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Mechanical ventilation affects alveolar fibrinolysis in LPS-induced lung injury

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Abstract

Objective: To determine the effects of mechanical ventilation on alveolar fibrin turnover in lipopolysaccharide (LPS)-induced lung injury.

Materials and Methods: Randomized controlled trial. After intratracheal LPS (*Salmonella enteritidis*) instillations Sprague-Dawley rats (n=61) were allocated to 3 ventilation groups. Group I: positive inspiratory pressure (PIP) 16 cm H_2O/positive end-expiratory pressure (PEEP) 5 cm H_2O; group II: PIP 26 cm H_2O/PEEP 5 cm H_2O and group III: PIP 35 cm H_2O/PEEP 5 cm H_2O. Control rats (not mechanically ventilated) received LPS. Healthy rats served as a reference group. Thrombin anti-thrombin complex (TATc), D-dimer, plasminogen activator inhibitor (PAI) activity and PAI-1 antigen levels in broncho-alveolar lavage fluid (BALF) were measured.

Results: LPS-induced lung injury increased TATc, D-dimer and PAI activity and PAI-1 antigen levels vs. healthy animals. High pressures amplitude ventilation increased TATc concentrations ($p<0.05$). D-dimer concentrations were not significantly higher. Instead, PAI activity levels increased with the size of the pressure amplitude: group I 0.7 U/mL, group II 3.4 U/mL and group III 5.0 U/mL ($p<0.05$), with no change in PAI-1 antigen levels.

Conclusions: Mechanical ventilation creates an alveolar/pulmonary antifibrinolytic milieu in endotoxin-induced lung injury which, at least in part, might be due to an increase in PAI activity.

Key words Adult respiratory distress syndrome; mechanical ventilation; animal experimentation; fibrinolysis; endotoxin

INTRODUCTION

Intraalveolar fibrin depositions are the pathognomonic hallmark of acute lung injury (ALI) on lung microscopy (1-3). The fibrin matrix inactivates and incorporates surfactant leading to severe hypoxemic respiratory failure (4, 5). Alveolar fibrin is part of the inflammatory response seen in ALI and may initiate fibrotic repair with long-term compromised pulmonary function (6, 7). Intraalveolar fibrin formation in ALI occurs after capillary alveolar leakage of plasma fibrinogen, activation of coagulation and suppression of local fibrinolysis (7-10). Alveolar macrophages and alveolar epithelial cells are directly stimulated by bacterial endotoxins or indirectly by pro-inflammatory mediators (e.g., tumor necrosis factor (TNF)-α) to produce pro-coagulant and anti-fibrinolytic proteins. On the pro-coagulant side activated factor VII and tissue factor, and on the anti-fibrinolytic side plasminogen activator inhibitor (PAI)–1 are the main mediators of disturbed fibrin turnover. The degree of alveolar fibrin formation and the persistence of fibrin mainly depend on suppressed fibrinolytic capacity due to increased local production of PAI–1 (3, 7-9, 11-15). Under normal circumstances, intraalveolar fibrin is resolved within minutes by plasmin (16) and intact surfactant is released, restoring pulmonary function (17). However, during ALI alveolar fibrin turnover is disturbed and aggravated by additional insults (e.g., hemorrhagic shock, infections, ventilator-associated pneumonia) (18, 19). We recently demonstrated that injurious mechanical ventilation can depress alveolar fibrinolytic capacity in healthy rats after iatrogenic fibrin formation (20). In the present study, we examined the effects of different ventilation strategies on alveolar fibrinolysis in rats with “pre-injured” lungs due to endotoxin-induced lung injury.

MATERIALS AND METHODS

The study was approved by the Animal Committee of the Erasmus University Rotterdam. Care and handling of the animals were in accordance with the European Community guidelines. The experiments were performed at the Department of Anesthesiology, Erasmus MC-Faculty Rotterdam in male Sprague-Dawley rats (n=61) with a bodyweight (BW) of 283.4 ± 2.6 g (IFFA Credo, The Netherlands).

