Ethyl pyruvate preserves cardiac function and attenuates oxidative injury after prolonged myocardial ischemia

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Objective: Myocardial injury and dysfunction following ischemia are mediated in part by reactive oxygen species. Pyruvate, a key glycolytic intermediary, is an effective free radical scavenger but unfortunately is limited by aqueous instability. The ester derivative, ethyl pyruvate, is stable in solution and should function as an antioxidant and energy precursor. This study sought to evaluate ethyl pyruvate as a myocardial protective agent in a rat model of ischemia-reperfusion injury.

Methods: Rats underwent 30-minute ischemia and 30-minute reperfusion of the left anterior descending coronary artery territory. Immediately prior to both ischemia and reperfusion, animals received an intravenous bolus of either ethyl pyruvate (n = 26) or vehicle control (n = 26). Myocardial high-energy phosphate levels were determined by adenosine triphosphate assay, oxidative injury was measured by lipid peroxidation assay, infarct size was quantified by triphenyltetrazolium chloride staining, and cardiac function was assessed in vivo.

Results: Ethyl pyruvate administration significantly increased myocardial adenosine triphosphate levels compared with control (87.6 ± 29.2 nmol/g vs 10.0 ± 2.4 nmol/g, P = .03). In ischemic myocardium, ethyl pyruvate reduced oxidative injury compared with control (63.8 ± 3.3 nmol/g vs 89.5 ± 3.0 nmol/g, P < .001). Ethyl pyruvate diminished infarct size as a percentage of area at risk (25.3% ± 1.5% vs 33.6% ± 2.1%, P = .005). Ethyl pyruvate improved myocardial function compared with control (maximum pressure: 86.6 ± 2.9 mm Hg vs 73.5 ± 2.5 mm Hg, P < .001; maximum rate of pressure rise: 3518 ± 243 mm Hg/s vs 2703 ± 175 mm Hg/s, P = .005; maximal rate of ventricular systolic volume ejection: 3097 ± 479 μL/s vs 2120 ± 287 μL/s, P = .04; ejection fraction: 41.9% ± 3.8% vs 31.4% ± 4.1%, P = .03; cardiac output: 26.7 ± 0.9 mL/min vs 22.7 ± 1.3 mL/min, P = .01; and end-systolic pressure-volume relationship slope: 1.09 ± 0.22 vs 0.59 ± 0.2, P = .02).

Conclusions: In this study of myocardial ischemia-reperfusion injury, ethyl pyruvate enhanced myocardial adenosine triphosphate levels, attenuated myocardial oxidative injury, decreased infarct size, and preserved cardiac function.
Myocardial dysfunction and injury following ischemia are attributed to multiple factors, of which metabolic depletion of high-energy phosphates and formation of reactive oxygen species (ROS) are perhaps most predominant. During ischemia, without citric acid cycle contribution, energy requirements are poorly met by anaerobic glycolysis. Finely controlling glycolysis are multiple regulators including substrate limitation, oxidized nicotinamide adenine dinucleotide (NAD\(^+\)), and allosterically governed enzymes. Researchers have attempted various approaches toward glycolytic substrate enhancement to augment adenosine triphosphate (ATP) production. Pyruvate, one such extensively studied efficacious glycolytic intermediate, yields increased cytosolic NAD\(^+\), the obligate electron acceptor for glyceraldehyde-3-phosphate dehydrogenase, which oxidizes glyceraldehyde-3-phosphate to 1,3 bisphosphoglycerate.\(^1^4\) In turn, phosphoglycerate kinase can then catalyze 1,3 bisphosphoglycerate to 3-phosphoglycerate generating ATP, augmenting cardiomyocyte energetics.\(^1\)

The second primary mechanism of injury involves the generation of ROS during reperfusion via multiple reactions, most notably:

- xanthine oxidase: \(2\text{O}_2 + \text{NADPH} \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+\)
- superoxide dismutase: \(2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2\)
- nitric oxide: \(\text{O}_2^- + \text{NO}^- \rightarrow \text{ONOO}^-\)
- ionized iron: \(\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{3+}\)

