# Activated Protein C Protection from Lung Inflammation in Endotoxin-Induced Injury

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**We studied the protection of recombinant human activated protein C (rhAPC) in endotoxin-induced lung inflammation and injury and whether this effect is correlated with modulation of lung matrix metalloproteinase (MMP) activity. We randomly assigned 12 Large White pigs to receive intravenous *Escherichia coli* lipopolysaccharide (LPS; 40 μg/kg/hr), rhAPC (24 μg/kg/hr), or both. We monitored respiratory mechanics and function, cell counts, and cytokine concentrations in bronchoalveolar lavage fluid (BALF). Lung samples were collected for the zymography of MMP-2 and MMP-9 activities and for histology. In septic pigs, rhAPC decreased proMMP-9 release as well as MMP-9 activation, and increased proMMP-2 presence without any evident activation compared with specimens that were given LPS alone. In addition, lung injury in rhAPC-treated animals was significantly attenuated, as shown by higher respiratory compliance, delayed increase in tumor necrosis alfa and interleukin-1β as well as neutrophil recruitment in the BALF, reduced lung edema, and histologic changes. In conclusion, rhAPC is beneficial in acute lung injury, and the protection may depend, at least in part, on modulation of MMP-2/9 activity.** Exp Biol Med 233:1462–1468, 2008

**Key words:** activated protein C; sepsis; acute lung injury; matrix metalloproteinases; swine

## Introduction

Sepsis is among the major causes of death in noncoronary intensive care units worldwide, and the incidence is on the rise (1). Sepsis is associated with a progressive depletion of activated protein C (APC), a physiologic anticoagulant generated from protein C by thrombin coupled with thrombomodulin on endothelial cells (2). The extent of the decrease is correlated with the risk of mortality (3). The use of recombinant human APC (rhAPC) is one of the hottest topics in sepsis therapy. The effect of APC very likely depends on its combined anticoagulant, profibrinolytic, anti-inflammatory, and anti-apoptotic activities (4, 5, 6). Nevertheless, the results of *in vivo* studies are less explicit and the exact mechanism(s) by which APC exerts its clinical effects remains partially unclear. The lung is a prime target organ, as the direct damage causes pulmonary edema, respiratory dysfunction, and, in the worst case, adult respiratory distress syndrome. In the pathogenesis of acute lung injury, degradation of the extracellular matrix is considered to be one of the crucial events (7). Extracellular matrix degradation is controlled primarily by matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes that are capable of degrading the major components of the matrix, such as collagens, gelatins, and proteoglycans. With regard to the lung, activation of MMPs has been implicated in the pathophysiology of acute and chronic inflammatory diseases, including adult respiratory distress syndrome (8, 9, 10), suggesting a role in basement membrane disruption. Furthermore, studies in various lung injury models show that MMP inhibitors decrease the extent of lung injury (11, 12, 13); therefore, inhibition of MMPs is currently considered an appealing target and a promising strategy for pharmacologic intervention. This investigation was designed to shed some light on rhAPC mechanism(s) of action in regulating the lung's response to lipopolysaccharide (LPS)-induced injury, with attention to lung MMP-2 and MMP-9 enzymatic activity.