Induction of intraalveolar fibrin formation by local lung inflammation. Lipopolysaccharide (LPS)-induced lung inflammation, adapted from the model originally described by Wheeldon et al. (21) and van Helden et al. (22), was induced in 51 animals by intratracheal instillation of 16 mg/kg LPS, derived from *Salmonella enteritidis* (Sigma L6761. St. Louis, Mo., USA). The procedure was performed after orotracheal intubation under gaseous anesthesia (65% nitrous oxide/33% oxygen/2% isoflurane; Pharmchemie, Haarlem, The Netherlands), using a miniature nebulizer (Penn-Cenntury, Philadelphia, Pa., USA). After the procedure rats were extubated.

Experimental protocol. Twenty four hours after LPS instillation, rats were anesthetized with nitrous oxide, oxygen and isoflurane (65/33/2%), tracheotomized and a catheter was inserted into a carotid artery. Anesthesia was maintained with hourly intraperitoneal injections of pentobarbital sodium (60 mg/kg, Nembutal; Algin BV, Maassluis, The Netherlands). Muscle relaxation was attained with hourly 2 mg/kg pancuronium bromide (Pavulon; Organon Technika, Boxtel, The Netherlands) intramuscular injections. After muscle relaxation all animals were connected to a ventilator (Servo Ventilator 300; Siemens-Elema, Solna, Sweden) set in a pressure-controlled mode with positive inspiratory pressure (PIP) of 12 cm H_2O and positive end-expiratory pressure (PEEP) of 2 cm H_2O, frequency of 30 breaths/min, I/E ratio of 1:2, and fractional inspired oxygen tension of 1.0. Body temperature was kept within normal range by means of a heating pad. After 15 min of stabilisation, arterial blood gases were taken from a carotid artery catheter and PIP was adjusted according to the

allocation of the animals to the different ventilation groups. Further, arterial blood gases were sampled every 30 min thereafter using conventional methods (ABL555, Radiometer, Copenhagen, Denmark). Mean arterial blood pressure (MAP) was monitored throughout the experiment by the intra-arterial carotid artery catheter every 30 min for 3 hours.

Study groups. We had three ventilation groups, each with different pressure amplitude. A PEEP of 5 cm H_2O was selected for all groups, but the PIP level was different in each group. Group I was ventilated with a PIP of 16 cm H_2O (N=12); group II with a PIP of 26 cm H_2O (N=13) and group III with a PIP of 35 cm H_2O (N=13). The ventilation period was designed to last 3 hours, after which final measurements were made. FiO_2 was kept at 1.0 during the whole study period to prevent hypoxemia in any of the groups.

For the control group we included 13 animals which received LPS instillations as described previously (i.e., "LPS controls") which, however, were not mechanically ventilated. Further, to create reference values, we included 10 healthy rats which did not receive LPS and which were not ventilated (i.e., "healthy"). At the end of the ventilation period all rats (including LPS controls and healthy rats, n=61) were killed with an intra-arterial overdose of pentobarbital sodium (600 mg/kg BW) and all measurements were then made.

Broncho-alveolar lavage. After the rats were killed, the thorax and diaphragm were opened and lungs removed. Lung weight/kg body weight (BW) as the parameter of lung injury was measured. Then, broncho-alveolar lavage (BAL) was performed with normal saline (30 ml/kg heated to 37ºC) and re-aspirated three times. BAL fluid (BALF) was centrifuged (400 x g for 10 minutes at 4^{o} c) and the recovered supernatant fluid was then snap frozen and stored at -80ºC until further processing. Measurements were not completed in 2 rats of group II and in 3 rats of group III due to air leakage during the test.

Measurements.

Coagulation activation as assessed by thrombin-antithrombin complexes (TATc) were measured in BALF with an ELISA-based method. Briefly, rabbits were immunized with

mouse thrombin or rat antithrombin. Antithrombin antibodies were used as capture antibody, digoxigenin-conjugated anti-antithrombin antibodies were used as detection antibodies, horseradish peroxidase–labeled sheep antidigoxigenin (anti-DIG) F(ab') fragments (Boehringer Mannheim, Mannheim, Germany) were used as staining enzyme, and o-phenylene-diamine dihydrochloride (OPD, Sigma) was used as substrate. Dilutions of mouse serum (Sigma) were used for the standard curve, yielding a lower detection limit of 0.3 ng/mL (23).

Fibrin breakdown of LPS-induced alveolar fibrin formation was determined by measurements of D-dimers (cross-linked fibrin degradation products) in BALF (24). D-dimers were quantitated by a sandwich-type ELISA (Asserachrom D-dimer, Diagnostica Stago, Asnières-sur-Seine, France). This assay shows cross-reactivity with rat D-dimers.