Superoxide anion (\(\text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)) are moderately reactive, whereas peroxynitrite (\(\text{ONOO}^-\)) and hydroxyl radical (\(\text{OH}^-\)) are highly toxic and produce a spectrum of cellular injury via multiple mechanisms including membrane destabilization, mitochondrial disruption, and metabolic derangement. There exists a battery of native antioxidant enzyme systems that sequentially convert ROS to water and oxygen:

- superoxide dismutase: \(2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2\)
- catalase: \(2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2\)
- glutathione peroxidase: \(\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}\)

Where GSH is reduced glutathione and GSSG is oxidized glutathione. Multiple strategies for augmenting these native systems have likewise been investigated.\(^5^8\)

Interestingly pyruvate, in the presence of hydrogen peroxide, will decarboxylate to yield acetate, water, and carbon dioxide.\(^9\) Pyruvate is also capable of scavenging hydroxyl radical.\(^10\) Studies have shown that exogenous pyruvate administration diminishes ischemic myocardial oxidative injury,\(^1^1^4\) apparently in a dose-dependent manner.\(^1^5\) Furthermore, independent of its metabolic and antioxidant features, pyruvate may possess myocardial inotropic properties. Pyruvate directly increases sarcoplasmic reticular ATPase activity and hence calcium cycling efficiency.\(^1^5\) In addition, pyruvate also indirectly augments beta agonist-mediated inotropy.\(^1^6\) Unfortunately, the therapeutic potential of exogenously administered pyruvate is significantly limited by extreme aqueous instability. Pyruvate can undergo spontaneous condensation to parapyruvate, which can further undergo cyclization and either dehydration or reduction to yield a purported mitochondrial inhibitor.\(^1^7^1^8\)

Ethyl pyruvate, a commercial food additive, is a lipophilic ester derivative of pyruvate. Although poorly soluble in water or saline, ethyl pyruvate is highly soluble in calcium solutions, forming a dimer where the anionic enolate form stabilized with divalent cationic calcium.\(^1^9\) In this form, ethyl pyruvate possesses far greater stability than pyruvate and hence may serve as a practical pyruvate precursor.

We therefore hypothesized that in a model of myocardial ischemia-reperfusion injury, ethyl pyruvate administration would provide a cardioprotective effect.

**Methods**

**Animal Care and Biosafety**

Male Wistar rats weighing 250 to 350 g were obtained from Charles River (Boston, Mass). Food and water were provided ad libitum. This study was performed in accordance with the standard humane care guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania, which conform to current federal guidelines.

**Preparation of Treatment Solutions**

Experimental treatment groups were divided into animals receiving Ringer’s solution as a control or Ringer’s solution containing 28 mmol/L ethyl pyruvate obtained from Sigma Chemicals (St Louis, Mo). Ringer’s solution contained 130 mmol/L Na\(^+\), 4.0 mmol/L K\(^+\), 2.7 mmol/L Ca\(^{2+}\), and 109 mmol/L Cl\(^-\) at pH 7.0.

**Acute in Vivo Ischemia-Reperfusion Model**

Animals (n = 52) underwent induction of general anesthesia with ketamine (75 mg/kg) and xylazine (7.5 mg/kg), endotracheal intubation with a 14-gauge angiocatheter, and mechanical ventilation with 2% isoflurane maintenance anesthesia with a respirator (Hallowell EMC, Pittsfield, Mass). Venous access for administration of treatment solutions was obtained via exposure of the right femoral vein and insertion of a 24-gauge intravenous catheter. A left thoracotomy was performed through the fourth intercostal space and the pericardium was reflected, exposing the heart. A 7-0 polypropylene suture was then placed around the left anterior descending (LAD) coronary artery and briefly snared to visually verify the territory of myocardial ischemia. Animals were then randomized to either the control (n = 26) or ethyl pyruvate group (n = 26) and received a 1.5 mL/kg intravenous bolus of either Ringer’s solution or ethyl pyruvate. Two minutes later, ischemia was initiated. The LAD was then occluded for 30 minutes. In a small subset of animals (control n = 5, ethyl pyruvate n = 5), hearts were harvested 10 minutes into the ischemia period for...
analysis of myocardial energetic state via ATP assay. In the other 42 animals, after the 30-minute ischemic period, just prior to reperfusion, the treatment solution was again intravenously bolused (3.0 mL/kg). Hearts were then reperfused for 30 minutes. At the end of this period, the animals were either put to death and hearts were harvested for lipid peroxidation analysis (control n = 6, ethyl pyruvate n = 6) or animals were prepared for cardiac functional assessment and subsequent heart explantation for infarct size determination (control n = 15, ethyl pyruvate n = 15).

