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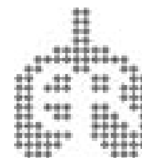

Natural anticoagulants limit lipopolysaccharide-induced pulmonary coagulation but not inflammation

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ABSTRACT: **Pulmonary coagulopathy and hyperinflammation may contribute to an adverse outcome in sepsis. The present study determines the effects of natural inhibitors of coagulation on bronchoalveolar haemostasis and inflammation in a rat model of endotoxaemia.**

Male Sprague-Dawley rats were randomised to treatment with normal saline, recombinant human activated protein C (APC), plasma-derived antithrombin (AT), recombinant human tissue factor pathway inhibitor (TFPI), heparin or recombinant tissue plasminogen activator (tPA). Rats were intravenously injected with lipopolysaccharide (LPS), which induced a systemic inflammatory response and pulmonary inflammation. Blood and bronchoalveolar lavage were obtained at 4 and 16 h after LPS injection, and markers of coagulation and inflammation were measured.

LPS injection caused an increase in the levels of thrombin–AT complexes, whereas plasminogen activator activity was attenuated, both systemically and within the bronchoalveolar compartment. Administration of APC, AT and TFPI significantly limited LPS-induced generation of thrombin–AT complexes in the lungs, and tPA stimulated pulmonary fibrinolytic activity. However, none of the agents had significant effects on the production of pulmonary cytokines, chemokines, neutrophil influx and myeloperoxidase activity.

Natural inhibitors of coagulation prevent bronchoalveolar activation of coagulation, but do not induce major alterations of the pulmonary inflammatory response in rat endotoxaemia.

KEYWORDS: **Acute lung injury, coagulation, fibrinolysis, protein C, sepsis**

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STATEMENT OF INTEREST
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Severe sepsis is a clinical syndrome characterised by a systemic inflammatory response and activation of coagulation, potentially leading to intravascular depositions of fibrin and microvascular thrombosis, or bleeding related to uncontrolled consumption of coagulation factors [1]. Under physiological circumstances, the activation of coagulation is regulated by natural inhibitors of coagulation, *i.e.* activated protein C (APC), antithrombin (AT) and tissue factor pathway inhibitor (TFPI). In sepsis, these anticoagulant systems are impaired, most likely due to massive consumption and downregulation by inflammatory mediators, which form the rationale for therapeutic restoration of these natural anticoagulant pathways [2]. Unfortunately, both plasma-derived AT [3] and recombinant human (rh)TFPI [4] have failed to reduce patient mortality in severe sepsis. The pivotal phase III clinical trial with rhAPC showed a significant increase in patient survival [5], but there is ongoing debate about the exact mechanisms by which APC prevents death.

One of the proposed mechanisms by which APC exerts its protective effects relates to pathways involving the lungs. The lungs are the most frequently involved organ system in sepsis-related multiple organ failure. Notably, APC treatment causes more rapid resolution of respiratory failure during sepsis [6] and limits both coagulation and influx of neutrophils in the pulmonary compartment in experimental lung injury [7, 8]. In a relatively limited number of patients with sepsis, lung-protective effects have also been suggested during treatment with AT [9] and TFPI [10], but these were not confirmed in larger phase III clinical trials [3, 4]. In the current study, using a rat model of endotoxaemia, it was hypothesised that APC, AT and TFPI would have differential effects on pulmonary coagulation and inflammation, potentially explaining different outcomes in human sepsis.

MATERIAL AND METHODS

Rats

Male Sprague-Dawley rats (200–250 g) were purchased from Harlan (The Hague, the Netherlands). The rats were allowed to acclimatise to laboratory conditions for ⩾7 days (12/12 h day–night cycle at 22°C). The Institutional Animal Care and Use Committee of the Academic Medical Centre (University of Amsterdam, Amsterdam, the Netherlands) 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.

