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Nebulized Anticoagulants Limit Pulmonary Coagulopathy, But Not Inflammation, in a Model of Experimental Lung Injury

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Abstract

Background: Pulmonary coagulopathy may contribute to an adverse outcome in lung injury. We assessed the effects of local anticoagulant therapy on bronchoalveolar and systemic haemostasis in a rat model of endotoxemia-induced lung injury.

Methods: Male Sprague–Dawley rats were intravenously challenged with lipopolysaccharide (LPS) and treated with nebulized normal saline (placebo), recombinant human-activated protein C (APC), plasma-derived antithrombin (AT), heparin, or danaparoid.

Results: Intravenous administration of LPS resulted in lung injury associated with elevated bronchoalveolar levels of thrombin–antithrombin complex (TATc), 6.9 ± 0.8 ng/mL (placebo) versus 0.5 ± 0.2 ng/mL (healthy control) ($p < 0.01$), and elevated bronchoalveolar levels of fibrin degradation products (FDP), 555 ± 74 ng/mL versus 27 ± 12 ng/mL ($p < 0.01$). Nebulized APC, AT, and danaparoid all significantly limited the rise of bronchoalveolar levels of TATc, 2.4 ± 0.7 ng/mL, 1.5 ± 0.2 , 3.8 ± 0.7 , and 3.2 ± 0.9 ng/mL, respectively (all $p < 0.01$ vs. placebo), and fibrin degradation products, 243 ± 77 , 113 ± 20 , 317 ± 74 , and 300 ± 42 ng/mL (all $p < 0.01$ vs. placebo). Heparin and danaparoid also significantly affected systemic coagulopathy. However, pulmonary inflammatory responses [neutrophil influx into the lungs, bronchoalveolar levels of myeloperoxidase, and bronchoalveolar levels of tumor necrosis factor (TNF), interleukin (IL)-6 and CINC-3], and histopathology of lungs were not affected by nebulization of anticoagulants.

Conclusions: In conclusion, local treatment with APC, AT, heparin, or danaparoid attenuate pulmonary coagulopathy, but not inflammation, in rats with endotoxemia-induced lung injury.

Key words: acute lung injury, activated protein C, administration by inhalation, antithrombin, danaparoid, heparin

Introduction

SEVERE SEPSIS IS CHARACTERIZED by systemic activation of coagulation, leading to intravascular depositions of fibrin and microvascular thrombosis while at the same time bleeding may occur, due to uncontrolled consumption of coagulation factors.⁽¹⁾ Under physiological circumstances,

activation of coagulation is regulated by natural inhibitors of coagulation, such as activated protein C (APC) and antithrombin (AT). In sepsis, anticoagulant systems are impaired, due to massive consumption and downregulation by inflammatory mediators. Restoration of anticoagulant pathways to diminish the procoagulant state forms the rationale for substitution therapy. Although infusion of plasma-derived

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AT failed to reduce patient mortality in severe sepsis,⁽²⁾ administration of recombinant-human APC reduced morbidity and mortality.⁽³⁾ There is ongoing debate, however, on the exact mechanisms by which APC prevents morbidity and mortality.

One can question whether the beneficial effect of APC is solely the result of APC on intravascular coagulopathy. Severe sepsis is an important cause of acute lung injury/acute respiratory distress syndrome (ALI/ARDS),⁽⁴⁾ and pulmonary coagulopathy is intrinsic to ALI/ARDS.⁽⁵⁾ APC exerts an anticoagulant effect in the human lung challenged with lipopolysaccharide (LPS).⁽⁶⁾ Notably, APC treatment caused more rapid resolution of respiratory failure during sepsis,⁽⁷⁾ and APC limited both coagulation and influx of neutrophils in the pulmonary compartment in experimental lung injury.⁽⁸⁾ In a recent study in patients with ALI, systemic recombinant human APC treatment decreased pulmonary dead space fraction but not ventilator-free days possibly due to lack of statistical power.⁽⁹⁾ In a limited number of patients with sepsis, lung-protective effects were suggested during treatment with AT⁽¹⁰⁾ but these were not confirmed in a larger phase III clinical trial.⁽²⁾ Continuous infusion of low-dose unfractionated heparin (UFH) did not affect mortality in patients with sepsis;⁽¹¹⁾ however, no subgroup analysis was performed on patients with respiratory failure or ALI/ARDS. In pediatric patients suffering from inhalation injury nebulized heparin seemed to reduce mortality.⁽¹²⁾

Previous animal studies specifically targeting pulmonary coagulopathy showed systemic treatment with anticoagulants to attenuate pulmonary coagulopathy with lung injury.^(13–15) However, systemic treatment with anticoagulants, of course, also affected systemic coagulation. In the present study we investigated the effect of locally administered anticoagulants on pulmonary procoagulant and proinflammatory responses to LPS in a rat model of sepsis-induced lung injury. We hypothesized that nebulized anticoagulants reduce pulmonary coagulopathy while not influencing systemic coagulation, and attenuate pulmonary inflammation in terms of pulmonary neutrophil influx, pulmonary levels of myeloperoxidase (MPO), and proinflammatory cytokines and histopathology.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments. All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care, and handling of laboratory animals.

