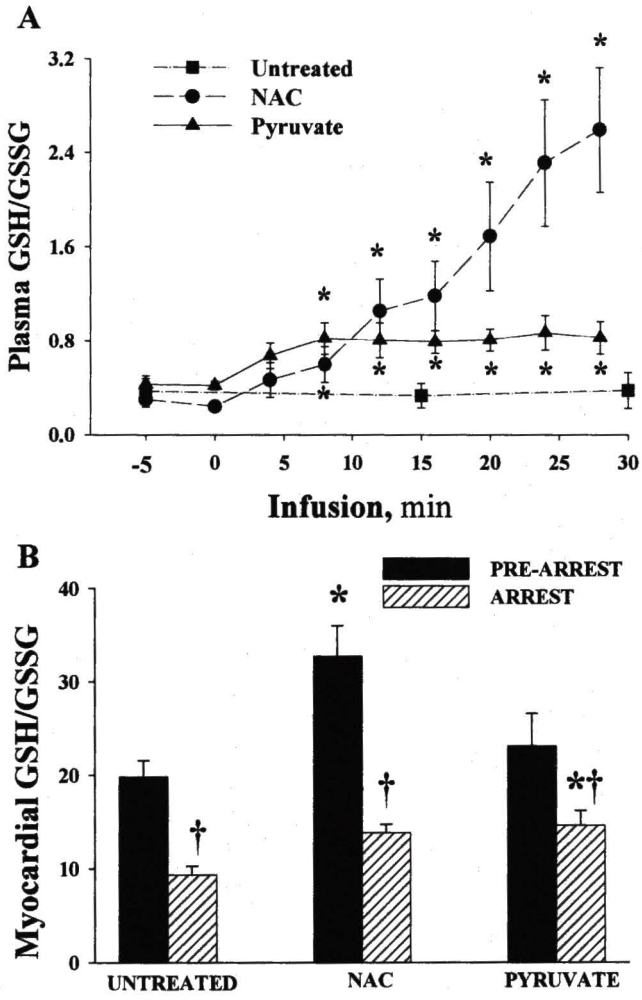
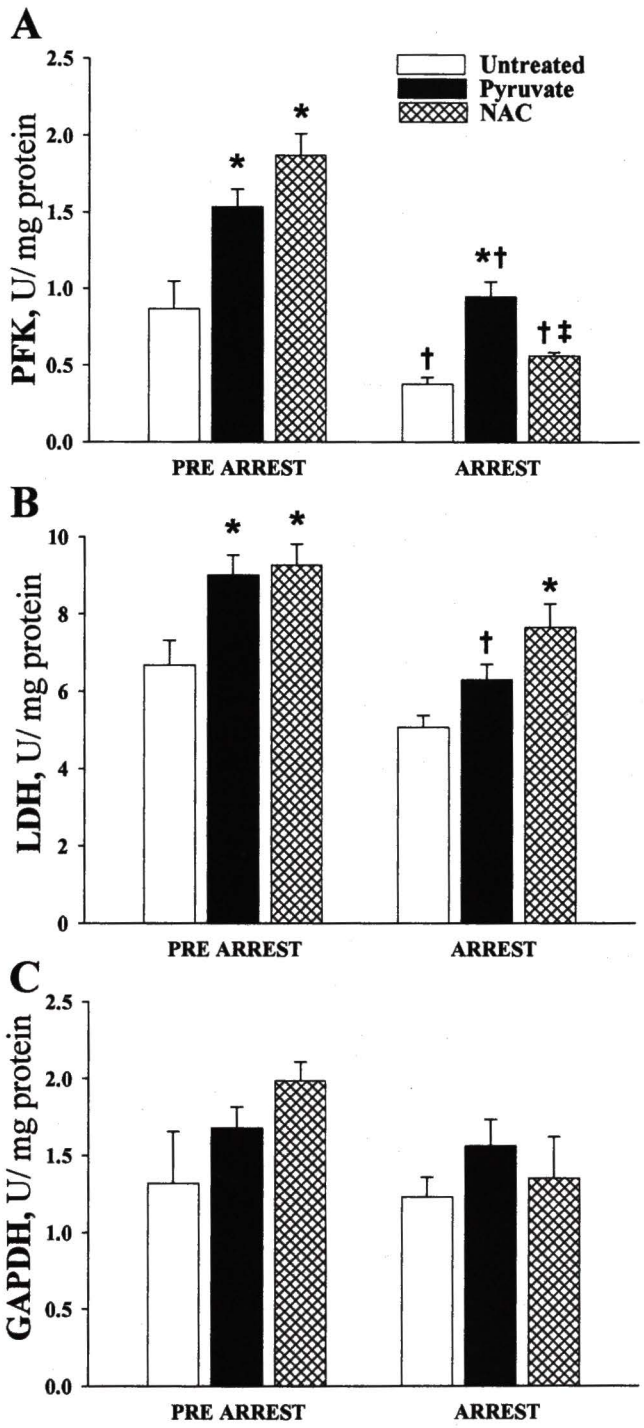

A
Untreated
NAC
Pyruvate
Plasma GSH/GSSG
3.2
2.4
1.6
0.8
0.0
-5
0
5
10
15
20
25
30
Infusion, min
B
PRE-ARREST
ARREST
Myocardial GSH/GSSG
40
30
20
10
0
UNTREATED
NAC
PYRUVATE

Figure 7.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Untreated
Pyruvate
NAC
PFK, U/ mg protein
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2.0
1.5
1.0
0.5
0.0
*
*
*†
†
†‡
PRE ARREST
ARREST
B
LDH, U/ mg protein
10
8
6
4
2
0
*
*
†
*
PRE ARREST
ARREST
C
GAPDH, U/ mg protein
2.5
2.0
1.5
1.0
0.5
0.0
PRE ARREST
ARREST

Figure 8.

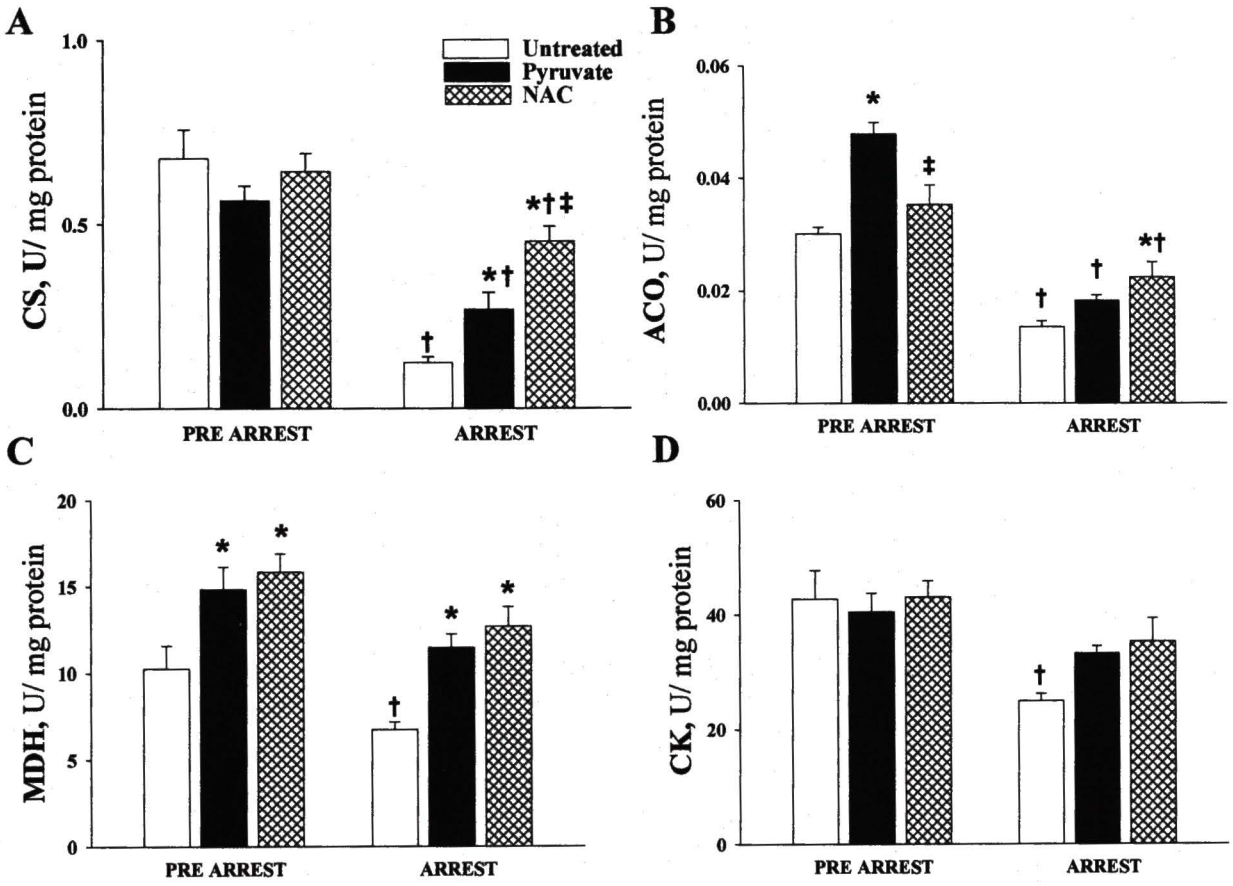

A
CS, U/ mg protein
1.0
0.5
0.0
Untreated
Pyruvate
NAC
*†‡
*†
†
PRE ARREST
ARREST
B
ACO, U/ mg protein
0.06
0.04
0.02
0.00
*
‡
*†
†
†
PRE ARREST
ARREST
C
MDH, U/ mg protein
20
15
10
5
0
*
*
*
*
†
PRE ARREST
ARREST
D
CK, U/ mg protein
60
40
20
0
†
PRE ARREST
ARREST

Figure 9.