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## Materials and Methods

**Animals and Experimental Procedures.** Twelve Large White pigs of both sexes (20.7 ± 0.63 kg, mean ± SEM) were used. The animals were fed a standard diet with free access to water and were deprived of food 12 hrs before use. Experiments were conducted according to the Institutional Guidelines for the Care and Use of Laboratory Animals (University of Milan). Pigs were randomly assigned to 3 groups of 4: (1) LPS (*Escherichia coli* LPS serotype 055:B5; Sigma Chemical, Milan, Italy; infused at a dose of 40 μg/kg/hr for 2 hrs, followed by a 3-hr observation period); (2) rhAPC (24 μg/kg/hr by intravenous [iv] infusion for 5 hrs); and (3) LPS+rhAPC (using the same protocols). Pigs were sedated (medetomidine intramuscular 0.03 mg/kg and tiletamine-zolazepam intramuscular 4 mg/kg) and anesthetized with a bolus of thiopental sodium (15 mg/kg iv) followed by a continuous infusion of 9 mg/kg/hr. Pigs were tracheostomized, intubated, paralyzed with pancuronium bromide (0.2 mg/kg iv), and mechanically ventilated (Servo-Ventilator 900C, Siemens-Elema, Solna, Sweden) with 0.21 fraction of inspired oxygen, tidal volume 10 ml/kg, respiratory rate 18 breaths/min, and positive end-expiratory pressure 5 cm $H_2O$. A supplementary paralyzing agent was administered hourly. A polyethylene catheter was inserted into the right femoral vein for administration of drugs and fluids. At the end of the experiments, all animals were euthanized by barbiturate overdose.

**Functional Studies.** *Bronchoalveolar Lavage Fluid (BALF) Analysis.* BALF was collected at baseline and just before euthanasia and placed in EDTA-coated tubes. The fluid collected was quantified, and total nucleated cells, tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β concentrations were determined using an automated cell counter (Hemat 8, SEAC, Florence, Italy), calibrated with threshold values specific for swine blood cells for measurement of the nucleated cells, and porcine-specific enzyme-linked immunoabsorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) for determination of TNF-α and IL-1β. A differential cell count was done on samples cytocentrifuged (300 $g \times 3$ mins) in a sedimentation chamber (Neuroprobe, Gaithersburg, MD). Slides were then stained with May Grunwald-Giemsa for microscopic evaluation. A minimum of 200 cells per sample were counted.

**Respiratory Function Profile.** Mean pulmonary arterial pressure (MPAP), extravascular lung water (EVLWI), and respiratory compliance (Crs) were monitored as described elsewhere (14), at baseline and every 30 mins.

**Lung Tissue Zimography.** Lung samples were frozen in isopentan cooled with liquid nitrogen and stored at –80°C for the detection of MMP-2 and MMP-9 activities by zymography, as described previously (15). Briefly, tissues were homogenized in 10 m*M* Tris-HCl, 150 m*M* NaCl, 20 m*M* EDTA, pH 7.5, and the protein content was assessed using the Bradford method. The same amount of protein in each extract (5 μg) was loaded onto sodium dodecyl sulfate polyacrylamide gels containing 1 mg/ml gelatin, and the samples were run at 150 V for 1 hr in a minigel apparatus. The samples were loaded into the gels without heat denaturation and reducing agents. After the run, the gels were washed at room temperature for 2 hrs in 2.5% Triton X-100 and incubated overnight at 37°C in 10 m*M* $CaCl_2$, 150 m*M* NaCl, and 50 m*M* Tris-HCl, pH 7.5 buffer. The gel was stained in 2% (v/v) Coomassie Blue G-250 in fixing solution and photographed on a light box after appropriate destaining. Proteolysis was detected as white bands in a dark blue field. The gels were then scanned and imaged in black and white, and gelatinolytic bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and Adobe Photoshop (Adobe Software, Seattle, WA). Densities were expressed as arbitrary units. The examination was performed by a pathologist blinded to study groups.

**Lung Tissue Light Microscopy.** For histologic examination, lung specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. The examination was performed by a pathologist blinded to study groups. The intensity of inflammatory lesions was assessed with a predetermined scoring system on 5 different microscopic fields at ×20 magnification. A 0–3 scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe) was used to evaluate and grade the following pathologic findings: septal and alveolar neutrophilic infiltration, thickening of alveolar septa, and interstitial or intra-alveolar edema. An overall histologic score was calculated by totalling the scores.

