Published in final edited form as:

J Surg Res. 2008 March ; 145(1): 130–134. doi:10.1016/j.jss.2007.03.067.

Ethyl pyruvate inhibits hypoxic pulmonary vasoconstriction and attenuates pulmonary artery cytokine expression

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Abstract

Hypoxic pulmonary vasoconstriction is a common consequence of acute lung injury and may be mediated by increased local production of proinflammatory cytokines. Ethyl pyruvate is a novel anti-inflammatory agent that has been shown to downregulate proinflammatory genes following hemorrhagic shock; however, its effects on hypoxic pulmonary vasoconstriction are unknown. We hypothesized that ethyl pyruvate would inhibit hypoxic pulmonary vasoconstriction and downregulate pulmonary artery cytokine expression during hypoxia. To study this, isometric force displacement was measured in isolated rat pulmonary artery rings (n=8/group) during hypoxia (95% N₂/5% CO₂) with or without prior ethyl pyruvate (10 mM) treatment. Following 60 minutes of hypoxia, pulmonary artery rings were analyzed for TNF- α and IL-1 mRNA via RT-PCR. Ethyl pyruvate inhibited hypoxic pulmonary artery contraction (4.49 $\pm$ 2.32% vs. 88.80 $\pm$ 5.68% hypoxia alone) and attenuated the hypoxic upregulation of pulmonary artery TNF and IL-1 mRNA (p<0.05). These data indicate that: 1) hypoxia increases pulmonary artery vasoconstriction and proinflammatory cytokine gene expression; 2) ethyl pyruvate decreases hypoxic pulmonary vasoconstriction and downregulates hypoxia-induced pulmonary artery proinflammatory cytokine gene expression; and 3) ethyl pyruvate may represent a novel therapeutic adjunct in the treatment of acute lung injury.

Keywords

hypoxia; pulmonary hypertension; inflammation; signal transduction

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INTRODUCTION

Perioperative pulmonary hypertension is a challenging clinical problem in cardiac surgery patients. Hypoxia is a potent stimulus for pulmonary vasoconstriction [1–4], which may be an adaptive mechanism to match lung perfusion with ventilation. Sustained hypoxic pulmonary vasoconstriction (HPV) may have detrimental effects, such as vascular remodeling, activation of proinflammatory pathways, and eventual right heart failure. Hypoxic injury may also activate proinflammatory signaling pathways [5], leading to production of inflammatory mediators such as tumor necrosis factor (TNF- α). It is now known that TNF- α can be produced by local tissues and can exert deleterious effects on these tissues in an autocrine fashion [6]. This has been demonstrated in myocardium following various forms of acute injury [7], and we have correlated increased TNF- α and IL-1 expression with pulmonary artery dysfunction following acute hypoxia [2].

Pyruvic acid is an end product of glycolysis, but it is also a potent antioxidant and free radical scavenger. Ethyl pyruvate is a derivative of pyruvic acid and it has demonstrated anti-inflammatory effects in animal injury models [8–10]. Specifically, treatment with ethyl pyruvate attenuated damage to rat intestinal mucosa during mesenteric ischemia-reperfusion [11], and cardiac function in rats was preserved by ethyl pyruvate treatment prior to prolonged myocardial ischemia [12]. There is evidence that ethyl pyruvate downregulates inflammatory gene expression following acute injury [13]. The effects of ethyl pyruvate on hypoxic pulmonary vasoconstriction and hypoxia-induced inflammatory signaling have not been studied. Therefore, we hypothesized that ethyl pyruvate would inhibit hypoxic pulmonary vasoconstriction and downregulate pulmonary artery cytokine expression during hypoxia.

MATERIALS AND METHODS

Animals

All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 85-23, revised 1985). All animal protocols are approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were allowed *ad libitum* access to food and water up to the time of experimentation.