PAI activity in BALF was determined on an automated coagulation analyzer (Behring Coagulation System, Marburg, Germany) with reagents and protocols from the manufacturer. This assay determines the urokinase-inhibiting activity of the sample. The remaining urokinase is then assayed by activating plasminogen to plasmin and subsequent determination of plasmins chromogenic activity. The assay is independent of variable concentrations of plasminogen, α-2-antiplasmin, and fibrinogen in the sample. The upper detection limit of this test is set at 6.9 U/mL. Protein concentration in BALF was measured using the Bradford method (Biorad protein assay, Munich, Germany (25).

To determine PAI-1 antigen in BALF, a rat PAI-1 ELISA was developed using a rabbit polyclonal antibody (Abcam Ltd, Cambridge, UK) as coating antibody and a biotinylated rabbit IgG antibody (Molecular Innovations Inc., Southfield, MI, USA) as developing antibody. Rat PAI-1 (Calbiochem, La Jolla, CA, USA) was used as standard.

To illustrate alveolar fibrin depositions, samples were taken from all lung lobes and 30 fields were analyzed. The analyzing pathologist was not informed about the study purpose and only asked to prepare rat lungs for illustration of alveolar fibrin deposition. Histological

analysis was performed as previously described (20). Briefly, lungs were fixed at 10 cmH_2O mean airway pressure, and slices were stained for fibrinogen. Due to the washing procedure, fibrinogen would have been washed out leaving solely fibrin attached to the alveolar wall. Slides of lung tissue were deparaffinized and endogenous peroxydase activity was quenched by a solution of methanol/0.03% H2O2 (Merck, Darmstadt, Germany). After digestion with a solution of pepsine 0.25% (Sigma, St Louis, MO, USA) in 0.01M HCl, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to biotin-labelled goat anti-human fibrinogen antibody (Ixell, Accurate Chemical & Scientific Corp., Westbury, NY, USA). After washes, slides were then incubated in a streptavidin-ABC solution (Dako) and developed using 1% H_2O_2 and 3.3'-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were mounted in glycerin gelatin and counterstained with hematoxylin.

Statistical analysis.

Statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, IL, USA). Data are presented as means ± standard error of means (SEM). Group differences were analysed with ANOVA, differences between the 3 ventilated groups were analyzed with a Kruskall-Wallis test. Differences between the healthy and LPS control group, and between the ventilated groups and the LPS control group were analyzed with a Mann-Whitney test. A p-value < 0.05 was considered significant.

In Table 1 all values for TATc, D-dimer, PAI activity and PAI-1 antigen were corrected for their individual corresponding protein levels. Analysis was further performed as described above.

RESULTS

All animals survived the study period. LPS instillation caused severe respiratory distress with tachypnea and significant weight loss, from 283.4 ± 2.6 g before LPS instillation to a mean of 260.3 ± 2.6 g ($p<0.001$) 24 hours after LPS instillation.

Mechanical ventilation with high pressure amplitudes caused lung injury with compromised partial arterial oxygen tension (PaO_2): group III showed lower values compared to group I at 120 min, at 150 min and at 180 min (Figure 1, $p<0.05$). Furthermore, animals ventilated with high pressure amplitudes also showed lower MAP values at the end of the study period: ventilation group II (pre-study 124 ± 6 mm Hg vs. end of study 105 ± 6 mm Hg, $p<0.05$) and ventilation group III (pre-study 126 ± 4 mm Hg vs. end of study 73 ± 5 mm Hg, $p<0.001$). At the end of the experiment, the extent of lung injury was also evidenced by a significant increase in median lung weight/kg BW in group II of 9.2 ± 0.4 g/kg BW and in group III of 13.3 ± 0.8 g/kg BW vs. 7.8 ± 0.6 g/kg BW in animals from group I (Table 1)

Coagulation activation.

LPS instillation resulted in activation of coagulation, TATc increased from 3.3 ± 1.0 ng/ml in healthy animals to 78.6 ± 15.2 in LPS-treated animals (Figure 2). Increased pressure amplitude resulted in a significant increase of TATc between the 3 ventilated groups (Figure 2). Only TATc levels in group I animals were statistically significantly lower compared to LPS controls (Figure 2).

Fibrinolysis.

