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ANNOUNCEMENT

Effective with all new manuscripts submitted after January 1, 1990 the $50 manuscript processing charge will no longer be required. Instead, the AMERICAN REVIEW OF RESPIRATORY DISEASE will require page charges for original manuscripts of $40 per printed page. Authors will be billed for the charges.

Effect of Recombinant Hirudin, a Specific Inhibitor of Thrombin, on Endotoxin-induced Intravascular Coagulation and Acute Lung Injury in Pigs[1-4]

HANS HOFFMANN, MATTHIAS SIEBECK, MICHAEL SPANNAGL, MARION WEIS, REINHARD GEIGER, MARIANNE JOCHUM, and HANS FRITZ

SUMMARY We hypothesized that thrombin activation may play a prominent role in endotoxin-induced secondary organ failure, such as acute lung injury. To test this hypothesis, we administered a thrombin-specific inhibitor, recombinant hirudin, in endotoxemic pigs. The pigs were anesthetized, mechanically ventilated, and prepared with Swan-Ganz and extravascular lung water (EVLW) catheters. A total of 18 randomly selected animals received a pretreatment of 1,000 U/kg of hirudin, followed by a continuous infusion over 6 h of 500 U/kg/h given simultaneously with the infusion of 10 μg/kg/h of *Salmonella abortus equi* endotoxin. Another 18 animals received a continuous infusion over 6 h of endotoxin but did not receive hirudin. All animals were fluid resuscitated with 17 ml/kg/h of saline for the duration of the experiment. Data are expressed as the mean (95% confidence interval). Hirudin reduced the endotoxin-induced consumption of plasma fibrinogen from −110 (−138 to −82) mg/100 ml to −39 (−67 to −12) mg/100 ml ($p = 0.0001$) and endotoxin-induced increases in the soluble fibrin in plasma from 434 (369 to 499) ng/ml to 236 (171 to 300) ng/ml ($p = 0.0002$). These data suggest an effective inhibition of the endotoxin-generated thrombin by hirudin. Furthermore, hirudin significantly reduced endotoxin-induced increases in pulmonary vascular resistance from 32 (27 to 37) kdyn × s × cm^{-5} × kg to 20 (15 to 25) kdyn × s × cm^{-5} × kg ($p = 0.0015$) and increases in EVLW from 15.4 (13.2 to 17.6) ml/kg to 12.2 (10.0 to 14.4) ml/kg ($p = 0.0299$). The administration of hirudin also attenuated the endotoxin-induced loss of protein-rich fluid from the vasculature and reduced the plasma PMN elastase concentration in plasma. These results indicate that the administration of the thrombin-specific inhibitor hirudin can attenuate endotoxin-induced acute lung injury, suggesting an important role of thrombin in endotoxin-induced acute lung injury.

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Introduction

Septicemia is frequently associated with intravascular coagulation and secondary organ failure, such as acute lung injury (1-3). Infusion of endotoxin (lipopolysaccharide [LPS]) into animals has been shown to cause an activation of the coagulation cascade and to cause acute lung injury characterized by pulmonary hypertension, pulmonary endothelial injury, increased pulmonary vascular permeability, and pulmonary edema (4-8). Serine proteases, such as thrombin, plasmin, or plasma kallikrein, generated during activation of the coagulation cascade have been shown to activate polymorphonuclear leukocytes (PMN) as well as to directly increase pulmonary vascular permeability (9-12). Thus, these proteases are thought to play a major role in the pathophysiology of LPS-induced acute lung injury. The response of thrombin infusion in animals resembles that seen after LPS administration in several respects, including pulmonary hypertension and increased pulmonary vascular permeability (10). The direct effects of thrombin *in vitro* include increases in endothelial permeability (13), vascular smooth muscle contraction (14), activation of multiple PMN functions (15), and thromboxane generation from various cells (16-18). *In vivo*, the thrombin-induced cleavage of fibrinogen to form soluble fibrin, which subsequently polymerizes to form fibrin clots, may also be a causative factor in LPS-induced acute lung injury. Soluble fibrin causes pulmonary hypertension in isolated perfused rabbit lungs (19). In addition, thrombin may induce the release of tissue plasminogen activator from the endothelial cells and thereby activate plasmin (20). Fibrin degradation products (FDP) generated by plasmin are noxious agents in the lung and may increase vascular permeability (21). Plasmin may also lead to complement activation resulting in PMN chemotaxis (22). The sum of these actions suggests a prominent role of thrombin in the pathogenesis of LPS-induced acute lung injury.