Myocardial ATP Levels
ATP levels were quantified using the commercially available EN-LITEN ATP luciferin/luciferase bioluminescence assay system (Promega, Madison, Wis). Myocardial tissue specimens from the ischemic region were excised, immediately frozen in liquid nitrogen, and individually pulverized into a fine powder by hand grinding with a dry ice–chilled steel mortar and pestle. Ten milligrams of myocardium were homogenized with 1 mL of pre-cooled extractant (0.1% trichloroacetic acid) and centrifuged at 4500 revolutions per minute (rpm) for 10 minutes.21 Supernatant (100 µL) was diluted 10-fold with 50 mmol/L Tris-acetate buffer containing 2 mmol/L ethylenediaminetetraacetic acid (pH 7.75). Then 100 µL of sample extract or reference standard solution was placed in a tube luminometer (Turner Designs Luminometer TD-20/20, Promega), followed by the auto-injection of 100 µL of ATP luciferin/luciferase assay mix for ATP quantification. Luminescence was measured at a set lag time of 1 second and integration time of 10 seconds.

Myocardial Lipid Peroxidation Measurements
Myocardial lipid peroxidation was quantified using the commercially available PeroxiDetect KIT (Sigma Chemicals). Myocardial tissue specimens were obtained from the ischemic anterolateral left ventricular wall and from the nonischemic remote basal postero-septal left ventricle and immediately frozen in liquid nitrogen. Lipid soluble components were extracted from the myocardial tissue specimens using a CHCl₃/methanol extraction protocol.22 Briefly, 100 mg of tissue specimen was homogenized in a 2:1 volume mixture of CHCl₃ and 100% methanol at a ratio of 1 g tissue to 15 mL of the CHCl₃/methanol mixture. The homogenized material was centrifuged at 10,000 rpm for 10 minutes, after which the supernatant was clarified with 0.3 mL of 0.9% NaCl per 1 g of tissue and then removed. The CHCl₃ layer was then evaporated with nitrogen gas to leave the lipid peroxides from the specimens contained in the methanol solvent. Specimen samples (100 µL) were added to a 1-mL mixture of Fe²⁺ ion and xylene orange. Peroxides convert Fe²⁺ to Fe³⁺, which forms a color adduct with xylene orange spectrophotometrically detectable at 560 nm. Lipid peroxide levels were then calculated using a reference standard curve generated with defined quantities of tert-butyl hydroperoxide.

Cardiac Functional Assessment Following Ischemia and Reperfusion
Following the 30-minute reperfusion period, a median sternotomy was performed and myocardial performance was assessed in vivo (control n = 15, ethyl pyruvate n = 15). A fully calibrated miniature pressure/volume conductance catheter (MIKRO-TIP catheter and ARIA Pressure Volume Conductance System, Millar Instruments, Houston, Tex) was inserted into the left ventricular cavity through the apex. Calibration consisted of the cuvette 2-point linear interpolation process and parallel conductance subtraction via the hypertonc saline method. The digitized pressure and volume signals were displayed and recorded using Chart v4.1.2 software (AD Instruments, Colorado Springs, Colo). Multiple cardiac functional parameters were measured in the 2 groups using Cardiac Pressure Volume Analysis Software-PVAN 2.9 (Millar Instruments). Additionally, a flow probe monitor (Transonic Systems, Ithaca, NY) was placed around the ascending aorta to measure cardiac output. To provide a reference of baseline rat myocardial function, a separate group of 15 noninfarcted native animals underwent pressure-volume and cardiac output analysis.

