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Ethyl Pyruvate Reduces Acute Lung Injury *Via* Regulation of iNOS and HO-1 Expression in Endotoxemic Rats

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Background. **Ethyl pyruvate (EP) has been shown to attenuate lipopolysaccharide (LPS)-induced acute lung injury (ALI). Induction of heme oxygenase-1 (HO-1) and suppression of inducible nitric oxide synthase (iNOS) expression provide cytoprotection in lung and vascular injury. The aim of this study is to evaluate whether the beneficial effect of EP on lung inflammation is related to HO-1 induction in a rat model of LPS-induced ALI.**

Materials and Methods. **Rats were administered LPS (30 mg/kg) by intravenous infusion for 4 h to induce ALI. EP (20, 40, and 60 mg/kg/4 h i.v. infusion) or vehicle was given 1 h after LPS initiation.**

Results. **EP 40 and 60 mg/kg attenuated plasma levels of TNF-α and IL-6 caused by LPS, and further increased IL-10 levels compared with the LPS group. At 6 h after LPS initiation, iNOS protein expression in lungs and plasma NO metabolite levels were markedly increased, which were reduced by EP 60 mg/kg. LPS caused a significant HO-1 induction, whereas administration of EP 60 mg/kg significantly induced higher HO-1 expression compared with the LPS group. The beneficial effects of EP on cytokines and iNOS expression were reversed by HO-1 inhibitor SnPP. EP significantly suppressed phosphorylated p38 MAPK and increased phosphorylated ERK1/2 protein levels in the lung tissue. The edema and infiltration of neutrophils into lungs was reduced by EP.**

Conclusion. **EP reduced LPS-induced ALI, which may be mediated by induction of HO-1. The underlying mechanisms are associated with suppression of p38 MAPK and increase of ERK1/2 signaling pathway activation.**

Key Words: **ethyl pyruvate; iNOS; heme-oxygenase-1; lipopolysaccharide; acute lung injury.**

INTRODUCTION

Acute lung injury (ALI) induced by lipopolysaccharide (LPS) is the most commonly used experimental model, in which the lung parenchyma is damaged by the generation and release of proteases and reactive oxygen and nitrogen species produced by activated lung macrophages and transmigrated neutrophils in the interstitial and alveolar compartments, leading to microvascular injury and diffuse alveolar damage with intrapulmonary hemorrhage, edema, and fibrin deposition [1]. These features are also found in patients with ALI and the acute respiratory distress syndrome (ARDS) [2]. The pathogenesis of ALI has been suggested to mediate by pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and high-mobility mobility group box (HMGB) 1, which are released from macrophages and neutrophils [3].

It is well known that administration of LPS results in the increased expression and activity of inducible nitric oxide synthase (iNOS), with the concomitant increase in nitric oxide (NO) generation [4]. The iNOS induction may be a regulator for leukocyte recruitment by interfering with leukocyte–endothelium interactions [5, 6]. The induction of sepsis in mice by cecal ligation and puncture revealed a prominent role of pulmonary iNOS induction in ALI secondary to sepsis, and

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iNOS-knockout animals were completely protected, showing attenuated lung injury and inflammation [7]. Moreover, beneficial effects of iNOS inhibition or deletion have been shown in different acute lung inflammation models [8, 9].

The relationship between the NOS/NO and heme oxygenase (HO)/carbon monoxide (CO) pathways and their contributions to various biological functions have been discussed [10]. HO-1 degrades heme into three products: Fe^{2+}, biliverdin, and CO. Past studies point out that heme catabolism and HO-1 overexpression exert profound inhibitory effects on the cascade of host inflammatory responses mediated by neutrophils, macrophages, and lymphocytes [11]. HO-1 can be induced by heme, and inflammatory cytokines such as HMGB1, IL-6, IL-1β, and IL-8, as well as oxidative stress in patients with severe sepsis or septic shock [12]. In HO-1-deficient mice, increased oxidative stress is related to organ dysfunction and the high mortality of endotoxemia [13]. Induction of HO-1 by administration of heme or exposure to a low concentration of CO protects against LPS-induced lung injury and lethal endotoxic shock in rats [14]. Bilirubin, a metabolite generated by HO-1 degrading heme, has been reported to inhibit iNOS expression in response to endotoxin in rats [15]. LPS-induced NF-κB activation was blocked by CO at the level of IκBα phosphorylation in the RAW 264.7 macrophage [14].

Early administration of high-dose ethyl pyruvate (EP) significantly protected against ALI lethality. Low dose EP or delayed administration gave no advantage in terms of survival [16]. The intrapulmonary delivery of EP attenuates LPS- and lipoteichoic acid-induced lung inflammation *in vivo* [17]. EP has been confirmed to improve survival and reduce the lung permeability index in mice with LPS-induced ALI [18]. Furthermore, EP inhibits activation of NF-κB in a variety of *in-vitro* and *in-vivo* systems [16, 19–21]. It is possible that EP is an antioxidant and is possible to inhibit activation of NF- κB by scavenging ROS [21, 22]. However, it is still unknown whether the anti-inflammatory property of EP is related to induction of HO-1 expression, leading to reduction in NOS expression. Therefore, the aim of this study is to explore whether the anti-inflammatory effect of EP is associated with HO-1 induction in LPS-induced ALI animal model and its possible mechanisms.