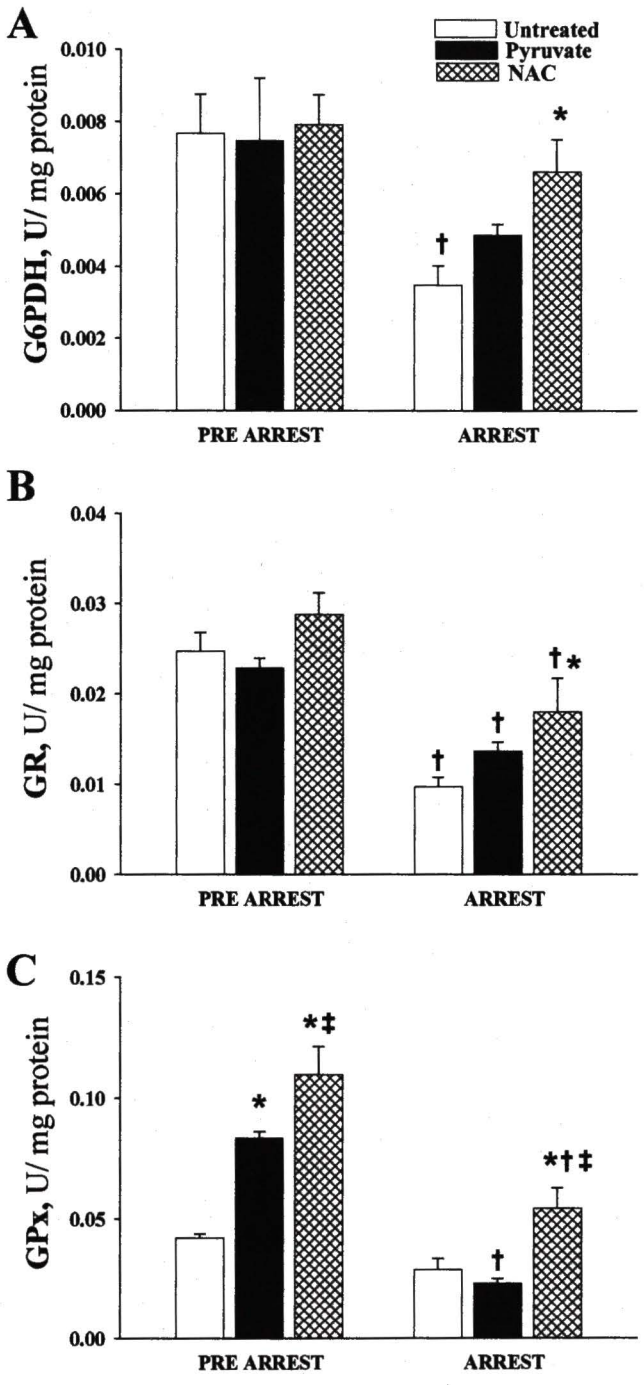

A
Untreated
Pyruvate
NAC
G6PDH, U/ mg protein
0.010
0.008
0.006
0.004
0.002
0.000
*
†
PRE ARREST
ARREST
B
GR, U/ mg protein
0.04
0.03
0.02
0.01
0.00
†*
†
†
PRE ARREST
ARREST
C
GPx, U/ mg protein
0.15
0.10
0.05
0.00
*‡
*
*†‡
†
PRE ARREST
ARREST

Figure 10.

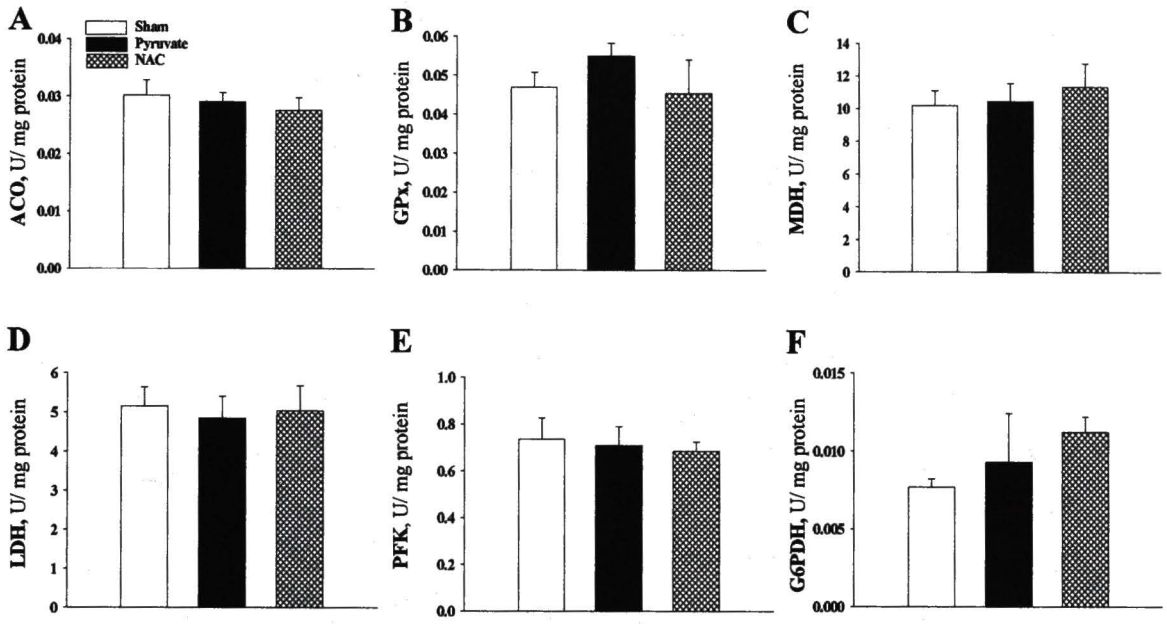
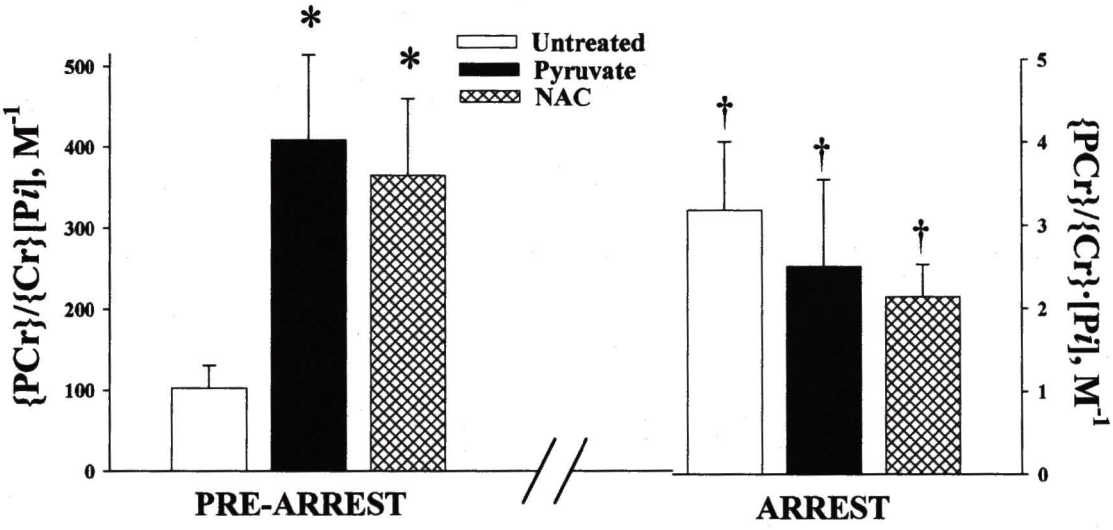

A
Sham
Pyruvate
NAC
ACO, U/ mg protein
0.04
0.03
0.02
0.01
0.00
B
GPx, U/ mg protein
0.06
0.05
0.04
0.03
0.02
0.01
0.00
C
MDH, U/ mg protein
14
12
10
8
6
4
2
0
D
LDH, U/ mg protein
6
5
4
3
2
1
0
E
PFK, U/ mg protein
1.0
0.8
0.6
0.4
0.2
0.0
F
G6PDH, U/ mg protein
0.015
0.010
0.005
0.000

Figure 11.


Untreated
Pyruvate
NAC
{PCr}/{Cr}[Pi], M-1
{PCr}/{Cr}·[Pi], M-1
500
400
300
200
100
0
5
4
3
2
1
0
*
*
†
†
†
PRE-ARREST
ARREST

Chapter IV

PYRUVATE ENHANCES NEUROLOGICAL RECOVERY FOLLOWING CARDIOPULMONARY ARREST AND RESUSCITATION

Arti B. Sharma,[1] Matthew A. Barlow,[1] Shao-Hua Yang,[2] James W. Simpkins,[2] Robert T. Mallet[1]

ABSTRACT

Purpose: Cerebral oxidative stress and metabolic dysfunction impede neurological recovery from cardiac arrest-resuscitation. Pyruvate, a potent antioxidant and energy-yielding fuel, has been shown to protect against oxidant- and ischemia-induced neuronal damage. This study tested whether acute pyruvate treatment during cardiopulmonary resuscitation (OCCC) can prevent neurological dysfunction and cerebral injury following cardiac arrest. **Methods:** Anesthetized, open-chest mongrel dogs underwent 5 min cardiac arrest, 5 min cardiac massage, defibrillation and 3 d recovery. Pyruvate (n = 9) or NaCl volume control (n = 8) were administered (0.125 mmol/kg/min *iv*) throughout OCCC and the first 55 min recovery. Sham dogs (n = 6) underwent surgery and recovery without cardiac arrest-resuscitation. **Results:** Neurological deficit score (NDS), evaluated at 2 d recovery, was sharply increased in NaCl-treated dogs (10.3 ± 3.5) *vs.* shams (1.2 ± 0.4), but pyruvate treatment mitigated neurological deficit (NDS = 3.3 ± 1.2; $P < 0.05$ *vs.* NaCl). Brain samples were taken for histological examination and evaluation of inflammation and cell death at 3 d recovery. Loss of pyramidal neurons in the hippocampal CA1 subregion was greater in the NaCl controls than in pyruvate treated dogs (11.7 ± 2.3% *vs.* 4.3 ± 1.2%; $P < 0.05$). Cardiac arrest increased caspase 3 activity, matrix metalloproteinase activity, and DNA fragmentation in the CA1 subregion; pyruvate prevented caspase-3 activation and DNA fragmentation, and suppressed matrix metalloproteinase activity. **Conclusion:** Intravenous pyruvate therapy during cardiopulmonary resuscitation prevents initial oxidative stress and neuronal injury and enhances neurological recovery from cardiac arrest.

Keywords: brain ischemia; cardiac arrest; cardiopulmonary resuscitation (CPR); cerebral blood flow; inflammatory response; metabolism; neurologic dysfunction; post-resuscitation period

Abbreviations: ANOVA, analysis of variance; ARR, cardiac arrest; BL, pre-arrest baseline; CA1, *cornus ammonis* region 1 of hippocampus; DAPI, 4',6-diamidino-2-phenylindole; MCT: monocarboxylate transporter; MMP, matrix metalloproteinases; MPO, myeloperoxidase; NDS, neurological deficit score; OCCC, open chest cardiac compressions; Q, regional blood flow; RFU, relative fluorescence units; ROS, reactive oxygen species; ROSC, return of spontaneous circulation; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling

Introduction

Neurological damage inflicted by cardiac arrest-resuscitation contributes to high mortality and residual neurological dysfunction after cardioversion.[1,2] Cardiac arrest-resuscitation imposes global cerebral ischemia-reperfusion. Interruption of cerebral blood flow during arrest depletes high energy metabolites required to maintain neuronal function and integrity. Cerebral oxidative stress during resuscitation and following cardioversion[3] can exacerbate structural and functional impairment. Markers of systemic inflammation are elevated after cardiopulmonary resuscitation,[4, 5] and inflammatory mediators, *e.g.* matrix metalloproteinases[6] are activated in the brain. Oxidative and inflammatory cascades initiated during cerebral ischemia-reperfusion culminate in cell injury and death[7,8] which can continue for several days following transient ischemic insult.[9-11] Neuronal death following transient global ischemia involves both necrosis and apoptosis[12] and correlates with clinical impairment of function.[10]

Pyruvate, a well documented natural antioxidant and metabolic fuel,[13] bolsters energy reserves and antioxidant redox state in post-ischemic and oxidant-injured myocardium.[13] Pyruvate readily traverses the blood brain barrier via monocarboxylate transporters[14] and provides an excellent energy substrate for neurons and astroglia.[15] Pyruvate's antiacidotic,[16,17] anti-inflammatory[18] and anti-apoptotic[19] properties could minimize neuronal death following cerebral ischemia-reperfusion. Indeed, pyruvate treatment preserves neurons following exposure to oxidants.[20,21] Pyruvate also improves neuronal survival following hypoglycemic insult[22] and minimizes neuronal loss 72 h after transient forebrain ischemia in rats.[23] Intravenous pyruvate stabilized cerebral energy reserves and preserved electroencephalographic activity in pigs subjected to hemorrhagic shock.[24] Collectively, these reports suggest that application of

pyruvate during cardiopulmonary resuscitation could minimize ischemic injury inflicted on the central nervous system by cardiac arrest.