**Statistical Analysis.** SPSS 14.0 for Windows (SPSS Inc., Chicago, IL) was used for statistical analysis, and $P < 0.05$ was deemed statistically significant. Data were subjected to one-way ANOVA followed, if justified by the resulting statistical probability value (i.e., $P < 0.05$), by Tukey's Honest Significant Difference (HSD) test.

## Results

**Pulmonary Function Study.** Crs was significantly higher ($P < 0.05$) in LPS+rhAPC-treated animals than in those receiving LPS alone throughout most of the experiment (Fig. 1A). EVLWI increased after LPS infusion was started, but rhAPC attenuated the LPS-induced rise and kept EVLWI more stable throughout the experiment (Fig. 1B). MPAP increased significantly in the first 30 mins of endotoxin challenge in both the LPS and LPS+rhAPC groups (Fig. 1C). None of the variables in the group receiving only rhAPC changed significantly during the experiment (Figs. 1 A–C).

**BALF Analysis.** The amount of BALF (Fig. 2A) and the percentage of neutrophils in BALF (Fig. 2B) were significantly higher ($P < 0.05$) at the end of the experiment in both groups receiving LPS than at baseline. However, at the end of the experiment, the percentage of neutrophils in BALF from the LPS+rhAPC group was significantly lower

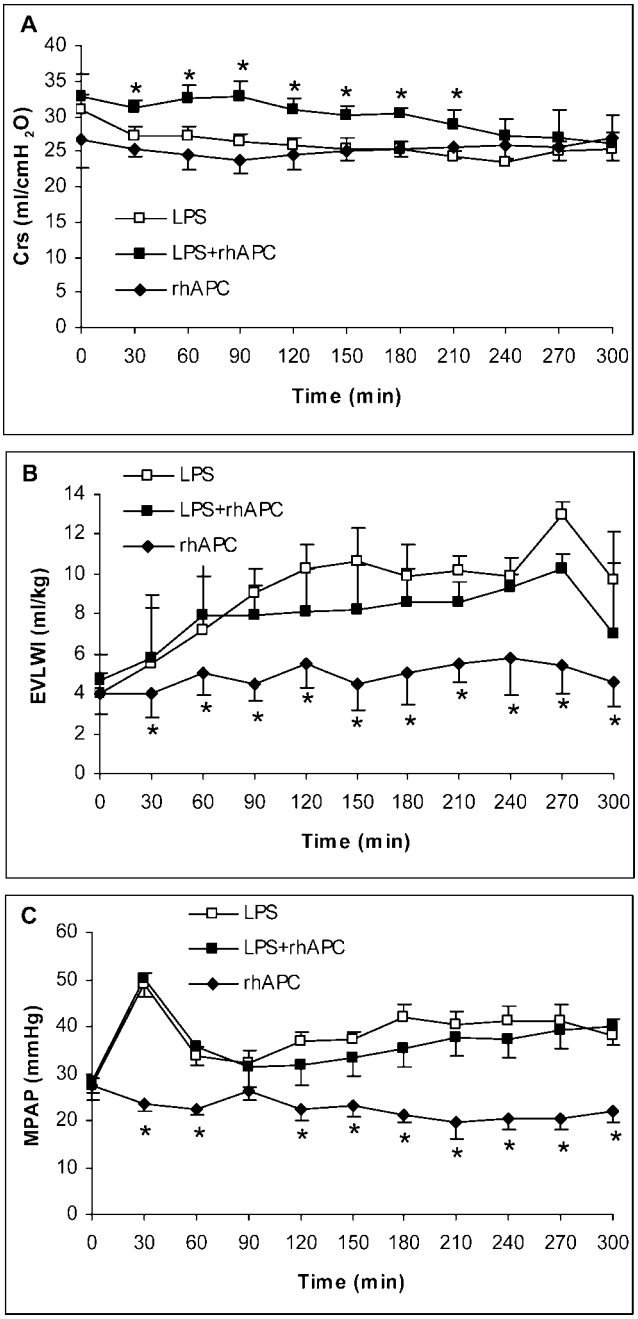



**Figure 1.** (A) Trend of the Crs in the LPS, LPS+rhAPC, and rhAPC groups. The data are mean ± SD and were analyzed by one-way ANOVA, followed by Tukey's HSD test ($P < 0.05$). * = statistical significance of the LPS+rhAPC group versus the other two studied groups. (B) EVLWI was determined as the index of lung edema. Values are mean ± SD and were analyzed by one-way ANOVA. * = $P < 0.05$, statistical significance of the rhAPC group versus the other two groups. (C) Trend of the MPAP. The data are mean ± SD and were analyzed by one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). * = statistical significance of the rhAPC group versus the other two groups.