Isolated pulmonary artery ring preparation

Rats were anesthetized with pentobarbital (150 mg/kg IP). Median sternotomy was performed and the heart and lungs were removed *en bloc* and placed in modified Krebs-Henseleit (KH) solution at 4°C. Under a dissecting microscope, extralobar pulmonary artery (PA) branches were dissected and cleared of surrounding tissue. Right and left main branch PA were cut into 2–3 mm wide rings (4 per animal) and suspended on steel hooks connected to force transducers (ADInstruments, Colorado Springs, CO) for measurement of isometric force displacement. Care was taken during this process to minimize endothelial injury by avoiding contact with the luminal surface of the arteries. Pulmonary artery rings were immersed in individual water-jacketed organ chambers containing modified Krebs-Henseleit (KH) solution bubbled with 95% O₂/5% CO₂ at 37°C. Krebs-Henseleit solution is a physiologic balanced salt solution containing (in mmol/L): NaCl 127, KCl 4.7, NaHCO₃ 17, MgSO₄ 1.17, KH₂PO₄ 1.18, CaCl₂ 2.5, and D-glucose 5.5. Force displacement was recorded using a PowerLab (ADInstruments) eight-channel data recorder on an Apple iMac PowerPC G4 Computer (Apple Computer Co., Cupertino, CA).

Experimental protocol and groups

Prior to starting experimental protocols, PA rings were stretched to a predetermined [14] optimal passive tension of 750 mg and allowed to equilibrate for 60 min, during which time KH solution was changed every 15 min. Viability of each PA ring was then checked by measuring contractile response to 80 mmol/L KCl. This dosage was determined to produce maximal contractile response to KCl in previous experiments [14]. Following washout of KCl, endothelial integrity of each PA ring was assessed with relaxation to acetylcholine (1 μ mol/L) after phenylephrine (1 μ mol/L) precontraction. These concentrations were derived from preliminary experiments to produce optimal contraction and relaxation. Rings demonstrating less than 50% vasorelaxation to acetylcholine were discarded. Following washout of acetylcholine, PA rings were precontracted with phenylephrine and hypoxia was induced by changing the gas to 95% N₂/5% CO₂. Hypoxia was induced in PA rings (n=8/group) with or without ethyl pyruvate (10 mM) treatment 20 minutes prior to the onset of hypoxia. Experiments were terminated after 60 minutes of hypoxia and rings were immediately snap frozen in liquid nitrogen for subsequent mRNA analysis.

RT-PCR

Semiquantitative RT-PCR was used to assess TNF- α and IL-1 gene expression in PA rings. Following tissue homogenization, total RNA was extracted from each pulmonary artery segment using RNA STAT-60 (TEL-TEST, Friendswood, TX). 0.1 μ g of total RNA was subjected to cDNA synthesis using a cloned Avian Myeloblastosis Virus first-strand cDNA synthesis kit (Maxim Biotech, South San Francisco, CA). cDNA from each sample was used for polymerase chain reaction of cytokines using message screen rat PCR kits (Maxim Biotech). PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. Gels were digitally photographed under ultraviolet illumination with a FotoAnalyst Luminary cooled camera electronic documentation system (Fotodyne Inc, Hartland, WI). All gel lanes were then subjected to densitometric analysis (NIH Image).

Chemicals and reagents

All chemical reagents were obtained from Sigma (St. Louis, MO), unless otherwise specified. All reagents were dissolved in deionized distilled water unless otherwise specified. All drug concentrations were expressed as final molar concentration in the organ bath. Final pH of all solutions was 7.35–7.45.

Statistical analysis

Vasodilation was expressed as the percentage difference from the force caused by phenylephrine precontraction. Force displacement during hypoxia was expressed as percentage change from the amount of phenylephrine-precontraction. All reported values were mean $\pm$ SEM. Experimental groups were compared using two-way analysis of variance (ANOVA) with post-hoc Bonferroni test or unpaired student's t-test (Prism 4, Graphpad Software, San Diego, CA). P<0.05 was considered statistically significant.