In this study, we used the thrombin-specific inhibitor, recombinant hirudin, to investigate the role of thrombin in a porcine model of LPS-induced acute lung injury. Hirudin, the anticoagulant from the medical leech, is a single-chain polypeptide composed of 65 amino acid residues with a M_r of 7,000 (23). Recently, recombinant hirudin (r-hirudin) has become available (24). r-Hirudin has the same inhibitory properties as the natural protein from the leech (25, 26). Hirudin reacts with thrombin in an equimolar ratio to form an enzyme-inhibitor complex that blocks all the proteolytic activity of thrombin (27, 28). In this respect, hirudin functions differently from other therapeutic anticoagulants such as heparin. Heparin, the antithrombin III (AT III) cofactor, accelerates the reaction of AT III with thrombin and other clotting factors (29). Thus, the anticoagulant effectiveness of heparin depends on the concentration of AT III in plasma, which is low in cases of septic shock (30, 31).

The objectives of this study were (1) to examine whether hirudin administration would inhibit the action of throm-

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[2] Supported by the Deutsche Forschungsgemeinschaft (Ge 378/2-2), the Bundesministerium für Forschung und Technologie (FIW 0284), the Ciba-Geigy AG, and the Plantorgan KG.

[3] Presented in part at the Annual Meeting of the American Thoracic Society, Las Vegas, Nevada, 1988.

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bin generated during LPS infusion, and (2) to determine the effect of hirudin on acute lung injury following LPS infusion.

Methods

Animals

Anesthetized weaned miniature pigs of either sex (Minipig; Medical Service GmbH, Munich, FRG), weighing 18 to 23 kg, were used. The animals were fasted overnight, except for unlimited access to water. Only animals without signs of infection and with a baseline body temperature below 38.6° C, Hb above 7.0 g/100 ml, WBC 5,000 to 18,000/mm³, and lung compliance/kg body weight greater than 1 were included. All animal procedures were approved by the Tierschutzreferat, Regierung von Oberbayern.

Anesthesia and Surgical Procedures

The animals were premedicated with 3 mg/kg of azaperone (Stresnil; Janssen, Neuss, FRG) and 3.75 mg/kg of metomidate hydrochloride (Hypnodil; Janssen, Neuss, FRG) injected intramuscularly. Anesthesia was induced with 24 mg/kg of pentobarbital (Narcoren; Iffa Merieux, Laupheim, FRG) injected intravenously and maintained throughout the experiment by a continuous infusion of 0.5 mg/kg/h of piritramide (Dipidolor; Janssen, Neuss, FRG), 0.3 mg/kg/h of pancuronium bromide, and 4 mg/kg/h of pentobarbital. The animals were orally intubated (Tubus 6.5 mm, Portex, Hythe, UK) and mechanically ventilated (volume cycled) with 40% O_2, a respiratory rate of 20 breaths/min, and a tidal volume of about 12 ml/kg adjusted to Pa_{CO_2} 29 to 33 mm Hg using a Servo Ventilator® 900C (Siemens-Elema, Solna, Sweden). To prevent atelectasis, we hyperinflated the animals every 2 min. A large-bore cannula and two central venous lines (Cavafix 0.8 × 1.4 mm × 70 cm; Braun, Melsungen, FRG) were placed in the left external jugular vein. A 7F Swan-Ganz catheter (7F thermodilution catheter; American Edwards Laboratories, Anasco, PR) was introduced via the right external jugular vein into a main branch of the pulmonary artery. A 5F lung water catheter (American Edwards Laboratories, Irvine, CA) was inserted through the right femoral artery into the abdominal aorta. After preparing the animals, we allowed a stabilization period of 60 min before taking baseline measurements. For fluid resuscitation, the animals received 25 ml/kg of Ringer's lactate during the stabilization period and a continuous infusion of 17 ml/kg/h during the observation period (this volume included the fluid needed for cardiac output (CO) and extravascular lung water (EVLW) measurements and for flushing the catheters). If needed, sodium bicarbonate (8.4%) was given according to the hourly blood gas analysis.

