# Captopril decreases plasminogen activator inhibitor-1 in rats with ventilator-induced lung injury

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***Objective:*** **To test the hypotheses that high tidal-volume ventilation increases plasminogen activator inhibitor (PAI)-1, and the angiotensin-converting enzyme inhibitor, captopril (CAP), may attenuate these effects.**

***Setting:*** **University research facility.**

***Subjects:*** **Twenty adult male Sprague-Dawley rats.**

***Interventions:*** **All rats were randomized to receive two ventilation strategies for 2 h: 1) a high-volume zero positive end-expiratory pressure (PEEP) (HVZP) group at a tidal volume of 40 mL/kg, a respiratory rate of 25 breaths/min, and an $F_{IO_2}$ of 0.21; and 2) an HVZP + CAP group which received an intraperitoneal injection of CAP (100 mg/kg) 30 min before HVZP ventilation. Another group that was not subjected to ventilation served as the control.**

***Measurements and main results:*** **Total protein recovered from bronchoalveolar lavage fluid was significantly higher in rats ventilated with the HVZP protocols than in control rats. Rats treated with HVZP ventilation had significantly higher lung angiotensin (ANG) II and PAI-1 messenger RNA expression levels and a higher plasma active PAI-1 level than did the control and HVZP + CAP groups. Lung ANG II levels were positively correlated with plasma PAI-1. Representative lung tissue of the HVZP + CAP group showed mild inflammatory cell infiltration and less hemorrhage and fibrin deposition than did the HVZP group. The HVZP and HVZP + CAP groups had significantly higher lung injury scores than did the control group and rats treated with HVZP + CAP ventilation exhibited significantly lower lung injury scores than did the HVZP group.**

***Conclusions:*** **Mechanical ventilation with a high tidal volume and no PEEP increases alveolar fibrin deposition and systemic PAI-1 activity, which are attenuated by captopril, an angiotensin-converting enzyme inhibitor. These results imply that local ANG II is involved in the pathogenesis of disordered coagulation in ventilator-induced lung injury (VILI) and suggest that the protective mechanism of captopril's attenuation of VILI is related to a reduction in PAI-1. (Crit Care Med 2008; 36:1880–1885)**

**KEY WORDS: angiotensin; bronchoalveolar lavage; coagulation; fibrinolysis**

Mechanical ventilation has been used to support acutely ill patients for several decades. Regardless of the life-saving potential of this support, it has several potential disadvantages and complications (1). Mechanical ventilation with high tidal volumes causes lung hemorrhage and edema and activates inflammatory pathways. This course is referred to as *ventilator-induced lung injury* (VILI) (2,3). The spectrum of VILI includes disruption of endothelial and epithelial cells, and increases in endothelial and epithelial permeability and in pulmonary inflammatory mediators (2–4). Research has revealed a broad range of VILI that is physiologically and histopathologically indistinguishable from acute lung injury (ALI). Disordered coagulation and fibrinolysis and fibrin deposition in the alveolar space are important features of ALI (5). Given the similarities between the inflammatory responses in ALI and VILI, it is appealing to speculate that similar changes in coagulation and fibrinolysis may occur in VILI.

A growing body of evidence suggests that mechanical ventilation may influence pulmonary fibrin turnover in VILI (6,7). Abnormal fibrin turnover is important to evolving ALI. Transitional fibrin deposition in the alveolar space can accelerate the fibrotic process (8), which results in a remodeling of alveolar fibrin and ultimately pulmonary fibrosis. This can lead to surfactant dysfunction, poor gas exchange, decreased lung compliance, and increased ventilatory dependence (5). Alveolar fibrin deposition is the net result of an alteration in the balance of coagulation and fibrinolytic protease and antiprotease (9,10). Plasminogen activator inhibitor-1 (PAI-1), a fibrinolytic antiprotease, is the major plasminogen inactivator in the plasma and the primary inhibitor of the tissue-type and urokinase-type plasminogen activator resulting in decreased plasmin activity and fibrinolytic potential (11). The initiation and mechanisms of pulmonary fibrin deposition in VILI remain unclear. Angiotensin (ANG) II can be generated locally in lung tissues and may have autocrine and paracrine actions at the cellular level (12). This renin-angiotensin system has been reported to regulate the fibrinolytic balance in experimental and clinical studies (13–16). ANG II may induce PAI-1 production in endothelial and smooth muscle cells (14,15). We have demonstrated that captopril may attenuate VILI and the efficacy is related to reduction of inflammatory cytokines and inhibition of apoptosis (17). However, there is little information on the influence of mechanical ventilation on the pulmonary coagulation status *in vivo*. Captopril is a common angiotensin-converting enzyme (ACE) inhibitor used to treat hypertension, heart failure, and other cardiovas-