Study design

Endotoxaemia was induced by administration of 7.5 $mg \cdot kg^{-1}$ lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO, USA) into the penile vein under isoflurane (3%) anaesthesia. Pilot studies with this model demonstrated that LPS injection led to a transient hyperacute systemic inflammatory response, with systemic tumour necrosis factor (TNF)-α, interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-3 levels peaking within 1–2 h. Thereafter, these pro-inflammatory mediators were undetectable in the circulation, with prolonged effects on systemic and pulmonary coagulopathy ⩽24 h. No major histopathological changes in lung tissue were observed, other than neutrophil infiltration at 16 h after LPS injection. Rats were randomised to placebo (normal saline) or treatment with one of the natural anticoagulants: APC, AT or TFPI (n=8 per group). Additionally, heparin and tissue plasminogen activator (tPA) were involved in the randomisation process. Four healthy rats without endotoxaemia were used as controls. All agents were administered in bolus injections of 2 $mL \cdot kg^{-1}$, 30 min before injection of LPS. Therapeutic doses were determined using data from previous studies [4, 10–14]. All the agents and doses that were used are described in table 1. Considering the plasma clearance, rats sacrificed at 16 h after LPS injection received additional injections of APC, TFPI, heparin or tPA at 6 and 12 h. All rats were administered the same volume of fluid (2 $mL \cdot kg^{-1}$) at 6 and 12 h.

Rats were sacrificed with intraperitoneal injections of ketamine 80 $mg \cdot kg^{-1}$ (Eurovet, Bladel, the Netherlands) and medetomidine 0.5 $mg \cdot kg^{-1}$ (Novartis, Arnhem, the Netherlands) at 4 and 16 h after LPS injection. Blood was collected from the inferior vena cava 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 homogenised in four volumes (*i.e.* four times lung weight (in mg), in μL) of sterile saline using a tissue homogeniser (Biospec Products, Bartlesville, OK, USA). Total cell numbers in each lavage sample were determined by an automated cell counter (Coulter Counter; Coulter Electronics, Hialeah, FL, USA). Neutrophil counts in lavage fluids were performed on Giemsa-stained cytospin preparations.

For coagulation assays, plasma and cell-free supernatants from bronchoalveolar lavage were used. For cytokine and chemokine measurements in lungs, supernatants were used from lung homogenates which were diluted 1:1 in lysis buffer (150 $nmol \cdot L^{-1}$ NaCl; 15 $mmol \cdot L^{-1}$ Tris; 1 $mmol \cdot L^{-1}$ $MgCl_2$-H_2O; 1 $mmol \cdot L^{-1}$ $CaCl_2$; 1% Triton X-100; and 100 $\mu g \cdot mL^{-1}$ pepstatin A, leupeptin and aprotinin).

Assays

Thrombin-antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (FDP; Asserachrom D-Di; Diagnostica Stago, Asnières-sur-Seine, France) were measured using ELISA. AT, plasminogen activator activity (PAA) and plasminogen activator inhibitor (PAI)-1 activity were measured by automated amidolytic assays [15–17]. Levels of TNF-α, IL-6 and CINC-3 were measured using ELISA (R&D Systems, Abingdon, UK). Myeloperoxidase (MPO) activity was determined by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine, and was expressed as activity per gram of lung tissue [18].

Statistical analysis

All data are expressed as mean±SEM or median (interquartile range), as appropriate. Comparisons between the experimental groups and the saline-treated placebo group were performed using one-way ANOVA or the Kruskal–Wallis test, followed by *post hoc* Dunnett's or Dunn's tests, depending on data distribution. A p-value <0.05 was considered statistically significant.

RESULTS

Coagulation and fibrinolysis

Compared with controls which were not injected with LPS, endotoxaemia caused increased generation of TATc (fig. 1), an effect which was attenuated by APC, AT and TFPI at both 4 and 16 h after LPS injection (fig. 1a). Plasma PAA was significantly decreased after LPS injection; tPA increased PAA to levels exceeding assay maximum at both time-points ($p<0.001$ *versus* saline; fig. 1b). With AT treatment, plasma PAA was also significantly higher than with saline at both time-points ($p<0.01$ *versus* saline; fig. 1b).

TABLE 1 Pharmaceutical agents and doses used

Biological equivalent	Abbreviation	Agent	Manufacturer	Dose	[Ref.]
Activated protein C	APC	Drotrecogin alpha (activated)	Eli Lilly (Indianapolis, IN, USA)	500 $\mu g \cdot g^{-1}$	[12]
Antithrombin III	AT	Plasma-derived antithrombin III	Baxter (Vienna, Austria)	250 $U \cdot kg^{-1}$	[12, 13]
Tissue factor pathway inhibitor	TFPI	Tifacogin	Chiron (Emeryville, CA, USA)	1.5 $mg \cdot kg^{-1}$	[4, 10, 11]
Heparan sulfate	Heparin	Unfractionated heparin	Leo Pharma (Ballerup, Denmark)	300 $U \cdot kg^{-1}$	[12]
Tissue-type plaminogen activator	tPA	Tenecteplase	Boehringer Ingelheim (Ingelheim, Germany)	1.25 $mg \cdot kg^{-1}$	[14]