Planimetric Determination of Left Ventricular Infarction Size
Following the cardiac functional analysis, control and ethyl pyruvate hearts were excised and rinsed in normal saline solution (pH 7.4). The LAD was again snared and Evans blue dye was infused into the aortic root to facilitate determination of area at risk. The specimens were cut perpendicular to the long axis into 5 sections and incubated in 1% triphenyltetrazolium chloride (TTC) (Sigma Chemicals) in phosphate-buffered saline solution (pH 7.4) at 37°C for 20 minutes. Following the incubation period, the TTC was rinsed from the sections and a 10% formalin solution was added for tissue fixation. Sections were photographed using a digital camera and were downloaded onto a desktop computer containing digital planimetry software (OpenLab, Lexington, Mass). Infarct size as a percentage of area at risk was then measured.

Statistical Methods
Statistical analyses were performed using unpaired, 1-tailed Student t tests. All results were expressed as mean ± standard error of the mean (SEM).
Results

ATP Quantification
Analysis of ATP levels in ischemic hearts revealed a marked increase in animals receiving ethyl pyruvate compared with control animals (Figure 1). The ischemic region of control hearts contained 10.0 ± 2.4 nmol/g, whereas the ischemic region of ethyl pyruvate hearts contained 87.6 ± 29.2 nmol/g (P = .03).

Lipid Peroxidation Analysis
Spectrophotometric quantification of lipid peroxides in myocardium exposed to ischemia and reperfusion in nonischemic myocardium, which served as an internal control, is represented in Figure 2. Lipid peroxide levels in the nonischemic myocardium did not differ significantly between animals treated with ethyl pyruvate (42.3 ± 4.8 nmol/g; n = 6) and the control, Ringer’s solution (37.5 ± 5.1 nmol/g; n = 6), implying equivalent assay conditions between control and ethyl pyruvate hearts. Ischemia significantly increased the level of lipid peroxidation from 37.5 ± 5.1 nmol/g to 89.5 ± 3.0 nmol/g in control hearts (P < .001). When comparing ischemic myocardium in ethyl pyruvate hearts with control hearts, there was a statistically significant decrement in lipid peroxidation with ethyl pyruvate administration (63.8 ± 3.3 vs 89.5 ± 3.0 nmol/g, P < .001).

Infarction Analysis
Macroscopic analysis of TTC-stained cross sections following 30 minutes of ischemia and 30 minutes of reperfusion demonstrated attenuated left ventricular infarction sizes in animals treated with ethyl pyruvate (n = 15) as compared with the control group (n = 15; Figure 3). Ethyl pyruvate animals had 25.3% ± 1.5% of the left ventricular area at risk infarcted as compared with 33.6% ± 2.1% in control animals (P = .005).

Cardiac Function
Table 1 displays heart rate and multiple pressure and volume parameters for native animals (n = 15) as a reference,
CSP

Discussion
Ischemia-reperfusion injury of myocardium is a significant entity in many clinical situations, including coronary thrombolysis, percutaneous coronary interventions, cardiac surgery, and heart transplantation. Myocardial dysfunction and cellular injury occurs in part due to metabolic depletion during ischemia followed by ROS formation during reperfusion. Significant research efforts have investigated means of protecting myocardium against ischemia-reperfusion injury. This article describes our utilization of ethyl pyruvate as an intravenous myocardial protection agent. Rats received bolus ethyl pyruvate before temporary coronary ligation and just before reperfusion and demonstrated statistically significantly increased myocardial ATP levels, decreased tissue oxidative injury, decreased infarct sizes, and preserved myocardial function. To our knowledge, this is the first report of a cardiovascular application of this agent.