($P < 0.05$) than the percentage in the LPS group. Endotoxin infusion steeply increased both TNF-α (Fig. 3A) and IL-1β (Fig. 3B) concentrations in BALF, but not in pigs given rhAPC.

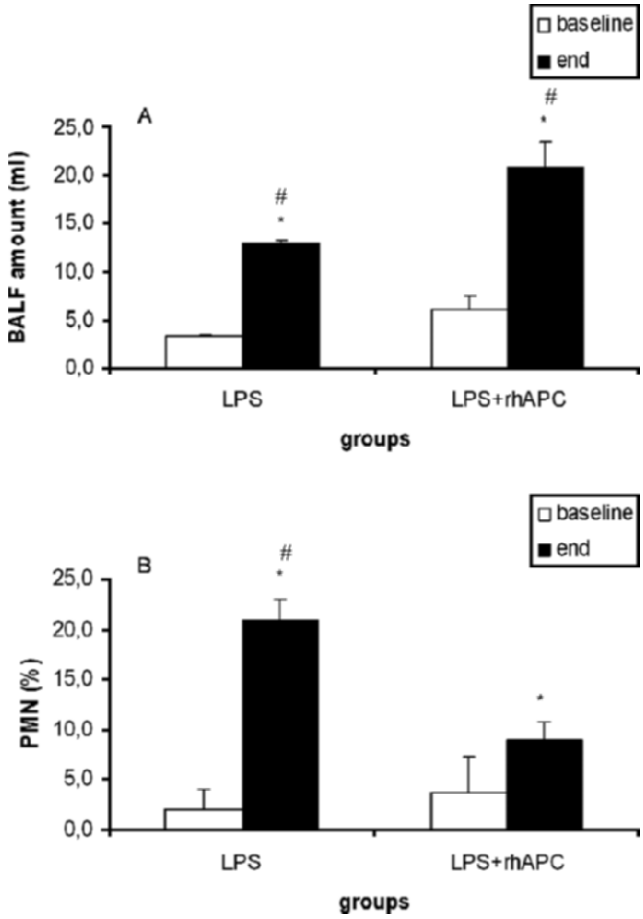



**Figure 2.** (A) BALF amounts. (B) Percentage of polymorphonuclear cells (PMN). Results are expressed as mean ± SD and were analyzed by one-way ANOVA. * = statistical significance between treatments ($P < 0.05$); # = statistical significance of posttreatment values versus controls ($P < 0.05$).

**Lung MMP Analysis.** Zymography showed marked MMP-9 activity in specimens administered LPS alone, whereas the samples from pigs treated with rhAPC showed a consistent decrease in MMP-9 presence and activation. The bands corresponding to proMMP-2 (72 kDa) in the LPS group were markedly smaller than the bands in the LPS+rhAPC-treated samples (Fig. 4), even if the samples showed no significant activation level (MMP-2 band). Quantitative measurements by densitometry confirmed the visual findings, with proteolytic activity reported as arbitrary units of area. In particular, no activated MMP-2 was seen in either the LPS or LPS+rhAPC group, while proMMP-2 was higher in the LPS+rhAPC group than in the LPS group (852 ± 502.75 vs. 103.5 ± 48.79; mean ± SD). ProMMP-9 was higher in the LPS group than in the LPS+rhAPC group (1529.5 ± 146.37 vs. 1090 ± 141.19; mean ± SD), and this difference was statistically significant for MMP-9 (433.5 ± 108.19 vs. 0 ± 0; mean ± SD, for LPS vs. LPS+rhAPC).