This study tested whether intravenous administration of sodium pyruvate during cardiopulmonary resuscitation prevented neurological deficit and neuronal injury 2-3 days after cardiac arrest. Open chest dogs underwent 5 min cardiac arrest, 5 min of open chest cardiac compressions and cardioversion. Neurological recovery was evaluated over the next 3 days, then brain tissue was harvested for assessment of inflammation and neuronal injury. Administration of pyruvate during cardiac massage and the first 55 min of recovery prevented neurological deficit and neuronal loss 3 days after global cerebral ischemia imposed by cardiac arrest.

Methods

Instrumentation and protocol surgery

All animal experimentation was conducted according to the *Guide to the Care and Use of Laboratory Animals* (NIH Publication 85-23, revised 1996), and was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center. Twenty-three mongrel dogs weighing 22 ± 4 kg were randomly assigned to sham (3♂, 3♀), pyruvate (4♂, 5♀) or vehicle control NaCl (4♂, 4♀) groups. Pyruvate and NaCl groups underwent an open chest cardiac arrest-CPR-defibrillation protocol[17] under sterile conditions. Sham controls underwent the same surgical procedures and 3 days recovery without cardiac arrest-resuscitation. The left saphenous vein was cannulated for infusion of pyruvate or NaCl, and the left antecubital vein was cannulated to administer drugs during resuscitation. Rectal temperature was maintained at 36-37°C using circulating water heating pads. The dogs were sedated with acepromazine (0.1 mg kg^{-1} *i.m.*), anesthetized with thiopentathal (15 mg kg^{-1} *iv*), and then maintained with 1-2% halothane in O_2 (1 L min^{-1}) enriched air. Following endotracheal intubation, the dogs were artificially ventilated to maintain arterial O_2 saturation, monitored with a pulse oximeter, within physiological limits of 94-96%. Under sterile conditions the chest cavity was opened via left lateral thoracotomy through the 5^{th} intercostal space, and the pericardium was divided and reflected to expose the left ventricular epicardium. A 17 gauge catheter was implanted in the thoracic aorta to monitor blood pressure and sample arterial blood. This catheter was externalized through the chest layers and fixed to the skin. Animals were then allowed to stabilize for at least 15 min before starting the arrest-resuscitation protocol.

Cardiac arrest- resuscitation protocol

To initiate cardiopulmonary arrest, ventricular fibrillation was induced by applying a 9V current to the epicardium, and ventilation was suspended. At 5 min arrest, 1 mg epinephrine was injected into the left ventricular lumen, intravenous infusion of pyruvate or NaCl was begun, and mechanical ventilation was resumed at 12 cycles min^{-1}. Open chest cardiac compressions (OCCC; 60 min^{-1}) were administered at 5-10 min arrest to maintain mean aortic pressure ~ 40 mmHg. Sterile 2M solutions of pyruvate or NaCl were continuously infused (0.125 $mmol \cdot kg^{-1} \cdot min^{-1}$) into the left saphenous vein from the beginning of OCCC until 55 min after recovery of spontaneous circulation (ROSC). The heart was defibrillated with direct current countershocks applied to the epicardium with sterile internal paddles. Up to 8 (3 · 10 J, 3 · 20 J, 2 · 30 J) countershocks were administered, with 15 s intervening OCCC, until spontaneous rhythm was reestablished. ROSC was defined as sustained mean aortic pressure of 60 mmHg[25] at normal sinus rhythm. Sodium bicarbonate (10 mEq, *iv*) was administered to counter metabolic acidosis, and lidocaine (1 mg/kg, *iv*) was injected for treatment of severe ventricular dysrhythmias. Controlled norepinephrine infusion (~1 $\mu g\ min^{-1}\ kg^{-1}$) was given as needed to maintain mean aortic pressure between 60-80 mm Hg. After ROSC and 30 min cardiac stabilization, the chest was closed in layers and the dog was allowed to recover.

Plasma 8-isoprostane measurement

Aortic whole blood (10 ml) was collected in heparinized syringes at baseline, 3 min arrest, 3 min OCCC and 5, 15, 25, 60, 90, 120 and 150 min ROSC, and centrifuged to obtain clear plasma. 8-isoprostane, an indicator of lipid peroxidation,[3] was measured in flash frozen plasma[26,27] using competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI).

Neurological deficit evaluation

A simplified assessment was used to evaluate neurological recovery on the first post-surgical day. Deficit score was assigned based on six criteria: level of consciousness (0/1/2/3), respiration (0/1), response to oral commands (0/1), walking (0/1), limb movements (0/1), and chewing and drinking (0/1). An aggregate score of zero indicated normal function, and a score of eight, profound impairment.

Neurological function was assessed the day before cardiopulmonary arrest and on recovery day 2 using a standard canine neuro-deficit scoring (NDS) protocol.[28, 29] This procedure evaluates 22 criteria in five categories: level of consciousness, respiratory pattern, cranial nerve function, sensorimotor function, and behavior. A score of zero represents normal neurological function, and 100 represents brain death. Two investigators blinded to the treatments scored each dog and the mean of the two scores was taken as the result.

Tissue sampling for analyses of brain injury

On recovery day 3, dogs were pre-anesthetized with acepromazine (0.1 mg kg^{-1} *i.m.*), anesthetized with α-chloralose (100 mg kg^{-1} *i.v.*), intubated and mechanically ventilated. The brain was removed via craniotomy. The cerebrum was divided by a mid-sagittal cut. The left hemisphere was dissected, and hippocampus and cerebellum were excised and flash-frozen in liquid N_2 for further analyses. The right hippocampus and cerebellum were fixed in 10% neutral buffered formalin, rinsed with 70% ethanol, and then dehydrated in series. Sections (8 μm) were processed, paraffin-embedded, and stained with hematoxylin-eosin.

Caspase-3 activity

Activity of caspase-3 was measured in flash-frozen brain tissue according to Wen *et al.*[30] Brain tissue was extracted in ice cold lysis buffer for 1 h and homogenized. The extract was diluted in an equal volume of assay buffer containing caspase-3 fluorogenic substrate (Ac-DEVD-AMC; 20 μM; Pharmingen, San Diego, CA), and incubated for 60 min at 37°C. Fluorescence was measured in a microplate fluorospectrometer (FL600, Boi-tek Instruments, Winooski, VT), at 380 nm excitation/440 nm emission wavelengths. Purified caspase-3 (Pharmingen, San Diego, CA) provided positive control.

Myeloperoxidase activity

Myeloperoxidase activity, an indicator of neutrophil infiltration,[31] was measured in frozen brain samples as described by Koh *et al.*,[32] with modifications. 100 mg of frozen brain tissue was pulverized in a liquid N_2-precooled mortar, homogenized in 1 ml 20 mM K_2HPO_4 buffer, pH 7.4, and then centrifuged (20,000 g, 15 min, 4°C). The pellet was re-extracted 3 times in 150, 100 and 100 μl of 0.5% hexadecyltrimethylammonium bromide/ethylene-diamine-tetraacetate, and the four supernatants were combined. 50 μl of extract was combined with 250 μl of 50 mM K_2HPO_4/0.017% o-dianisidine hydrochloride/0.0005% H_2O_2, pH 6.0. Enzyme activity was computed from the rate of absorbance change at 460 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT), by comparison with a horseradish peroxidase standard curve.[33] Myeloperoxidase activity was undetectable in the pellets.

Matrix metalloproteinase (MMP) activity

The aggregate activities of MMPs in the brain samples were assayed fluorometrically.[34] Brain tissue was extracted in 20 mM Tris/150 mM NaCl/0.5% Triton-X 100 (pH 7.4). Protein (50 μg) was diluted in assay buffer (50 mM Tris base, 200 mM NaCl, 5 mM $CaCl_2$, Brij35 0.02%, pH 7.6) to a final concentration of 1 mg protein/ml. The fluorescence energy transfer peptide substrate TNO211 (Calbiochem) was added to a concentration of 10 μM.[35] The samples were incubated at 37°C for 3 h, and fluorescence was measured at 340 nm excitation and 485 nm emission wavelengths.

Histological examination

Hematoxylin-eosin-stained sections were examined by light microscopy for structural hallmarks of neuronal cell death, including cytoplasmic hypereosinophilia, absence of visible Nissl substance, nuclear condensation (pyknosis) and lack of a discrete nucleolus.[10,36] Viable and nonviable, hypereosiniphilic neurons were counted manually by an investigator blinded to treatments, and percentages of nonviable neurons were computed. Three sections at least 15 μm apart were examined for each experiment and mean value was taken. Cerebellar sections were examined for neuronal loss in the same manner.

Paraffin embedded hippocampal sections were terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL)-stained[37] to detect DNA fragmentation due to apoptotic cell death.[38] Necrotic and non-neuronal cell death can also give TUNEL positivity, but such staining is more diffuse than that of apoptotic cells, and characteristic apoptotic bodies are absent.[38] For negative controls, TdT enzyme was replaced with double-distilled H_2O.

Brain regional blood flow measurements

In a separate series of 4 sham, 5 pyruvate and 5 NaCl experiments, dogs were surgically prepared as described above, and then underwent the cardiac arrest-resuscitation protocol with 3 hr ROSC. Brain blood flow was measured using radioactive microspheres.[39] Briefly, $5 \cdot 10^6$ microspheres (15 μm diameter) radiolabeled with ^{46}Sc, ^{103}Ru or ^{141}Ce were injected into the aorta at pre-arrest baseline and at 15 and 180 min ROSC. Duplicate arterial reference samples were collected at a constant rate for 3 min after microsphere injection to confirm adequacy of microsphere mixing in the blood. At the end of the experiment brain was dissected to obtain 1 g sections of hippocampus, cerebellum and frontal, parietal, occipital and temporal cortex. Radioactivities of tissue and arterial reference samples were measured in a gamma counter (Packard Instruments, Meridian, CT). Regional blood flow was expressed as $ml \cdot min^{-1} \cdot g^{-1}$ of brain tissue.

Statistical analysis

All data are presented as mean ± SEM. Between-group comparisons of brain blood flow, neurodeficit scores, caspase-3, matrix metalloproteinase and myeloperoxidase activities, and TUNEL analyses were accomplished by one way analysis of variance (ANOVA). Within group differences in plasma 8-isoprostane and cerebral blood flow were analyzed by repeated measures ANOVA. Student-Newman-Keul's multiple comparison test was applied when ANOVA detected significant differences. P values <0.05 were taken to indicate statistical significance. Statistical analyses were performed with Sigma Stat software version 3.1.

Results

8-isoprostane

Arterial plasma 8-isoprostane concentration, a global measure of oxidative stress, did not change during arrest, but almost doubled by 5 min ROSC (Figure 1) and remained elevated until 60 min of recovery before subsiding. Pyruvate treatment during OCCC and the first 55 min of ROSC blunted the post-arrest increase in 8-isoprostane.

Brain blood flow

Regional brain blood flow was measured using radioactive microspheres. At 15 min of ROSC blood flow was depressed in frontal cortex (Figure 2C), while the decline in other regions was not appreciable (Figure 2). Pyruvate treatment increased flow in occipital (Figure 2D) and parietal cortex (Figure 2F) and partially prevented the drop in frontal cortical flow (Figure 2C) at 15 min ROSC. However, by 180 min of ROSC all brain regions were underperfused, regardless of treatment, in comparison with baseline or sham control flows.

