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Effects of Ethyl Pyruvate and Other α-Keto Carboxylic Acid Derivatives in a Rat Model of Multivisceral Ischemia and Reperfusion

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Background. **Ethyl pyruvate (EP) has been shown to ameliorate hepatic, renal, and intestinal mucosal injury and down-regulate expression of several pro-inflammatory mediators in a wide variety of preclinical models of critical illnesses, such as sepsis, burn injury, acute pancreatitis, stroke, and hemorrhagic shock. The molecular mechanisms responsible for the therapeutic effects of EP remain poorly understood, but might be related to the compound's structure as the ester of an α-keto carboxylic acid. Herein, we tested the hypothesis that EP and other α-keto carboxylic acid derivatives can modulate organ injury after lower torso ischemia/reperfusion (I/R).**

Methods. **Rats were subjected to 50min of supraceliac aortic occlusion. Over a 20-min period, starting 2min before the release of the aortic clamp, the animals received 2μL/g of Ringer's lactate solution (RL, $n = 5$) or an equivalent volume of a solution containing EP ($n = 5$), benzoyl formate (BF, $n = 5$), parahydroxyphenyl pyruvate (PHPP, $n = 5$) or sodium pyruvate (NaPyr, $n = 5$). The total dose of each compound was 0.86 mMol/kg. After 1h of reperfusion, we measured ileal mucosal permeability to fluorescein-labeled dextran (mw 4000 Da), liver malondialdehyde (MDA) content, and plasma levels of alanine aminotransferase (ALT) and TNF. Rats in the control group (CT, $n = 4$) were subjected to laparotomy and surgical isolation of the supraceliac aorta, but not visceral I/R.**

Results. **Ileal mucosal permeability, plasma levels of ALT and TNF, and hepatic MDA content increased significantly in the RL group relative to the CT group. Both EP and BF significantly ameliorated the development of systemic arterial hypotension, mucosal hyperpermeability, and significantly decreased plasma levels of TNF. MDA content was significantly decreased by EP, PHPP, BF, and NaPyr.**

Conclusions. **In general, EP is more efficacious in this model than is NaPyr. Although more remains to be learned about the pharmacologic differences between EP and pyruvate, one important factor may the greater lipophilicity of the former compound. This insight may permit the development of even more effective cytoprotective and anti-inflammatory agents based on the pyruvoyl moiety.**

Key Words: **ethyl pyruvate; ischemia; reperfusion; permeability; intestinal; resuscitation; pyruvate.**

INTRODUCTION

Ethyl pyruvate (EP), a simple aliphatic ester derived from pyruvic acid, has been shown to improve survival and ameliorate organ dysfunction in a variety of animal models of critical illness, including acute endotoxemia [1, 2], polymicrobial bacterial sepsis [2, 3], postsurgical ileus [4], burn injury [5, 6], acute pancreatitis [7], mesenteric ischemia and reperfusion (I/R) injury [8, 9], hepatic I/R injury [10], extrahepatic biliary tract obstruction [11], and hemorrhagic shock [12, 13]. EP is an effective anti-inflammatory agent that inhibits activation of key pro-inflammatory signaling pathways, such as NF-κB and p38 mitogen activated protein kinase [2, 7, 9, 13, 14], and down-regulates the secretion of multiple pro-inflammatory proteins, including IL-6, TNF, and high mobility group box 1 (HMGB1) [2, 7, 9, 13, 15]. EP also has been shown to be an effective reactive oxygen species (ROS) scavenger [10, 12, 16].

EP is an ester of an α-ketocarboxylic acid (pyruvic acid), whereas sodium pyruvate (NaPyr) is a salt of

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the same carboxylic acid. In prior studies of inflammation-induced or I/R-induced injury, EP was more effective then NaPyr [8, 16], suggesting the possibility that the ester linkage is required for optimal pharmacologic activity. EP, however, is considerably more lipophilic than NaPyr, which raises the possibility that differences in the pharmacologic effects of these two compounds may be related to differences in their solubility in lipids. Alternatively, EP and NaPyr might display distinct pharmacologic properties for other reasons, such as differences in their tendencies to form enol tautomers.

In order to further investigate the differences in the pharmacology of EP *versus* NaPyr, we evaluated the effects of these two compounds in a rodent model of abdominal visceral and lower extremity I/R-injury induced by temporary aortic occlusion. In addition to studying the effects of EP and NaPyr, we also studied two other compounds, namely benzoyl formate (BF) and para-hydroxyphenylpyruvate (PHPP). Like pyruvate, BF is the anion of an α-keto carboxylic acid. But, unlike pyruvate, BF cannot undergo a keto-enol tautomerization reaction. Like pyruvate and BF, PHPP is also the anion of an α-keto carboxylic acid. When pyruvate is dissolved in an aqueous solvent, the keto tautomer predominates; however, in fresh aqueous solutions of PHPP, the enol tautomer is the predominant species of the compound [17]. Both BF and PHPP are more lipophilic than pyruvate but less lipophilic than EP.