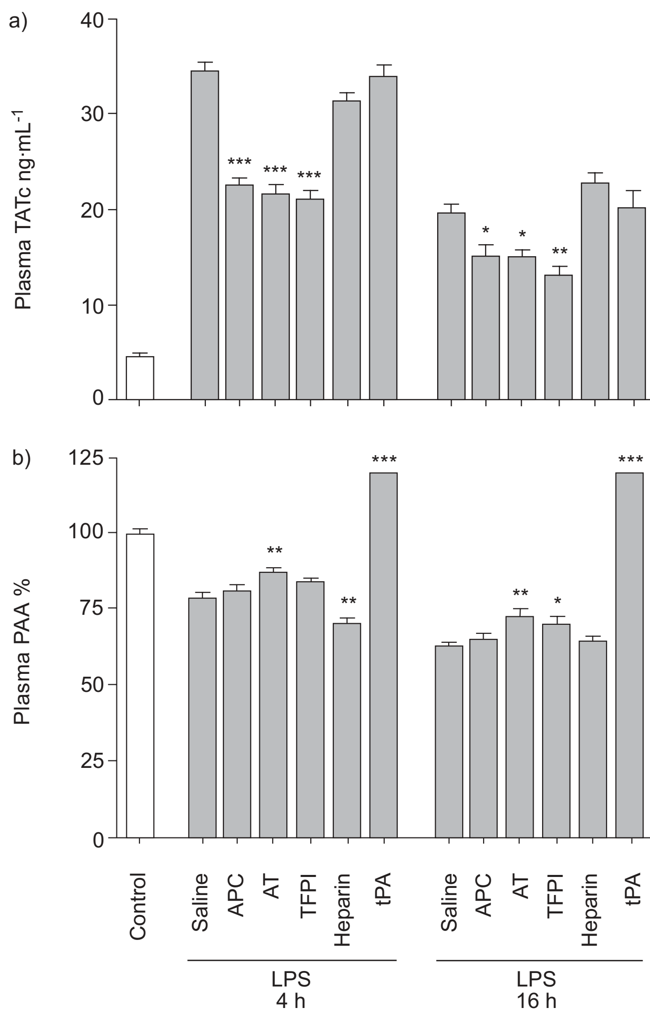

FIGURE 1. The effects of anticoagulants 4 and 16 h after injection of lipopolysaccharide (LPS; *Escherichia coli* O111:B4) on plasma levels of a) thrombin-antithrombin complexes (TATc) and b) systemic plasminogen activator activity (PAA). Control, n=4; saline, n=11 in the LPS 4 h group and n=12 in the LPS 16 h group; recombinant human activated protein C (APC), n=8; plasma-derived human antithrombin (AT), n=8; recombinant human tissue factor pathway inhibitor (TFPI), n=8; heparin, n=8; tissue-type plasminogen activator (tPA), n=8. Error bars represent SEM. The p-values were calculated according to Dunnett's test. *: $p<0.05$ *versus* saline; **: $p<0.01$ *versus* saline; ***: $p<0.001$ *versus* saline.

Furthermore, bronchoalveolar levels of TATc were increased by endotoxaemia (fig. 2). APC, AT and TFPI limited LPS-induced generation of bronchoalveolar TATc ($p<0.001$ *versus* saline for both time-points; fig. 2a), thereby preventing a decrease in AT activity and limiting FDP generation (figs 2b and c). Heparin treated rats also showed less bronchoalveolar TATc but only at 4 h (fig. 2a), while AT activity was not different from saline treated rats (fig. 2b). Pulmonary PAA in lungs was significantly reduced by endotoxaemia, with concurrently enhanced PAI-1 activity in lungs (fig. 3). tPA increased bronchoalveolar PAA and FDP levels (both $p<0.001$ *versus* saline for both time-points; figs 3a and 2c), while abolishing enhanced PAI-1 activity ($p<0.001$ *versus* saline for both time-points; fig. 3b).