Post-arrest neurological deficit

A simple evaluation was used to assess neurological function on day 1 post-arrest (Figure 3A). The non-arrested, sham-operated dogs completely recovered from the surgery. All shams were fully conscious, responsive, breathing normally, able to walk, eat and drink; accordingly, each sham dog scored zero. Appreciable neurological impairment was evident in the NaCl group on the day following arrest, but pyruvate treatment during OCCC and the first 55 min ROSC minimized subsequent impairment 24 h later (Figure 3A).

The dogs were evaluated by standard, comprehensive neurological examination [26, 27] before the arrest-resuscitation experiment and on the 2nd day of recovery (Figure 3B). Marked neurological deficit was detected in the NaCl group on day 2 post-arrest. Pyruvate treatment during OCCC and initial recovery mitigated subsequent neurological impairment. Figure 4 presents scores for each of the 22 functions examined on recovery day 2. Modest abnormalities in carinal, muscle stretch and cleaning reflexes were noted in the sham group. Cardiac arrest-resuscitation appreciably impaired the ciliospinal reflex, response to painful stimuli, standing and walking. Pyruvate treatment minimized subsequent impairment of these responses.

Caspase-3

Activity of caspase-3, the effector protease in apoptotic cell death mechanisms, was measured in hippocampus (Figure 5A) and cerebellum (Figure 5B) at 3h and 3 d ROSC. Caspase-3 activity increased in the NaCl group *vs.* sham-arrested controls (Figure 5A) at 3 d recovery. Pyruvate treatment during OCCC and initial ROSC suppressed activation of the cell death effector. Caspase-3 activity did not differ among the sham-arrested, NaCl and pyruvate groups in the cerebellum at 3 h recovery (Figure 5B), nor did pyruvate prevent subsequent activation of caspase 3.

Hippocampal matrix metalloproteinase (MMP) and myeloperoxidase (MPO)

Matrix metalloproteinase and MPO activities were measured in hippocampus at 3 d ROSC. Appreciable MMP activity was evident in the NaCl-treated dogs, but was attenuated when pyruvate was administered during initial resuscitation (Figure 6A). MPO activity, a measure of inflammatory cell infiltration, tended to be higher in NaCl dogs *vs.* shams, which also had

detectable MPO activity (Figure 6B) following 3 d post-operative recovery. In pyruvate-treated dogs, hippocampal MPO activity was similar to sham controls, although the decrease *vs.* the NaCl group was not statistically significant.

Hematoxylin-eosin staining

Cardiopulmonary arrest-resuscitation caused appreciable neuronal injury in the pyramidal cell layer of the hippocampal CA1 subregion of NaCl-treated dogs (Figure 7A). Damaged neurons were hypereosinophilic, and lacked normal dendritic structure. Pyruvate treatment during OCCC and early recovery preserved neuronal structure (Figure 7A). By 3 d recovery, nearly 12% of the CA1 pyramidal cells exhibited structural hallmarks of irreversible injury in the dogs treated with NaCl during resuscitation (Figure 7B). Pyruvate treatment substantially improved neuronal survival.

Cerebellar cortical sections were also examined at 3 d recovery. Structure of Purkinje cells remained intact in a non-arrested sham dog (Figure 8A). Degeneration of Purkinje cells was evident in a NaCl-treated dog (Figure 8B) with loss of granular chromatin and indistinct nucleolus and dendritic processes. In contrast, pyruvate treatment during early ROSC minimized neuronal degeneration at 3 d recovery (Figure 8C).

TUNEL assay of DNA fragmentation

DNA fragmentation in the hippocampal CA1 subregion was detected by TUNEL at 3 d recovery. Light microscopic examination of the CA1 region revealed nuclear condensation and evidence of pyknosis. TUNEL-positive nuclei were identified by green fluorescent nuclei with overlapping

blue fluorescent DAPI stain (Figure 9A). Percentages of TUNEL-positive nuclei in the three groups are presented in Figure 9B. The NaCl-treated cardiac arrest group displayed considerably higher percentage of TUNEL-positive nuclei than shams. Pyruvate tended to lower the percentage of TUNEL-positive nuclei, although the difference was not statistically significant [P = 0.056].

Discussion

Cellular energy depletion and oxidant stress activate mechanisms leading to eventual neuronal loss and neurological dysfunction following cardiac arrest-resuscitation. This study tested whether intervention during the immediate resuscitative period with intravenous pyruvate, a potent energy-yielding fuel and antioxidant, could ameliorate post-ischemic neurological deficit, neuronal death and inflammation during the 3 days following cardiac arrest and cardiopulmonary resuscitation.

Cardiac-arrest resuscitation resulted in significant neurological impairment during the first 2 days of recovery. Intravenous pyruvate during OCCC and the first 55 min of recovery from cardiac arrest attenuated subsequent neurological impairment. During its infusion pyruvate also suppressed systemic arterial oxidative stress. Hippocampal caspase-3 was activated 3 d after arrest; pyruvate treatment suppressed activation of this cell death effector. Pyruvate also suppressed pro-inflammatory myeloperoxidase and matrix metalloproteinase activities in hippocampus. Histological examination at 3 d recovery revealed severe structural damage to hippocampal CA1 pyramidal neurons and TUNEL-detectable DNA fragmentation, both of which were prevented by pyruvate treatment. Although pyruvate moderately increased perfusion at 15 min ROSC in the occipital and frontal cortex, it didn't increase perfusion of the hippocampus at any time point. Accordingly, pyruvate's salutary effects on neurons in this region could not be ascribed to improved perfusion following ROSC. Instead, by bolstering metabolism and antioxidant state in the first 60 min of reperfusion, pyruvate may have prevented inflammatory injury and neuronal loss, and thereby improved neurological recovery.

Pyruvate transport in brain

Within 10-30 s of intravenous administration pyruvate concentration can increase to therapeutically effective levels in the brain.[14,15] Pyruvate traverses the blood-brain barrier via monocarboxylate transporters (MCTs) which co-transport pyruvate and a proton.[40] This transport is enhanced in acidemic conditions such as post-ischemia, due to increased plasma H^+ concentration. Increased circulating pyruvate and H^+ concentrations also would augment entry of pyruvate into neurons and astrocytes, which are equipped with monocarboxylate transporters MCT-2 and 4.[20,40] MCT-4 is abundant in ischemia-reperfusion susceptible areas such as the hippocampus.[40]

Antioxidant and anti-apoptotic actions of pyruvate during early ROSC

Caspase-3 in brain is activated following transient global ischemia with cardiac arrest.[41] In this study, caspase-3 activity increased appreciably at 3 d ROSC *vs.* sham controls, but pyruvate restrained caspase-3 activity to sham levels. Inhibition of caspase-3 prevents delayed neuronal death after transient ischemia[42] and improves neurological outcome.[41] Brain tissue contains high concentrations of polyunsaturated fatty acids that are targets of oxyradical attack. In dogs, 10 min cardiac arrest results in increased cerebrocortical lipid peroxidation products, which remain elevated even after 24 h recovery.[43] Moreover, arterial 8-isoprostane, an indicator of cerebral lipid peroxidation, increases following cardiac arrest.[3] Pyruvate infusion blunted the increase in plasma 8-isoprostane during OCCC and early reperfusion, indicating decreased post-ischemic oxidative stress. Reactive oxygen species (ROS) can trigger inflammation and apoptosis following ischemia-reperfusion in the brain.[8] Pyruvate has been shown to dampen ROS formation in brain.[44,45] Pyruvate and other α-keto carboxylates can directly neutralize oxidants such as H_2O_2 and $ONOO^-$ and thus reduce oxidative stress.[13,46] Pyruvate also acts as an

antioxidant by enhancing NADPH formation which in turn increases glutathione redox state.[13] Pyruvate concentration-dependently protected cultured human neuroblastoma cells from H_2O_2 toxicity by suppressing H_2O_2-induced ROS formation and attenuating oxidative stress-induced collapse of the mitochondrial membrane potential.[21] By dampening oxidative stress at the onset of cerebral reperfusion pyruvate treatment may have prevented activation of inflammatory and cell death pathways triggered by ROS.

Anti-inflammatory effects of pyruvate

Both oxidative stress[3] and inflammation[11] following cardiac arrest contribute to neuronal death. The CA1 subregion of the hippocampus is particularly susceptible to ischemia and exhibits delayed neuronal death[11,47] following acute ischemic insult. Matrix metalloproteinases, activated for several days following transient cerebral ischemia,[48,49] have been implicated in neuronal death in the hippocampal CA1 subregion.[50,51] ROS[52,53] and inflammatory mediators activate MMP following cerebral ischemia.[54] Myeloperoxidase activity increases up to 3 days after cerebral ischemia and correlates with ischemic damage in the brain[55] and neutrophil infiltration.[31] Pyruvate suppressed the marked MMP activation evident in the CA1 region at 72 h ROSC. This effect of pyruvate may have helped maintain integrity of the blood brain barrier and thereby minimized neutrophil infiltration, although the trend in the pyruvate group toward lower CA1 myeloperoxidase activity, a neutrophil marker enzyme, didn't achieve statistical significance. The anti-inflammatory effect of pyruvate may have contributed to its ability to prevent neuronal death and neurological dysfunction following cerebral ischemia-reperfusion.

Pyruvate prevents neuronal loss following cardiac arrest-resuscitation

Activated microglia produce NO, oxyradicals and cytokines, [56, 57] and are instrumental in post-ischemic inflammation. Ethyl pyruvate prevented lipopolysaccharide-induced inflammation and nuclear factor-κB activation in cultured microglia.[18] Intraperitoneal pyruvate, administered at 1 h reperfusion following 12 min ischemia, prevented hippocampal neuronal loss 72 h later.[23] Collectively, pyruvate may have protected neurons during initial cerebral reperfusion following cardiac arrest by minimizing acute energy depletion, oxidative stress and inflammation.

Clinical significance

As early as 1984 pyruvate was infused *iv* to humans without adverse effects.[58] Pyruvate has proven safe and efficacious as a major component of cardioplegia solutions for cardiopulmonary bypass.[59] Intravenous pyruvate during resuscitation exerts powerful antioxidant and energetic effects in myocardium that improve immediate cardiac recovery.[17] Thus, pyruvate may be therapeutically effective in ischemia-reperfusion scenarios involving the heart and brain.

Limitations

This study examined neurological injury at 72 h ROSC, a time when hippocampal CA1 neuronal death would be manifest.[60] However, cell death processes following transient ischemic insult can continue for a month.[47] Indeed, Lee *et al.* demonstrated neuroprotection by pyruvate even at 30 d of recovery from transient forebrain ischemia in rats.[23] Evaluation of neurodeficit required a high success rate of resuscitation, so cardiac arrest was limited to 5 min with an additional 5 min of OCCC, and an open chest preparation was used to facilitate cardiac massage. Increasing

the arrest interval and use of closed-chest OCCC could jeopardize successful ROSC, although it would more closely model clinical scenarios, especially cardiac arrests outside the hospital.

Conclusions

Cardiac arrest-resuscitation results in cerebral inflammation, neurological damage and neuronal loss 3 d later. Acute treatment with intravenous pyruvate prevents oxidative stress during initial ROSC, attenuates inflammatory response, preserves neurological function and prevents neuronal loss. An antioxidant and energy-yielding fuel, pyruvate could provide a safe and effective adjunct to conventional resuscitation to preserve neurological function following cardiopulmonary arrest.