MATERIALS AND METHODS

Animals were maintained according to standards of animal care and experimentation published by National Laboratory Animal Breeding and Research Center National Science Council, Taiwan. Male Wistar rats, 8 to 10 wk old and weighing 260 to 290 g, were acclimatized for at least 3 d in our animal quarters prior to use. All animals were housed at an ambient temperature of 23 ± 1°C and 55 ± 5% humidity. All experimental protocols were evaluated and approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center.

In Vivo ALI Model

The rat was anaesthetized by an intraperitoneal injection of urethane (1.2 g/kg). The trachea was cannulated to facilitate respiration. The left cervical artery was cannulated with polyethylene-50 (PE-50) and connected to a pressure transducer (P231D; Statham, Oxnard, CA) for measuring the mean arterial pressure and heart rate, displayed on a Gould model TA5000 polygraph recorder (Gould, Valley View, OH). The right cervical and left femoral vein were cannulated for administering drugs. After the surgical procedure was complete, all cardiovascular parameters were allowed to stabilize for 30 min. LPS (*Escherichia coli*, serotype o127: B8 lipopolysaccharide B; Sigma Chemical Co., St. Louis, MO) was freshly dissolved in physiologic saline solution before each experiment. Blood was withdrawn from the cervical artery into plastic syringes at 0, 2, 4, and 6 h. Each rat was sacrificed immediately after blood sampling under deep anesthesia. Citrated plasma samples were obtained by centrifugation of whole blood and stored at –80°C until assay.

Experimental Groups

Animals were randomly divided into six groups for treatment as follows: (1) Control group: rats were administered a sustained i.v. infusion of 9 mL physiologic saline for 4 h *via* the cervical vein. Concurrently, rats received the vehicle of EP (Sigma, St. Louis, MO), 2 mL of Ringer's solution infusion for 4 h after 1 h saline infusion initiation *via* femoral vein, ($n = 8$); (2) LPS group: the sustained i.v. infusion of 30 mg/kg LPS diluted in 9 mL saline for a maximum of 4 h *via* the cervical vein. After 1 h LPS initiation, rats received the vehicle of EP, 2 mL of Ringer's solution infusion for 4 h ($n = 10$); (3) three LPS + EP (LE) groups: One hour after LPS initiation, EP 20, 40, or 60 mg/kg dissolved in 2 mL of Ringer's solution was given in a sustained i.v. infusion for 4 h ($n = 10$). The doses of EP (20, 40, and 60 mg/kg) were used according to our preliminary study. (4) SnPP + LE60 group: Tin protoporphyrin IX (SnPP; 30 mg/kg, i.p.) (Tocris Bioscience, Bristol, UK), a potent HO inhibitor, was given 6 h ahead of the LPS challenge. One hour after LPS initiation, EP 60 mg/kg dissolved in 2 mL of Ringer's solution was given in a sustained i.v. infusion for 4 h ($n = 5$). The arterial blood pressure and heart rate were continuously monitored throughout the experimental period in all groups.

Measurement of Pro-Inflammatory and Anti-Inflammatory Cytokines

For determining pro-inflammatory cytokines TNF-α, IL-6, and anti-inflammatory cytokines IL-10 concentration in plasma, blood samples (0.5 mL) were collected at 0, 2, 4, and 6 h after the injection after LPS initiation, and were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R and D System, Inc., Minneapolis, MN).

Plasma Nitrite/Nitrate Determination

Blood samples were collected from rats 1, 2, and 6 h after LPS administration and centrifuged at 4°C to prepare plasma, which were stored at –20°C. Plasma (30 μL) was thawed and deproteinized by incubation with 60 μL of 95% ethanol (4°C) for 30 min. Samples were then centrifuged for 5 min at 14,000 × g. With this method, nitrate is reduced to NO *via* nitrite. The amount of nitrate in the plasma (2 μL) was measured by adding a reducing agent (0.8% VCl_3 in 1 N HCl) to the purge vessel to convert nitrate to NO, which was stripped from the plasma using helium purge gas. The NO was then drawn into a nitric oxide analyzer (Sievers, Boulder, CO). Nitrate concentrations

were calculated by comparison with standard solutions of sodium nitrate (Sigma) [23].

Quantitation of Heme Oxygenase-1 mRNA (HO-1 mRNA) by Quantitative Real-Time RT-PCR Analysis

Cytoplasmic RNA was extracted from rat lung using the Trizol Total RNA isolation kit (GIBCO BRL, Gaithersburg, MD). RNA concentration was determined spectrophotometrically at 260 nm. RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Relative expression levels of mRNA were determined using an Applied Biosystems 7300 Real-Time PCR System with a TaqMan Universal PCR Master Mix (Applied Biosystems) and gene-specific primers (*HO-1*: Rn01536933_m1). Expression levels were calculated as the ratio of mRNA level for a given gene relative to the mRNA level for glyceraldehyde-3-phosphate dehydrogenase (Rn99999916_s1) in the same cDNA sample.