LPS-induced lung injury

In Male Sprague–Dawley rats (200–250 g) (Harlan, The Hague, The Netherlands) endotoxemia was induced by 7.5 mg/kg LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO), administered into the penile vein under isoflurane (3%) anesthesia.

Study groups

Rats were randomized to placebo (normal saline) ($n=8$) or treatment with one of the following agents: APC

[Drotrecogin alpha (activated), Eli Lilly, Indianapolis, IN] ($n=8$), AT (plasma-derived AT III, Baxter, Vienna, Austria) ($n=6$), heparin (unfractionated heparin, Leo Pharma, Ballerup, Denmark) ($n=7$) or danaparoid (Orgaran, Organon, Oss, The Netherlands) ($n=7$); healthy animals served as controls without endotoxemia ($n=4$). All agents, except for AT, were administered in a volume of 0.7 mL per rat via nebulization 30 min before and at 6 and 12 h after LPS injection. AT was administered only once because of its longer pharmaceutical half-life. Total doses of each agent was based using data from previous studies⁽¹⁴⁾ and pilot studies with LPS-induced lung injury while taking into account the efficacy of a nose-only exposure system. APC, 5000 $\mu\text{g}/\text{kg}$;⁽¹⁶⁾ AT, 500 units/kg;⁽¹⁶⁾ heparin, 1000 IU/kg;⁽¹⁶⁾ Danaparoid 250 anti-Xa E/kg.⁽¹⁷⁾

Nebulization

For local administration of saline or study medication we used an exposure system that allowed direct exposure of nebulized agents to the noses of experimented animals. This system includes a concentric manifold connected to the necks of bottle-like restraint tubes (CHT 249 restraint tube, CH technologies Inc., Westwood, NJ) in which the animals were confined with their noses adjacent to the bottlenecks. The bottles are readily removable and the device can be disassembled, for example, for cleaning, by removing the bottles and removing the manifold. The inhalation chamber is suitable to accommodate several rats at once. One extra outlet is available for measuring the pressure and atmosphere sampling inside the inhalation chamber.

The aerosol atmosphere was generated using the Aeroneb Pro Nebulizer (Aerogen Ltd., Galloway, Ireland). The Aeroneb Pro Nebulizer uses a vibrating mesh with multiple apertures to generate a fine-particle, low-velocity aerosol and produces aerosols with an average size of 2.1 μm . This device has a high efficiency of delivering aerosol to the lung and the volume of drug solution left in these devices when the nebulization had ceased was negligible. The aerosols were directed to the inhalation chamber by a constant oxygen flow (2 L/min).

The animals were accommodated to restraint tubes at several occasions in the week before the experiments. During the experiment spontaneously breathing rats were simultaneously exposed for 10 min to the designated aerosol atmosphere.

Blood and tissue sampling

At 16 h after LPS injection, rats were sacrificed with an intramuscular injection of ketamine 45 mg/kg (Eurovet, Bladel, The Netherlands) and medetomidine 0.25 mg/kg (Novartis, Arnhem, The Netherlands). Blood was collected from the *vena cava inferior* in citrated (0.109 M) vacutainer tubes. The right lung was ligated, and the left lung was lavaged three times with 2-mL ice-cold normal saline. Right lungs were weighed and homogenized in 4 volumes [i.e., 4 $\times$ lung weight (mg) in (L) of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK).

Total cell numbers in each lavage sample were determined by an automated cell counter (Z2 Coulter Particle Counter, Beckman Coulter Corporation, Hialeah, FL). Neutrophil counts in lavage fluids are performed on Giemsa-stained cytospin preparations.

FIG. 1. The effects of anticoagulants on levels of thrombin–antithrombin complexes (**A**, TATc), antithrombin activity (**B**, AT), fibrin degradation products (**C**, FDP), plasminogen activator activity (**D**, PAA), and plasminogen activator activity (**E**, PAI-1) in bronchoalveolar lavage fluid (BALF), 16 h after injection of lipopolysaccharide (LPS, *Escherichia coli* O111:B4). APC, recombinant human activated protein C; AT, plasma-derived human antithrombin; Hep, heparin; Dan, danaparoid. Data represent mean $\pm$ SEM. ** $p < 0.01$ versus placebo.