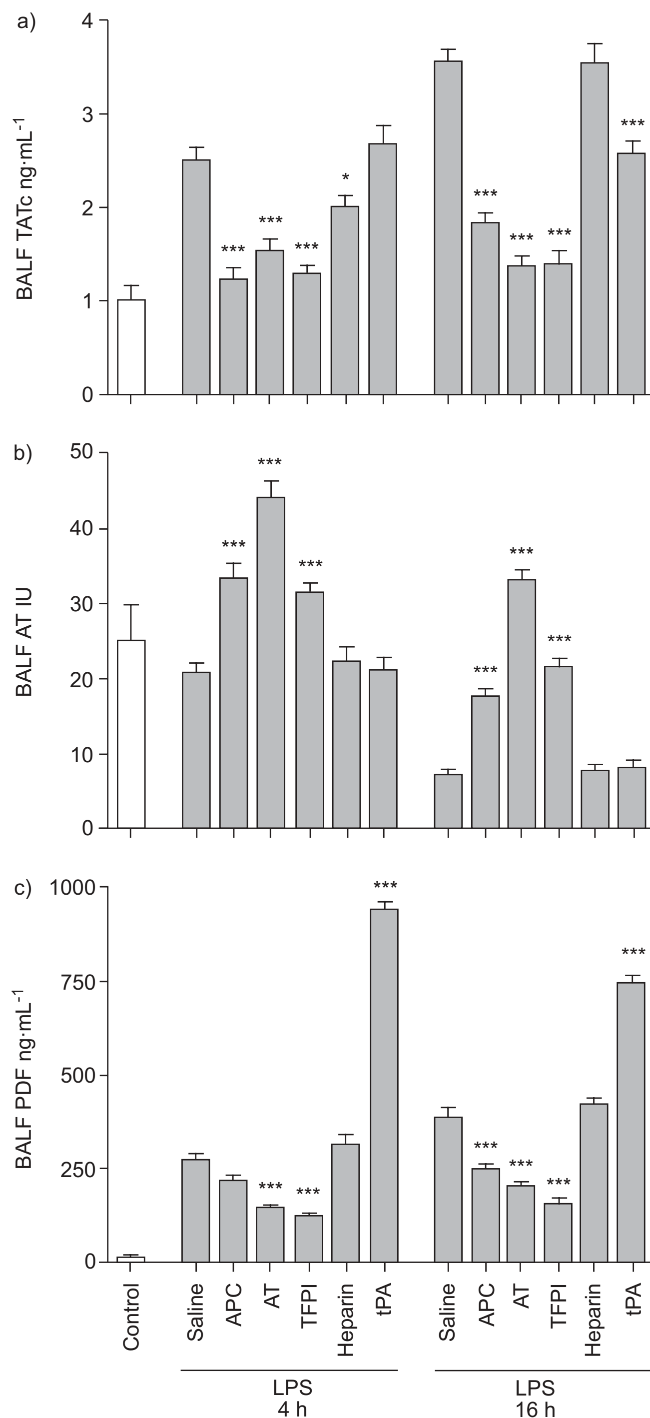

FIGURE 2. The effects of anticoagulants 4 and 16 h after injection of lipopolysaccharide (LPS; *Escherichia coli* O111:B4) on levels of a) thrombin-antithrombin complexes (TATc), b) antithrombin activity (AT) and c) fibrin degradation products (FDP) in bronchoalveolar lavage fluid (BALF). Control, n=4; saline, n=11 in the LPS 4 h group and n=12 in the LPS 16 h group; recombinant human activated protein C (APC), n=8; plasma-derived human antithrombin (AT), n=8; recombinant human tissue factor pathway inhibitor (TFPI), n=8; heparin, n=8; tissue-type plasminogen activator (tPA), n=8. Error bars represent SEM. The p-values were calculated according to Dunnett's test. *: $p<0.05$ *versus* saline; ***: $p<0.001$ *versus* saline.

