Activated Protein C Inhibits Local Coagulation after Intrapulmonary Delivery of Endotoxin in Humans

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Rationale: Acute lung injury and pneumonia are associated with pulmonary activation of coagulation and suppression of fibrinolysis, resulting in fibrin deposition in the lung. Activated protein C (APC) has systemic anticoagulant effects in patients with sepsis. **Objective:** To determine the effect of systemic administration of recombinant human APC on endotoxin-induced hemostatic alterations in the bronchoalveolar space in humans. **Methods:** Healthy humans received intravenous APC (24 $\mu\text{g/kg/hour}$; $n = 8$) or vehicle ($n = 7$); all subjects were administered saline in one lung subsegment and endotoxin (4 ng/kg) into the contralateral lung. Bronchoalveolar lavage was performed 16 hours after saline and endotoxin administration. **Measurements and Main Results:** Endotoxin induced local activation of coagulation, as reflected by elevated levels of thrombin-antithrombin complexes ($1.9 \pm 0.1 \text{ ng/ml}$) and soluble tissue factor ($15.0 \pm 0.6 \text{ pg/ml}$) in bronchoalveolar lavage fluid, which was inhibited by APC ($1.4 \pm 0.1 \text{ ng/ml}$ and $12.3 \pm 0.4 \text{ pg/ml}$, respectively; both $p < 0.01$). Concurrently, endotoxin suppressed fibrinolysis, as indicated by reduced bronchoalveolar levels of plasminogen activator activity accompanied by elevated levels of plasminogen activator inhibitor type I activity. APC diminished the rise in plasminogen activator inhibitor type I activity (from 3.9 ± 0.1 to $3.0 \pm 0.2 \text{ ng/ml}$, $p = 0.002$), while not significantly influencing plasminogen activator activity levels. Endotoxin reduced bronchoalveolar protein C concentrations, which was prevented by APC. Protein C did not influence the endotoxin-induced rise in local soluble thrombomodulin levels. **Conclusion:** APC exerts an anticoagulant effect in the human lung challenged with endotoxin.

Keywords: fibrinolysis; lipopolysaccharide; lung

Intravenous infusion of recombinant human activated protein C (rhAPC) has been shown to reduce mortality in patients with severe sepsis (1). The favorable effect of rhAPC on the outcome of severe sepsis has been attributed to its capacity to inhibit the activation of several pathways implicated in the pathogenesis of sepsis, including coagulation, the interaction between leukocytes and the vascular endothelium, and the production of proinflammatory cytokines (2, 3). However, the exact mechanisms by which rhAPC improves the outcome of severe sepsis in humans are not fully elucidated.

Because patients with sepsis represent a highly heterogeneous population, several investigators have tried to examine the *in vivo* effects of rhAPC on coagulation and inflammation in a standardized controlled setting (i.e., in the well established model of human endotoxemia) (4, 5). Remarkably, in this model, infusion of rhAPC at a dose also administered in the pivotal clinical

sepsis trial (1) did not influence a variety of host responses, including activation of coagulation (4, 5). Recently, we expanded these studies in healthy humans by an in-depth investigation on the effects of intravenous rhAPC on lung inflammation induced by pulmonary segmental instillation of LPS (6). rhAPC reduced neutrophil accumulation in the bronchoalveolar space without influencing cytokine or chemokine release. The effect of rhAPC on local activation of coagulation in the lung was not reported in that study. A possible anticoagulant effect of intravenous rhAPC in the lung is of considerable interest for several reasons. Both pneumonia, the most frequent cause of sepsis, and acute lung injury, a frequent complication of sepsis, are associated with fibrin deposition in the pulmonary compartment (7–9), and disturbances in the local hemostatic balance are considered to impact on the outcome of such patients (10, 11). Therefore, the present investigation sought to determine the effect of intravenous rhAPC on LPS-induced coagulation activation in the lung.