For coagulation assays, plasma and cell-free supernatants from bronchoalveolar lavage were used. For cytokine and chemokine measurements in lungs, lung homogenates were diluted 1:1 in lysis buffer (150 nmol/L NaCl; 15 mmol/L Tris; 1 mmol/L $\text{MgCl}_2 \cdot \text{H}_2\text{O}$; 1 mmol/L CaCl_2 ; 1% Triton X-100; and 100 $\mu\text{g/mL}$ pepstatin A, leupeptin, and aprotinin).

Assays

Thrombin–antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (FDP; Diagnostica Stago, Asnières-sur-Seine, France) were measured in BALF using ELISA. AT, plasminogen activator activity (PAA), and plasminogen activator inhibitor (PAI)-1 activity were measured by an automated amidolytic assays.⁽¹⁸⁾ Levels of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and cytokine-induced neutrophil chemoattractant (CINC)-3 (R&D Systems, Abingdon, UK) and MPO (HyCult biotechnology b.v., Uden, The Netherlands) were measured in lungs using ELISA.

Histopathology

Sections, 4 μm in thickness, were stained with hematoxylin and eosin and analyzed and scored by two investigators who were blinded for group identity. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following variables: interstitial inflammation, endothelialitis/vasculitis, bronchitis, edema, pleuritis, and thrombus formation, as described previously.⁽¹⁹⁾ Each variable was graded on a scale of 0 to 4 (0, absent; 1, mild; 2,

moderate; 3, severe; 4, very severe). The total histopathology score was expressed as the sum of the scores for all variables.

Statistical analyses

A sample size of seven rats per group was calculated using a power calculation in which the anticipated difference of BALF TATc is 40% with an alpha of 0.05 and a power of 90% and a standard deviation of 30%.

Data were expressed as mean $\pm$ standard error of the mean (SEM) or median with interquartile ranges, as appropriate. Comparisons between the experimental rat groups and saline-treated placebo rat group were performed using one-way analysis of variance (ANOVA) or Kruskal-Wallis test, followed by post hoc Dunnett's or Dunn's tests, depending on data distribution. A p -value < 0.05 is considered statistically significant.

Statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL) and Prism 4.0 (GraphPad Software, San Diego, CA).

Results

None of the animals died before 16 h after LPS administration.

Pulmonary coagulation and fibrinolysis

Bronchoalveolar levels of TATc increased during endotoxemia (Fig. 1A). Nebulization of APC, AT, heparin, danaparoid all limited LPS-induced generation of bronchoalveolar

FIG. 2. The effects of anticoagulants on plasma levels of thrombin-antithrombin complexes (A, TATc) and systemic plasminogen activator activity (B, PAA), 16 h after injection of lipopolysaccharide (LPS, *Escherichia coli* O111:B4). APC, recombinant human activated protein C; AT, plasma-derived human antithrombin; Hep, heparin; Dan, danaparoid. Data represent mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ versus placebo.

TATc ($p < 0.01$ vs. placebo) and FDP ($p < 0.01$ vs. placebo, Fig. 1B–C). Administration of AT increased pulmonary AT levels by more than twofold compared to baseline ($p < 0.01$ vs. placebo). Pulmonary PAA was significantly reduced by endotoxemia, with concurrently enhanced PAI-1 activity in lungs (Fig. 1D). None of the treatments altered bronchoalveolar fibrinolysis (PAA, Fig. 1D; PAI-1 activity, Fig. 1E).

Systemic coagulation and fibrinolysis

Compared to controls, LPS injection caused an increase of plasma levels of TATc 16 h after LPS injection. Bronchoalveolar treatment with APC and AT did not affect plasma TATc levels (Fig. 2A). However, both heparin and danaparoid attenuated plasma TATc levels 16 h after LPS injection ($p < 0.05$ and $p < 0.01$ vs. placebo, respectively). Compared to controls, plasma PAA was significantly decreased after LPS injection. Treatment with APC, AT, heparin, or danaparoid left plasma PAA unaltered (Fig. 2B).