Experimental Protocol

After baseline measurements, 36 animals received a continuous infusion of 10 μg/kg/h of *Salmonella abortus equi* endotoxin (LPS, batch M6-N1 TEN, 5 mg/ml, kindly provided by Dr. C. Galanos, Max-Planck Institut für Immunologie, Freiburg i.Br., FRG). These animals were randomly assigned into two groups: 18 animals (with hirudin) received a pretreatment of 1,000 U/kg of recombinant hirudin (batch: rBH5-7001/2, specific activity 11,114 U/mg; Plantorgan, Bad Zwischenahn, FRG), followed by a continuous infusion over 6 h of 500 U/kg/h given simultaneously with the infusion of LPS. Another 18 animals (w/o hirudin) did not receive hirudin but received a continuous infusion over 6 h of LPS and placebo. All treatments were given as an equal volume of 0.3 ml/kg/h. In addition, 6 control animals received not LPS but saline (n = 3) or r-hirudin (n = 3) according to the protocol of the corresponding LPS groups. Hemodynamic measurements and blood gas analysis were performed hourly. EDTA-anticoagulated or heparinized blood samples were drawn every hour and citrated (1:10 with 3.8% citrate) blood samples every 2 h. The blood was centrifuged at 1,500 × *g* for 10 min and the plasma aliquoted and kept at −80° C until measurement. After the end of the 6-h observation period, the surviving animals were sacrificed by the injection of 2.0 g embutramide + 0.5 g mebezonium-jodine + 0.05 g tetracainhydrochloride (=10 ml of T 61; Hoechst Pharmaceuticals, Munich, FRG).

Physiologic and Biochemical Methods

Mean aortic pressure ($\overline{Pa}$), mean pulmonary arterial pressure ($\overline{Ppa}$), and pulmonary arterial wedge pressure (Ppaw) were continuously monitored using Bentley Trantec Model 800 transducers, which were referred to the level of the atrium. Pressure recordings were made on a Sirecust® 404 monitor (Siemens, Munich, FRG). Cardiac output and extravascular lung water were determined in triplicate by the single-indicator (10 ml of iced 5% glucose solution) thermal dilution technique using a 9310 lung water computer (American Edwards Laboratories, Santa Ana, CA). The thermal signals from the catheters in the pulmonary artery and in the aorta were also plotted on a two-channel strip chart recorder (BD 41; Kipp & Zonen, The Netherlands) for visual control. Pulmonary vascular resistance (PVR) was calculated as $(\overline{Ppa} - Ppaw)/CO \times$ kg body weight) × 79.9. Airway opening pressure (Pao) and the tidal volume (VT) were measured using a 900C ventilator. Lung dynamic compliance was determined as CLdyn = VT/ Pao at the moment of zero airflow (end-inspiration). Blood gases were measured from heparinized arterial and mixed venous blood samples (Model 168 pH/blood gas analyzer; Corning, Medfield, MA). From the same samples, we determined O_2 saturation and Hb using an OSM 2 hemoximeter (Radiometer, Copenhagen, Denmark). Alveolar-arterial O_2 difference ($AaPo_2$) was calculated from the alveolar gas equation according to Thal's formula. Total intravascular protein (IVP) was calculated from measurements of plasma volume (PV) and total plasma protein concentration (TP) at baseline and 4 h:

$$IVP\ (g/kg) = \frac{PV\ (ml) \times TP\ (g/ml)}{body\ weight\ (kg)}$$

PV was measured by a dye dilution technique:

$$PV_x\ (ml) = \frac{V_i\ (ml) \times A_i}{A_x}$$

PV_x = plasma volume at time x
V_i = volume of injected dye solution
A_i = absorbance of injected dye solution
A_x = absorbance of dye in plasma sample at time x