In LPS controls, absolute levels of D-Dimers in BALF were 13.4 ± 2.0 µg/L compared with 1.8 ± 0.6 µg/L in healthy rats ($p<0.001$), indicating alveolar fibrin formation caused by endotoxin (Figure 2). Mechanical ventilation increased D-dimer levels in BALF compared to LPS control animals. Ventilation with the different pressure amplitudes did not significantly

alter D-dimer levels between groups I-III; group I (36.1 ± 8.3 µg/L) and 65.8 ± 16.9 µg/L in group III.

Anti fibrinolysis

PAI-activity levels were undetectable in BALF of healthy rats and 1.24 ± 0.8 U/ml in LPS controls (Figure 2). Between the ventilated groups PAI activity levels were significantly increased: median PAI activity concentrations in BALF in group I; 0.7 ± 0.5 U/mL, in group II; 3.4 ± 0.8 U/mL and in group III; 5.0 ± 0.7 U/mL (Figure 2). PAI activity in group III was also significantly different from LPS control animals. Minimal levels of PAI-1 antigen were detected in healthy animals (0.03 ± 0.02 ng/ml), instillation of LPS led to a significant rise of PAI-1 antigen (13.9 ± 3.6 ng/ml; $p<0.05$) (Figure 2). There was no difference in PAI-1 antigen levels between the 3 ventilated groups, or between the ventilated groups and the LPS control animals (Figure 2).

Histology

In Figures 3 and 4, microscopy of representative rat lung tissue shows lung inflammation due to LPS. In all ventilated animals unresolved fibrin depositions (arrows) were observed. More fibrin deposits were observed in animals ventilated with the highest pressure amplitude, there were no differences in levels of lung inflammation. Atelectasis was also more pronounced in animals of group II and especially group III.

DISCUSSION

The present study investigated the effect of mechanical ventilation on alveolar fibrinolysis in LPS-induced lung injury. Intratracheal LPS instillation caused local activation of coagulation increase in TATc levels with fibrin formation as documented by the appearance of D-dimer in BALF of animals of the LPS control group. The occurrence of alveolar fibrin formation after LPS-induced lung inflammation has previously been documented in a comparable experimental model (24). Mechanical ventilation aggravates this endotoxin-induced lung inflammation and might therefore influence alveolar fibrin turnover (26). Therefore, the experimental model of the present study may be considered an appropriate model to study the effects of mechanical ventilation on alveolar fibrinolysis.

Acute lung injury is characterized by alveolar flooding especially ventilator-induced lung injury aggravates or may even be the cause of this flooding (27). In the present study we also observed pulmonary edema formation as characterized by the BALF protein levels and increased lung weights of the animals ventilated with higher pressure amplitudes.

D-dimer concentrations in BALF of the ventilated animals were higher than in the LPS controls, however, between the ventilation groups there were no significant differences. In contrast, PAI activity increased with the size of the pressure amplitude without a change in PAI-1 antigen levels. Our results for the first time link injurious ventilation settings in inflamed lungs with depressed fibrinolysis. Furthermore, independent of protein leakage into the lung, injurious mechanical ventilation increased the BALF levels of PAI-activity.

We observed an increase in PAI activity but did not see an increase in PAI antigen levels. Conversion of PAI-1 between its active and latent form is regulated by vitronectin, which circulates in plasma but is also a major constituent of the extracellular matrix (28, 29). In the adjusted data we still observe an increase in activity without a change in antigen levels,

suggesting that PAI activity is dependent on protein/vitronectin influx and subsequent stabilization of PAI activity occurring during high pressure amplitude ventilation.

Surprisingly, increased PAI activity did not result in smaller amounts of D-dimer in BALF. There might be two possible explanations. First, 3 hours of mechanical ventilation might not be long enough to demonstrate a larger effect on the downregulation of alveolar fibrin breakdown due to PAI production. Secondly, the high D-dimer levels in BALF of group III (although not significantly different compared to the other ventilated groups despite high PAI activity levels) might be explained by additional alveolar fibrin formation, triggered by traumatic mechanical ventilation and influx of plasma proteins. It is impossible in the current experiment to distinguish whether the increased levels of D-dimers reflect increased fibrinolysis only, or perhaps also reflect a higher level of pro-coagulation resulting in increased formation of fibrin, which will translate into increased D-dimer levels.

