Original Research

Ethyl pyruvate reduces ventilation-induced neutrophil infiltration and oxidative stress

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Abstract

Mechanical ventilation with high tidal volume causes intense inflammatory responses and oxidative stress, including the release of high-mobility group box-1 (HMGB1), plasminogen activator inhibitor-1 (PAI-1) and heme oxygenase-1 (HO-1). The mechanisms regulating ventilator-induced lung injury (VILI) are unclear. We hypothesized that ethyl pyruvate attenuated acute lung injury as adjunctive pharmacological strategy by down-regulating neutrophil infiltration, oxidative stress and HMGB1 mRNA expression. C57BL/6 mice, weighing 20–25 g, were exposed to either low-tidal-volume (6 mL/kg) or high-tidal-volume (30 mL/kg) mechanical ventilation with room air for 2–5 h and subjected to 100 mg/kg ethyl pyruvate administration intraperitoneally. Non-ventilated mice served as the control group. Evans blue dye, lung wet-to-dry weight ratio, lung neutrophil infiltration and myeloperoxidase, free radicals, gene expression of HMGB1, active PAI-1 and HMGB1 production and HO-1 expression were measured. The expression of HO-1 was studied by immunohistochemistry. High-tidal-volume ventilation induced microvascular leak, neutrophil recruitment, oxidative injury, HMGB1 and active PAI-1 protein production, and upregulation of HMGB1 mRNA and stress-inducible protein HO-1. In contrast, administration of ethyl pyruvate before high stretch mechanical ventilation prevented lung edema formation, inflammatory cytokine production, neutrophil accumulation, oxidative stress and HMGB1 and HO-1 expression. High-tidal-volume mechanical ventilation increased microvascular permeability, neutrophil influx and inflammatory cytokines. Ethyl pyruvate is capable of suppressing the VILI related to the reduction of HMGB1 and our findings support the potential use of ethyl pyruvate as a therapeutic agent for the prevention of VILI.

Keywords: ethyl pyruvate, heme oxygenase-1, high mobility group box-1, plasminogen activator inhibitor-1, ventilator-induced lung injury

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Introduction

Acute respiratory distress syndrome (ARDS) is an inhomogeneous lung disease characterized by non-cardiogenic pulmonary edema, release of cytokines, influx of neutrophils and changes in the coagulation system, leading to platelet-fibrin thrombi in small vessels and impaired fibrinolysis and hyaline membrane formation within the distal air spaces of the injured lung.[1,2] High-tidal-volume (V_T) mechanical ventilation has been proved to induce the activation of proinflammatory cytokines, cascades of coagulopathy, and thus leads to ventilator-induced lung injury (VILI).[3–5] It has been shown that high stretch mechanical ventilation can increase both high-mobility group box-1 (HMGB1) and plasminogen activator inhibitor-1 (PAI-1).[6–8] Either antibody blocking HMGB1 or anticoagulant blocking PAI-1 has proved to improve microvascular permeability, neutrophil influx into the alveolar lumen and proinflammatory cytokines.[6–8]

In a variety of *in vitro* and *in vivo* model systems, ethyl pyruvate administration ameliorates multiple-organ-system damage.[9–14] Ethyl pyruvate has both anti-inflammatory effects, related to decreased influx of inflammatory cells and inhibition of HMGB1 secretion, and anticoagulant effects relating to decreasing tissue factor, PAI-1, and mRNA for urokinase-like plasminogen activator.[15,16]

Heme oxygenase-1 (HO-1), a stress responsive protein, degrades heme into three products: ferrous iron, biliverdin and carbon monoxide, and can be induced by inflammatory cytokines such as HMGB1, interleukin (IL)-8, heat shock protein and oxidants.[17] In the lungs, HO-1 is highly

expressed in alveolar macrophages, but is also found in epithelial cells, fibroblasts and endothelial cells. The HO-1 system exerts antioxidant, antiapoptotic and immunomodulatory functions in various situations.[18] In patients with ARDS, increased HO-1 expression has been demonstrated in lung tissue and bronchoalveolar lavage (BAL) fluid.[19] The increase in HO-1 gene and protein expression after mechanical ventilation suggests that HO-1 may play an important role in defense against VILI.[20–22] We hypothesized that ethyl pyruvate administration decreased ventilator-induced microvascular permeability, recruitment of neutrophils, oxidative injury and HMGB1 mRNA expression. To test the hypothesis, we ventilated mice with high tidal volume and examined the inhibitory effects of ethyl pyruvate in acute lung injury (ALI), inflammatory cells and chemokines.

Materials and methods

Experimental animals

Male C57BL/6 mice, aged between six and eight weeks, weighing between 20 and 25 g, were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (Permit number: 2008090102). All surgery was performed under ketamine and xylazine anesthesia and all efforts were made to minimize suffering.