V_i was determined by weighing the syringe before and after the injection of 0.25 ml/kg of 1% Evans blue (Sigma) dye solution intravenously. A_i and A_x were determined at 620 nm using a spectrophotometer with appropriate corrections for plasma background absorbance (32). TP was measured with the standard biuret method. Leukocyte (WBC) and platelet counts were determined on EDTA-anticoagulated blood samples using a hemacytometer (Neubauer Chamber). The activities of antithrombin III (AT III), prothrombin (PT), and antiplasmin (AP) in citrated plasma samples were determined using amidolytic assays with the specific chromogenic peptide substrates S-2238 (H-D-Phe-Pip-Arg-pNA, Kabi Diagnostica, Stockholm, Sweden), Chromozym® TH (Tos-Gly-Pro-Arg-pNA, Boehringer, Mannheim, FRG), and S-2251 (H-D-Val-Leu-Lys-pNA, Kabi Diagnostica, Stockholm, Sweden), respectively. The manufacturer's instructions were modified as previously described (4). Baseline plasma samples of all 36 animals were pooled and served as reference. AT III, PT, and AP activities are given as a percentage of the pool plasma value. Plasma fibrinogen was determined on citrated plasma samples using a functional turbidimetric test with batroxobin (Fibrinogen Kinetic; Boehringer, Mannheim, FRG). Fibrinogen data are presented as a percentage of the individual baseline value because we observed a considerable variance in baseline values. Soluble fibrin was measured on citrated plasma samples using a functional test employing the plasmin-specific chromogenic substrate S-2251 (33). Leukocyte neutral proteinase inhibitor (LNPI) and LNPI-bound lysosomal PMN elastase (PMN elastase) in plasma were measured on citrated plasma samples using enzyme-linked immunosorbent assays (ELISA) as recently described (34, 35). r-Hirudin in plasma was measured on citrated plasma samples using an ELISA modified for porcine plasma (36).

Statistical Analysis

We used a factorial analysis of variance to analyze the effect of hirudin administration on the parameters that we measured (37). We employed the paired Student's *t* test to make comparisons within groups. Differences were

TABLE 1

CLOTTING PARAMETERS DURING ENDOTOXIN INFUSION IN PIGS*

		Absence of Hirudin (*n* = 18)	Presence of Hirudin (*n* = 18)	p
Prothrombin, %	Base	106 (100–112)	105 (99–111)	0.8179
	2 h	95 (90–100)	90 (84–95)	0.1655
	Last	67 (61–73)	63 (57–69)	0.6375
AT III, %	Base	99 (93–104)	101 (96–107)	0.4782
	2 h	94 (87–100)	94 (88–101)	0.9245
	Last	81 (75–86)	83 (77–88)	0.5663
Antiplasmin, %	Base	98 (92–104)	96 (90–102)	0.5745
	2 h	91 (85–96)	88 (83–94)	0.5800
	Last	73 (67–79)	71 (64–77)	0.6828
r-Hirudin, nmol/L	Base	–	–	–
	2 h	–	72 (62–83)	–
	Last	–	104 (89–119)	–

Definition of abbreviation: AT III = antithrombin III.

* Values are mean (95% confidence interval) given as a percentage of the standard porcine pool plasma; p = factorial ANOVA.

considered significant at $p < 0.05$, and exact p values are given. We detected no significant differences in baseline levels of any parameter that we examined except in baseline WBC counts. Data are presented as mean (95% confidence interval) in the absence and presence of hirudin. When last measurements are presented, the last valid measurements of each animal were compared and the time of the measurement period was included as a covariate.

Results

Clotting Parameters

Infusion of LPS caused a steady decrease in prothrombin and antithrombin III activities in plasma. In the absence of hirudin, PT fell to approximately 65% of baseline by the last measurement; AT III decreased to approximately 80% of baseline (last measurement). Hirudin had no significant effect on the LPS-induced changes in PT or AT III activities in plasma (table 1). Plasma fibrinogen was 323 (295 to 350) mg/100 ml at baseline and decreased steadily following LPS infusion. Hirudin reduced the consumption of plasma fibrinogen from −110 (−138 to −82) mg/100 ml in the absence of hirudin to −39 (−67 to −12) mg/100 ml in the presence of hirudin. By the last measurement, plasma fibrinogen was 64 (57 to 71)% of baseline in the absence of hirudin and 87 (80 to 94)% of baseline in the presence of hirudin (p = 0.0001). The data are shown in figure 1. Fibrinogen consumption was paralleled by an increase in the soluble fibrin in plasma. In the absence of hirudin, the soluble fibrin in plasma increased from 94 (65 to 124) ng/ml at baseline to 434 (369 to 499) ng/ml (last measurement), whereas in the presence of hirudin the increase in soluble fibrin was reduced to 236 (171 to 300) ng/ml (p = 0.0002; figure 2). Antiplasmin activities in plasma decreased gradually during LPS infusion to approximately 70% of baseline by the last measurement; no significant effect of hirudin was detected (table 1). The plasma level of hirudin was 72 (62 to 83) nmol/L at 2 h. A gradual further increase in the hirudin concentration in plasma was noted during the continuous hirudin infusion, with values of 104 (89 to 119) nmol/L by the last measurement. In control animals that did not receive LPS but received saline or hirudin, we detected no significant changes in PT, AT III, plasma fibrinogen, soluble fibrin in plasma, or AP activities in plasma during the 6-h observation period.