The idea of extra fibrin formation in a second hit model of lung injury (endotoxin plus mechanical ventilation) is plausible. Under both circumstances, similar inflammatory mediators are expressed (e.g., TNF-α), which may activate the intraalveolar coagulation system and fibrin formation (18, 30). LPS induces coagulation activation as indicated by the increased TATc levels, whereas mechanical ventilation with low pressure amplitudes significantly reduced this. A possible explanation for this phenomenon could be that, with higher pressure amplitudes, resolution of coagulation is impaired.

Despite these questions concerning the results of D-dimer in BALF, the interpretation of the PAI values is striking: aggressive mechanical ventilation can upregulate PAI, the strongest antifibrinolytic mediator which might contribute to the persistence of alveolar fibrin and aggravate lung injury (e.g., lung fibrosis). The clinical importance of PAI-1 upregulation in mechanically ventilated patients has only recently been reported (31). In adult ALI patients high levels of PAI-1 in pulmonary edema fluid were associated with an increase in mortality (31).

The fact that mechanical ventilation may affect the alveolar fibrinolytic milieu has been demonstrated earlier (20). However, in that study, alveolar fibrin formation was generated by instillations of fibrinogen/thrombin in healthy rats, whereas in the present study the amount of alveolar fibrin formation was dependent on the endogenous capability of each animal to build alveolar fibrin after the LPS challenge. A recent report on early stress response genes demonstrated that coagulation genes are upregulated during VILI (32).

The major limitation of the present study is that we can not determine the origin of the fibrin deposits, or more accurately the breakdown product D-dimers in the BALF. Our immunohistochemistry data does not allow us to distinguish between fibrin or fibrinogen in the alveolar space or the capillary or the interstitial space. Despite this the D-dimer values in the lavage fluid clearly demonstrate increased brake down of intra-alveolar fibrin deposits during mechanical ventilation, suggesting increased fibrin deposits in the alveolar space.

In summary, we provide new information showing that mechanical ventilation influences alveolar PAI-activity after endotoxin exposure and can influence alveolar fibrionolysis. Future experimental studies are needed to elucidate the underlying mechanisms.