**Lung Histology.** Microscopy of lung specimens from LPS+rhAPC-treated animals showed normal blood and vessels, normal subpleural lymphatic vessels, mild occa-

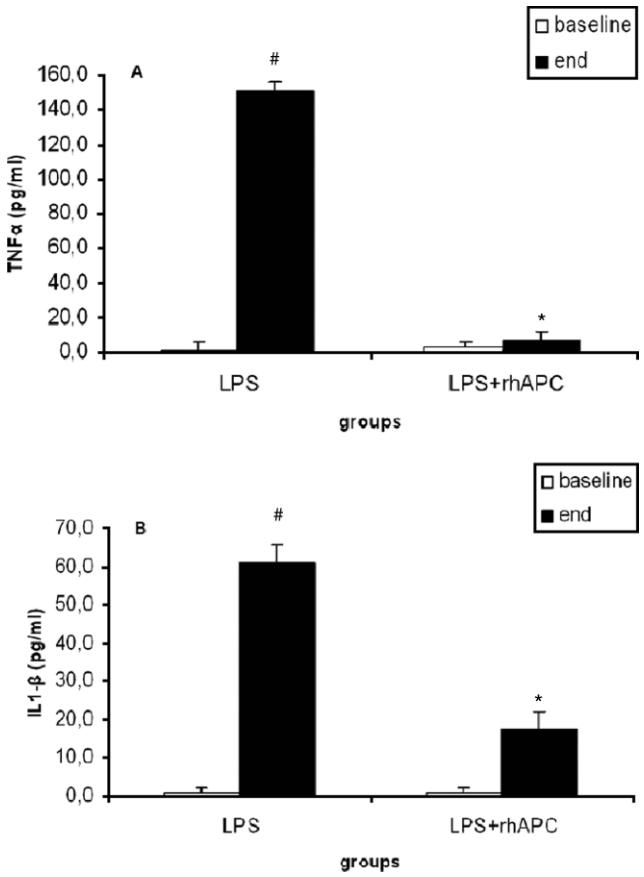



**Figure 3.** (A and B) BALF concentrations of TNF-α and IL-1β determined by porcine-specific ELISA kits. The data are mean ± SD and were analyzed by one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). * = statistical significance between treatments; # = statistical significance of posttreatment values versus controls.

sional thickening of alveolar septa (Fig. 5D, arrowheads), and only mild and occasional neutrophilic infiltration of alveolar septa (Fig. 5B, arrow). This is in sharp contrast to the extensive morphologic lung damage observed in the LPS group, in which thickened interlobular septa, mild edema (Fig. 5E, arrow), and mild to severe neutrophilic infiltration of alveolar spaces (Fig. 5E, arrowheads), with thickened alveolar septa due to massive cellular inflammatory infiltrate, were found (Fig. 5A, arrows; Fig. 5C, arrowheads). Mild to moderate neutrophil margination in most of the blood vessels and subpleural ectatic lymphatic vessels was also present (Fig. 5C, arrows). The median score in the LPS+rhAPC group was significantly lower than the score in the LPS group ($P < 0.05$): 6.33 ± 1.53 vs. 17.67 ± 3.79; mean ± SD.