Western Blotting

To elucidate the effect of EP on the protein induction of iNOS and HO-1, as well as activation of p38 MAPK and ERK1/2 signaling pathways elicited by LPS, lung tissue was immediately frozen in liquid nitrogen, and stored at –80°C until processed. Detection of the proteins by Western blotting was performed as described previously [23]. The primary antibodies probed in this experiment were rabbit anti-HO-1 polyclonal antibody (Stressgen Bioreagents, Plymouth Meeting, PA, USA, 1:1000 dilution), mouse monoclonal anti-phospho-p38 MAPK antibody, mouse monoclonal anti-phospho-ERK1/2 (for reflecting p38 MAPK and ERK 1/2 activation; Cell Signaling; 1:1000 dilution) and anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000 dilution). To standardize densitometry measurements between individual samples, the rations of phospho-p38, HO-1, iNOS to β-actin, and phospho-ERK 1/2 to total ERK, were calculated for statistical analysis.

Histopathology

Lung tissue was fixed in 10% buffered formalin and embedded in paraffin and cut into 4 μm sections that were subsequently stained with hematoxylin and eosin. We used modified scoring systems from published articles for quantifying lung injury [24]. The lung injury score was assessed by two components, edema and neutrophil infiltration. Tissue injury was graded from 0 (normal) to 3 (severe) in the two categories. The individual scores for edema and infiltration were added together to get a final score, ranging from 0 to 6.

Determination of Plasma Levels of Lactate Dehydrogenase

Plasma levels of lactate dehydrogenase (LDH) were measured to observe tissue damage 6 h after LPS administration. These were measured by enzymatic determination using an automatic analyzer (Fuji DRI-CHEM FDC 3000; Fuji Photo Film, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical evaluation was performed with one-factor analysis of variance (ANOVA) followed by the Newman-Keuls method. $P < 0.05$ was assumed statistically significant.

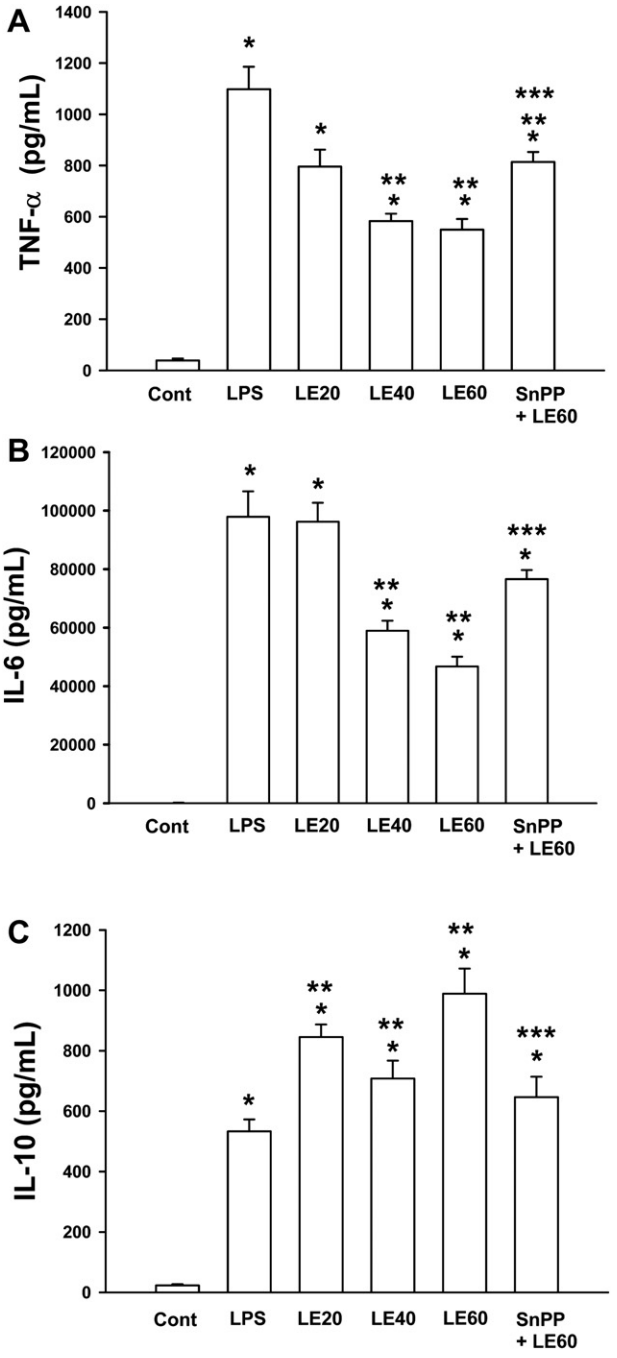

FIG. 1. Effect of post-treatment with ethyl pyruvate (EP; 20–60 mg/kg/4 h, i.v. infusion) on plasma levels of TNF-αIL-6, and IL-10 collected at 2, 4, and 6 h after LPS administration (30 mg/kg/4 h, i.v. infusion), respectively. LE20, LE40, and LE60: EP 20, 40, and 60 mg/kg/4 h was given 1 h after initiation of LPS administration, respectively. SnPP: HO-1 inhibitor; data are shown as the mean ± SEM ($n = 5$–10). *$P < 0.05$ *versus* control; **$P < 0.05$ *versus* LPS; ***$P < 0.05$ *versus* LE60.