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Figure legends

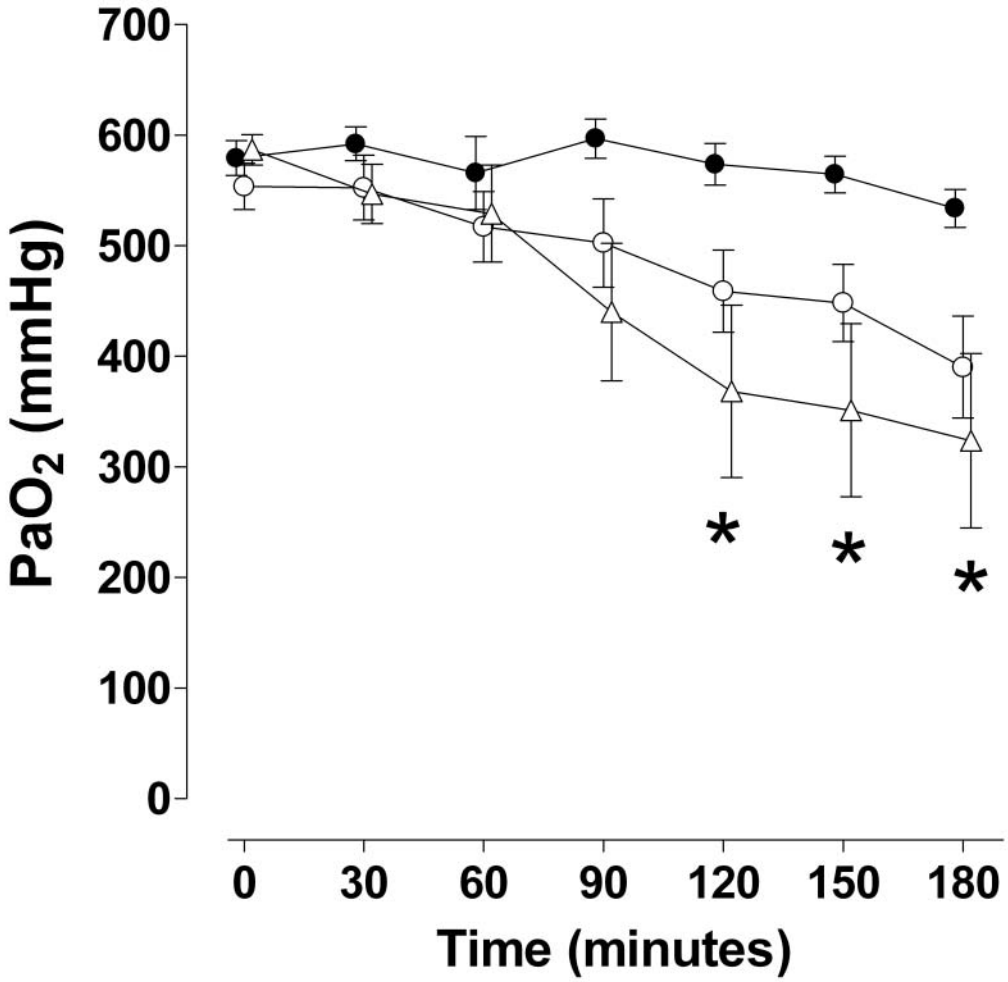


Figure 1.

Effect of mechanical ventilation on oxygenation (PaO_2, mm Hg) overtime in the 3 ventilated groups. Closed circles; group I, open circles; group II, triangles; group III.