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cular and renal diseases (18). ACE converts the inactive peptide, ANG I, to the active vasoconstrictor, ANG II, while inactivating the vasodilator bradykinins. We hypothesized that high tidal volume ventilation may increase lung ANG II and PAI-1 levels and decrease fibrinolytic activity in rats, and these deleterious effects can be attenuated with the ACE inhibitor, captopril. The aims of this study were to investigate the mechanism of decreased fibrinolytic activity in lung injury induced by a high tidal volume and to find a potential treatment modality against VILI.

## MATERIALS AND METHODS

This experimental protocol was approved by the Institutional Animal Use Committee at Taipei Medical University and was performed with 20 adult male Sprague Dawley rats weighing 250–300 g. Rats were maintained on a 12-h light-dark cycle with free access to food and water.

### Preparation of the Rats

The rats were anesthetized intraperitoneally with pentobarbital (50 mg/kg, Abbott, North Chicago, IL, USA). A tracheostomy was performed, and a 14-gauge plastic canula was inserted into the trachea. The animals were then ventilated with a high-volume zero positive end-expiratory pressure (PEEP) (HVZP) protocol by a volume-cycled ventilator (Small Animal Ventilator, Model SAR-830/AP; CWE Inc., Ardmore, PA, USA) for 2 h at a tidal volume of 40 mL/kg, zero PEEP, a respiratory rate of 25 breaths/min, and an $F_{IO_2}$ of 0.21. The HVZP + CAP group received a 1-mL intraperitoneal injection of the ACE inhibitor, captopril (CAP) (100 mg/kg, Sigma, St. Louis, MO, USA), 30 min before the HVZP ventilation. The dose of CAP was based on recommendations by Gavin et al. (19). Rats were randomized to receive one of these two ventilation strategies. Another group that received no ventilation served as the control. All animals were kept supine for the duration of the experiment.

### Experimental Protocols

*Bronchoalveolar Lavage.* After 2 h of ventilation, the chest was opened and the lung was removed intact from the animal with the tracheostomy tube in place. The lungs were instilled with 7 mL of 0.9% saline at 4°C which was washed in and out of the lungs three times and then recovered. This washing procedure was repeated two more times for each animal, with the three washes finally being pooled, and the total volume recorded. There were no differences in the total volume of saline infused or recovered after the lavage procedure between the three experimental groups. An aliquot of the bronchoalveolar lavage fluid (BALF) from each animal was used to measure the total protein content with bovine serum albumin as the standard, and the value was expressed as mg/kg body weight.

*Measurements of Lung ANG II and Plasma Active PAI-1.* Lung tissue was homogenized in lysis buffer and centrifuged at speeds according to the manufacturer's instructions. The supernatant solution was used for measuring ANG II levels with an enzyme-linked immunosorbent assay kit (SPI-BIO, May Cedes, France). The protein content was measured by the Lowry method (20). All blood samples were placed on ice and spun at 4°C, and the resulting plasma was stored at −70°C until analyzed for active PAI-1. Plasma samples were assayed for active PAI-1 using a commercially available assay kit that measures active PAI-1 (Innovative Research, Southfield, MI, USA).