METHODS

Design

This study was performed simultaneously with an investigation that evaluated the effects of rhAPC on neutrophil influx and activation in the lung, the results of which have been published previously (6). Study design and subjects have been described in detail (6). Fifteen subjects received either intravenous rhAPC (drotrecogin α [activated]; 24 $\mu\text{g/kg/hour}$; $n = 8$) or normal saline (vehicle for drotrecogin α [activated]; $n = 7$) starting 2 hours before the initial bronchoscopy and continuing for 16 hours; the infusion of rhAPC or placebo was discontinued 2 hours before the second bronchoscopy to lessen the risk of hemorrhage resulting from anticoagulant properties of rhAPC. At the time of the first bronchoscopy, 10 ml of saline was instilled into a lung subsegment (either the right middle lobe or lingula) followed by instillation of reference *Escherichia coli* O:113 endotoxin 4 ng/kg in 10 ml saline (obtained from the National Institutes of Health, Bethesda, MD) into the contralateral lung. The subjects were randomized to left or right lungs for LPS or saline instillation. At the time of the second bronchoscopy, both LPS- and placebo-instilled subsegments were lavaged with 150 ml of normal saline. Bronchoalveolar lavage fluid (BALF) was centrifuged and supernatants were stored at -80°C until analyzed. BAL specimens were collected in normal saline without any inhibitors or preservatives. The experimental procedures did not induce clinically relevant signs or symptoms (Reference 6 and data not shown), and chest radiographs did not show evidence of pulmonary infiltration at the site of LPS administration. Informed consent using a General Clinical Research Center and Institutional Review Board–approved consent form was obtained in all cases.

Assays

APC was measured with an enzyme capture assay using monoclonal antibody HAPC 1555 and chromogenic substrate Spectrozyme Pca (American Diagnostica, Greenwich, CT) (12); this assay does not cross-react with the assay for protein C (PC) activity (12). PC activity was measured with an amidolytic assay using chromogenic substrate S2366 (Chromogenix, Milan, Italy), which correlates well with PC antigen levels (13, 14). Thrombin-antithrombin complexes (TATc), soluble tissue factor (TF), plasminogen activator inhibitor type I (PAI-1), tissue-type plasminogen activator (tPA), and soluble thrombomodulin (TM) were measured using ELISAs (TATc: Behringwerke AG, Marburg,

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Germany; soluble TF: American Diagnostics; PAI-1: TintElize PAI-1; Biopool, Umea, Sweden; tPA: Asserachrom tPA; Diagnostica Stago, Asnieres-sur-Seine, France; soluble TM: Diagnostica Stago). Urokinase plasminogen activator (uPA) was measured by ELISA (15). PAI-1 activity (16) and plasminogen activator activity (17) were measured by amidolytic assays.

Statistical Analysis

All data are presented as means $\pm$ SE. Overall differences between the datasets (APC/LPS, APC/control, vehicle/LPS, vehicle/control) were analyzed by repeated-measurements one-way analysis of variance, which was followed by Tukey's multiple-comparison *post hoc* test. Residuals from the different models were normally distributed (Shapiro-Wilk $W > 0.90$), and variances between the datasets were equal (Levene's test). These analyses were performed using SPSS 12.0.2 (SPSS, Chicago, IL). A p value less than 0.05 was considered to represent a statistically significant difference.