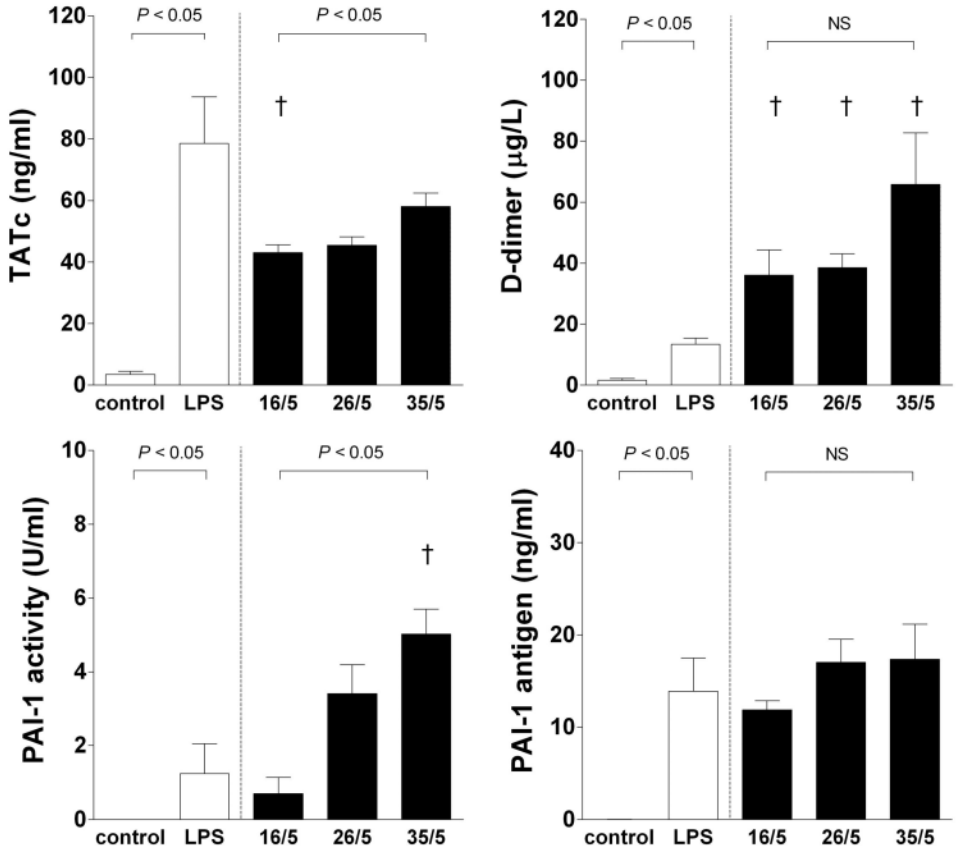
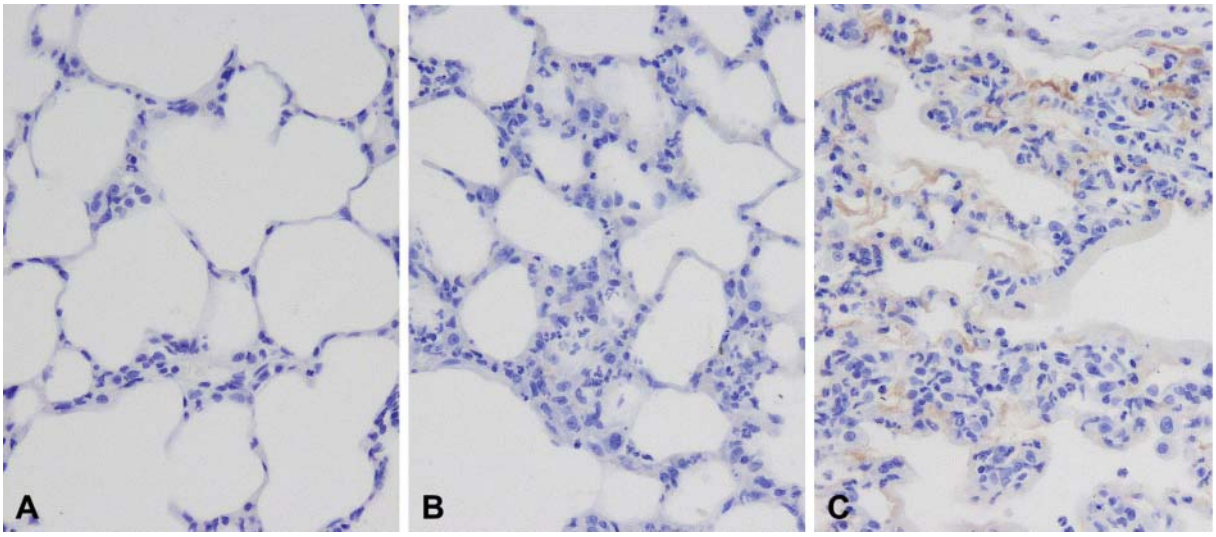
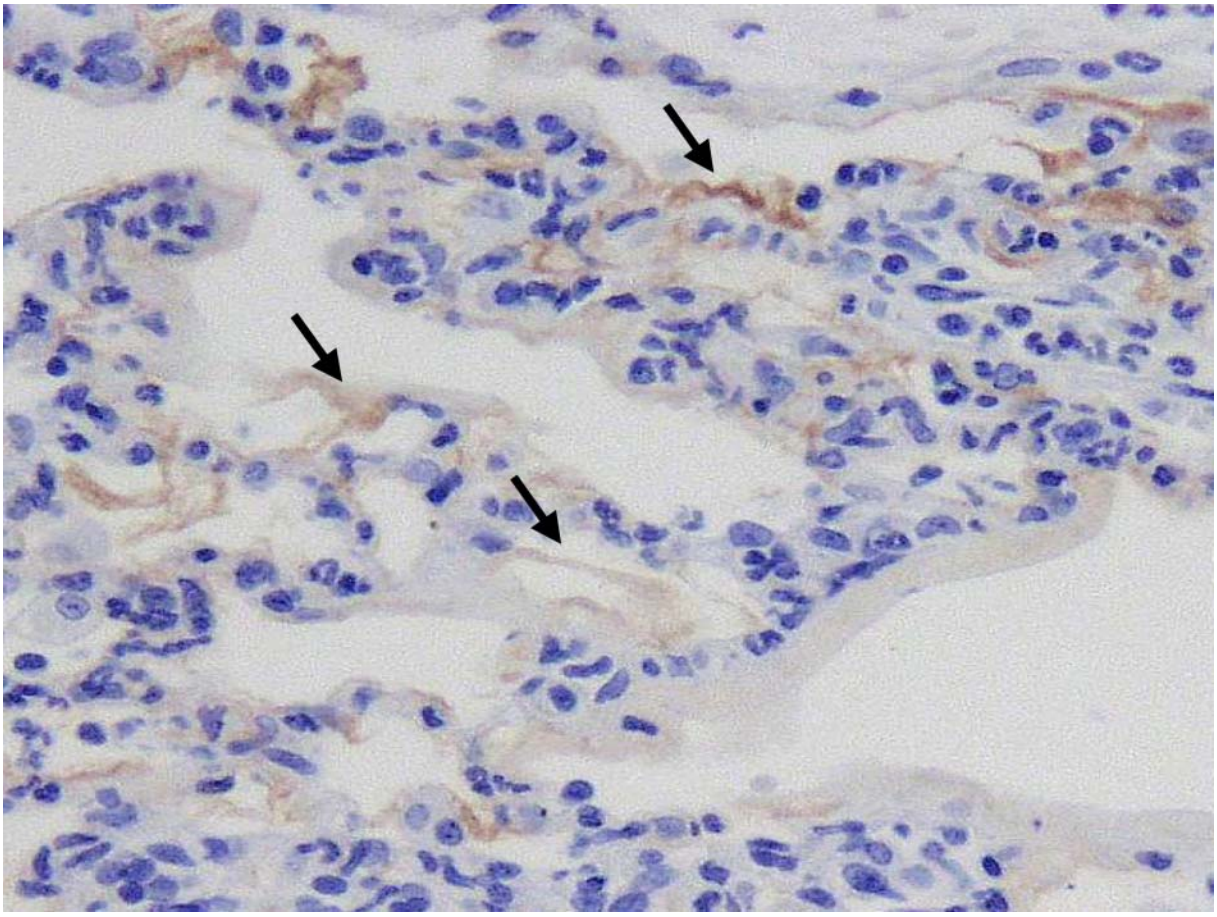