*Lung PAI-1 messenger RNA Expression by Real-Time Polymerase Chain Reaction (PCR).* Lung tissue was ground into a powder in liquid nitrogen, and PAI-1 messenger RNA (mRNA) expression was measured using a real-time PCR. Total RNA was extracted using the TRIzol Reagent (Invitrogen Life Technologies, Paisley, UK). Reverse transcription was performed on 1 μg of RNA with oligo-dT primers and avian myeloblastosis virus reverse transcriptase (Roche, INpolis, IN, USA). Primer sequences for SYBR green real-time PCR included: PAI-1 sense (5′-ATGGCTCAGAACAACAAGTTCAAC-3′) and antisense (5′-CAGTTCCAGGATGTCGTACTCG-3′), and GAPDH rRNA sense (5′-ATGATTCTACCCACGGCAAG-3′) and antisense (5′-CTGGAAGATGGTGATGGGTT-3′). Gene expression was quantitatively analyzed using the comparative CT (ΔCT) method, in which CT is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the ΔCT method is the difference in the number of threshold cycles for a target (PAI-1) and an endogenous reference (the GAPDH rRNA housekeeping gene). The amount of target normalized to an endogenous reference and relative to a calibration normalized to an endogenous reference is given by 2ΔΔCT. Value of the control group was normalized to a value of 1, and values of HVZP and HVZP + CAP groups were normalized to this.

*Immunohistochemistry of PAI-1 and Fibrin(ogen).* Immunohistochemical staining for PAI-1 and fibrin(ogen) were performed on paraffin sections with immunoperoxidase visualization. After deparaffinization in xylene and rehydration in an alcohol series, sections were first preincubated for 1 h at room temperature in 0.1 M PBS containing 10% normal goat serum and 0.3% $H_2O_2$ to block endogenous peroxidase activity and nonspecific binding of the antibody before being incubated for 20 h at 4°C with a rabbit polyclonal antibody against rat PAI-1 or monoclonal antibody against human fibrin(ogen) (1: 50; American Diagnostica Inc., Stamford, CT, USA). Sections were then treated for 1 h at room temperature with biotinylated goat anti-rabbit immunoglobinG (1: 200, Vector, CA, USA). This was followed by reaction with the reagents from an ABC kit (Avidin-Biotin Complex, Vector Laboratories, Burlingame, CA, USA) per the manufacturer's recommendations, and the reaction products were visualized by 3,3 diaminobenzidine and 0.003% $H_2O_2$ in 0.5 M TRIS buffer (pH 7.6) before the sections were mounted on gelatin-coated slides using Permount (Fisher Scientific, Pittsburgh, PA, USA). The sections for PAI-1 were mounted in glycerine gelatin and counterstained with hematoxylin.

*Quantification of PAI-1 and Fibrin(ogen) Immunoreactivities.* A minimum of four random lung fields of immunohistochemistry-stained sections per animal were captured with a digital camera and imported into the computerized image analysis system Image-Pro Plus 5.1 for Windows. The automatic object counting and measuring process was used to quantify the immunoreactivity in the sections (21). We used the "count/size" and "density" commands to perform cell number and density counting operation for PAI-1 and fibrin(ogen), respectively. These generated a percentage of positive stained cells and fibrin(ogen) and the values were expressed as labeling index (%) and density (%).

*Histology.* Immediately after the bronchoalveolar lavage was finished, the right lung was fixed by instillation of a 10% formaldehyde solution at 20 cm $H_2O$. Specimens were embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined by a pathologist who was blinded to the protocol and experimental groups. Lung injury was scored according to the following items: 1) alveolar congestion, 2) hemorrhage, 3) infiltration of neutrophils into the airspace or the vessel wall, and 4) thickness of the alveolar wall (22). Each item was graded according to a five-point scale: 0, minimal (little) damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, maximal damage.

### Statistical Analysis

The lung injury score data are given as the median (range), whereas other data are presented as the means ± SEM. Statistically significant differences were analyzed by ANOVA followed by Scheffe's post hoc analysis. Differences were considered significant at $p < .05$.