RESULTS

Hypoxic pulmonary vasoconstriction

Acute hypoxia resulted in a biphasic PA contraction: an initial transient contraction followed by a delayed sustained contraction (Fig. 1). Ethyl pyruvate treatment had no effect on the magnitude of the early transient contraction (23.39 $\pm$ 7.71 vs. 24.50 $\pm$ 8.89% hypoxia alone). However, delayed hypoxic contraction was significantly decreased by ethyl pyruvate treatment (4.49 $\pm$ 2.32% vs. 88.80 $\pm$ 5.68% hypoxia alone).

TNF- α and IL-1 expression by pulmonary artery tissue during hypoxia

PA rings exposed to hypoxia were homogenized and subjected to RT-PCR for measurement of TNF- α and IL-1 mRNA expression. For comparison, control PA rings were incubated in the organ baths under the same conditions and maintained on normoxic gas for an equivalent time period. Hypoxia resulted in increased TNF- α and IL-1 expression compared to normoxic controls. Ethyl pyruvate inhibited the hypoxic upregulation of TNF- α and IL-1 mRNA (Figures 2 and 3).

DISCUSSION

As demonstrated in this and previous studies, acute hypoxia resulted in a biphasic pulmonary artery contraction. Hypoxic pulmonary vasoconstriction correlated with an upregulation of proinflammatory cytokine gene expression. Ethyl pyruvate pretreatment abolished the sustained pulmonary artery contraction during hypoxia, and downregulated hypoxia-induced TNF- α and IL-1 mRNA expression from pulmonary artery tissue.

In isolated pulmonary artery rings submaximally precontracted with phenylephrine, exposure to acute hypoxia resulted in an immediate vasodilation followed by a rapid contraction. The contraction diminished rapidly and was followed by relaxation. With continued hypoxia, there was a second, sustained contraction. The initial transient contraction may represent a compensatory perfusion-ventilation mechanism, whereas the secondary sustained contraction may be responsible for the maladaptive effects of pulmonary vasoconstriction. The mechanisms responsible for the biphasic contraction are not completely understood, but there are likely multiple interrelated pathways involved in hypoxic pulmonary contraction [3,15]. These include inhibition of voltage-gated potassium channels and activation of voltage-gated calcium channels with subsequent release of calcium from the sarcoplasmic reticulum. In addition, several intracellular signaling pathways such as RhoA/Rho-kinase, protein kinase C, and p38 MAPK are involved as well [15].

Pyruvate is the conjugate anion of pyruvic acid. Pyruvic acid is the final product of glycolysis and the initial substrate for the tricarboxylic acid (TCA) cycle. The effects of pyruvate on scavenging reactive oxygen radicals have long been known. It became of clinical interest since reactive oxygen species have been implicated in numerous models of acute injury. However, the therapeutic application of pyruvate is limited because it is not stable in solution and spontaneously forms potentially toxic metabolites. Not until the derivation of ethyl pyruvate, which is stable in solution and non-toxic [8–10,16], has the clinical utility of pyruvate been recognized. Ethyl pyruvate has demonstrated protective effects in necrotizing pancreatitis [17], extrahepatic biliary obstruction [18], hemorrhagic shock [19], and H₂O₂-induced renal injury [20], as well as mesenteric [21] and myocardial ischemia-reperfusion injury [22]. In addition, there is evidence that ethyl pyruvate can ameliorate hepatic ischemia-reperfusion injury by inhibiting lipid peroxidation, downregulating inflammatory mediators, and decreasing apoptosis [23].

The role of ethyl pyruvate in sepsis has been examined in several studies. Hauser et al. demonstrated improved PaO₂/FiO₂ ratios in endotoxemic pigs treated with ethyl pyruvate, and these effects were correlated with decreased oxidative stress and nitric oxide production [24]. Ethyl pyruvate has also been demonstrated to decrease circulating levels of TNF- α , high-motility group box-1 (HMGB1), IL-1, and IL-6 [19,25] during septic shock. Interestingly, ethyl pyruvate shares many anti-inflammatory and anti-oxidant effects with insulin, and insulin has been demonstrated to increase the mitochondrial uptake of pyruvate [26].