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9. FIGURE LEGENDS

Figure 1. *Plasma 8-isoprostane.* 8-isoprostane concentration was measured in arterial plasma sampled at pre-arrest baseline (BL), 3 min cardiac arrest (ARR), 3 min OCCC, and at several times during ROSC. Values are means ± SEM. *P < 0.05 *vs.* sham; †P < 0.05 *vs.* NaCl at the same time.

Figure 2. *Regional brain blood flows.* Blood flows (Q) were measured at baseline, 15 min ROSC and 180 min ROSC in hippocampus (Panel A), cerebellum (Panel B), and frontal (Panel C), occipital (Panel D), temporal (Panel E) and parietal cortex (panel F) of 5 NaCl (hatched bars) and 5 pyruvate (filled bars) experiments. Flows were measured in 4 sham experiments (open bars) at a time corresponding to 180 min ROSC. Values are means ± SEM. *P < 0.05 *vs.* baseline same treatment; ‡P < 0.05 *vs.* 15 min ROSC same treatment; †P < 0.05 *vs.* NaCl at the same time.

Figure 3. *Neurological deficit after cardiac arrest-resuscitation.* Panel A: a simplified neurological evaluation was used to assess recovery on day 1 post-arrest, scored between 0 (normal) to 8 (profound impairment). Data are means ± SEM, *P < 0.05 *vs.* sham; †P < 0.05 *vs.* NaCl. Panel B: Standard neurodeficit score pre-arrest (hatched bars) and on day 2 following recovery from cardiac arrest-resuscitation (filled bars), expressed as percentages of maximum score. Values are means ± SEM. * P<0.05 *vs* day 2 sham; † P<0.05 *vs* day 2 NaCl.

Figure 4. *Individual components of neurodeficit score.* Functions were examined at day 2 recovery in sham-arrested controls (open bars) and in dogs subjected to cardiac arrest-

resuscitation and treated with NaCl (hatched bars) or pyruvate (solid bars) during OCCC and ROSC. Values are means ± SEM. * P<0.05 *vs* sham; † P<0.05 *vs* day 2 pyruvate.

Figure 5. *Activation of cell death effector caspase-3 following cardiac arrest-resuscitation.* Caspase-3 activities, expressed as relative fluorescence units (RFU)/mg protein, in hippocampus (Panel A) and cerebellum (Panel B), at 3 h (hatched bars) and 3 day ROSC (solid bars), in sham, NaCl and pyruvate treated experiments. Values are means ± SEM. * P<0.05 *vs* sham; †P < 0.05 *vs*. NaCl.

Figure 6. *Pro-inflammatory enzymes in hippocampus 3 d after cardiac arrest-resuscitation.* Panel A: Hippocampal matrix metalloproteinase (MMP) activities in sham (n=6), NaCl (n=8) and pyruvate (n=8) treated dogs at 3 d ROSC. MMP activity is expressed as relative fluorescence units (RFU) per mg protein. Panel B: Myeloperoxidase (MPO) activity (U/g protein) measured in the same experiments as panel A. Values are means ± SEM. *P < 0.05 *vs.* sham; †P < 0.05 *vs.* NaCl.

Figure 7. *Neuronal loss in hippocampal CA1 subregion 3 days after cardiac arrest-resuscitation.* Panel A: Representative hematoxylin-eosin stained sections from sham, NaCl-treated and pyruvate-treated dogs. Panel B: Neuronal loss, expressed as % loss of neuronal structure. Values are means ± SEM. *P < 0.05 *vs.* sham; †P < 0.05 *vs.* NaCl.

Figure 8. *Cerebellar neuronal degeneration 3 days following cardiac arrest-resuscitation.* Representative hematoxylin-eosin sections depicting cerebellar cortex with inner granular, outer molecular and middle Purkinje cell layer. Hypereosinophilia and abnormal architecture of

Purkinje cells is visible in NaCl treated dogs (panel B), but not in the sham and pyruvate treated experiments. Insets: enlargements of single cell bodies of Purkinje cells in each condition.

Figure 9. *Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) of DNA fragmentation in the hippocampal CA1 region.* Panel A: representative TUNEL and DAPI stained images from sham, NaCl and pyruvate-treated dogs at 3 d ROSC. Green fluorescence: TUNEL-positive nuclei; blue fluorescence: DAPI-staining to detect all nuclei. Panel B: percentages of CA1 cells with TUNEL positive nuclei. Values are means ± SEM. *P < 0.05 *vs.* sham.

Figure 1.

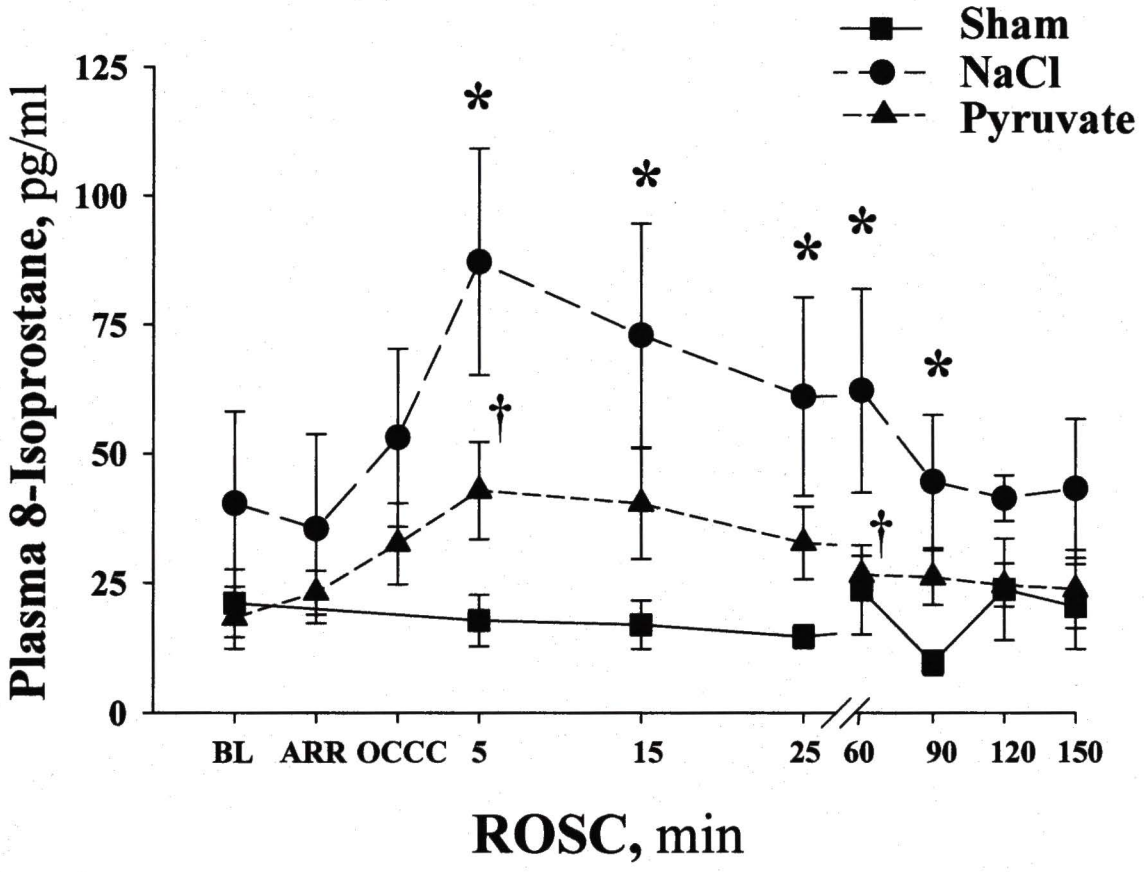

Sham
NaCl
Pyruvate
Plasma 8-Isoprostane, pg/ml
125
100
75
50
25
0
BL
ARR
OCCC
5
15
25
60
90
120
150
ROSC, min

Figure 2.

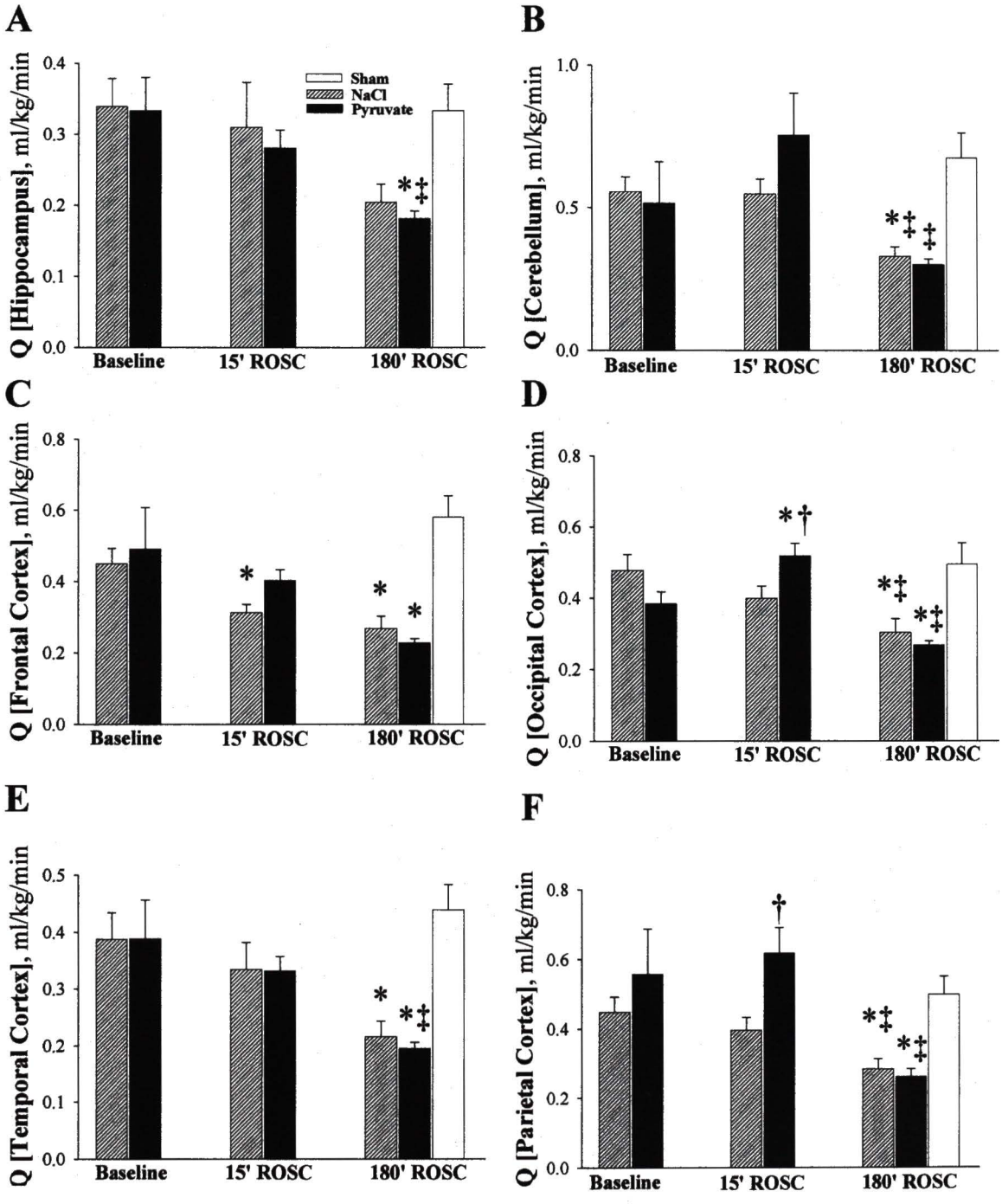

A
B
C
D
E
F
Sham
NaCl
Pyruvate
Q [Hippocampus], ml/kg/min
Q [Cerebellum], ml/kg/min
Q [Frontal Cortex], ml/kg/min
Q [Occipital Cortex], ml/kg/min
Q [Temporal Cortex], ml/kg/min
Q [Parietal Cortex], ml/kg/min
Baseline
15' ROSC
180' ROSC

Figure 3.