## RESULTS

### Total Protein in the BALF

Total protein contents recovered from the BALF were significantly higher in rats

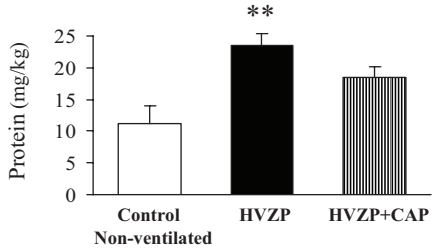


Figure 1. Total protein in bronchoalveolar lavage fluid in the control, high-volume positive end-expiratory pressure (PEEP) (*HVZP*), and HVZP + captopril (*CAP*) groups. All rats were randomly divided into three groups: a control group (n = 6) received no ventilation; HVZP group (n = 6) received 2 h of ventilation at a tidal volume of 40 mL/kg, a respiratory rate of 25 breaths/min, and an $F_{IO_2}$ of 0.21; and HVZP + CAP group (n = 8) received an intraperitoneal injection of CAP (100 mg/kg) 30 min before the HVZP ventilation. Total protein contents recovered from the lavage fluid were significantly higher in rats ventilated with HVZP than in control animals (**$p < .01$ vs. the control group).

ventilated with HVZP than in control animals (Fig. 1). Rats treated with HVZP and captopril showed decreased total protein content, but the difference was not statistically significant when compared with the HVZP group.

## Lung ANG II and Plasma Active PAI-1 Levels

Rats treated with HVZP ventilation had significantly higher lung ANG II and plasma active PAI-1 levels than did the control and HVZP + CAP groups (Fig. 2A, B). The control and HVZP + CAP groups had comparable lung ANG II levels. Lung ANG II levels were positively correlated with plasma active PAI-1 in all study animals ($r = .494$, $p < .05$).

## PAI-1 mRNA Expression

PAI-1 mRNA expression significantly increased ~4-fold in rats ventilated with the HVZP protocol than in control and HVZP + CAP animals, and the values were comparable between the control and HVZP + CAP groups (Fig. 3).

## Immunohistochemistry of PAI-1 and Fibrin(ogen)

PAI-1 immunoreactivities were mainly detected in airway epithelial and some mesenchymal cells, and the immunoreactivity markedly increased in rats

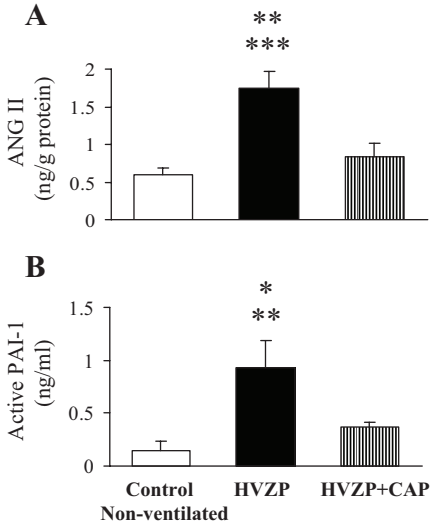


Figure 2. Lung angiotensin (*ANG*) II and plasma active plasminogen activator inhibitor (*PAI*)-1 levels in the control, high-volume end-expiratory pressure (*HVZP*), and HVZP + captopril (*CAP*) groups. Treatment details are given in the legend to Figure 1. *A*, Rats treated with HVZP ventilation had significantly higher lung ANG II concentrations than did the control and HVZP + CAP groups (***$p < .001$ vs. the control group, **$p < .01$ vs. the HVZP + CAP group). *B*, Rats treated with HVZP ventilation exhibited significantly higher plasma active PAI-1 levels than did the control and HVZP + CAP groups (**$p < .01$ vs. the control group, *$p < .05$ vs. the HVZP + CAP group).

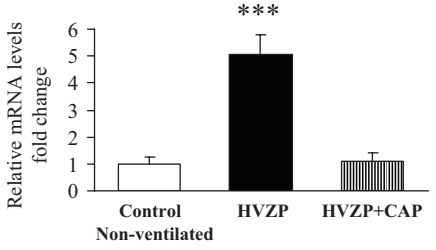


Figure 3. Lung plasminogen activator inhibitor (*PAI*)-*1* messenger RNA (*mRNA*) expressions in the control, high-volume end-expiratory pressure (*HVZP*), and HVZP + captopril (*CAP*) groups. Treatment details are given in the legend to Figure 1. Rats treated with HVZP ventilation had a significantly higher level of lung PAI-1 mRNA expression than did the control and HVZP + CAP groups (***$p < .001$).

treated with HVZP when compared with the control and HVZP + CAP groups (Fig. 4). Changes in PAI-1 immunoreactivities were similar to changes in their mRNA expressions in all three groups. Very few fibrin(ogen) was detected in control animals. Fibrin(ogen) immunoreactivity in the alveoli was diffuse and more intense in HVZP group than in the control and HVZP + CAP groups (Fig. 5).