Physiologic Findings

The response in mean arterial pressure to LPS was similar in the absence or presence of hirudin. The $\overline{Pa}$ of both groups increased to approximately 9 mm Hg above baseline by 30 min and then dropped to approximately 30 mm Hg below baseline by 2 h. Thereafter, both groups remained hypotensive, showing $\overline{Pa}$ approximately 25 mm Hg below baseline by the last measurement (data not shown). Cardiac output decreased to approximately 80% of baseline by 1 h and then returned to approximately baseline level by 2 h in the absence and in the presence of hirudin. Thereafter, the CO in both groups showed a steady fall toward approximately 55% of baseline by the last measurement. No significant hirudin effect on CO was detected (data not shown). PVR was 4.4 (4.2 to 4.7) kdyn × s × cm^{-5} × kg at baseline and

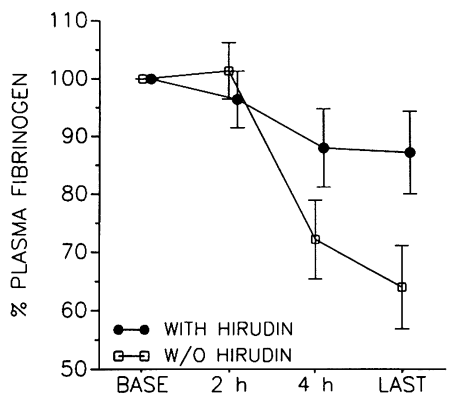

Fig. 1. Endotoxin-induced decreases in plasma fibrinogen in the presence (with hirudin, n = 18) and absence of hirudin (without hirudin, n = 18) in pigs. Endotoxin was given as a continuous intravenous infusion of 10 μg/kg/h. Hirudin was administered as an intravenous bolus injection of 1,000 U/kg before endotoxin, followed by a continuous infusion of 500 U/kg/h simultaneously with the endotoxin infusion. Hirudin significantly reduced the endotoxin-induced decrease in plasma fibrinogen (p = 0.0001). Data expressed as a percentage of baseline value (mean [95% confidence interval], factorial ANOVA).

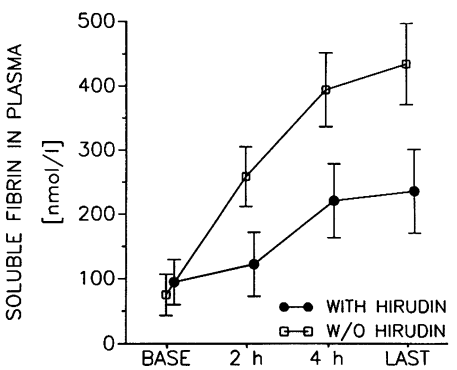

Fig. 2. Soluble fibrin in plasma during endotoxin infusion in the presence (with hirudin, n = 18) and absence of hirudin (without hirudin, n = 18) in pigs. The endotoxin-induced increase in the soluble fibrin was significantly reduced in the presence of hirudin (p = 0.0002). Data are mean and 95% confidence interval (factorial ANOVA).

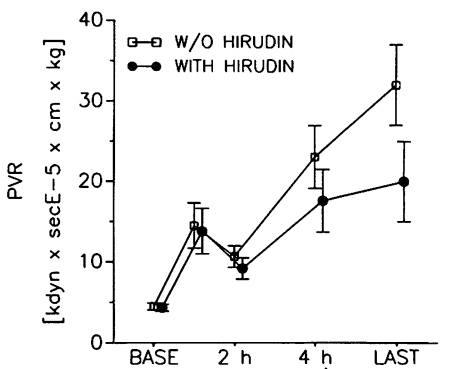

Fig. 3. Effect of endotoxin infusion on pulmonary vascular resistance (PVR) in the presence (with hirudin, n = 18) and absence of hirudin (without hirudin, n = 18) in pigs. Hirudin had no effect on the early (< 2 h) response but significantly (p = 0.0015) reduced the increase in the later phase. Data are mean and 95% confidence interval (factorial ANOVA).