RESULTS

APC Concentrations

To examine to what extent intravenously administered rhAPC penetrates in the bronchoalveolar space, we measured APC levels in BALF. Overall, APC levels were significantly different between groups ($p < 0.0001$). Administration of rhAPC resulted in readily detectable APC levels in BALF obtained 2 hours after the discontinuation of the infusion (APC/LPS: 15.6 ± 1.0 ng/ml; APC/control: 14.7 ± 0.8 ng/ml; both $p < 0.001$ vs. vehicle). In subjects who had not received rhAPC, LPS administration was associated with slightly lower APC levels in BALF when compared with control (vehicle/LPS: 0.4 ± 0.04 ng/ml vs. vehicle/control: 0.8 ± 0.05 ng/ml, nonsignificant). APC was not detectable in plasma at the time the second bronchoscopy was performed.

rhAPC Inhibits LPS-induced Bronchoalveolar Coagulation Activation

The local concentrations of TATc and soluble TF have been used to determine the extent of bronchoalveolar coagulation activation in patients with acute lung injury and/or pneumonia (7–9). TATc and soluble TF levels were significantly different between groups (both $p < 0.001$). Local instillation of LPS induced a rise in BALF concentrations of both coagulation markers (Figure 1; both $p < 0.0001$ vs. vehicle/control). Importantly, rhAPC reduced the rise in BALF TATc and soluble TF (Figure 1; $p < 0.001$ and $p < 0.01$ vs. vehicle/LPS, respectively), although

not to levels measured in BALF obtained from lungs not challenged with LPS. Hence, these data indicate that intravenous infusion of rhAPC at a dose also given to patients attenuates alveolar coagulation elicited by local administration of LPS.

Effect of rhAPC on the LPS-induced Suppression of Bronchoalveolar Fibrinolysis

Acute lung injury and pneumonia result in locally suppressed fibrinolysis as a result of strongly elevated BALF levels of PAI-1 (7–9). BALF PA activity ($p = 0.001$), PAI-1 activity ($p < 0.001$), and PAI-1 antigen ($p < 0.001$) demonstrated a difference between groups. LPS reproduced the suppressed fibrinolytic response observed in patients with lung injury (i.e., PA activity was reduced, whereas PAI-1 activity and antigen levels were elevated in BALF; Figures 2A and 2B; $p < 0.01$ vs. both control groups; PAI-1 antigen levels not shown). rhAPC did not influence the LPS effects on PA activity or PAI-1 antigen; however, rhAPC did attenuate the LPS-induced rise in PAI-1 activity levels ($p = 0.002$). Of note, BALF concentrations of tPA and uPA antigen also demonstrated a difference between groups ($p = 0.001$ and $p = 0.002$, respectively), and LPS instillation elicited rises of both proteins ($p < 0.05$ vs. both control groups), which were not significantly influenced by rhAPC (Figures 2C and 2D).

rhAPC Prevents the LPS-induced Decrease in BALF PC Concentrations

Patients with acute lung injury display decreased PC together with elevated soluble TM concentrations in their alveolar space (18). In the current study, PC and soluble TM levels were significantly different between groups (Figure 3; both $p < 0.001$). LPS induced a significant decrease in BALF PC concentrations when compared

Figure 2. Effect of rhAPC on the LPS-induced fibrinolytic response in the lung. (A) Plasminogen activator (PA) activity, (B) PA inhibitor 1 (PAI-1) activity, (C) tissue PA (tPA), and (D) urokinase (uPA) in BALF recovered 16 hours after endobronchial LPS (black bars) or saline (white bars) instillation from volunteers administered rhAPC ($n = 8$) or vehicle ($n = 7$). Data are mean $\pm$ SE. PA activity ($p = 0.001$), PAI-1 activity ($p < 0.001$), tPA ($p = 0.001$), and uPA ($p = 0.002$) demonstrated an overall difference between groups as tested by analysis of variance (ANOVA). * $p < 0.05$ versus control/vehicle and control/APC; † $p < 0.05$ LPS/APC versus LPS/vehicle; ‡ $p < 0.05$ versus control/APC (by Tukey's multiple-comparison test).