## Discussion

To our knowledge, the present study provides the first evidence of rhAPC protection from lung damage and failure through a mechanism involving the modulation of lung inflammatory response, in particular, MMP-2/9 enzymatic activity. Despite all of the potentially beneficial properties of rhAPC in the context of sepsis, its clinical use remains

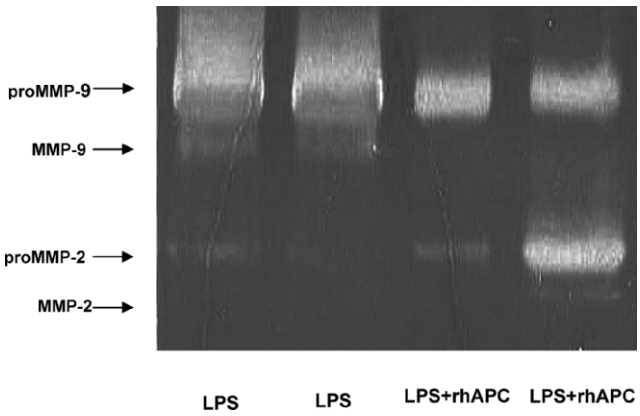
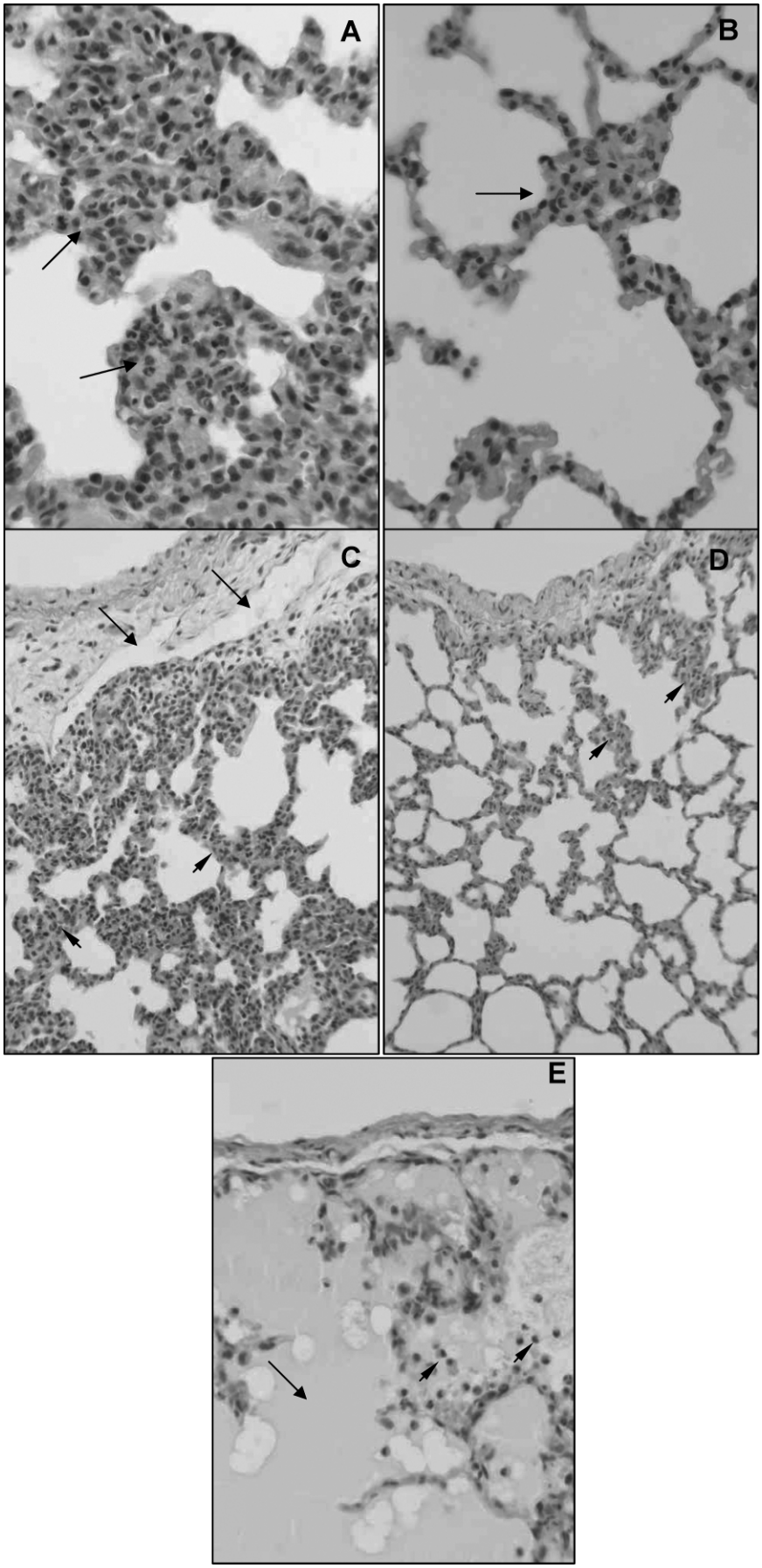