Inflammatory response

There was a significant increase in lung to body weight ratio after LPS administration compared to healthy animals (Fig. 3). However, no differences were observed in lung-to-body weight between treatments. There was an evident increase in total cell count in the lungs after endotoxemia, mostly attributed to neutrophil influx (Table 1). Only heparin

treatment led to an attenuated number of BALF cells. Although neutrophil counts in BALF were lower in the heparin group they did not significantly differ from placebo-treated animals (placebo vs. heparin, $p = 0.06$), nor did neutrophil counts in any other treatment group. Endotoxemia significantly increased MPO levels compared to healthy controls and was not affected by any treatment. Lung levels of TNF, IL-6, and CINC-3 were variable and no differences in pulmonary levels of these cytokines were seen with the tested anticoagulant treatments (Fig. 4). There were no statistically significant differences between the groups with regard to histopathology scores (Fig. 5).

Discussion

We show that nebulization of various other anticoagulant agents alters LPS-induced bronchoalveolar coagulation in rats. Although local treatment with APC or AT leaves systemic thrombin generation unaltered, nebulized heparin and danaparoid also significantly reduced systemic thrombin generation. The inflammatory response in the lungs as assessed by pulmonary neutrophil influx into the lungs, bronchoalveolar levels of myeloperoxidase, bronchoalveolar levels of TNF, IL-6, and CINC-3, and histopathology was left unaltered. Although it is possible other endpoints would have revealed anti-inflammatory effects, our data suggests that inflammation was not significantly directed by changes

FIG. 3. Weight loss (A) and lung weight/body weight ratio (B) after sacrifice, 16 h after injection of lipopolysaccharide (LPS, *Escherichia coli* O111:B4). Data represent median $\pm$ IQR.

TABLE 1. TOTAL CELL AND NEUTROPHIL COUNTS IN BRONCHOALVEOLAR LAVAGE FLUID OF RATS

	Total cells	Neutrophils	MPO ($\mu\text{g/mL}$)
Controls	21 (19–24)	0 (0–0)	32 (20–59)
Endotoxemia ($t = 16$ h)			
Placebo	89 (59–129)	35 (16–42)	353 (270–439)
APC	92 (53–123)	22 (0–41)	308 (224–471)
AT	64 (46–73)	19 (4–34)	354 (326–429)
Heparin	39 (21–46) ^a	12 (2–17)	323 (282–372)
Danaparoid	50 (43–67)	15 (6–16)	391 (318–467)

Cell counts 16 h after injection with lipopolysaccharide (*Escherichia coli* O111:B4). Controls are healthy rats ($n = 4$). Data are expressed as median (interquartile range) $\times 10^4$ per milliliter of bronchoalveolar lavage fluid.

^a $p < 0.01$ versus placebo according to Kruskal-Wallis and Dunn's posttests.

in pulmonary coagulation itself, at least not in this rat model of LPS-induced lung injury.

Various models of LPS-induced lung injury show that AT and APC are able to reduce pulmonary coagulopathy as well as inflammation and to improve oxygenation.^(8,20–22) In our experiments, the inflammatory response was left unaltered in all treatment groups. This discrepancy with previous studies may be caused by the fact that our model was designed to investigate the effects of nebulized anticoagulants on pulmonary coagulopathy. It may not have been suitable to pick up the effect on inflammation due to differences in LPS delivery, species, investigated time points, and the subsequent different roles for neutrophil involvement in the experimental models. Of note, in most previous investigations, inflammatory effects are investigated in the hyperacute phase after LPS challenge (e.g., after 1 h). Our current results illustrate that it is relevant to investigate later time points as well. It is also possible our study did not have sufficient statistical power to detect any effects on inflammation. However, no trend was observed favoring any treatment group, so if any effect on inflammation exists it is assumed to be relatively small and may be of limited clinical relevance.

We previously showed that systemic application of APC or AT alters bronchoalveolar coagulation during experimental endotoxemia in rats.⁽¹⁴⁾ In that study systemically administered heparin, however, did not alter pulmonary coagulopathy. Here we show that the effect of nebulized

heparin, or danaparoid, equals the anticoagulant effects in the lungs of nebulized APC or AT.

Besides theoretical considerations that intervening in pulmonary coagulopathy may be beneficial for patients with ALI/ARDS substantial *in vitro* evidence indicates that unfractionated heparin, besides potent anticoagulant effects, has a wide range of anti-inflammatory and immunomodulatory effects.^(23–25) A study in pediatric patients with inhalation injuries revealed that combined heparin nebulization significantly reduced mortality.⁽¹²⁾ However, in this study, patients were also treated with N-acetylcysteine, which may have contributed to the favorable outcome.