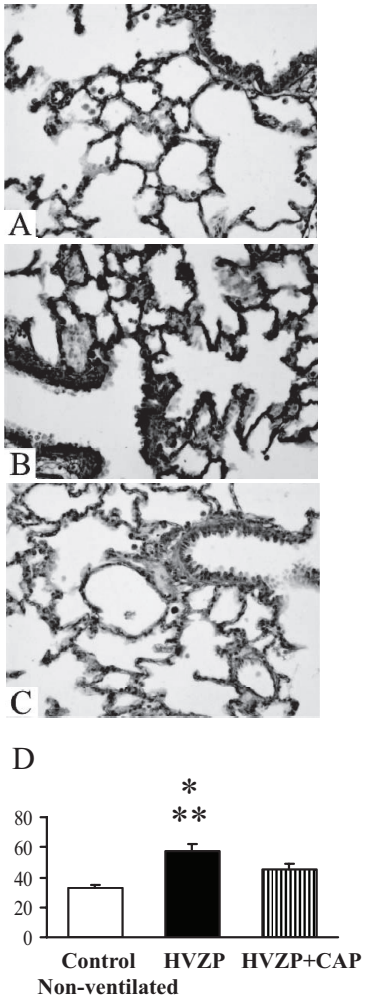


Figure 4. Immunohistochemical staining for plasminogen activator inhibitor (*PAI*)-*1* in the (*A*) control, (*B*) high-volume end-expiratory pressure (*HVZP*), and (*C*) HVZP + captopril (*CAP*) groups (×200) and (*D*) quantitative analysis of PAI-1 immunoreactivity. Positive staining is shown as brown. PAI-1 immunoreactivities were mainly detected in airway epithelial and some mesenchymal cells, and the immunoreactivity markedly increased in rats treated with HVZP when compared with the control and HVZP + CAP groups (**$p < .01$ vs. the control group, *$p < .05$ vs. the HVZP + CAP group).

## Histology

After 2 h of ventilation, the HVZP and HVZP + CAP groups had significantly higher lung injury scores than did the control group (Table 1). Rats treated with HVZP + CAP ventilation exhibited significantly lower lung injury score than did the HVZP group. Plasma active PAI-1 levels were positively correlated with total lung injury score ($r = .721$, $p < .01$). Lung injury was characterized by hemorrhage, thickened alveolar walls, and inflammatory cell infiltration (Fig. 6).

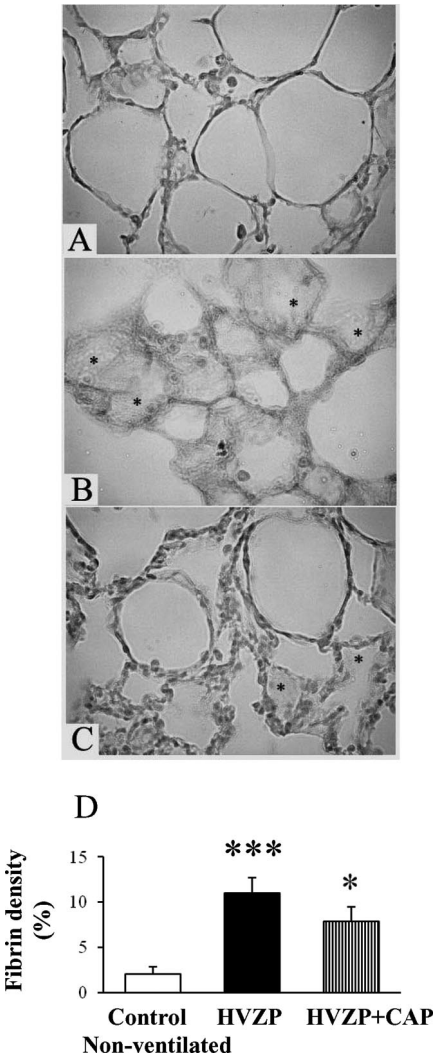


**Figure 5.** Immunohistochemical staining for fibrin(ogen) in the (*A*) control, (*B*) high-volume end-expiratory (*HVZP*), and (*C*) HVZP + captopril (*CAP*) groups (×400) and (*D*) quantitative analysis of fibrin(ogen). Fibrin(ogen) deposits stained as light brown and appeared as strands in alveolar spaces (stars). Very few fibrin(ogen) is detected in control animals. Fibrin(ogen) immunoreactivity in the alveoli is diffuse and more intense in the HVZP and HVZP + CAP groups than in the control group (***$p < .001$, *$p < .05$ vs. the control group).