**Figure 4.** Zymography of MMP activities in lung samples from LPS- and LPS+rhAPC-treated pigs. A color version of the figure is available in the online version of the journal.

controversial. Past efforts have been focused on the effects of APC on neutrophil chemotaxis, endothelial leakage, or effects other than anti-inflammatory, such as anticoagulant and anti-apoptotic. The rationale of our research was that the effects of rhAPC on other target tissues and processes might contribute significantly to lung protection during infection. The extracellular matrix is a critical component of the pulmonary interstitium and is important to maintain lung compliance. Therefore, degradation of the extracellular matrix is a crucial event in the pathophysiology of acute lung injury (7). Various lung injury models have evidenced the biological function of MMPs: MMP-9 and MMP-2 have been shown to increase in acute lung injury and to be correlated with tissue inflammation and damage (16, 17, 18). Furthermore, MMP inhibitors have been reported to decrease the extent of lung injury (11, 12, 13). Based on these critical observations, we hypothesized that rhAPC-related protection might be mediated by processes that modulate tissue remodeling after parenchymal damage, such as attenuation of lung extracellular matrix degradation, alveolar edema, and liberation of damaging mediators by inflammatory cells. Our results support the initial hypothesis; in fact, MMP-9 activation was lower in septic animals receiving rhAPC compared with those given LPS alone. This MMP trend closely paralleled a significant decrease in the recognized signs of acute endotoxin-induced pulmonary inflammation and injury. MMP-2 was not activated in either group, but greater amounts of the inactive zymogen were present in samples from the LPS+rhAPC group than from the LPS group. Exposure to endotoxin induced a marked inflammatory response of the lungs, with low respiratory compliance, high cytokine levels in BALF, massive cellular inflammatory infiltrates, and pulmonary edema. Therefore, it is not surprising that levels of MMP-9, which is secreted most by activated inflammatory cells and is considered a marker of tissue degradation during pulmomary edema (19, 20), rose in the lung tissue of those animals, thereby modifying the organ's biomechanical properties. In contrast,