MATERIALS AND METHODS

This research protocol complied with the regulations regarding animal care published by the National Institutes of Health, and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh School of Medicine. The animals were maintained at the University of Pittsburgh Animal Research Center under a 12-h light-dark cycle with free access to standard laboratory feed and water. Animals were not fasted before the experiments. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted.

Experimental Protocol

Male rats weighing 200 to 225 g (Charles River Laboratories, Wilmington, MA), were anesthetized using intramuscular pentobarbital sodium (50 mg/kg). The left femoral artery and vein were surgically prepared and cannulated. The arterial catheter was used for continuous mean arterial pressure (MAP) monitoring. The venous catheter was used to administer the test substances. Animals were randomized in six groups. Rats in the control group were anaesthetized, subjected to laparotomy, and surgical isolation of the supraceliac aorta, although they were not subjected to splanchnic ischemia (CT, $n = 4$). Five groups of rats underwent a midline celiotomy and were subjected to 50 min of multivisceral ischemia by applying a non-crushing vascular clamp across the supraceliac aorta. In a 20-min period, starting 2 min before the aortic clamp release, the rats received 2 μL/g of Ringer's lactate solution (RL group, $n = 5$) or an equivalent volume of a solution containing ethyl pyruvate (EP group, $n = 5$), benzoyl formate (BF group, $n = 5$), parahydroxyphenyl pyruvate (PHPP group, $n = 5$), or sodium pyruvate (NaPyr group, $n = 5$). All of these solutions were prepared in order to administer 0.86 mMol/kg of each compound over a 20-min period. EP and NaPyr were dissolved in a Ringer's lactate type crystalloid solution containing 130 mEq/L Na, 109 mEq/L Cl, 4 mEq/L K, 2.7 mEq/L Ca, and 28 mEq/L lactate. Parahydroxyphenyl pyruvic acid and benzoyl formic acid were dissolved in a 1.1:8.9 (vol/vol) mixture of 10 N NaOH and Ringer's lactate solution (final pH $\sim$8.0). After the release of the clamp, the animals were observed for 60 min. At the end of the experiment, a segment of ileum was harvested for the determination of mucosal permeability, blood was aspirated from the heart for the measurement of the plasma concentrations of alanine aminotransferase (ALT) and TNF, and a portion of liver was removed for determination of malondialdehyde content (MDA). Hepatic tissue to be used for the MDA assay was immediately frozen at -80 °C, whereas the intestinal tissue for histologic examination was fixed in 10% formalin.

Determination of Intestinal Mucosal Permeability

We determined ileal mucosal permeability to the fluorescent tracer, fluorescein isothiocyanate-labeled dextran (molecular mass 4000 Da; FD4) using an everted gut sac method, as previously described [18]. In brief, everted gut sacs were prepared in ice-cold modified Krebs-Henseleit buffer solution (KHBB; pH 7.4). One end of the gut segment was ligated with suture, and then the segment was everted onto a thin plastic rod. The resulting sac was secured with another suture to the grooved tip of a 3-mL plastic syringe containing KHBB. We gently distended the sac by injecting 1.5 mL of KHBB. The sac was then suspended for 30 min in a 50-mL beaker containing 40 mL of KHBB plus FD4 (40 mg/mL). The solution in the beaker was temperature-jacketed at 37 °C and continuously bubbled with a gas mixture containing 95%O_2–5%CO_2. The FD4 concentration of the fluid in the beaker and the inside of the sac was determined spectrofluorometrically, and permeability was expressed as the mucosal-to-serosal clearance of FD4.

Serum TNF Measurement

Blood samples were collected in 1.5 mL tubes and centrifuged for 10 min at 5000 *g*; plasma was then removed and stored at -80 °C until analyses were performed. TNF concentration was measured with a specific ELISA kit (R and D System, Minneapolis, MN) and compared with a TNF standard curve that demonstrated a direct relationship between optical density and cytokine concentration.

Lipid Peroxidation Assay

The assay for lipid peroxidation was performed as described previously [12]. In brief, after tissue specimens were thawed, 0.4 mL of phosphate buffer (0.05 mol/L, pH 7.4) was added to 200 mg of tissue. The tissue was homogenized. Trichloroacetic acid (20% vol/vol solution; 1.25 mL) and thiobarbituric acid (0.67% wt/vol solution; 0.50 mL) were added to 0.25 mL of the tissue homogenate. The color of the thiobarbituric acid pigment was developed by incubating the mixture in a 100°C water bath for 30 min. After cooling the mixture to room temperature by immersion in tap water, 2 mL of n-butanol were added and shaken vigorously. After centrifugation, absorbance of the butanol layer was determined at 535 nm. Samples were run in duplicate and the results were averaged. We used 1,1,3,3-tetraethoxypropane to generate a standard curve. Results were expressed as nanomoles of MDA per gram of tissue.

Serum ALT Measurement

The blood obtained by means of cardiac puncture was placed in a 1.5 mL centrifugation tube. The samples were centrifuged for