Experimental groups

Animals were randomly distributed into five groups: (1) control, non-ventilated wild-type mice with normal saline; (2) control, non-ventilated wild-type mice with ethyl pyruvate (Evans blue dye [EBD] assay, lung water, BAL total protein); (3) V_T 6 mL/kg wild-type mice with normal saline; (4) V_T 30 mL/kg wild-type mice with normal saline; and (5) V_T 30 mL/kg wild-type mice with ethyl pyruvate. In each group except for group 2, five mice underwent measurements for Western blot, reverse transcription polymerase chain reaction, EBD assay, myeloperoxidase (MPO), lung water, cell counts, BAL total protein, HMGB1, active PAI-1, malondialdehyde (MDA), hematoxylin and eosin (H&E) stain and immunohistochemical assay.

Ventilator protocol

We used our established mouse model of VILI as previously described.[7] A 20-gauge angiocatheter was introduced into the tracheotomy orifice of mice under general anesthesia with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg). The mice were placed in a supine position on a heating blanket and then attached to a Harvard apparatus ventilator, model 55-7058 (Harvard Apparatus, Holliston, MA, USA), set to deliver either 6 mL/kg at a rate of 135 breaths/min or 30 mL/kg at a rate of 65 breaths/min, for 2–5 h while breathing room air with zero end-expiratory pressure. The mice then received 0.9% saline containing maintenance ketamine (0.1 mg/g/h) and xylazine (0.01 mg/g/h) at a rate of 0.09 mL/10 g/h by a continuous intraperitoneal fluid pump. Continuous monitoring of end-tidal CO_2 by a microcapnograph (Columbus Instruments, Columbus, OH, USA) was performed and respiratory frequencies of 135 breaths/min for 6 mL/kg and 65 breaths/min for 30 mL/kg were chosen in the experiment, with end-tidal CO_2 at 30–40 mm Hg. Airway peak inspiratory pressure was measured with a pressure-transducer amplifier (Gould Instrument Systems, Valley View, OH, USA) connected to the tubing at the proximal end of the tracheostomy. Mean arterial pressure was monitored every hour during mechanical ventilation by using the same pressure-transducer amplifier connected to a 0.61-mm outer diameter polyethylene catheter ending in the common carotid artery. Our present and previous studies have shown that increased expressions of HO-1 and HMGB1 mRNA occurred around two hours after mechanical stretch, whereas changes in cytokine production occurred later. Therefore, two hours of ventilation was used for collection of samples of total RNA, protein for Western blot analysis of HO-1 and five hours of ventilation for collection of samples of HMGB1, PAI-1, EBD assay, lung water, MPO assay, H&E stain and immunohistochemistry.[7] At the end of the study period, heparinized blood was taken from the arterial line for analysis of arterial blood gas and the mice were sacrificed. Control, non-ventilated mice were anesthetized and sacrificed immediately.

Ethyl pyruvate administration

Two doses of ethyl pyruvate (100 mg/kg; Sigma, St Louis, MO, USA) were given intraperitoneally. The first dose was given 30 min before the mice were subjected to mechanical ventilation and the second dose was given after the mice were subjected to two hours of mechanical ventilation. The dose was chosen on the basis of previous *in vivo* studies that showed 100 mg/kg ethyl pyruvate inhibited HMGB1 activity.[23]

Analysis of lung water

Lungs were removed *en bloc* and large airways were removed. Both lungs were weighed and then dried in an oven at 80°C for 48 h. If no changes were found in the dry lung weight at 24 and 48 h, the weight at 48 h was used. Lung wet-to-dry weight ratio was used as an index of pulmonary edema formation.

Histopathological grading of VILI

The lung tissues from control, non-ventilated mice and mice exposed to high-tidal-volume ventilation for five hours while breathing room air were removed *en bloc* and filled with 10% neutral buffered formalin (pH 6.8–7.2) at 30 cm H_2O pressure via polyethylene tubing inserted into the trachea. The lungs were paraffin-embedded, sliced at 4 μm, stained with H&E and reviewed from five non-

overlapping fields by a single investigator blinded to the therapeutic category of the mouse. Lung injury was scored using an average score of the following items: alveolar congestion, hemorrhage, infiltration of neutrophils into the airspace or the vessel wall and thickness of the alveolar wall.[24] A score of 0 represented normal lungs; 1, mild, <25% lung involvement; 2, moderate, 25–50% lung involvement; 3, severe, 50–75% lung involvement; and 4, very severe, >75% lung involvement.

BAL total protein

Total protein concentration in the BAL was determined with the Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Immunoblot analysis

The lungs were homogenized in 0.5 mL of lysis buffer as previously described.[7] Crude cell lysates were matched for protein concentration, resolved on a 10% bis-acrylamide gel and electrotransferred to immobilon-P membranes (Millipore Corp., Bedford, MA, USA). For assay of HO-1 protein expression, Western blot analysis was performed with antibody of HO-1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were developed by enhanced chemiluminescence (NEN Life Science Products, Boston, MA, USA).