Figure 2.

Effect of mechanical ventilation on thrombin-anti-thrombin complex (TATc), D-dimer, plasminogen activator inhibitor (PAI) activity and PAI-1 antigen levels in broncho-alveolar lavage fluid in LPS pre-treated animals. LPS alone significantly increased all parameters compared to healthy unventilated animals. Mechanical ventilation with increasing pressure amplitudes significantly increased TATc and PAI activity. † $p<0.05$ vs. LPS controls, ANOVA followed by a Mann-Whitney test.





Figures 3 & 4.

Microscopy of representative fibrin-stained paraffin sections of rat lung tissue of lipopolysacchride-treated rats. A: Normal lung tissue. B Lung tissue of a representative sample of the LPS control group (24 hours after lipoploysaccharide instillation) showing predominately interstitial infiltrates with neutrophils C: Representative lung tissue of group III (ventilation pressure 35/5 cm H_2O) showing interstitial infiltrates with neutrophils and unresolved fibrin/fibrinogen depositions (brown, counterstained with hematoxylin, arrows) after three hours of mechanical ventilation. Figure 4. Close-up of group 35/5

Table 1. Lung weight, total BALF protein levels and values for TATc, D-dimer, PAI-1 activity and PAI-1 antigen adjusted for protein levels in BALF

	Healthy (n=8)	LPS controls (n=11)	Group I (n=11)	Group II (n=12)	Group III (n=12)	P between ventilated groups
Lung weight (g/kg BW)	6.0 ± 0.4	8.1 ± 0.3*	7.8 ± 0.6	9.2 ± 0.4	13.3 ± 0.8†	0.01
Total protein (mg/mL)	0.09 ± 0.01	0.7 ± 0.11*	0.54 ± 0.06	0.67 ± 0.07	1.02 ± 0.12	0.01
TATc/protein (ng/mg)	42.6 ± 13.7	116 ± 15.8*	85.1 ± 6.6	74.7 ± 6.9	67.6 ± 9.7	0.23
D-dimer/protein (µg/mg)	21.8 ± 7.2	22.2 ± 4.2	88.6 ± 35.2†	62.7 ± 7.9†	56.3 ± 13.4	0.76
PAI activity/protein (U/mg)	0.0 ± 0.0	0.9 ± 0.6*	1.1 ± 0.7	4.4 ± 0.9†	5.3 ± 0.7†	0.005
PAI-1 antigen/protein (ng/mg)	0.3 ± 0.2	19 ± 2.3*	25.3 ± 4.2	24.9 ± 2.6	18.3 ± 3.1	0.23

TATc: Thrombin-antithrombin complex; **PAI**: Plasminogen activator inhibitor**; BALF**: Broncho-alveolar lavage fluid. Values are means ± SEM.

Adjustments were made for protein content in BALF per individual sample.

* $p<0.05$ vs. healthy controls, ANOVA followed by a Mann-Whitney test.

† $p<0.05$ vs. LPS controls, ANOVA followed by a Mann-Whitney test

P-values between the ventilated groups were analysed with ANOVA, followed by a Kruskall-Wallis test.