concomitant administration of rhAPC substantially lowered the activity of MMP-9 and, at the same time, successfully mitigated inflammatory cell sequestration, reduced neutrophil accumulation in BALF, and prevented increased secretion of TNF-α and IL-1β, two potent inducers of MMPs. The lung function derangement caused by LPS was also consistently ameliorated by rhAPC, as supported by the higher respiratory compliance and lower, although not significantly lower, and more stable EVLWI values that treated animals displayed compared with nontreated ones. A remarkable presence of inactive zymogen proMMP-2 was seen in rhAPC-treated tissue, but no trace of activated MMP-2 was detected, indicating that proteolytic activity had not started: MMP-2 activation, in fact, is an early event that triggers the cascade of proteolytic enzyme activation. However, although it is traditionally considered to be an important effector of inflammation, MMP-2 has been recently reported to also play a role in cessation of the inflammatory response through the proteolysis of chemokines and the inhibition of chemoattractant signals, which in turn result in restricted new cell recruitment and promoted clearance of the inflammatory infiltrate (21). Hence, the increased release of proMMP-2 occurring in LPS+rhAPC-treated lungs might be considered an early step preceding the enzymatic activation induced by the drug for protection. Further studies, including those with longer experimental duration, are needed to confirm this hypothesis. However, the picture of MMP activities that we observed helps to explain the protective effect of rhAPC on tissue integrity. Because of the intricacy of these enzymes and their substrates, and because of the still unacceptably high mortality rate associated with sepsis and septic lung injury, which is mostly due to the complex pathophysiology and inadequate overall efficacy of antimicrobial therapy and supportive care, the use of carefully crafted models of lung injury and repair is crucial. Actually, the effects of rhAPC treatment in sepsis-induced lung injury have not been extensively investigated. Until now, the effects have been studied only in rodent models, which do not closely reflect human clinical infections, or in healthy human volunteers with localized infections, and these studies have yielded controversial results. Among the other studies that should be discussed is one of Murakami and coworkers (22), which showed that APC treatment prevented LPS-induced pulmonary vascular permeability in systemic endotoxemia in rats, while Robriquet and colleagues (23) reported an increase in lung water and other measures of injury with rhAPC in bacteria-challenged rats. Looney and Matthay (24) reported preliminary data from mice given acid intratracheally; in rhAPC-treated mice, lung injury was worsened, with increased pulmonary edema. Nick and colleagues (25) hypothesized that rhAPC may decrease neutrophil recruitment in tissues. In their study, rhAPC or saline was administered by iv infusion to healthy human volunteers, and a bolus of bacterial LPS was delivered to the air spaces of the lungs by instillation. rhAPC decreased neutrophil recruitment to the alveolar air spaces. However, a single instillation of LPS into the lungs differs significantly from severe systemic infection, and these results do not confirm whether neutrophil recruitment is indeed reduced by rhAPC during sepsis. Finally, among all of the rhAPC clinical trials, data from the PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) study (26) showed that the drug seems to have a beneficial effect on lung function: treated patients had significantly less respiratory dysfunction over time. However, patients eligible for participation in PROWESS had to meet very specific inclusion criteria, based on a strictly quantitative approach that may not always be adaptable to the real-life setting. In addition, different retrospective subgroup analyses provided conflicting results on rhAPC overall efficacy. To our knowledge, ours is one of the first studies investigating the effects of rhAPC administration in the pig, and we have chosen this model of endotoxin-induced lung injury because it bears close resemblance to the human clinical entity. We believe that the well-known similarities between the pig model and human adult respiratory distress syndrome help to validate our data.

In summary, there is growing conviction that, in severe sepsis, rhAPC may help to prevent lung injury and improve outcomes, but the mechanisms by which rhAPC exerts its protection remain speculative. In our model, iv infusion of rhAPC diminished LPS-induced pulmonary inflammation and failure. Our findings support the view that this protection is, at least in part, mediated by the modulation of lung MMP-2/9 enzymatic activity, thereby providing novel and interesting proof of rhAPC's ability to help preserve lung integrity and respiratory mechanics.

The study drug (APC) was freely provided by Eli Lilly & Co. for this research only.

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**Figure 5.** The histologic appearance of lung sections from the LPS group (A, C, and E) and LPS+rhAPC-treated pigs (B and D) stained with hematoxylin and eosin. (A) (×400): Severe inflammatory cell infiltration and thickening of alveolar septa (arrows). (B) (×400): Mild neutrophilic infiltration of alveolar septa (arrow). (C) (×250): Dilated lymphatic vessel in subpleural space (arrows) and moderate thickening of alveolar septa (arrowheads). (D) (×250): Normal appearance of subpleural lymphatic vessels; mild occasional thickening of alveolar septa (arrowheads). (E) (×400): Alveolar edema (arrow) and neutrophilic infiltration of alveolar spaces (arrowheads). A color version of the figure is available in the online version of the journal.

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