Systemic administration of anticoagulant compounds substantially increases the risk of bleeding. Indeed, in the pivotal APC trial in patients with severe sepsis the incidence of serious bleeding was higher in the treatment group than in the placebo group (3.5 vs. 2.0%).⁽³⁾ The effect of systemically administered high-dose AT on the occurrence of bleeding events in patients with severe sepsis, especially when combined with heparin was even more outspoken (22.0 vs. 12.8%).⁽²⁾ With local delivery as employed in our experiments, higher local drug concentrations may be attained while reducing the risk of systemic bleeding. The fact that locally administered APC and AT in our experiments significantly reduces pulmonary coagulopathy without systemic effects on coagulation or fibrinolysis may be of clinical importance for future studies in patients with ALI/ARDS.

The effects on systemic coagulation seem to be dependent on the dose and or agent. It seems nebulized heparin and danaparoid leak from the pulmonary compartment into the circulation. Because their molecules are relatively small (8 and 5.5 kDa, respectively) they are more likely to leak into the circulation than APC (56 kDa) and AT (58 kDa). In a clinical study published previously nebulization of heparin dose lengthened clotting time only after > 24 h,⁽²⁶⁾ which suggests heparin may be stored in pulmonary endothelial cells and metabolized by heparinases, initially limiting heparin reaching the systemic circulation. These processes may become saturated with repeated heparin doses.⁽²⁷⁾

Our study has important limitations. First, the chosen dosages for each agent were determined based on data from previous studies and pilot studies combined with the efficacy of our nose-only exposure system, the expected biological availability, and possibility of dissolving each agent to an acceptable volume for nebulization. For all studied agents this

FIG. 4. Tumor necrosis factor- α (TNF- α) (A), interleukin (IL)-6 (B), and cytokine-induced neutrophil chemoattractant (CINC)-3 (C), determined in lung homogenates 16 h after injection of lipopolysaccharide (LPS, *Escherichia coli* O111:B4). APC, recombinant human activated protein C; AT, plasma-derived human antithrombin; Hep, heparin; Dan, danaparoid. Data represent median $\pm$ interquartile range, IQR.

FIG. 5. Histopathologic changes. Representative hematoxylin and eosin-stained photomicrographs (original magnification, $\times 100$) of lung tissue from rats treated with saline (placebo) (A), recombinant human activated protein C (APC) (B), plasma-derived antithrombin (AT) (C), heparin (Hep) (D), danaparoid (Dan) (E), at 16 h after injection of lipopolysaccharide (LPS, *Escherichia coli* O111:B4). (F) Total histopathologic scores are presented as median with interquartile range.

approach lead to a significant attenuation of pulmonary coagulopathy; however, lower dosages may also have been sufficient while further preventing the systemic effects on coagulation, in particular of heparin and danaparoid. However, anti-inflammatory effects by these agents may require higher doses than required for anticoagulant use. Moreover, due to differences between the studied drugs, aerosol characteristics may differ between the treatment groups. Second, in our study the agents were administered intermittently in 6-h intervals. It may well be that this regimen is able to limit bronchoalveolar coagulation; however, for anti-inflammatory effects (e.g., neutrophil migration) continuous administration is needed to maintain constant pulmonary drug levels.

Another limitation of the study is the rat model of endotoxemia. Lung injury models with either direct endotracheal instillation of endotoxin or via endotoxemia are routinely used in experimental studies due to their relative ease and good reproducibility. However, LPS-induced lung injury is a simplified model of patients with pneumonia or sepsis with ALI/ARDS. Despite the limitations of our model, our results are in line with previous investigations from our group⁽¹⁴⁾ and data from a study in patients after abdominal surgery for secondary peritonitis. In that study, patients demonstrate a short-lived systemic inflammatory response syndrome after the surgical procedure, followed by a profound procoagulant response in the lungs.⁽²⁸⁾ Our model mimics this clinical pattern.

In general, our animal model does not account for the heterogeneous etiology of ALI/ARDS in patients as well as clinical factors, such as antimicrobial therapy, mechanical ventilation, fluid management, and other supportive interventions.

Possibly due to its design, our study did not reveal clinically relevant benefit of local treatment with nebulized anticoagulants; however, our study did demonstrate nebulization of anticoagulants to be feasible. Clinical trials are required to

further investigate the safety and efficacy of nebulized anticoagulants in patients with ALI/ARDS. In these trials, especially when using anticoagulant compounds with a low molecular weight, due consideration must be given to systemic (anticoagulant) effects.

In conclusion, recombinant-human activated protein C, plasma-derived antithrombin, heparin, or danaparoid attenuate pulmonary coagulopathy, but not inflammation, in rats challenged with LPS.

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Author Disclosure Statement

No conflicts of interest exist.

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