RESULTS

Effects of EP on Plasma Levels of TNF-α, IL-6, and IL-10

There was no significant difference in baseline plasma concentration of TNF-α, IL-6, and IL-10 among all

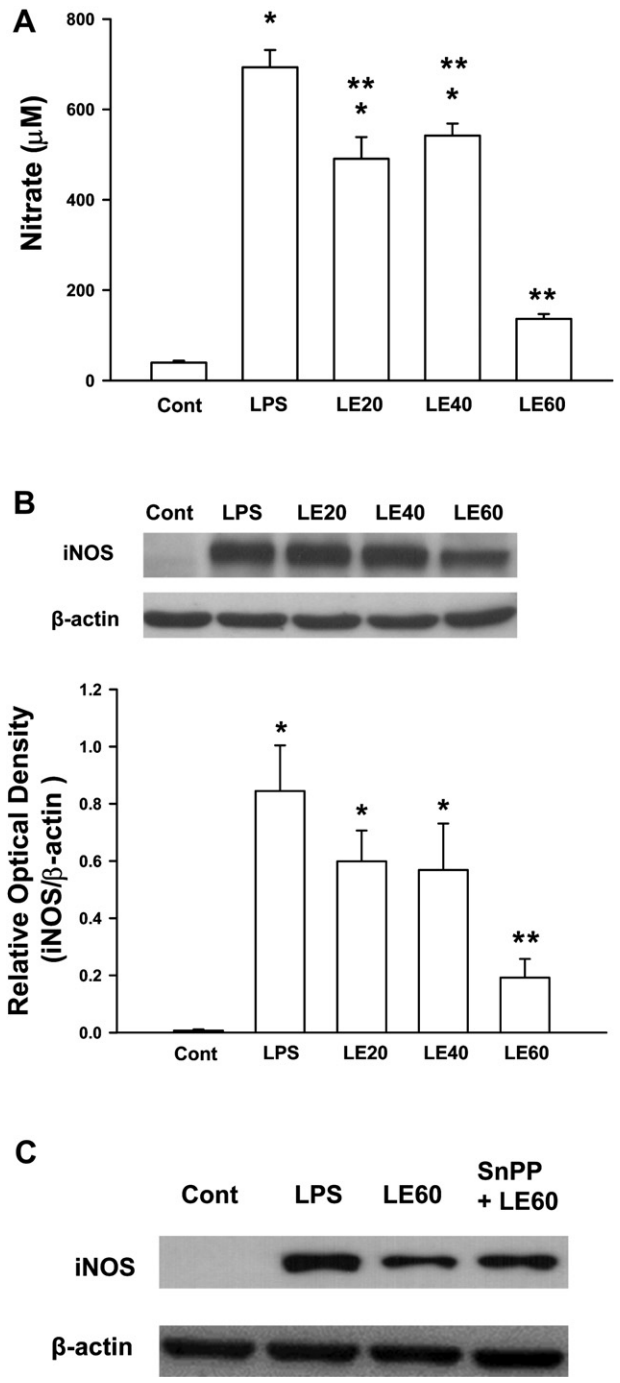

FIG. 2. Effect of post-treatment with ethyl pyruvate (EP; 20-60 mg/kg/4 h, i.v. infusion) on plasma nitrate (as NO indicator) levels (A) and inducible nitric oxide synthase (iNOS) protein expression in the lung (B), (C) 6 h after LPS administration (30 mg/kg/4 h, i.v. infusion). The depicted is a typical display of iNOS protein expression (B upper panel), (C) and a statistical analysis of the changes in iNOS protein (B lower panel). LE20, LE40, and LE60: EP 20, 40, and 60 mg/kg/4 h was given 1 h after initiation of LPS administration, respectively. SnPP: HO-1 inhibitor; data are shown as the mean ± SEM ($n = 8$–10). *$P < 0.05$ *versus* control; **$P < 0.05$ *versus* LPS.

groups. Plasma levels of TNF-α and IL-6 were measured 2 and 4 h after LPS, respectively. Post-treatment with EP (40 and 60 mg/kg), but not 20 mg/kg, significantly decreased the LPS-induced increase of plasma TNF-α levels compared with the LPS group (LPS: 1004.0 ± 89.3; LE40: 582.9 ± 28.8; LE60: 549.3 ± 41.7 ng/mL, $n = 8$–10) ($P < 0.05$) (Fig. 1A). Similarly, post-treatment with EP (40 and 60 mg/kg) significantly decreased the LPS-induced increase of plasma IL-6 level at 4 h compared with the LPS group (LPS: 6071.6 ± 12000; LE40: 58931.6 ± 3445.7, LE60 49950.7 ± 6045.6 ng/mL, $n = 8$–10) ($P < 0.05$) (Fig. 1B). Pretreatment with SnPP to inhibit HO-1 activity significantly reversed the inhibitory effect of EP 60 mg/kg on plasma TNF-α and IL-6 levels after LPS challenge, compared with the LE60 group ($P < 0.05$). LPS caused a significant increase in the plasma IL-10 level compared with the control group at 6 h (Control: 23.02 ± 4.1 ng/mL, $n = 8$; LPS: 528.2 ± 37.3 ng/mL, $n = 10$) ($P < 0.05$). The plasma levels of IL-10 in the three EP-treated groups (20, 40, and 60 mg/kg) were significant higher than that of LPS group at 6 h after LPS initiation (LE20: 927.9 ± 60.3, LE40: 707.9 ± 59.4, LE60: 988.9 ± 83.2 ng/mL, $n = 10$) ($P < 0.05$). Pretreatment with SnPP significantly reduced the elevation of IL-10 levels caused by EP 60 mg/kg compared with the LE60 group ($P < 0.05$) (Fig. 1C).