These findings are consistent with changes in alveolar damage found in acute lung injury. No major histologic abnormalities were present in control animals.

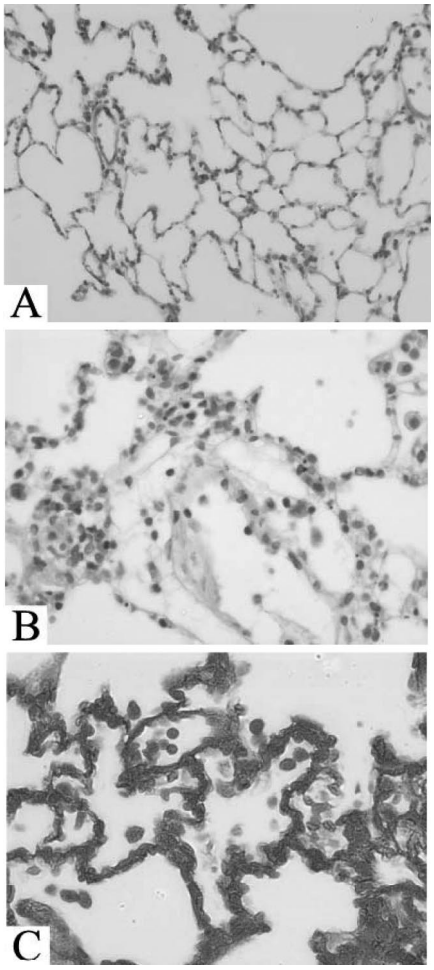


**Figure 6.** Representative lung tissue photomicrographs (×200). *A*, Control group showing no major histologic abnormalities. *B*, The high-volume end-expiratory pressure (HVZP) group showing patchy areas of hemorrhage and thickened alveolar walls with inflammatory cells infiltration. *C*, The HVZP + captopril (*CAP*) group showing less hemorrhage and mild inflammatory cell infiltration.

## DISCUSSION

Our *in vivo* model showed that mechanical ventilation at a high tidal volume increased the total protein in the BALF and the lung injury score. These phenomena are consistent with alterations known to occur in VILI. The main findings of this study are that VILI is associated with increased lung PAI-1 mRNA expression and plasma active PAI-1 level, and an ACE inhibitor (captopril) decreased these deleterious effects and attenuated lung injury. These data indicate that high tidal volume ventilation may decrease local fibrinolytic activity in the lungs and suppress systemic fibrinolytic activity and suggest that the angiotensin in local tissues mediates these events.

In this study, we found that rats treated with HVZP ventilation had the highest scores for neutrophil infiltration into the airspace. These results suggest that mechanical ventilation has a major influence on the inflammatory environment of normal lungs and can initiate or augment lung injury. There were linear relationships of the lung ANG II level with neutrophil infiltration and total lung injury score ($r = .816$, $p < .01$ and $r = .827$, $p < .001$, respectively). These data imply that ANG II is involved in neutrophil recruitment into the alveolar compartment in VILI. These results are consistent with observations by Nabah et al. who found that intraperitoneal administration of ANG II induces neutrophil accumulation in peritoneal exudate fluid in rats (23). Our study also found increased lung ANG II and plasma active PAI-1 levels in the ventilated groups. PAI-1 has been shown to regulate cell migration *in vitro* in addition to its function in the fibrinolytic pathway (24,25). However, the role of PAI-1 in pulmonary neutrophil recruitment *in vivo* is poorly understood. Lung inflammation and pulmonary neutrophil recruitment were improved in PAI-1-deficient mice in hyper-