The clinical process that causes hypoxia is often associated with an inflammatory stimulus, such as sepsis or trauma, resulting in the acute respiratory distress syndrome. Hypoxia alone,

in the absence of blood loss or tissue injury, has been shown to induce release of proinflammatory cytokines [27]. This process may occur via activation of inflammatory cells. Primed and activated neutrophils disrupt the integrity of the endothelial layer and cause dysfunction of endothelium-dependent and -independent cGMP-mediated vasorelaxation. Neutrophils have also been shown to mediate endotoxin-induced acute lung injury [28]. Hypoxemia in the clinical setting enhances cytotoxic function of neutrophils [29]. Furthermore, activated resident macrophages release inflammatory mediators which likely disrupt endothelium-dependent vasorelaxation [30]. Proinflammatory cytokines, specifically TNF- α , IL-1, and IL-6, contribute to pulmonary vasoconstriction and pulmonary hypertension [31, 32], and pretreatment with either endotoxin or TNF- α potentiates HPV [33,34]. Inflammatory mediators themselves are not vasoactive, but are thought to augment hypoxic contraction via inhibition of constitutive nitric oxide synthase (eNOS) activity or via endothelial “stunning”. Moreover, it has been demonstrated that TNF- α , IL-1, or lipopolysaccharide augmented the downregulation of eNOS expression associated with hypoxia [35]. The upregulation of cytokine expression from pulmonary artery may occur at the pretranscriptional level. In this regard, nuclear factor-kappa B (NF- κ B), a transcription factor involved in the transcription of proinflammatory molecules, may be involved in the signaling pathway of HPV. Indeed, ethyl pyruvate attenuates NK- κ B signaling pathways and increases plasma levels of the anti-inflammatory cytokine IL-10 [19,25,36]. Therefore, we speculate that ethyl pyruvate exerts its protective effects against hypoxic pulmonary vasoconstriction by inhibiting NK- κ B signaling and attenuating cytokine-induced vasoconstriction.

In summary, these data demonstrate that ethyl pyruvate decreases hypoxic pulmonary vasoconstriction and downregulates hypoxia-induced pulmonary artery proinflammatory cytokine gene expression. To our knowledge, this is the first study to demonstrate the beneficial effects of ethyl pyruvate on the pulmonary vasculature. Ethyl pyruvate may represent a novel therapeutic adjunct in the treatment of acute lung injury and pulmonary hypertension.

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Figure 1.

Effect of ethyl pyruvate pretreatment on hypoxic pulmonary vasoconstriction. PA rings subjected to 60 minutes of hypoxia ($pO_2 = 30\text{--}35$ mmHg) demonstrated a biphasic contraction, expressed as a percentage of phenylephrine precontraction. Compared to controls ($n=8$), pretreatment with ethyl pyruvate (10 mM) ($n=8$) significantly decreased hypoxic pulmonary artery contraction. Mean $\pm$ SEM. * $P<0.001$ vs. control.

Figure 2.

TNF- α expression in PA tissue following hypoxia. Representative gel photographs and gel densitometry of TNF- α mRNA from PA tissue exposed to 60 minutes of hypoxia. Positive control (PC) was provided by the PCR manufacturer (Maxim Biotech). 18S housekeeping gene was used as an internal standard. Control PA rings were given DMSO vehicle and subjected to 60 minutes of normoxia. Hypoxia alone increased TNF- α expression, whereas hypoxia-induced upregulation of TNF- α was decreased by ethyl pyruvate pretreatment. † $P < 0.01$ vs. control, * $P < 0.05$ vs. hypoxia alone.

Figure 3.

IL-1 expression in PA tissue following hypoxia. Representative gel photographs and gel densitometry of IL-1 mRNA from PA tissue exposed to 60 minutes of hypoxia. Positive control (PC) was provided by the PCR manufacturer (Maxim Biotech). 18S housekeeping gene was used as an internal standard. Control PA rings were given DMSO vehicle and subjected to 60 minutes of normoxia. Hypoxia increased IL-1, whereas hypoxia-induced upregulation of IL-1 was decreased by ethyl pyruvate pretreatment. ‡ P<0.001 vs. control, †P<0.01 vs. hypoxia alone.