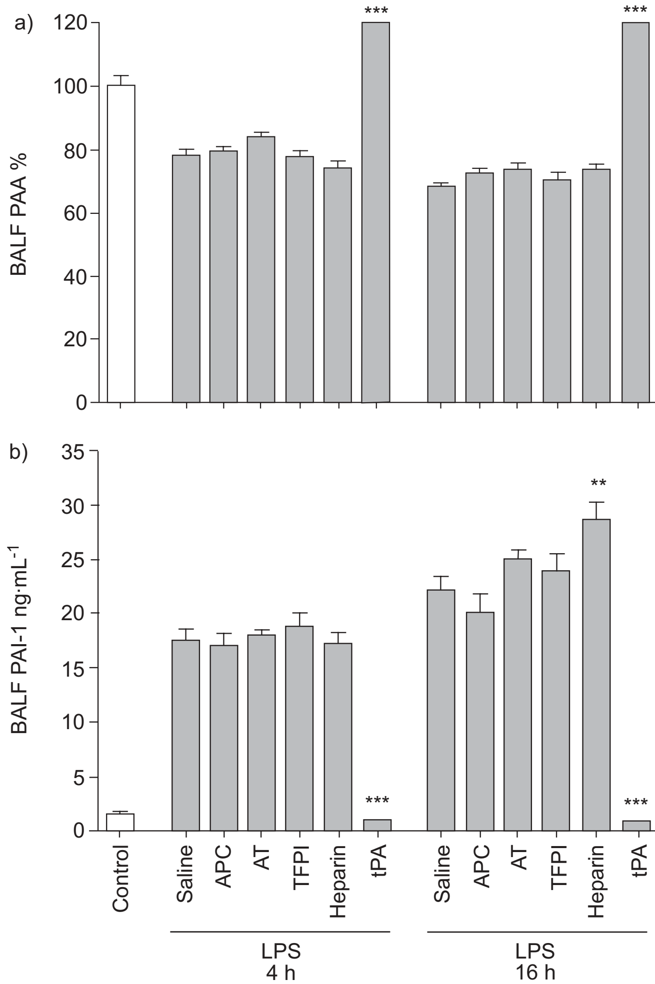

FIGURE 3. The effects of anticoagulants 4 and 16 h after injection of lipopolysaccharide (LPS; *Escherichia coli* O111:B4) on a) plasminogen activator activity (PAA) and b) levels of plasminogen activator inhibitor type 1 (PAI-1) in bronchoalveolar lavage fluid (BALF). Control, n=4; saline, n=11 in the LPS 4 h group and n=12 in the LPS 16 h group; recombinant human activated protein C (APC), n=8; plasma-derived human antithrombin (AT), n=8; recombinant human tissue factor pathway inhibitor (TFPI), n=8; heparin, n=8; tissue-type plasminogen activator (tPA), n=8. Error bars represent SEM. The p-values were calculated according to Dunnett's test. **: p<0.01 *versus* saline; ***: p<0.001 *versus* saline.