Ethyl pyruvate’s parent compound, the glycolytic product pyruvate, has been shown to attenuate myocardial ischemic injury through both metabolic augmentation and antioxidant mechanisms but is limited in potential therapeutic efficacy by extreme aqueous instability.1,12,17 Specific studies into the metabolic activity of the related pyruvate ester, methyl pyruvate, in pancreatic islet cells have demonstrated cellular and mitochondrial membrane permeation, deesterification liberating free pyruvate for glycolytic substrate utilization, and subsequent augmentation of ATP generation.23 These studies, which were performed on nonischemic cells, also found a disproportionate increase in ATP generation compared with glucose utilization. This enhanced production of ATP is felt to result from rapid intracellular deesterification of methyl pyruvate to pyruvate and thus the generation of a significant transcellular gradient favoring further intracellular influx of methyl pyruvate. This results in the rapid accumulation of an intracellular excess pool of pyruvate generated free of glycolytic regulators and without the obligatory initial ATP investment at the glucokinase and phosphofructokinase reactions. Pyruvate is then available for pyruvate dehydrogenase conversion to acetyl CoA and citric acid cycle substrate provision.2 Such pyruvate stores may be particularly useful in the myocardial reperfusion phase when the citric acid cycle becomes available again, yet ATP levels are very low and require rapid repletion.

Biologic investigation of ethyl pyruvate was first described in ophthalmologic research aimed at the prevention of free radical–induced lens injury, which normally leads to cataract formation.24,25 Ethyl pyruvate has subsequently been studied in the trauma/critical care literature with models of mesenteric ischemia, hemorrhagic shock, and endotoxemic sepsis, all demonstrating a cytoprotective effect.19,26-29 Another postulated role of ethyl pyruvate is related to its anti-inflammatory properties. In a murine hemorrhagic shock model, ethyl pyruvate decreased expression of tumor necrosis factor and interleukin-6.26 This could be explained as an indirect finding related to ethyl pyruvate–mediated attenuation of multiorgan ischemic injury from the induced state of hemorrhagic shock. In vitro, ethyl pyruvate

Figure 4. Composite representation of end-systolic pressure-volume relationships (ESPVR) in native animals (n = 15) and in control (n = 15) and ethyl pyruvate–treated (n = 15) animals following ischemia and reperfusion. For native animals, mean ± SEM slope, x-axis intercept (x-int), and correlation coefficient (r) were: 1.27 ± 0.12, 10.4 ± 2.0, and 0.97 ± 0.07, respectively. For control animals, the values were: slope = 0.59 ± 0.2, x-int = 9.9 ± 24.0, r = 0.68 ± 0.13; for ethyl pyruvate–treated animals, these values were: slope = 1.09 ± 0.22, x-int = −4.1 ± 19.9, r = 0.89 ± 0.03. Comparing the slopes of the control and ethyl pyruvate hearts demonstrates a statistically significant improvement in contractility with the administration of ethyl pyruvate (P = .02). Composite ESPVR for the native animals (n = 15) was 1.27 (end-systolic volume [ESV]-10.39), control (n = 15) was 0.59 (ESV-9.88), and ethyl pyruvate (n = 15) was 1.09 (ESV-4.06). Significant differences in the slope of the ESPVR were found between ethyl pyruvate and control animals.
does appear to directly inhibit the nuclear factor κB– and p38 mitogen–activated protein kinase pathways of inflammatory cytokine activation. To date, there has not been a report of the application of ethyl pyruvate to the cardiovascular system.

This study sought to investigate the potential effect of exogenous ethyl pyruvate administration on reducing myocardial injury and contractile dysfunction after ischemia.

The overall design of this study evaluated the effects of ethyl pyruvate at biochemical, cellular, and physiologic levels. Myocardial high-energy phosphate levels, free radical injury, myocardial infarction, and cardiac mechanics were examined in a model of myocardial ischemia-reperfusion injury. The proportionate degree of myocardial injury induced is more pronounced than what occurs in a typical cardiac surgical procedure. Nevertheless, the model was selected for its predictability and functionality.