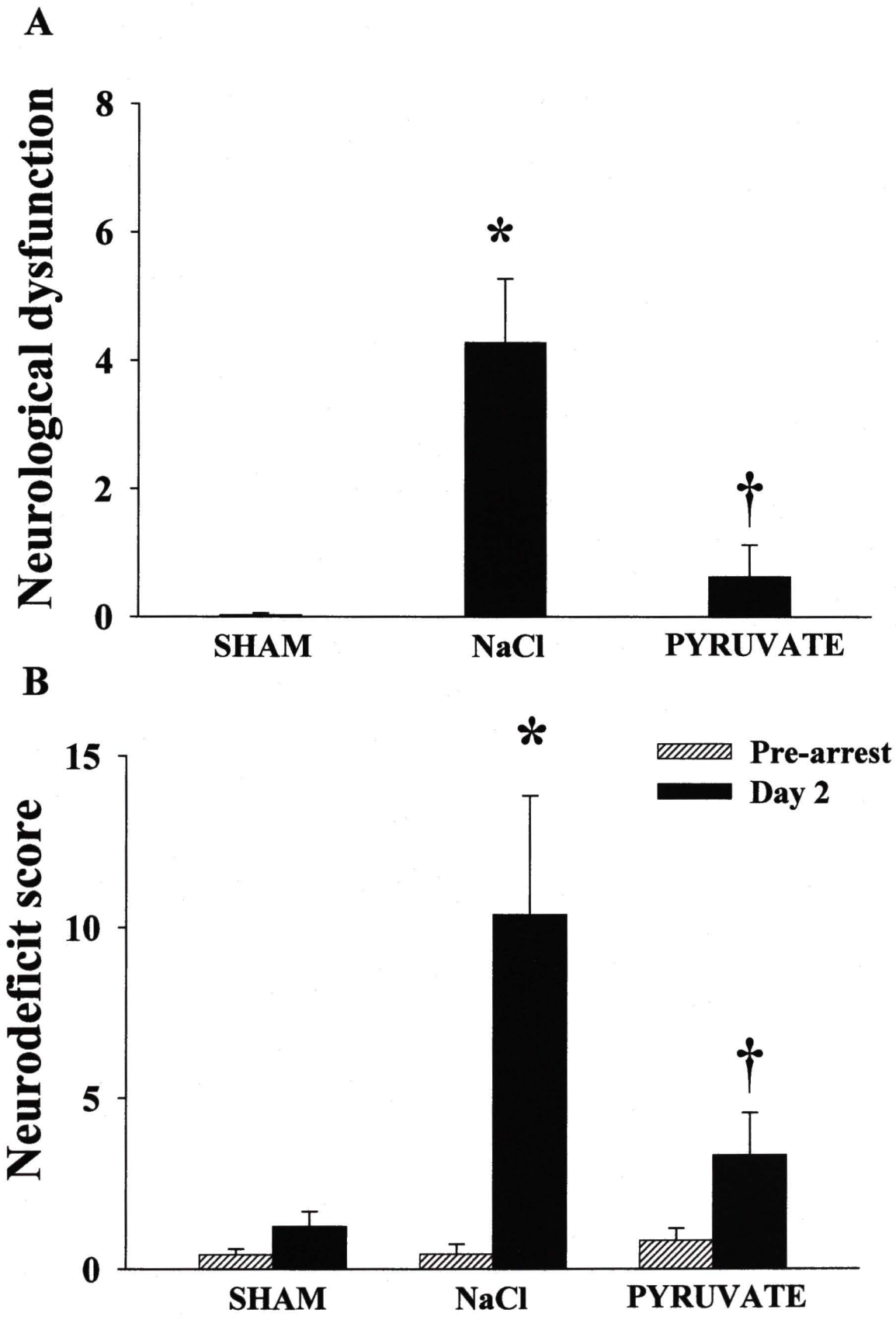

A
Neurological dysfunction
8
6
4
2
0
*
†
SHAM
NaCl
PYRUVATE
B
Neurodeficit score
15
10
5
0
*
†
Pre-arrest
Day 2
SHAM
NaCl
PYRUVATE

Figure 4.

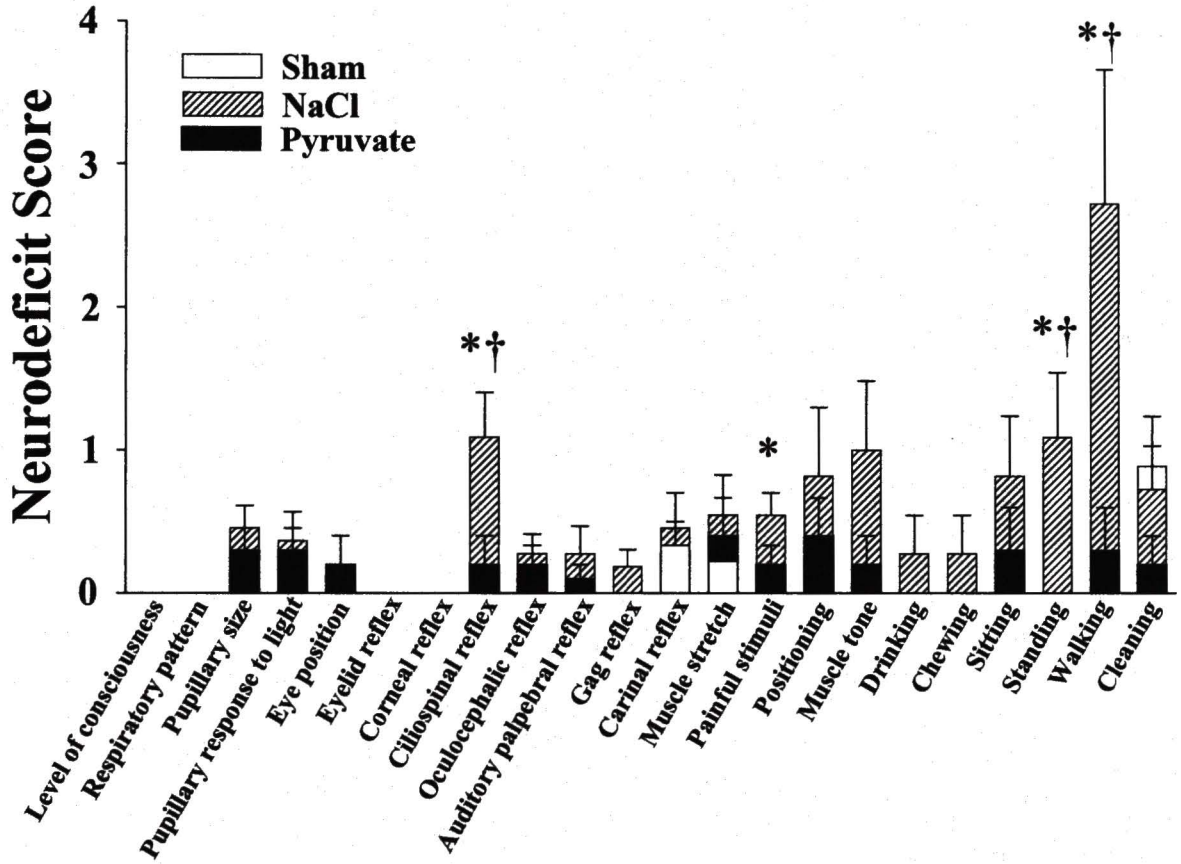

Neurodeficit Score
4
3
2
1
0
Sham
NaCl
Pyruvate
*†
*
*†
*†
Level of consciousness
Respiratory pattern
Pupillary size
Pupillary response to light
Eye position
Eyelid reflex
Corneal reflex
Ciliospinal reflex
Oculocephalic reflex
Auditory palpebral reflex
Gag reflex
Carinal reflex
Muscle stretch
Painful stimuli
Positioning
Muscle tone
Drinking
Chewing
Sitting
Standing
Walking
Cleaning

Figure 5.

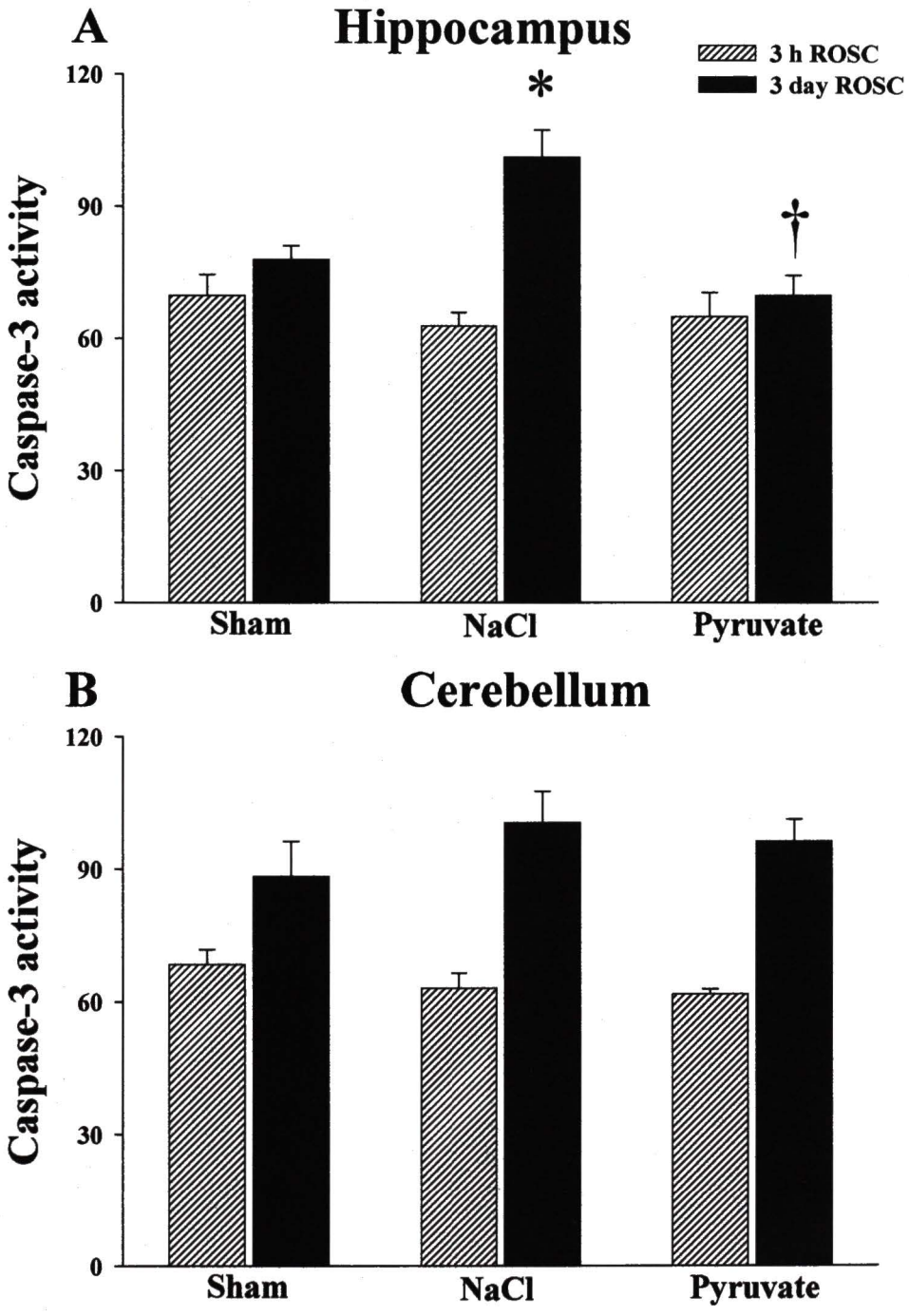

A
Hippocampus
3 h ROSC
3 day ROSC
*
†
Caspase-3 activity
120
90
60
30
0
Sham
NaCl
Pyruvate
B
Cerebellum
Caspase-3 activity
120
90
60
30
0
Sham
NaCl
Pyruvate

Figure 6.