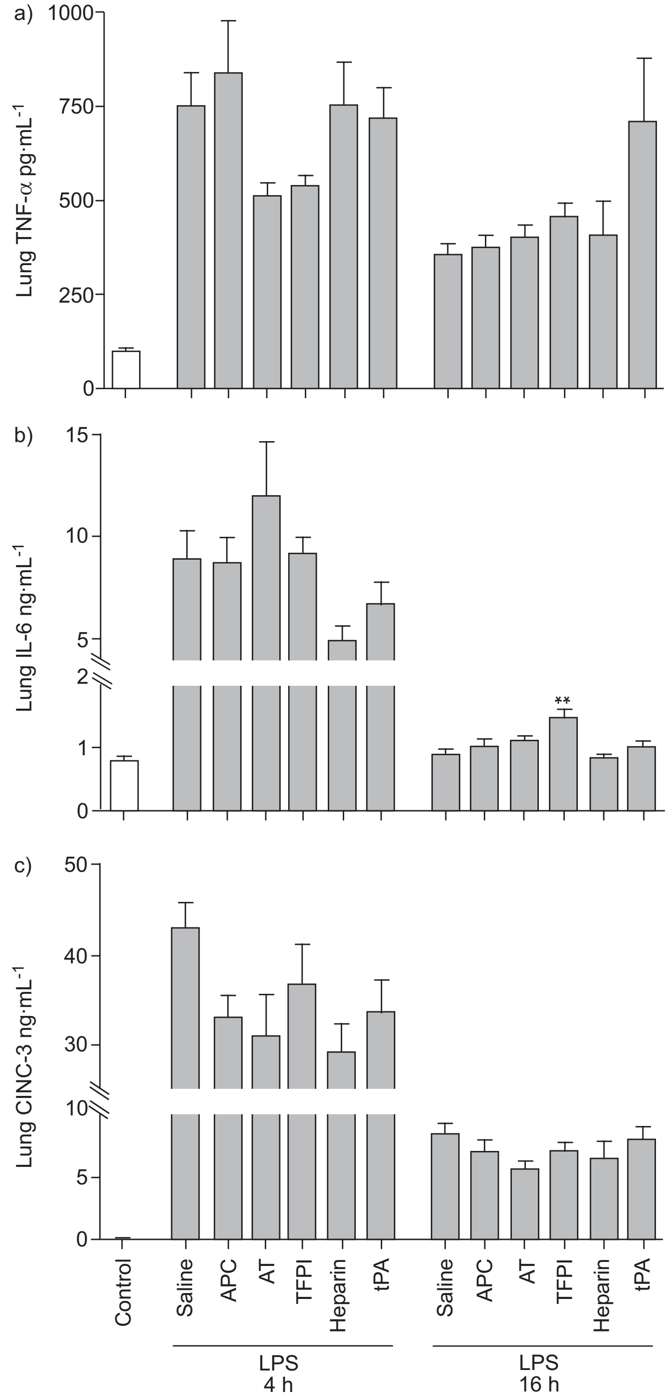

FIGURE 4. a) Tumour necrosis factor (TNF)-α, b) interleukin (IL)-6 and c) cytokine-induced neutrophil chemoattractant (CINC)-3, determined in lung homogenates 4 and 16 h after injection of lipopolysaccharide (LPS; *Escherichia coli* O111:B4). Control, n=4; saline, n=11 in the LPS 4 h group and n=12 in the LPS 16 h group; recombinant human activated protein C (APC), n=8; plasma-derived human antithrombin (AT), n=8; recombinant human tissue factor pathway inhibitor (TFPI), n=8; heparin, n=8; tissue-type plasminogen activator (tPA), n=8. Error bars represent SEM. The p-values were calculated according to Dunn's test. **: p<0.01 *versus* saline.

Inflammatory response

During rat endotoxaemia, TFPI treatment enhanced IL-6 generation in lungs at 16 h (p<0.01 *versus* saline; fig. 4b); all other agents did not induce significant changes in pulmonary levels of TNF-α, IL-6 and CINC-3. Inflammatory cell influx into the lungs was not observed at 4 h after LPS injection. There was an increase in neutrophil counts in the lungs during endotoxaemia at 16 h, but there were no differences in the number of neutrophils between the anticoagulant groups and the saline group (table 2). Finally, MPO in lung tissue was not influenced by any treatment (table 2).

DISCUSSION

The present authors have shown that the application of systemic anticoagulant agents alters bronchoalveolar coagulation during endotoxaemia. APC, AT and TFPI had major effects on limiting thrombin generation, both systemically and in the bronchoalveolar compartment. However, the various agents

TABLE 2 Total cell[#] and neutrophil counts in bronchoalveolar lavage fluid (BALF)/myeloperoxidase (MPO) activity in lungs

	Subjects n	Total cells	Neutrophils	MPO[¶]
Controls[+]	4	18.2 (15.4–20.5)	0	1.8 (1.1–2.8)
Endotoxaemia (t=16 h)				
Saline	12	38.2 (27.7–55.4)[§]	2.7 (0.1–20.2)	14.0 (11.7–16.2)
APC	8	32.0 (26.3–47.3)	8.9 (4.0–37.2)	11.5 (8.5–14.7)
AT	8	50.1 (32.9–71.9)	4.3 (3.8–23.8)	12.0 (10.4–13.9)
TFPI	8	39.1 (36.9–46.7)	6.1 (4.1–24.1)	14.6 (13.2–14.8)
Heparin	8	22.5 (20.5–41.9)	7.4 (2.1–14.6)	13.4 (12.3–18.8)
tPA	8	21.5 (18.1–28.9)	5.3 (1.3–11.6)	14.2 (12.4–15.7)

Data are expressed as median (interquartile range) $\times 10^4$ per mL of BALF. APC: recombinant human activated protein C; AT: plasma-derived antithrombin; TFPI: recombinant human tissue factor pathway inhibitor; tPA: recombinant tissue-type plasminogen activator. [#]: cell counts were performed 16 h after injection with lipopolysaccharide (*Escherischia coli* O111:B4); [¶]: measured in units per lung weight (in g); [+]: controls are uninfected rats; [§]: p=0.008 and p>0.05 (all *versus* saline) according to Kruskal–Wallis and Dunn's post-tests, respectively.

had very limited effects on pulmonary inflammation, suggesting that the inflammatory response is not significantly directed by changes in pulmonary coagulation itself, at least not in the present rat model of endotoxaemia. The question ''what mechanisms account for differences in clinical outcome with various anticoagulant strategies?'' remains unresolved.