**Table 1.** Lung injury scores

| Treatment | *n* | Alveolar Congestion | Hemorrhage | Neutrophil Infiltration | Alveolar Wall Thickness | Lung Injury Score |
|---|---|---|---|---|---|---|
| Control | 6 | 0 (0–1) | 1 (0–1) | 1 (1–2) | 0 (0–1) | 2 (1–4) |
| HVZP | 6 | 3 (3–3) | 3 (2–3) | 3 (3–3) | 1 (0–2) | 9 (9–11)[a] |
| HVZP+CAP | 8 | 1 (1–2) | 1 (1–2) | 2 (2–3) | 1 (1–2) | 6 (5–8)[a,b] |

[a]$p < .001$ vs. the control group, [b]$p < .01$ vs. the high-volume positive end-expiratory pressure (HVZP) group.

Data given as median (range). The control group (n = 6) received no ventilation; the high-volume zero PEEP (HVZP) group (n = 6) received 2 hrs of ventilation at a tidal volume of 40 mL/kg, a respiratory rate of 25 breaths/min, and an $F_{IO_2}$ of 0.21; and the HVZP+captopril (CAP) group ($n = 8$) received an intraperitoneal injection of CAP (100 mg/kg) 30 min before the HVZP ventilation.

oxia- and lipopolysaccharide-induced lung injury (26,27). Our studies and those of others suggest that PAI-1 may modulate cellular recruitment during the acute inflammatory process.

PAI-1 expression is known to increase in the lung *in vivo* following systemic liposaccharide administration (28) and in alveolar macrophages (29), alveolar epithelium (30), and endothelial cells (31) *in vitro* after LPS stimulation. The bronchoalveolar lavage procedure itself may dilute the alveolar contents 100-fold, making it difficult to quantify PAI-1 in the BALF. Although we did not measure PAI-1 in the BALF, the correlation of PAI-1 in plasma with the histologically assessed degree of lung injury indicates that intrapulmonary injury is the main factor in determining the level of PAI-1 in plasma and suggests that the elevation of plasma PAI-1 is a consequence of local, rather than system factors. Activated protein C (APC) limits thrombin generation by inactivating clotting factors Va and VIIIa and reducing endothelial cell and monocyte tissue factor expressions (32,33). The antithrombotic activity of APC is also associated with profibrinolytic actions by inhibiting PAI-1 activity *in vitro* (34). APC has systemic anticoagulation effects and has been shown to reduce mortality in patients with severe sepsis (35). However, there have been no randomized, controlled studies to determine the effects of anticoagulant therapy on the course of VILI. Our study suggests that APC may have a potential therapeutic role in VILI.

The renin-angiotensin system plays an important role in regulating blood pressure, fluid, and electrolyte homeostasis (36). ANG II is released from its precursor, angiotensinogen, by enzymatic processing with renin and then by ACE. ANG II is the principal biologically active peptide that causes arteriolar vasoconstriction and stimulates aldosterone secretion. Although angiotensinogen is mainly synthesized in the liver and secreted into the circulating blood, angiotensin formation has also been shown to occur in diverse tissues other than the liver (37). ANG II can be generated locally in lung tissues and may have autocrine and paracrine actions at the cellular level (12). Ridker et al. reported that infusion of physiologic doses of ANG II promotes a rapid and dose-dependent increase in plasma PAI-1 levels in humans (13). This study provides evidence that a direct functional link exists between the renin-angiotensin system and the fibrinolytic system in humans. Captopril was the first ACE inhibitor designed for treating hypertension. Gavin et al. found that acute administration of captopril (100 mg/kg) significantly decreased the mean arterial pressure in initially normotensive rats (19). In this study, we found a significant reduction of ANG II in lung tissue after captopril administration; this result demonstrates that efficient ACE inhibition had occurred in our procedure.

In conclusion, we have demonstrated that mechanical ventilation with a high tidal volume and no PEEP increased lung PAI-1 mRNA expression and the plasma PAI-1 level, and the deleterious effects were attenuated by captopril treatment. These results imply that ANG II is involved in the pathogenesis of disordered coagulation in VILI and suggest that the protective mechanism of captopril's attenuation of VILI is related to a reduction in PAI-1. A full understanding of the mechanisms that mediate increased PAI-1 levels may permit possible strategies directed at preventing VILI to be instituted early in the course of the disease process.

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