Reverse transcription polymerase chain reaction

For isolating total RNA, the lungs were homogenized in 1.5 mL of TRIzol reagents and isolated according to the manufacturer's directions (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA (1 μg) was reverse transcribed using a GeneAmp polymerase chain reaction (PCR) system 9600 (PerkinElmer, Life Sciences, Inc., Boston, MA,USA) as previously described.[7] The following primers were used for PCR: HMGB1, forward primer 5′ TGGCAAAGGCTGAC AAGGCTC 3′ and reverse primer 5′ GGATGCTCGCCT TTGATTTTGG 3′ and glyceraldehyde phosphate dehydrogenase as internal control, forward primer 5′ AATGC ATCCTGCA CCACCAA 3′ and reverse primer 5′ GTAGCCATATTCATTGTCATA 3′ (Integrated DNA Technologies, Inc., Coralville, IA, USA).[25]

Measurement of MDA

The lungs were homogenized in phosphate-buffered saline containing butylated hydroxytoluene for MDA. The MDA in the protein extracts were measured using the Oxiselect TBARS assay kit containing thiobarbituric acid-reactive substances (Cell Biolabs, San Diego, CA, USA). Each sample was run in duplicate and expressed as μmol/g protein according to the manufacturer's instructions.

Measurement of HMGB1 and active PAI-1

At the end of the study period, the lungs were lavaged via tracheostomy with a 20-gauge angiocatheter (sham instillation) three times with 0.6 mL of 0.9% normal saline. HMGB1 with a lower detection limit of 1 ng/mL and active PAI-1 with a lower detection limit of 0.02 ng/mL were measured in BAL fluid using a commercially available immunoassay kit containing primary polyclonal anti-mouse antibody that was cross-reactive with rat and mouse HMGB1 (Shino-Test Corporation, Kanagawa, Japan) or active PAI-1 (Molecular Innovations, Inc., Southfield, MI, USA). Each sample was run in duplicate according to the manufacturer's instructions.

Immunohistochemistry

The lungs were paraffin-embedded, sliced at 4 μm, deparaffinized, antigen-unmasked in 10 mmol/L sodium citrate (pH 6.0), and incubated with goat HO-1 primary antibody (1:100; Santa Cruz Biotechnology) and biotinylated goat anti-goat secondary antibody (1:100; Santa Cruz Biotechnology) according to the manufacturer's instructions for an immunohistochemical kit (Santa Cruz Biotechnology). The specimens were further conjugated with horseradish peroxidase-streptavidin complex, detected with a diaminobenzidine (DAB) substrate mixture and counterstained by hematoxylin. A dark-brown DAB signal indicated positive staining of damaged epithelial cells, whereas shades of light blue signified non-reactive cells.

Statistical analysis

The Western blots and HMGB1 mRNA were quantitated using an NIH image analyzer, ImageJ 1.27z (National Institute of Health, Bethesda, MD, USA) and presented as arbitrary units. Values were expressed as the mean $\pm$ SD for at least five experiments. The data of EBD assay, lung wet-to-dry weight ratio, HMGB1, PAI-1, MPO assay, immunohistochemical staining and MDA were analyzed using Statview 5.0 (Abascus Concepts Inc. and SAS Institute, Inc., Cary, NC, USA). All results of Western blots and HMGB1 mRNA were normalized to control, non-ventilated mice. Analysis of variance was used to assess the statistical significance of the differences, followed by multiple comparisons with a Scheffe's test and a P value <0.05 was considered statistically significant. Regression coefficients were calculated using the simple regression test in Statview.

Additional details, including cell counts, EBD assay and MPO assays were performed as previously described.[7]

Results

Inhibition of high-tidal-volume-induced microvascular leak, lung edema and total lung injury with ethyl pyruvate

To determine the effects of mechanical ventilation on changes of microvascular permeability and lung water in VILI, we measured lung EBD, BAL total protein and wet-to-dry weight ratio (Figure 1). The levels of lung EBD, BAL total protein and wet-to-dry weight ratio significantly