Effect of EP on Plasma Nitrite/Nitrate Content and iNOS Protein Expression in Lungs

Plasma levels of NO metabolites (nitrite/nitrate) were low and not significantly different among groups at 1 and 2 h after LPS initiation. LPS administration significantly induced the elevation of plasma nitrite/nitrate content at 6 h compared with the control group ($P < 0.05$). Post-treatment with EP (20, 40, and 60 mg/kg) significantly suppressed the LPS-induced increase in plasma nitrite/nitrate levels compared with the LPS group ($P < 0.05$). The level of LE60 group did not show significantly different from the control group ($P > 0.05$) (Fig. 2A). As shown in Fig. 2B, iNOS protein expression was very low in lung homogenates obtained from the control rats, whereas a significant induction of iNOS protein was observed in rats 6 h after LPS administration ($P < 0.05$). Post-treatment with EP (60 mg/kg) significantly reduced the induction of iNOS challenged with LPS ($P < 0.05$), which did not show significant difference from the control group ($P > 0.05$). This inhibitory effect was markedly reversed by pretreatment with SnPP (Fig. 2C).

Effect of EP on HO-1 mRNA and HO-1 Protein Expression in Lungs

The expression of HO-1 mRNA in lungs increased substantially in rat subjected to LPS compared with the control group. Treatment with EP 60 mg/kg further significantly increased the levels of HO-1 mRNA

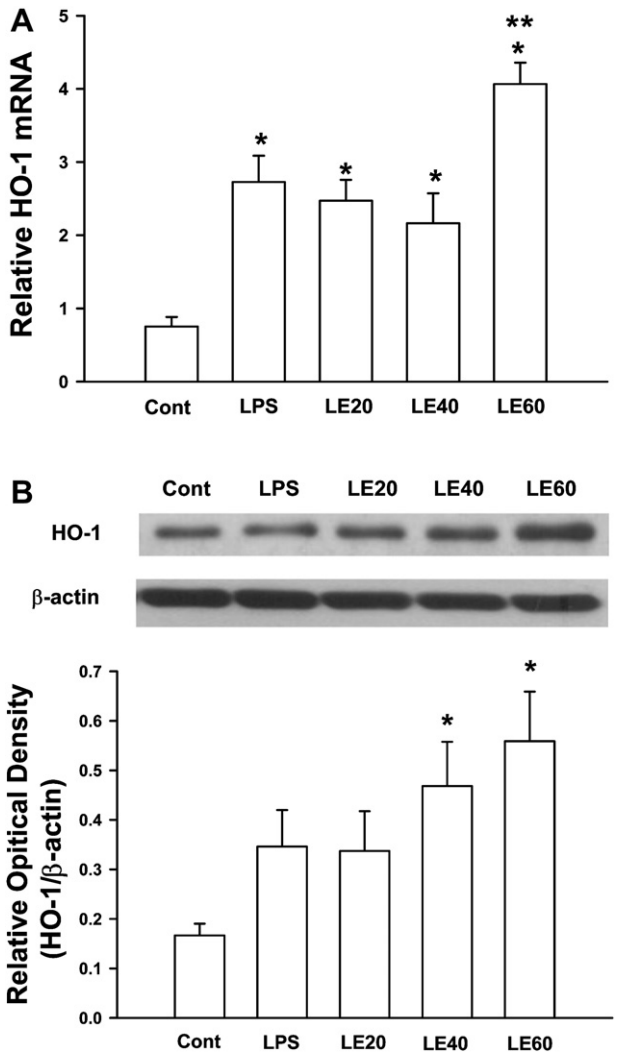

FIG. 3. Effect of post-treatment with ethyl pyruvate (EP; 20-60 mg/kg, i.v. infusion for 4 h) on heme oxygenase-1 (HO-1) mRNA expression (A) and HO-1 protein expression (B) in the lung 6 h after LPS administration (30 mg/kg/4 h, i.v. infusion). The depicted is a typical display of HO-1 protein expression (B, upper panel) and a statistical analysis of the changes in HO-1 protein (B, lower panel). LE20, LE40 and LE60: EP 20, 40, and 60 mg/kg/4 h was given 1 h after initiation of LPS administration, respectively. Data are shown as the mean ± SEM ($n = 8$–10). *$P < 0.05$ *versus* control; **$P < 0.05$ *versus* LPS.

compared with the LPS group ($P < 0.05$, Fig. 3A). Similar results observed in HO-1 protein expression, LPS challenge elicited HO-1 protein induction, and post-treatment with EP 60 mg/kg significantly induced the expression of HO-1 protein compared with the LPS group ($P < 0.05$) (Fig. 3B).