In the control group, Ringer’s solution was utilized to preclude any attribution of observed differences to electrolyte composition of solutions. Also, in the unlikely event that some acid-base buffering capacity of ethyl pyruvate was the only cause of observed differences, we also performed a small pilot series of control animals with lactated Ringer’s solution. These animals would have obtained any buffering benefit, yet myocardial function and infarct size were equivalent to Ringer’s solution controls. The dose of ethyl pyruvate selected for this study was based upon a concentration of ethyl pyruvate previously optimized in an intestinal ischemia model. The timing of administration of the ethyl pyruvate was designed to enhance the two purported mechanisms of action, glycolytic substrate augmentation and antioxidation.

An initial left thoracotomy was employed during the ischemic period for several reasons. This permitted ease of observation and antioxidation. This permitted ease of observation and antioxidation. This permitted ease of observation and antioxidation.

Alternate timing strategies should also be evaluated as an additive to transplant organ preservation solutions.

In this study, ethyl pyruvate—a nontoxic, inexpensive, intravenously administered antioxidant and potential glycolytic substrate—significantly preserved cardiac function, enhanced tissue ATP levels, attenuated myocardial oxidative injury, and markedly reduced infarct size after prolonged myocardial ischemia.

References
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**Discussion**

**Dr W. Randolph Chitwood, Jr** (Greenville, NC). What other mechanisms might be being involved here besides peroxidation? *Dr Woo.* The data that we show relates primarily to the antioxidant aspects and that’s been well shown with pyruvate. We are very interested in evaluating the potential ability to augment glycolysis. I just bought a luminometer, and we’re going to look at ATP levels through a luciferin-luciferase assay to see if there is any augmentation of the total cellular bioenergetic state.

**Dr Jakob Vinten-Johansen** (Atlanta, Ga). Can you tease out the functional recovery aspects that you demonstrated? Because you have an agent that exhibits positive inotropic effects, antioxidant effects, and also metabolic support effects; any 1 of those 3 could be responsible for the functional recovery you observed. The reduction in the infarct size by itself perhaps could be responsible for that improvement in function.

In addition, when you looked at the ejection fraction, did you have a control group to which you could compare that ejection fraction? In other words, was it actually an increase in the treated group relative to baseline, or does ejection fraction return to a baseline level? *Dr Woo.* In terms of your first question, that’s sort of what I think Dr Chitwood was alluding to, whether or not there was something else going on. You, of course, have accurately defined what we have proposed in our background as the 3 primary mechanisms. And again, we have the data for the antioxidant effect and we’re starting the bioenergetics aspect.

I haven’t really thought about how to get at the direct inotropy aspect. There is, I think, a lot more support for pyruvate’s antioxidant and metabolic aspects and less so for its direct inotropy, so I think that is perhaps a little less likely to yield something.

In terms of how to distinguish all 3, that’s obviously going to be of interest. We could, of course, try to block out 1 of those pathways, perhaps by augmenting free radical production to try to overwhelm the antioxidant effects and then see, so on and so forth.

In terms of your second question, we did not measure baseline levels of ejection fraction in these animals. But in our separate pilot studies where we were basically working out the details of the Millar catheter, also as part of also our heart failure studies, we do have a sense of where ejection fraction lies and it’s generally in the
40% to 50% range, so there is a decrement even in the treated animals. They do have an 8% infarct and they are just status post—significant ischemic injury. So we don’t necessarily expect them to get back to a baseline level, but it’s mostly the difference between the 2 groups that we’re looking at.

Dr Vinten-Johansen. You gave the injections or the administrations at ischemia and reperfusion to take advantage of both the metabolic effects as well as the antioxidant effects, is that the rationale?

Dr Woo. That’s right.

Dr Vinten-Johansen. How did you decide on the dosage, the concentration that you gave?

Dr Woo. We were guided somewhat by the intestinal ischemic-reperfusion work, although that’s just a starting point. There is no dose-dependent evaluation of ethyl pyruvate in the literature for any of the other trauma critical care work. There are only maybe 5 published studies, period, looking at anything physiologic.

In terms of pyruvate, there is a dose-dependent benefit. So we could potentially shoot for an equimolar concentration of presumed pyruvate release as another target point.