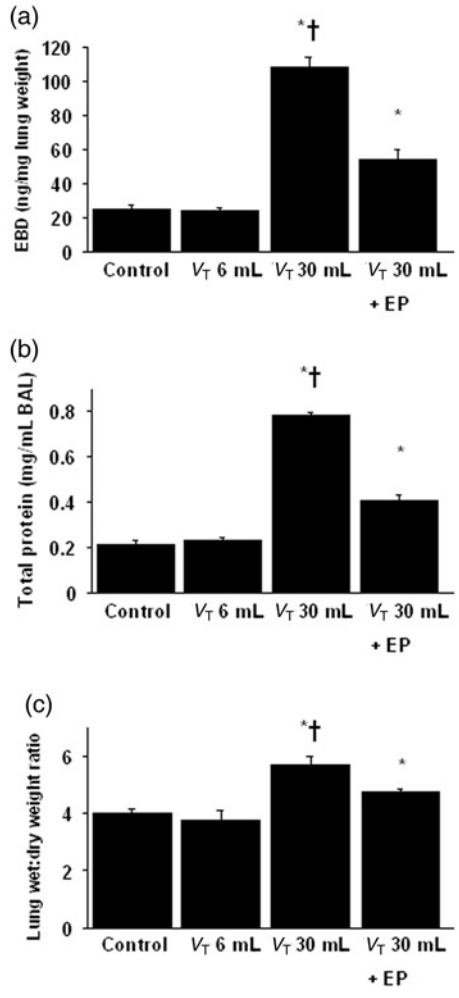

Figure 1 Ethyl pyruvate reduced lung stretch-induced microvascular permeability and lung edema. (a) Evans blue dye (EBD) analysis of lung tissue, (b) bronchoalveolar lavage (BAL) total protein concentration and (c) lung water measured by lung wet-to-dry-weight ratio were from control, non-ventilated mice and mice ventilated at a tidal volume (V_T) of 6 mL/kg (V_T 6 mL) or 30 mL/kg (V_T 30 mL) for five hours with room air. Ethyl pyruvate, 100 mg/kg, was given intraperitoneally 30 min before ventilation and two hours after ventilation ($n = 5$ per group). $^*P < 0.05$ versus control, non-ventilated mice; $^\dagger P < 0.05$ versus all other groups. EP, ethyl pyruvate

increased in mice receiving V_T 30 mL/kg mechanical ventilation compared with those of either V_T 6 mL/kg or control, non-ventilated mice. No significant elevation was found in mice ventilated with V_T 6 mL/kg compared with that of control, non-ventilated mice. No differences in the levels of EBD (24.7 ± 0.8 versus 25.3 ± 1.1 ng/mg lung weight; $P = 0.33$), BAL total protein (0.21 ± 0.02 versus 0.22 ± 0.03 mg/mL BAL; $P = 0.35$) and wet-to-dry weight ratio (3.9 ± 0.2 versus 4.0 ± 0.3; $P = 0.53$) were found between control, non-ventilated mice with or without administration of ethyl pyruvate. The increases of microvascular leak and lung edema with V_T 30 mL/kg mechanical ventilation were significantly reduced by pharmacological inhibition with ethyl pyruvate. Ethyl pyruvate also improved gas exchange (PaO_2/FiO_2 ratio: V_T 30 mL/kg mice with normal saline $= 413.5 \pm 5.1$ versus V_T 30 mL/kg mice with ethyl pyruvate $= 427.9 \pm 3.5$; $P = 0.01$) (Table 1). To further evaluate total lung injury, we measured alveolar congestion, hemorrhage, infiltration of neutrophils into airspace or the vessel wall and thickness of the alveolar wall on lung histopathology (Figure 2). The increases in lung injury with V_T 30 mL/kg mechanical ventilation were significantly reduced by pharmacological inhibition with ethyl pyruvate.

Inhibition of high-tidal-volume-induced neutrophil sequestration and oxygen radicals with ethyl pyruvate

Neutrophil counts were used to measure migration of neutrophils into the alveoli (Figure 3a). MPO assay was used to quantitate total lung neutrophils, i.e. neutrophils marginated in the vasculature, located in the parenchyma and in the alveoli (Figure 3b). The concentrations of MDA were measured to examine oxidant stress involved in VILI (Figure 3c). The neutrophil migration into lung lavage fluid, MPO and MDA concentrations were significantly elevated in mice ventilated with V_T 30 mL/kg compared with those of either V_T 6 mL/kg or control, non-ventilated mice. No significant elevation was found in mice ventilated with V_T 6 mL/kg compared with that of control, non-ventilated

Table 1 Physiological conditions at the beginning and end of ventilation

	Non-ventilated + normal saline	V_T 6 mL/kg + normal saline	V_T 30 mL/kg + normal saline	V_T 30 mL/kg + ethyl pyruvate
pH	7.41 ± 0.02	7.37 ± 0.02	7.39 ± 0.04	7.38 ± 0.01
PaO_2 (mmHg)	98.5 ± 0.3	85.9 ± 0.7	86.7 ± 3.9	89.5 ± 0.8
PaO_2/FiO_2 ratio	469.3 ± 2.5	409.7 ± 3.1	413.5 ± 5.1	427.9 ± 3.5
$PaCO_2$ (mmHg)	38.9 ± 0.2	40.2 ± 0.7	36.1 ± 1.8	35.9 ± 1.7
MAP (mmHg)				
Start	85 ± 1.1	84.6 ± 1.3	84.1 ± 3.8	85.2 ± 2.2
End	84.7 ± 0.5	81.9 ± 2.2	80.4 ± 2.6	82.8 ± 3.1
PIP				
Start		9.6 ± 1.2	23.1 ± 1.4	23.6 ± 1.5
End		11.9 ± 1.3	27.9 ± 0.9	27.8 ± 1.0

Arterial blood gases and mean arterial pressure were obtained from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for five hours ($n = 10$ per group). No statistical difference was found in pH, PaO_2, $PaCO_2$, mean arterial pressure and peak inspiratory pressure at the beginning versus the end of five hours of mechanical ventilation. The normovolemic statuses of mice were maintained by monitoring mean artery pressure. The physiological data of control groups were similar during the experiment and were used as the beginning data of mechanical ventilation. Although no differences were observed between control, non-ventilated mice with normal saline or ethyl pyruvate, the data were combined. FiO_2, fraction of inspired oxygen; MAP, mean arterial pressure; PIP, peak inspiratory pressure; V_T, tidal volume