Effects of EP on Phospho-p38 MAPK and Phospho-ERK1/2 Protein Expression in Lungs

After LPS treatment, phospho-p38 protein levels were significantly elevated compared with the control

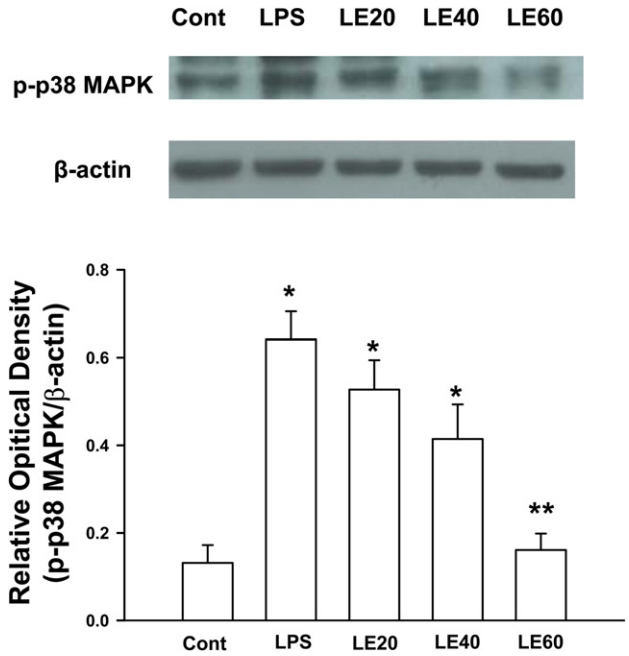

FIG. 4. Effect of post-treatment with ethyl pyruvate (EP; 20-60 mg/kg, i.v. infusion for 4 h) on phosphorylated p38 (p-p38) MAPK protein expression in the lung 6 h after LPS administration. LE20, LE40, and LE60: EP 20, 40, and 60 mg/kg/4 h was given 1 h after initiation of LPS administration, respectively. The depicted is a typical display of p-p38 MAPK protein expression (upper panel) and a statistical analysis of the changes in p-p38 MAPK protein (lower panel). Data are shown as the mean ± SEM ($n = 8$–10). *$P < 0.05$ *versus* control; **$P < 0.05$ *versus* LPS.

group. Treatment with EP 60 mg/kg, but not 20 and 40 mg/kg, significantly reduced LPS-induced elevation of phospho-p38 MAPK protein in lungs ($P < 0.05$, Fig. 4), which did not show significant difference from the control group.

The protein expression of phospho-ERK1/2 from lung tissue did not significantly increase after LPS treatment in LPS, LE20, and LE40 groups compared with the control group ($P > 0.05$). Interestingly, post-treatment with EP 60 mg/kg significantly increased the expression of phospho-ERK1/2 compared with the control and LPS groups ($P < 0.05$) (Fig. 5).

Effect of EP on Pathologic Changes in Lungs

LPS caused severe ALI characterized by edema formation and inflammatory cell infiltration. Light microscopy results showed the normal histology of lungs in Figure 6A with score 1. The injection of LPS resulted in not only diffuse edematous changes in alveolar interstitium and marked decreases in alveolar air space with scores 5 (Fig. 6B). In LPS rats treated with EP 60 mg/kg, these histologic changes in lungs were significantly reduced to score 3.

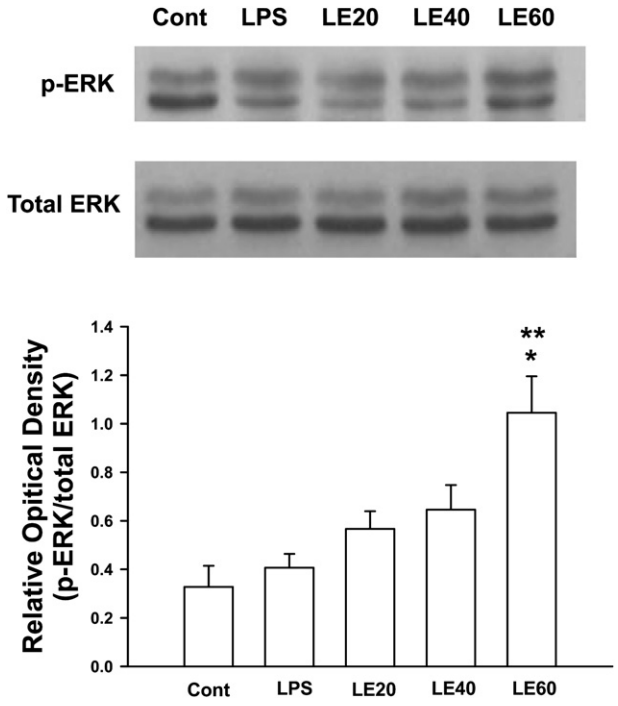

FIG. 5. Effects of post-treatment with ethyl pyruvate (EP; 20-60 mg/kg, i.v. infusion for 4 h) on phosphorylated ERK1/2 (p-ERK) protein expression in the lung 6 h after LPS administration. LE20, LE40 and LE60: EP 20, 40, and 60 mg/kg/4 h was given 1 h after initiation of LPS administration, respectively. The depicted is a typical display of p-ERK protein expression (upper panel) and a statistical analysis of the changes in p-ERK protein (lower panel). Data are shown as the mean ± SEM ($n = 8$–10). *$P < 0.05$ *versus* control; **$P < 0.05$ *versus* LPS.

Effects of EP on LPS-Induced Cell Damage

Plasma levels of LDH of the LPS group were significantly higher than those of control group at 6 h after LPS initiation ($P < 0.05$). Treatments with EP 20, 40, and 60 mg/kg significantly reduced the elevation of LDH levels caused by LPS, compared with the LPS group. However, these data in EP-treated groups were still significantly higher than the control group ($P < 0.05$), and there was no significant difference among groups. Pretreatment with SnPP significantly reversed the reduction in LDH levels by EP 60 mg/kg ($P < 0.05$) (Fig. 7).