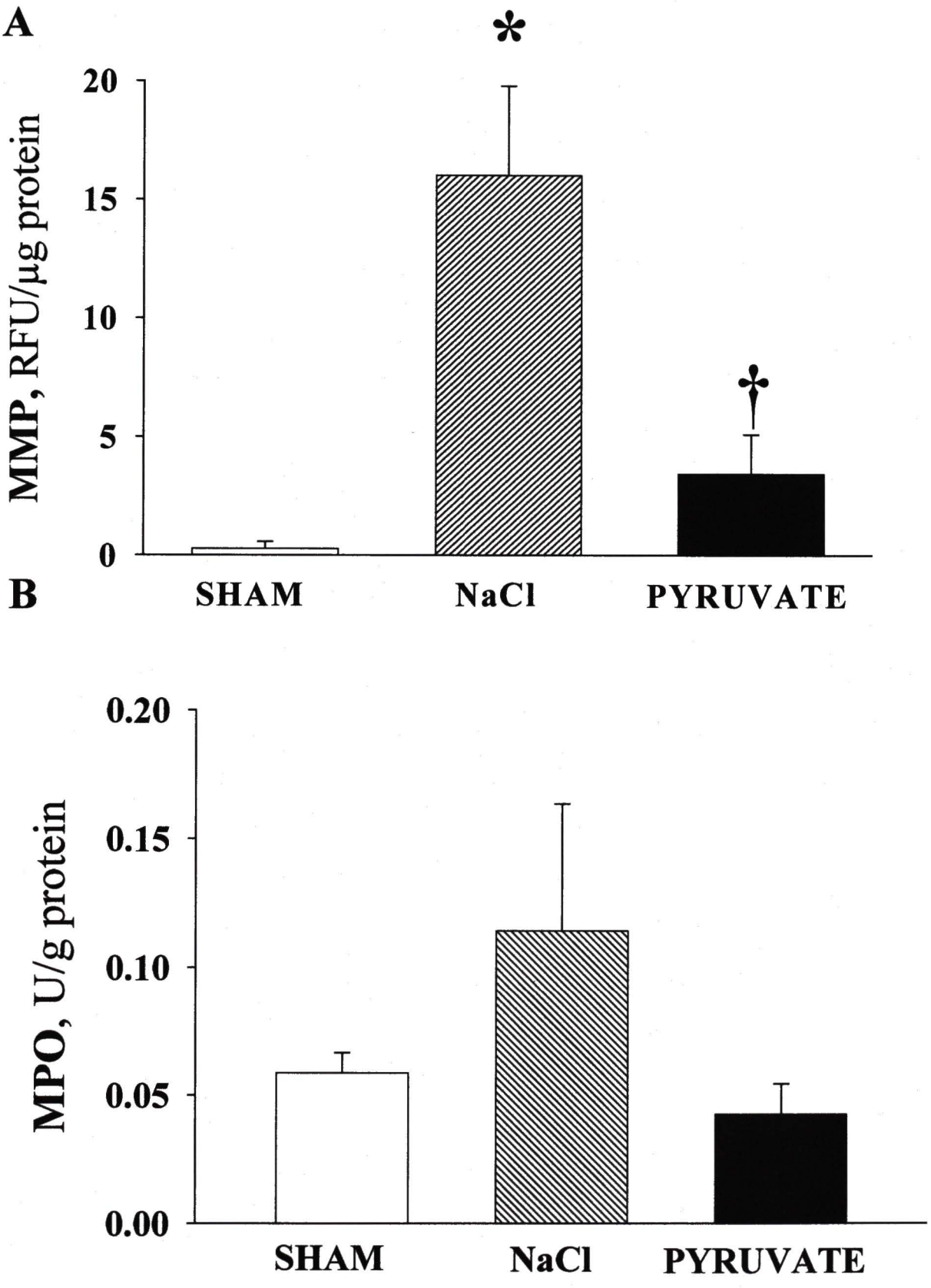

A
*
20
15
10
5
0
MMP, RFU/µg protein
†
B
SHAM
NaCl
PYRUVATE
0.20
0.15
0.10
0.05
0.00
MPO, U/g protein
SHAM
NaCl
PYRUVATE

Figure 7.

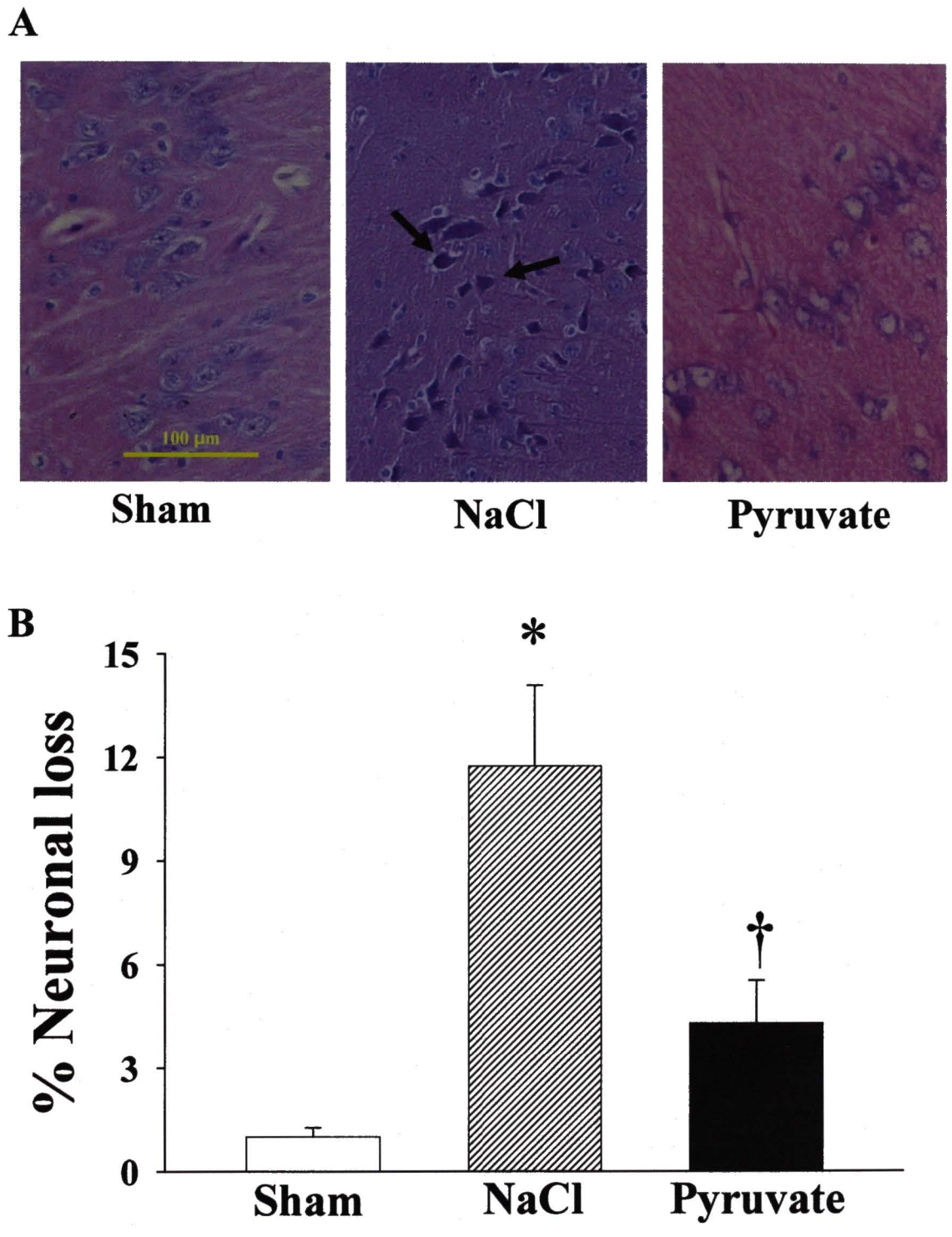

A
100 µm
Sham
NaCl
Pyruvate
B
% Neuronal loss
15
12
9
6
3
0
*
†
Sham
NaCl
Pyruvate

Figure 8.