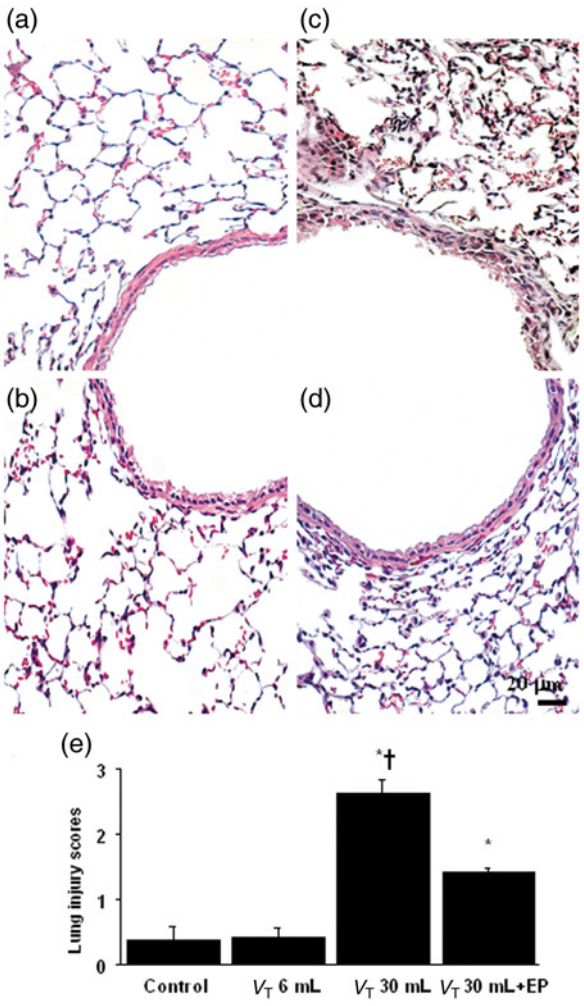

Figure 2 Ethyl pyruvate reduced lung stretch-induced total lung injury. Representative photomicrographs (×400) with hematoxylin and eosin (H&E) staining of paraffin lung sections were from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for five hours with room air. Ethyl pyruvate, 100 mg/kg, was given intraperitoneally 30 min before ventilation and two hours after ventilation ($n = 5$ per group). (a) Control, non-ventilated mice; (b) V_T 6 mL/kg mice; (c) V_T 30 mL/kg mice; (d) V_T 30 mL/kg mice treated with ethyl pyruvate; (e) total lung injury scores were measured as described in Materials and methods. $^*P < 0.05$ versus control, non-ventilated mice; $^\dagger P < 0.05$ versus all other groups. Scale bars represent 20 μm. EP, ethyl pyruvate. (A color version of this figure is available in the online journal)

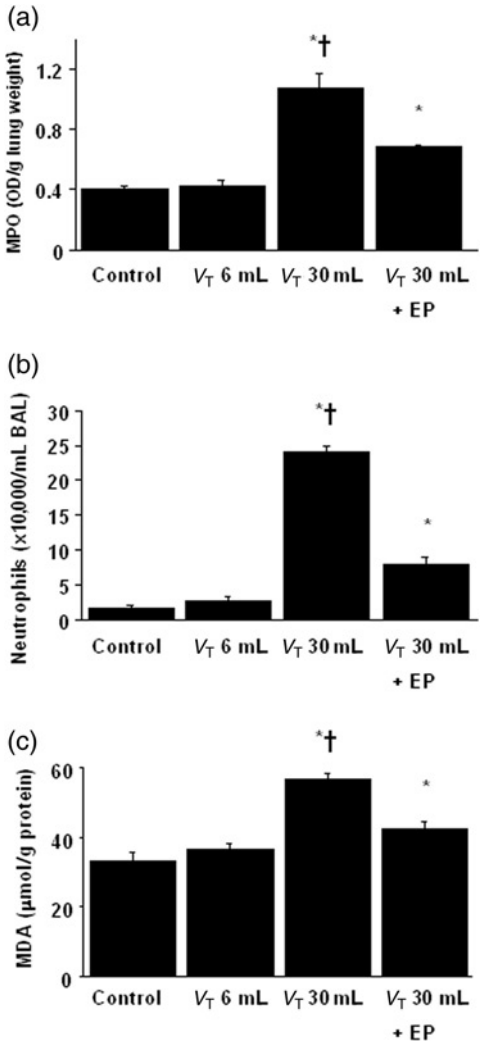

Figure 3 Ethyl pyruvate reduced lung stretch-induced neutrophil influx and oxidative stress. Neutrophil counts (a) in BAL fluid, myeloperoxidase (MPO) and malondialdehyde (MDA) of lung tissue (b, c) were from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for five hours with room air. Ethyl pyruvate, 100 mg/kg, was given intraperitoneally 30 min before ventilation and two hours after ventilation ($n = 5$ per group). $^*P < 0.05$ versus control, non-ventilated mice; $^\dagger P < 0.05$ versus all other groups. EP, ethyl pyruvate; OD, optical density

mice. The increases in neutrophil sequestration, lung MPO activity and oxidative stress with V_T 30 mL/kg mechanical ventilation were significantly reduced after pharmacological inhibition with ethyl pyruvate.