DISCUSSION

In this study, post-treatment with EP 60 mg/kg improved LPS-induced ALI, which is related to its anti-inflammatory activity evidenced by (1) inhibition of releases of pro-inflammatory cytokines, TNF-α and IL-6; (2) increase of anti-inflammatory cytokine, IL-10 production, and (3) reduction in iNOS protein expression and NO production. Moreover, EP increased HO-1 mRNA and protein expression in lung tissue, indicating HO-1 induction may contribute to its antioxidant and anti-inflammatory effects. EP also increased active form of ERK1/2 protein (phospho-ERK1/2) and decreased active form p38 MAPK (phospho-p38 MAPK), suggesting that increase in activation of ERK1/2 signaling pathway and inhibition of p38 MAPK activation are associated with the induction of HO-1.

EP exerts combined anti-inflammatory and anticoagulant effects on human monocytic cells [25]. Prevention of inflammatory mediator release (for example NO, TNF, and HMGB-1) emerged as a key mechanism underlying EP anti-inflammatory action and significantly prolonged survival time [16, 26]. Resuscitation with EP solution significantly decreased circulating concentrations IL-6 and increased circulating concentrations of IL-10 in a rat model of LPS-induced shock [26]. IL-6 is critical to the onset of inflammation and hypercoagulation [27]. IL-6 deficiency may result in increased levels of IL-10, which has been proposed to exert protective effects during sepsis and endotoxemia [28]. IL-10 is vitally important for modulating the inflammatory response to microbial products that can result in septic shock and other pathologies [29]. IL-10 attenuates macrophage expression of iNOS [30]. Suppression of TNF-α production in LPS-stimulated macrophages is involved in anti-inflammatory effects of IL-10. IL-10 has consistently been shown to induce HO-1 gene expression. A positive feedback circuit between IL-10 and HO-1 has been shown to be functional, which might amplify the anti-inflammatory effects of IL-10 in LPS-stimulated macrophages [31]. In the present study, EP suppressed IL-6 release caused by LPS, increased IL-10 levels, and induced HO-1 protein expression. Increased IL-10 levels by EP may contribute to induction of HO-1 and suppression of iNOS expression.

The HO-1/CO system may play an important role in sepsis [32]. HO-1 is not only induced by its substrate heme, but also by inflammatory cytokines and oxidative stress [12]. HO-1 acts as a potent anti-inflammatory agent and antioxidant through its products carbon monoxide (CO) and biliverdin [33]. HO-1-mediated CO and biliverdin suppress transcriptional iNOS expression and TNF-α production in LPS-stimulated macrophages [15, 34]. CO effectively inhibits the proinflammatory LPS-induced cytokine TNF-α, while simultaneously augmenting expression of the anti-inflammatory cytokine IL-10 [35]. Administration of CoPPIX, a HO-1 inducer, could significantly inhibit TNF-α and HMGB1 expression and thus attenuate the ALI induced by LPS in mice [36]. Furthermore, HO-1 provides a defense mechanism against endotoxemia by controlling the IL-6/IL-10 balance. While HO-1-deficient mice have reduced IL-10, but increased IL-6 production, and are sensitive to endotoxic challenges [37]. These findings