In the pathogenesis of endotoxin-induced lung injury, an important role has been attributed to neutrophils. UCHIBA *et al.* [19] demonstrated that endotoxin-induced pulmonary vascular injury in Wistar rats is mainly mediated by activated neutrophils. Using this model, it was shown that APC [20], AT with or without heparin co-administration [21], and TFPI [15] had inhibitory effects on activated neutrophils, thereby limiting neutrophil accumulation, MPO activity and cytokine generation in the lungs in the first hour after endotoxin administration. Also, it was shown that neutrophil influx is significantly inhibited by rhAPC infusion in healthy volunteers challenged with intrapulmonary endotoxin [7]. In the present rat model, neutrophil accumulation and MPO activity in the lungs were not significantly altered by any of the agents administered. This discrepancy with previous studies may have been caused by differences in endotoxin delivery, species, investigated time-points and the subsequent different roles for neutrophil involvement in the experimental models. It is noteworthy that in most previous studies, inflammatory effects were investigated in the hyperacute phase after endotoxin challenge (*e.g.* 1 h). The present results illustrate that it is relevant to include later time-points in which the local host response may significantly be altered, although systemic effects have subsided.

Heparin is a broadly used anticoagulant that acts by binding AT and facilitating its anticoagulant activity. Heparin had been shown to exert lung protective effects during endotoxaemia in sheep [16], while low molecular weight heparin had been able to limit lung injury in endotoxaemic pigs [17] and mice [18]. In the present experiment, heparin had a mild anticoagulant effect and a modest antifibrinolytic effect: pulmonary TATc and systemic PAA were decreased at 4 h after LPS injection, while pulmonary PAI-1 activity was increased after 16 h. This is in contrast with *in vitro* data suggesting that heparins have pro-fibrinolytic effects [22]. The limited anticoagulant effects of heparin may have been caused by inadequate dosing, but it is also possible that increased AT consumption had led to impaired anticoagulant activity. Notably, treatment with heparin did not result in altered inflammatory response within the lungs. Opposing the anticoagulant strategies, tPA was used to stimulate fibrinolysis. Increased fibrinolytic activity did not affect neutrophil influx into the lungs, nor did it alter generation of inflammatory mediators.

A most important limitation of the study is the rat model of endotoxaemia. Lung injury models with either direct endotracheal instillation of endotoxin or *via* endotoxaemia have routinely been used in experimental studies due to its relative ease and good reproducibility. It should be noted that endotoxin-induced lung injury is a simplified model of patients with pneumonia or sepsis with acute lung injury. If acute lung injury is related to infectious processes, interference with coagulation could theoretically limit containment of the primary infection, promoting microbial dissemination [23]. Furthermore, anticoagulants were delivered by bolus injections, instead of continuous intravenous infusion. It may well be that bolus injections of anticoagulants are able to limit bronchoalveolar coagulation; however, for anti-inflammatory effects (*e.g.* neutrophil migration) [7] continuous infusion is needed to maintain constant plasma levels. Despite the limitations of the present model, it should be noted that the current results are in line with data from a study in patients after abdominal surgery for secondary peritonitis [24]. In that study, patients demonstrated a short-lived systemic inflammatory response syndrome after the surgical procedure, which was followed by a profound pro-coagulant response in the lungs [24]. The present model mimics this clinical pattern, at least in part: rat endotoxaemia also caused a transient systemic inflammatory response, while inducing pulmonary coagulopathy at later time-points. This finding is of interest, especially in the context of multiple injurious processes that patients may encounter during critical illness.

In conclusion, it has been demonstrated that systemic application of natural anticoagulants significantly inhibits bronchoalveolar activation of coagulation in a rat model of

endotoxaemia, but does not lead to distinct effects on cytokine and chemokine production or neutrophil migration and activity. It remains uncertain whether controlled pulmonary coagulation significantly contributes to patient outcome and thus needs to be established in appropriate clinical trials.

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