Inhibition of high-tidal-volume-induced HMGB1 mRNA expression, active PAI-1 and HMGB1 production with ethyl pyruvate

To determine whether the increased neutrophil influx in mice receiving V_T 30 mL/kg mechanical ventilation was associated with upregulation of chemotactic factors for neutrophils, we measured HMGB1 mRNA expression, active PAI-1 and HMGB1 protein production (Figure 4). We found significantly increased ventilator-induced HMGB1 mRNA expression, active PAI-1 and HMGB1 protein production in the V_T 30 mL/kg mice compared with those of V_T 6 mL/kg or control, non-ventilated mice. No significant elevation was observed in the V_T 6 ml/kg mice compared with that of control, non-ventilated mice. The increases in HMGB1 mRNA expression, active PAI-1 and HMGB1 protein production with V_T 30 mL/kg mechanical ventilation were significantly reduced after pharmacological inhibition with ethyl pyruvate.

Inhibition of high-tidal-volume-induced HO-1 expression with ethyl pyruvate

In a previous study of an endotoxin-induced lung injury model in mice, HO-1 was shown to regulate the production of HMGB1.[26] To determine the roles of HO-1 upregulation in stretch-induced lung injury, we measured HO-1

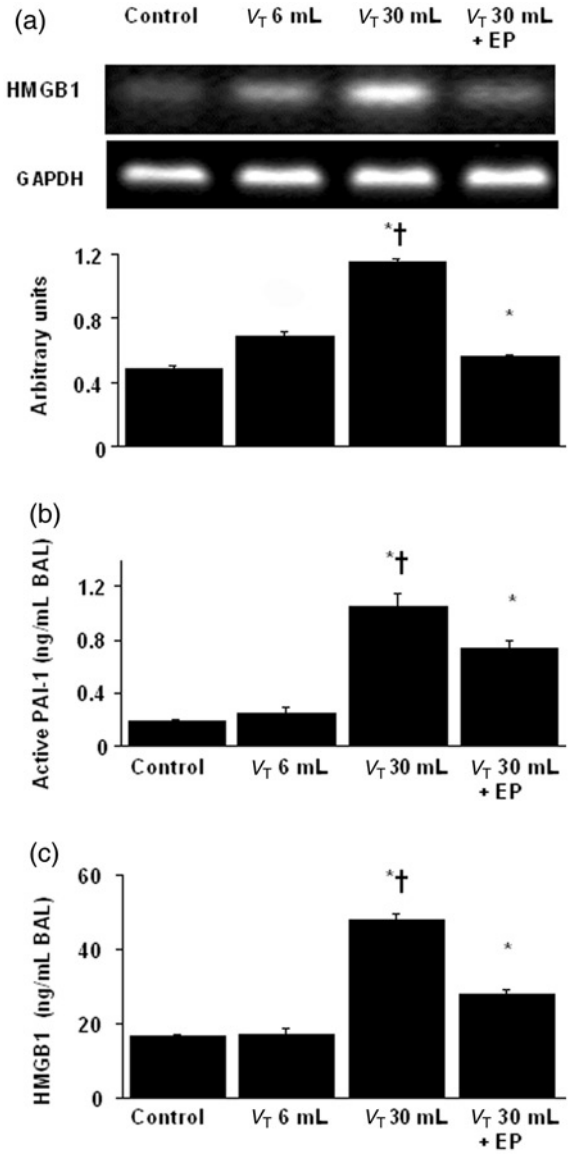

Figure 4 Ethyl pyruvate reduced lung stretch-induced high-mobility group box-1 (HMGB1) mRNA expression, active plasminogen activator inhibitor-1 (PAI-1) and HMGB1 protein production. Reverse transcription polymerase chain reaction (RT-PCR) assay of lung tissue was performed for HMGB1 mRNA (a, top panel), glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA (a, middle panel) and arbitrary units (a, bottom panel) from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for two hours with room air ($n = 5$ per group). Arbitrary units were expressed as the ratio of HMGB1 mRNA to GAPDH. Active PAI-1 (b) and HMGB1 (c) protein in BAL were from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for five hours with room air ($n = 5$ per group). Ethyl pyruvate, 100 mg/kg, was given intraperitoneally 30 min before ventilation and two hours after ventilation. $^*P < 0.05$ versus control, non-ventilated mice; $^\dagger P < 0.05$ versus all other groups. EP, ethyl pyruvate