Figure 1. Recombinant human activated protein C (rhAPC) inhibits LPS-induced coagulation activation in the lung. (A) Thrombin–antithrombin complexes (TATc) and (B) soluble tissue factor (TF) in bronchoalveolar lavage fluid (BALF) recovered 16 hours after endobronchial LPS (black bars) or saline (white bars) instillation from volunteers administered rhAPC ($n = 8$) or vehicle ($n = 7$). Data are mean $\pm$ SE. TATc and soluble TF demonstrated an overall difference between groups (both $p < 0.001$). * $p < 0.05$ versus control/vehicle and control/APC; † $p < 0.05$ LPS/APC versus LPS/vehicle (by Tukey's multiple-comparison test).

Figure 3. Effect of rhAPC on the LPS-induced changes in protein C (PC) and soluble thrombomodulin (TM) response in the lung. (A) PC and (B) soluble TM in BALF recovered 16 hours after endobronchial LPS (black bars) or saline (white bars) instillation from volunteers administered rhAPC ($n = 8$) or vehicle ($n = 7$). Data are mean $\pm$ SE. PC ($p < 0.001$) and soluble TM ($p < 0.001$) demonstrated an overall difference between groups as tested by ANOVA. * $p < 0.05$ versus control/vehicle and control/APC; † $p < 0.05$ LPS/APC versus LPS/vehicle (by Tukey's multiple-comparison test).

with saline ($p < 0.01$ vs. both control groups). Interestingly, rhAPC prevented this LPS-induced decline in PC levels (APC/LPS: $p < 0.05$ vs. vehicle/LPS, nonsignificant vs. both control groups), but did not influence the rise in BALF soluble TM.

DISCUSSION

Although several studies have indicated that APC may protect the lung from injury caused by inflammation (reviewed in Reference 19), knowledge of the potential anticoagulant effects of APC in the pulmonary compartment is highly limited. We here used a human model of lung inflammation induced by the subsegmental instillation of LPS via a bronchoscope (6, 20) to study the effect of intravenous rhAPC on pulmonary coagulation and fibrinolysis. The local challenge with LPS reproduced in a qualitative way the major features of the disturbed hemostatic balance in the alveolar space of patients with acute lung injury and/or pneumonia (i.e., activation of coagulation with concurrent suppression of fibrinolysis) (7–9). The main finding of our study is that intravenous infusion of rhAPC, at a dose also given to patients with severe sepsis, inhibits bronchoalveolar coagulation activation without influencing the inhibition of fibrinolysis.

APC is a natural anticoagulant by virtue of its capacity to inactivate clotting factors Va and VIIIa (2, 3). In addition, APC may further inhibit coagulation by decreasing the synthesis and expression of TF on leukocytes (21). We previously used soluble TF concentrations in BALF, together with TATc levels, as markers for local coagulation activation in patients with pneumonia (8, 9). In the current investigation, LPS elicited rises in the local concentrations of both soluble TF and TATc, and these increases were markedly reduced by rhAPC. In line with this finding, APC was readily detectable in BALF of subjects who had received rhAPC intravenously, even 2 hours after the discontinuation of rhAPC infusion. Of note, in the majority of patients with sepsis, APC is no longer detectable in the circulation within 2 hours after terminating the infusion (22). In this respect, it is interesting to note that mice had detectable APC levels in BALF up to 24 hours after inhalation of APC (23). Together, these data suggest that rhAPC enters the bronchoalveolar space after intravenous administration, that it is locally active, and that it is cleared from this compartment relatively slowly.