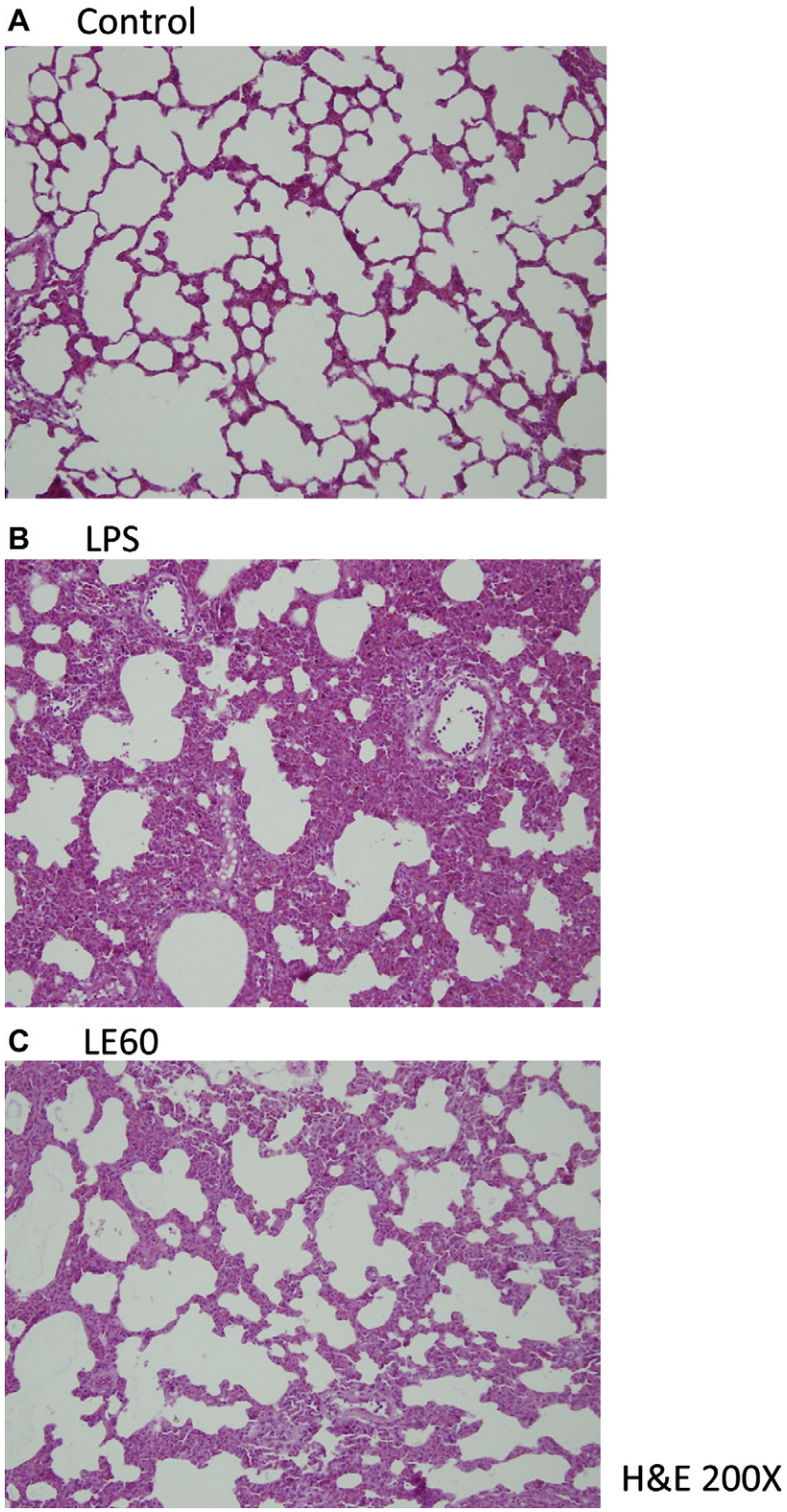

FIG. 6. Histologic studies of representative lung sections in the control group (A), LPS group (B), and LE 60 group (C) ($n = 3$ in each group). LE 60: EP 60 mg/kg/4 h was given 1 h after initiation of LPS administration. Sections were stained with hematoxylin and eosin (H and E) stain, each ×200 (original magnification).

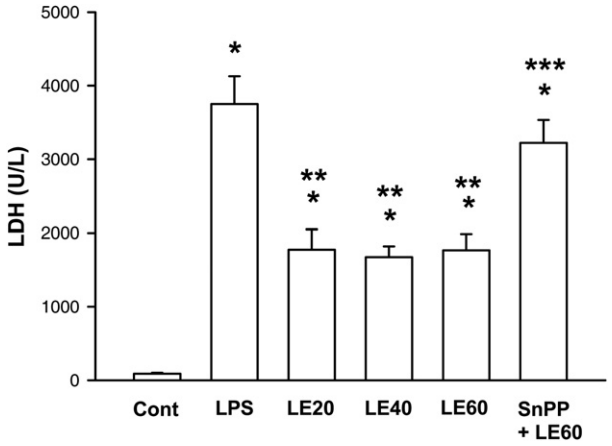

FIG. 7. Effects of post-treatment with ethyl pyruvate (EP 20-60 mg/kg, i.v. infusion for 4 h) on plasma levels of LDH 6 h after LPS administration. LE20, LE40, and LE60: EP 20, 40, and 60 mg/kg/4 h was given 1 h after initiation of LPS administration, respectively. SnPP: HO-1 inhibitor; data are expressed as mean ± SEM; $n = 5–10$; $^{*}P < 0.05$ *versus* sham; $^{**}P < 0.05$ *versus* LPS; $^{***}P < 0.05$ *versus* LE60.

demonstrate that HO-1 negatively regulates IL-6 production, and positively regulates IL-10 expression, thereby minimizing the onset of endotoxin-induced organ injuries. EP is an anti-oxidant, and inhibits activation of NF-κB by scavenging ROS [21, 22]. Furthermore, HO-1 induction has been suggested to involve in the beneficial effects of EP on colitis [38]. Therefore, in the present study, we also demonstrated EP induced HO-1 protein expression and simultaneously increased IL-10 levels, which are likely involved in the protective effect of EP on ALI.

Activation of MAPKs plays a central role for the induction of HO-1 gene expression [39]. The role of ERK1/2 for HO-1 gene activation *via* p38 inhibition is involved in HO-1 gene induction by numerous stimuli [40]. The p38 is activated by LPS stimulation in monocytes and has been shown to play a critical role for the regulation of proinflammatory genes such as cytokines [41]. Moreover, p38 MAPK has been previously shown to have a negatively regulatory effect on ERK1/2 activity [42]. Increased HO-1 activity degraded p38α in endothelial cells [43]. EP has been shown to inhibit phosphorylation of p38 MAPK in LPS-treated RAW 264.7 cells and mice [16, 44]. In the present study, EP also suppressed the activation of p38 MAPK and increased ERK1/2 activity, which may contribute to HO-1 induction, leading to the beneficial effect of EP on ALI.

However, dose range for EP treatment (20 to 60 mg/kg) is relatively narrow. Larger doses of EP or continuous infusion of EP to the end of experiment may be more effective. In conclusion, EP improved lung injury caused by LPS. Its cytoprotective action may be attributed to EP-mediated HO-1 induction *via* activation of ERK signaling pathway.

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