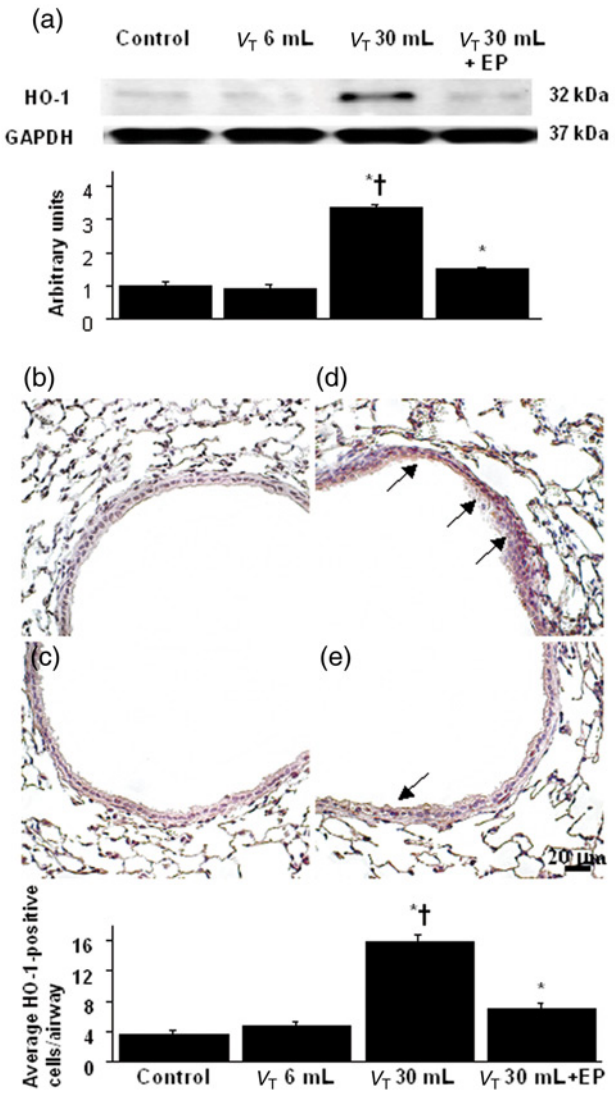

Figure 5 Ethyl pyruvate reduced lung stretch-induced heme oxygenase-1 (HO-1) expression. Western blot was performed using an antibody that recognizes HO-1 expression (a, top panel) and an antibody that recognizes GAPDH expression (a, middle panel) in lung tissue from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for two hours with room air. Arbitrary units were expressed as the ratio of HO-1 to GAPDH (a, bottom panel). Representative photomicrographs (×400) with HO-1 staining of paraffin lung sections with immunohistochemistry were from control, non-ventilated mice and mice ventilated at V_T 6 mL/kg or V_T 30 mL/kg for five hours with room air. (b) Control, non-ventilated mice; (c) V_T 6 mL/kg mice; (d) V_T 30 mL/kg mice; (e) V_T 30 mL/kg mice treated with ethyl pyruvate. Positive staining of airway epithelia is identified by arrows. HO-1-positive cells were quantified as the average number of epithelial cells with dark brown diaminobenzidine (DAB) signals per bronchiole, which were counted from 10 randomly chosen bronchioles of each section. Ethyl pyruvate, 100 mg/kg, was given intraperitoneally 30 min before ventilation and two hours after ventilation ($n = 5$ per group). $^*P < 0.05$ versus control, non-ventilated mice; $^\dagger P < 0.05$ versus all other groups. Scale bars represent 20 μm. EP, ethyl pyruvate. (A color version of this figure is available in the online journal)

expression in mice receiving mechanical ventilation (Figure 5a). We found that there was a significantly increased ventilator-induced HO-1 expression in the V_T 30 mL/kg mice compared with those of V_T 6 mL/kg or control, non-ventilated mice. No significant increase in HO-1 expression was observed in the V_T 6 mL/kg mice compared with that of control, non-ventilated mice. The increases in HO-1 expression with V_T 30 mL/kg mechanical ventilation were significantly reduced after pharmacological inhibition with ethyl pyruvate. With immunohistochemistry, we further confirmed the effectiveness of ethyl pyruvate in the inhibition of HO-1 expression involved in V_T 30 mL/kg VILI in bronchial epithelial cells (Figure 5).

Discussion

High tidal volumes in normal animals have been used to mimic the overdistension of the less injured and thus more compliant areas of lung found in ARDS patients. Besides offering adequate ventilation and oxygenation, mechanical ventilation with high tidal volume can also cause severe lung injury characterized by lung edema, inflammatory cell infiltration and cytokine production and enhanced pulmonary coagulopathy, thus leading to fibrin deposition and hyaline membrane formation. As the most common cause of death in ALI/ARDS is multiple-organ-system failure, the systemic abnormalities of coagulation and fibrinolysis may be an important therapeutic target over the local pulmonary abnormalities. In our previous study, we demonstrated that a short course of high stretch mechanical ventilation induced lung injury by increasing PAI-1 associated with dysregulation of intra-alveolar fibrinolysis.[7] In the present study, we found that high-tidal-volume ventilation in mice increased microvascular permeability, neutrophil influx, HMGB1 mRNA expression and production of HMGB1, which led to subsequent inflammatory cytokine cascades, including increased expression of HO-1 and production of PAI-1 and free radicals. Ethyl pyruvate, an HMGB1 inhibitor, reduced high-tidal-volume-induced lung inflammation and improved gas exchange.