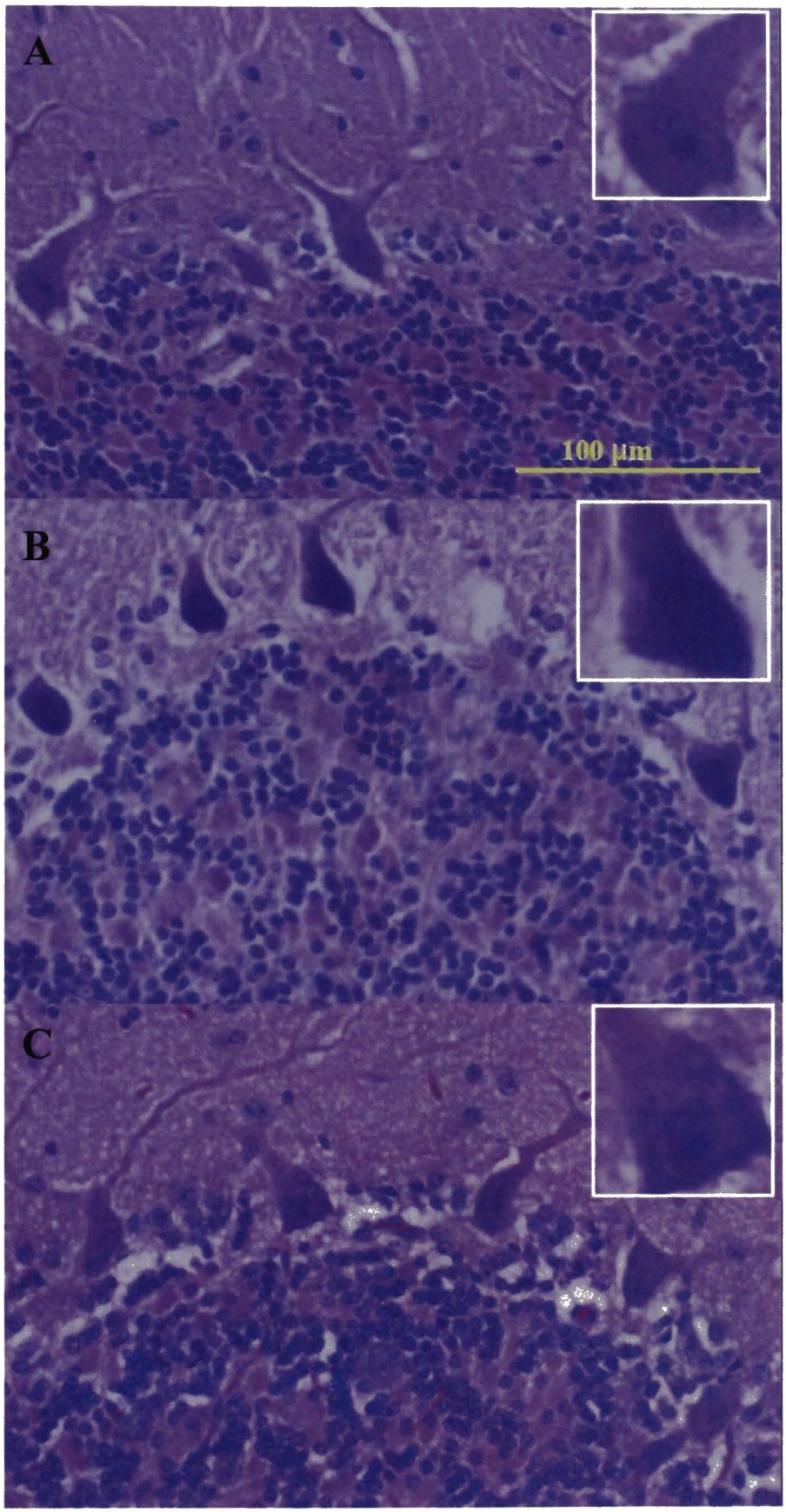
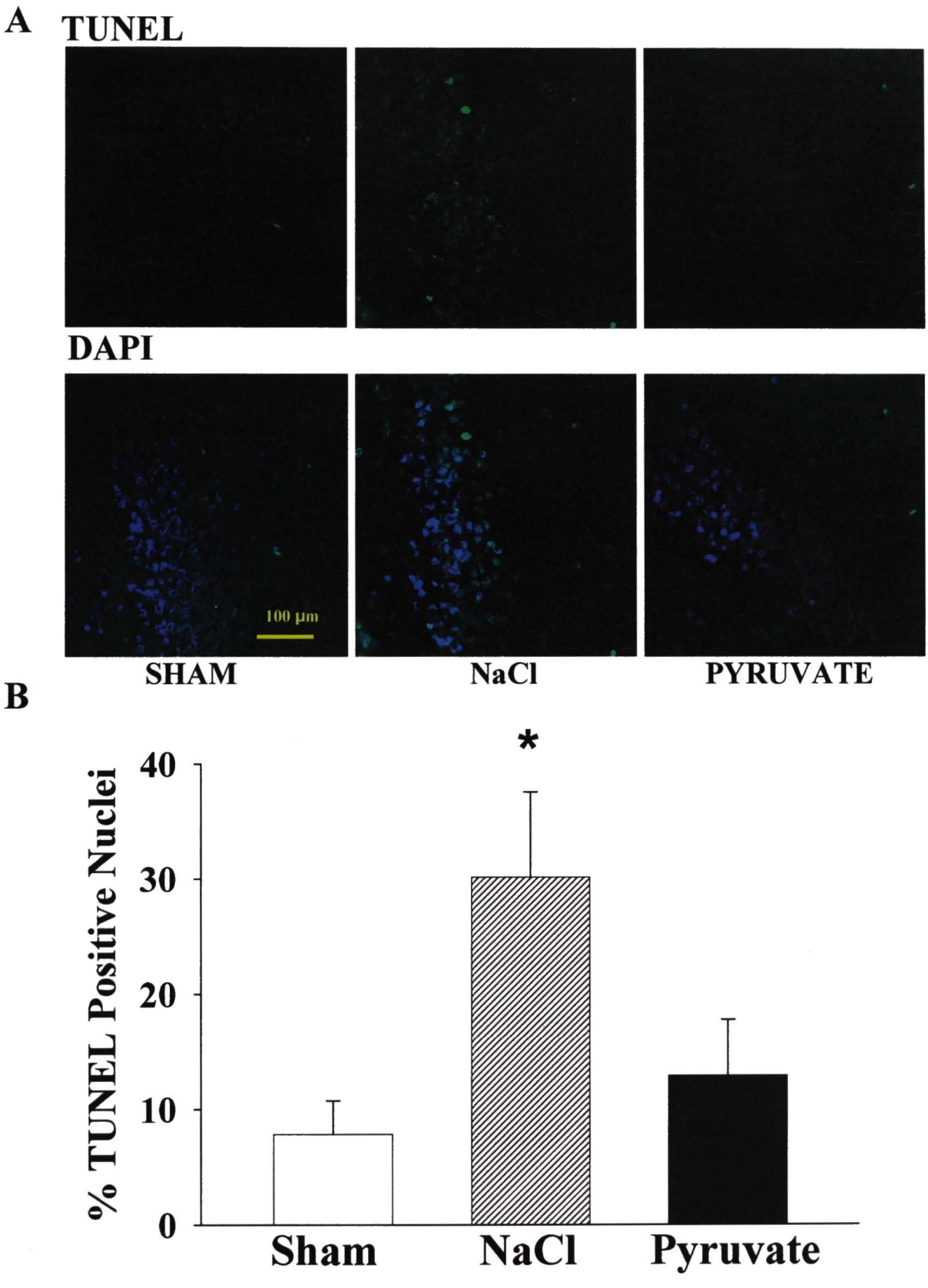


Figure 9.


A
TUNEL
DAPI
100 µm
SHAM
NaCl
PYRUVATE
B
% TUNEL Positive Nuclei
40
30
20
10
0
*
Sham
NaCl
Pyruvate

CHAPTER V

CONCLUSIONS

The investigations described herein support the concept that cardiopulmonary arrest and resuscitation is accompanied by metabolic impairments that eventually culminate in cardiac and neurological injury. Importantly, administration of pyruvate, a metabolic modulator that counters energy depletion and oxidative stress during the critical period of early reperfusion, augmented cardiac and neurological recovery.

The first investigation tested the ability of intravenous pyruvate to restore myocardial electromechanical function and energy and antioxidant resources during open-chest cardiac compression and defibrillation. This investigation demonstrated that (1) open-chest cardiac compression partially restores myocardial energy and antioxidant redox states; (2) myocardial energy and antioxidant reserves recover within the first 25 min following return of spontaneous circulation; (3) intravenous pyruvate infusion improves post-arrest myocardial electromechanical function; (4) pyruvate bolsters critical energy resources and glutathione redox state in resuscitated myocardium; (5) temporary pyruvate therapy to improve myocardial energy and antioxidant states in the peri-resuscitation period produces sustained improvements in left ventricular mechanical and electrocardiographic function, even though its metabolic effects subside post-infusion.

The second investigation supported the concept that cardiac arrest-reperfusion reversibly inactivates several important myocardial metabolic enzymes. Cardiopulmonary resuscitation facilitates partial recovery of some of these enzymes, and all of the enzymes eventually recover following return of spontaneous circulation, in parallel with the glutathione redox state. Maximum depletion of antioxidant redox state occurred during cardiac arrest, which supports the idea that ischemia alone can cause oxidative stress in the myocardium. Pretreatments with metabolic (pyruvate) and pharmacological (*N*-acetyl cysteine) antioxidants mitigated the fall in enzyme activities during arrest, in part by enhancing pre-arrest activities. These results implicated oxidative stress as a major contributor to reversible inactivation of myocardial enzymes during cardiac arrest.

The third investigation was conducted to examine the impact of temporary metabolic intervention during the first hour of ROSC, in mitigating delayed neuronal cell death and neurological impairment during 3 day recovery. Cardiac arrest-resuscitation results in neurological damage and neuronal loss evident at 3 days of recovery. Treatment with intravenous pyruvate during initial ROSC prevents immediate oxidative stress, preserves neurological function during the 3 days of recovery, attenuates inflammatory response and prevents neuronal loss from cardiac arrest-resuscitation. The following figure summarizes the role of pyruvate in myocardial and cerebral protection following resuscitation.

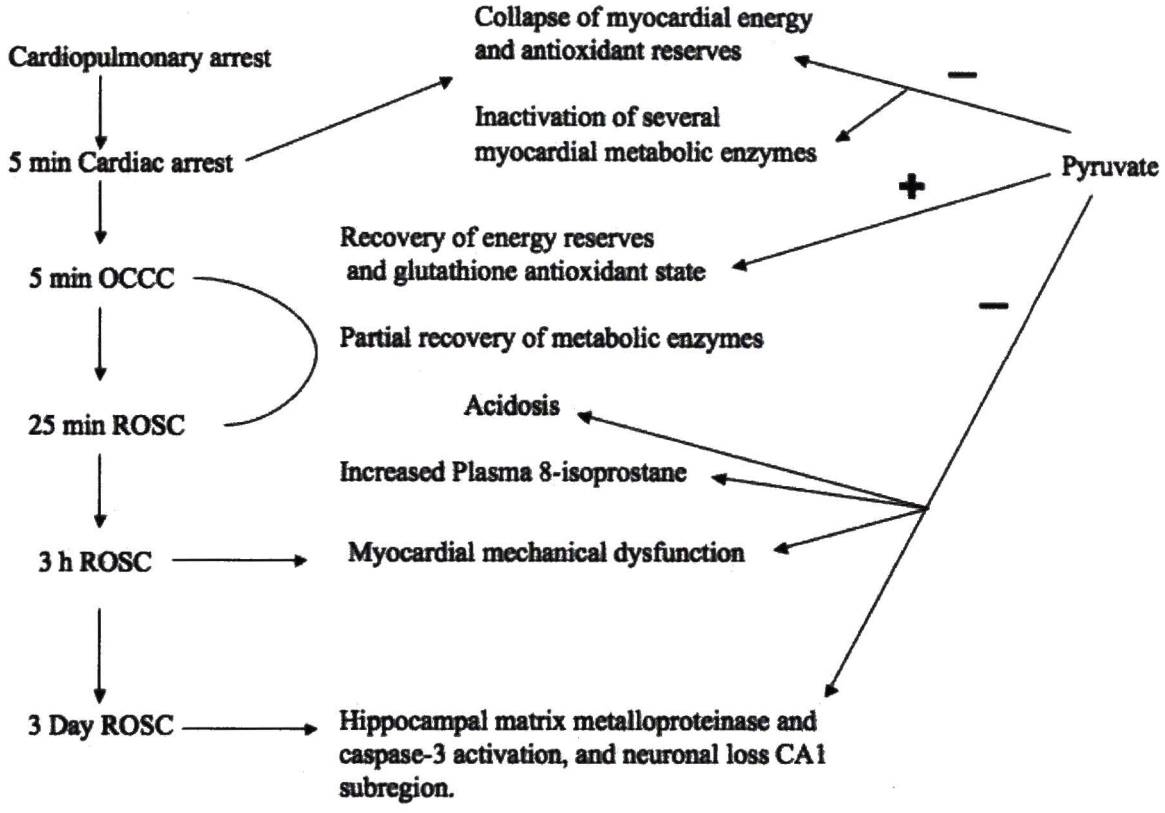


Figure: *Mechanism of pyruvate mediated myocardial and neuroprotection following cardiac arrest and resuscitation.*