Sepsis results in suppressed fibrinolysis in the circulation as a result of strongly elevated circulating levels of PAI-1 (24–26). Similar changes are found in the alveolar space of patients with acute lung injury and pneumonia (7–9). APC may enhance fibrinolysis by inhibiting PAI-1 (27), and therefore we considered it of interest to examine the effect of rhAPC on BALF PA activity and PAI-1 levels. Our results clearly show that rhAPC does not impact on the LPS-induced reduction in PA activity and enhanced release of PAI-1 antigen. Importantly, rhAPC did attenuate the LPS-induced rise in PAI-1 activity, which is in line with earlier findings by Sakata and colleagues (27). Notably, this effect of rhAPC on PAI-1 activity was not sufficient to influence PA activity. Although LPS reduced PA activity, tPA and uPA antigen levels were higher in BALF obtained from LPS-challenged segments. The assays used for tPA and uPA also detect tPA and uPA complexed to their inhibitor PAI-1 (not shown). Thus, although tPA and uPA were released into the alveolar space in increased quantities after LPS administration, net PA activity was decreased because of enhanced release of PAI-1. Similar observations have been made in the alveolar space of patients with pneumonia (8, 9) and in the circulation of patients with sepsis (28), healthy humans intravenously challenged with LPS (29) or tumor necrosis factor α (30), and primates infused with live bacteria (31).

Activation of PC requires binding to the TM–thrombin complex. Patients with sepsis have decreased plasma PC concentrations together with elevated circulating levels of soluble TM (18, 26, 32, 33). Recently, similar alterations in PC and soluble TM concentrations were found in the alveolar space of patients with acute lung injury (18). Considering that the shedding of TM from the surface of endothelial and epithelial cells reduces the availability of cell-surface TM for the activation of PC, reduced PC and increased soluble TM levels are indicative of a prothrombotic state. Hence, the fact that rhAPC prevented the drop in BALF PC concentrations after LPS instillation may further contribute to an anticoagulant effect of this treatment.

Our current data on the effect of rhAPC on pulmonary hemostasis after local LPS challenge are in line with the rhAPC effect on systemic coagulation and anticoagulation in patients with severe sepsis (26). In patients with sepsis, rhAPC not only improved survival (1) but also blunted coagulation activation, increased plasma PC concentrations, and did not significantly influence plasma soluble TM or PAI-1 antigen levels (26). We consider it likely that in the present (lung) study and the previous (systemic) patient study, the primary effect of rhAPC was inhibition of thrombin generation through inactivation of factors Va and VIIIa, possibly together with TF inhibition, which as a consequence in part prevented PC consumption. Moreover, these data combined argue against an important profibrinolytic effect of rhAPC *in vivo*, although in our study, rhAPC did diminish the rise in PAI-1 activity levels elicited by LPS. Of note, two earlier studies investigating the effect of rhAPC, given at the same dose as used here and in the clinical sepsis trial (1, 26) in human volunteers challenged with LPS intravenously, failed to demonstrate any influence of the treatment on coagulation activation (4, 5). Although a clear explanation for this discrepancy is lacking, these data suggest that rhAPC may have stronger and/or different effects on coagulation in the pulmonary than in the systemic compartment.

Our study has a number of limitations. The model used involves the administration of a relatively low LPS dose, one time and into a single lung segment, which differs significantly from the clinical scenario of acute lung injury or pneumonia. In addition, rhAPC was infused for 16 hours rather than for 96 hours, such as in patients with severe sepsis. Hence, our data should be interpreted with caution.

We here demonstrate for the first time an anticoagulant effect of intravenous rhAPC in the human lung. The beneficial effect of rhAPC on outcome of patients with severe sepsis and pneumonia has been documented previously (1, 34). In patients with sepsis, rhAPC treatment resulted in a better resolution of lung function in those with respiratory organ dysfunction at baseline (35). The present investigation, together with earlier animal data (19), identifies the lung as a potential target for APC and suggest that clinical studies are warranted to establish the effect of rhAPC on lung inflammation, coagulation, and damage in patients with acute lung injury and/or pneumonia.

Conflict of Interest Statement: T.v.d.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.A.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.A. received consulting fees from Eli Lilly in 2002 and 2003; he served as principal investigator for the Eli Lilly-sponsored ADDRESS study, an international multicenter clinical trial that investigated the utility of rhAPC in patients with sepsis at lower risk of mortality; he also served as principal investigator for an Eli Lilly-funded investigation of the effects of rhAPC in human volunteers given pulmonary endotoxin.

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