Neutrophils are the major inflammatory cells involved in the process of ALI and play a major role in the generation of reactive oxygen species (ROS).[27] We found that high-tidal-volume ventilation recruited the influx of neutrophils and increased the concentration of MDA, an aldehydic secondary product of lipid peroxidation used as a marker of oxidative damage. HMGB1 is expressed in various cells, including macrophages/monocytes, endothelial cells, neutrophils, epithelial cells, dendritic cells and smooth muscle cells.[28] Except as a late mediator of endotoxin lethality, HMGB1 can express its toxicity in a short period of time in VILI and increase its inflammatory activity by binding with proinflammatory mediators, such as PAI-1 and HO-1.[29–32] In our study, we found that mechanical ventilation increased HMGB1 mRNA expression and HMGB1 protein production after five hours of lung stretch. This is consistent with the notion that HMGB1 has two distinct functions in cellular systems, as an intracellular regulator of transcription and an extracellular inflammatory cytokine.

In addition to being a potent ROS scavenger, ethyl pyruvate also has anti-inflammatory effects through inhibition of the activation of nuclear factor-κappa B and p38 mitogen-activated protein kinase.[12,13] Previous studies of hemorrhagic shock and endotoxemia in mice showed that ethyl pyruvate inhibits the production of HMGB1 and PAI-1.[13,16] Treatment with ethyl pyruvate has been shown to improve survival and ameliorate organ dysfunction in a variety of critical illnesses, such as severe sepsis, intestinal mucosal injury and acute pancreatitis.[9] Besides the anti-inflammatory properties, ethyl pyruvate was shown to have anticoagulant effects by inhibiting lipopolysaccharide-induced expression of tissue factor in an *in vitro* study of cultured monocyte-like cells.[15] However, ethyl pyruvate used for treatment of VILI has not been studied before. In our study, we found that ethyl pyruvate improved high-tidal-volume ventilation-induced microvascular permeability, lung edema, neutrophil recruitment, production of free radicals and inflammatory cytokines.

HO-1 possesses anti-inflammatory and antioxidant properties and plays a vital role in defense against oxidant-induced lung injury during ARDS.[18] A previous study of ALI in mice showed that HO-1was induced as a stress response protein during lung stretch and that hydrogen sulfide prevented its upregulation by limiting the cellular stress associated with mechanical ventilation.[21] Inhaled carbon monoxide, a major byproduct of heme oxygenase catalysis of heme, has been found to prevent the upregulation of ventilation-induced expression of HO-1 mRNA and protein via the p38 mitogen-activated protein kinase pathway.[20,22] While moderate HO-1 induction is protective, overexpression can cause lung injury through the accumulation of iron-responsive protein.[19] In the study of patients with ARDS, ROS induced by cyclic stretch may up-regulate HO-1 and consequently the production of low molecular mass redox active iron.[19] In our study, we found that mechanical ventilation augmented the expression of HO-1 in lung homogenate and in bronchial epithelium. The effects of lung stretch blocked by pharmacological inhibition with ethyl pyruvate suggested that HO-1 may be induced by the production of HMGB1.

Our study does have limitations. In an *in vitro* study of MPO-induced inflammation of alveolar and bronchial epithelial cells, others found that MPO produced by neutrophils may increase the expression of HO-1, an indirect marker of oxidative stress.[32] Using immunohistochemistry, we found the increase of HO-1 staining in mice ventilated at V_T 30 mL/kg more in the bronchial epithelial cells than in the alveolar epithelial cells. The discrepancies of cell types involved may be due to the different physical forces of mechanical strain and immunohistochemistry method limitations. Previous studies of endotoxemia have shown that the increase of HO-1 may inhibit the release of HMGB1 and tumor necrosis factor-α.[26,33] In our and others' murine VILI model, we found that mechanical ventilation can increase both HO-1 expression and HMGB1 production. The discrepancy may be because HO-1 is an acute phase protein for defense against mechanical stretch or cellular injury and the degree of HO-1 expression varies in different timings.[19,20] HMGB1$^{-/-}$ mice are not used in this study because they die shortly after birth due to a defect in the transcriptional activation of the glucocorticoid receptor, consistent with the notion that HMGB1 functions as a regulator of transcription involving steroid hormone receptors.[34] Despite their small size, respiratory system mechanics are widely measured in studies with mice.[35]

Using an *in vivo* mouse model of ALI, we have found that high-tidal-volume mechanical ventilation induced epithelial cell injury, pulmonary neutrophil sequestration, oxidant stress, expression of HMGB1 and HO-1, PAI-1 and HMGB1 production. The deleterious effects were attenuated by ethyl pyruvate treatment. Our data suggest that effects of high-tidal-volume ventilation on lung inflammation occur in the first few hours of VILI. Understanding the protective mechanism of ethyl pyruvate related with the regulation of

HMGB1 may advance the growing knowledge of biomarkers to be monitored in the course of ALI and the effects of mechanical forces in the lung involved in the pathogenesis of biotrauma with VILI.

Author contributions: LF-L and YY-L conducted and designed the experiments. LF-L collected and analyzed the data. LF-L and YY-L reviewed the references and wrote the manuscript. KC-K, CT-Y and CC-H reviewed the references.

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