TABLE 2

LUNG FUNCTION DURING ENDOTOXIN INFUSION IN PIGS*

		Absence of Hirudin (*n* = 18)	Presence of Hirudin (*n* = 18)	p
$AaPO_2$, mm Hg	Base	90 (80–99)	85 (76–94)	0.4870
	2 h	127 (106–148)	116 (95–136)	0.4783
	Last	185 (165–205)	162 (142–181)	0.1027
CLdyn/kg, ml/mm Hg/kg	Base	1.07 (1.03–1.12)	1.12 (1.08–1.17)	0.1332
	2 h	0.75 (0.67–0.84)	0.81 (0.72–0.89)	0.3585
	Last	0.51 (0.45–0.56)	0.58 (0.53–0.64)	0.0863

Definition of abbreviations: $AaPO_2$ = aveolar-arterial O_2 difference; CLdyn/kg = lung dynamic compliance/body weight.

* Values are mean (95% confidence interval); p = factorial ANOVA.

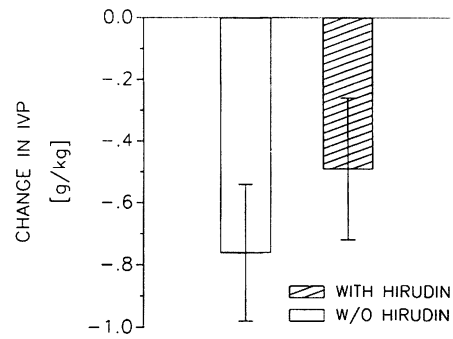

Fig. 5. Endotoxin-induced changes in total intravascular protein (IVP) in the presence (with hirudin, n = 18) and absence of hirudin (without hirudin, n = 18) in pigs. The IVP was calculated as a product of the plasma volume and the total protein concentration in serum. The differences between measurements at baseline and 4 h after onset of the endotoxin infusion are presented. Hirudin reduced the endotoxin-induced loss of IVP (p = 0.0448). Data are mean and 95% confidence interval (factorial ANOVA).

responded similarly initially to LPS in the absence and in the presence of hirudin. PVR increased to approximately three times baseline level by 1 h and then decreased to approximately 1.5 × baseline at 2 h in the absence and in the presence of hirudin. Thereafter, PVR increased again, reaching 32 (27 to 37) kdyn × s × cm^{-5} × kg (last measurement) in the absence of hirudin. This increase was significantly reduced to 20 (15 to 25) kdyn × s × cm^{-5} × kg in the presence of hirudin (p = 0.0015, figure 3). The LPS-induced changes in lung dynamic compliance (CLdyn) and in alveolar-arterial oxygen difference ($AaPO_2$) were similar in the absence and in the presence of hirudin; hirudin had no significant effect on either parameter. $AaPO_2$ increased to approximately two times baseline by the last measurement; CLdyn decreased to approximately 50% of baseline by the last measurement (table 2). The extravascular lung water increased from 10.1 (9.4 to 10.7) ml/kg at baseline to 15.4 (13.2 to 17.6) ml/kg (last measurement) in the absence of hirudin. In the presence of hirudin, the increase in EVLW was significantly reduced to 12.2 (10.0 to 14.4) ml/kg (p = 0.0299, figure 4). We calculated the total intravascular protein as the product of plasma volume times total protein concentration in serum, corrected for body weight. The IVP was 2.9 (2.7 to 3.1) g/kg at baseline. Hirudin reduced the loss of IVP during 4 h of LPS infusion from −0.76 (−0.98 to −0.54) g/kg to −0.49 (−0.72 to −0.26) g/kg. This effect was marginally significant (p = 0.0448, figure 5). In saline or hirudin control animals, no significant changes in $\overline{Pa}$, CO, PVR, CLdyn, $AaPO_2$, EVLW, or IVP were noted during the observation period.

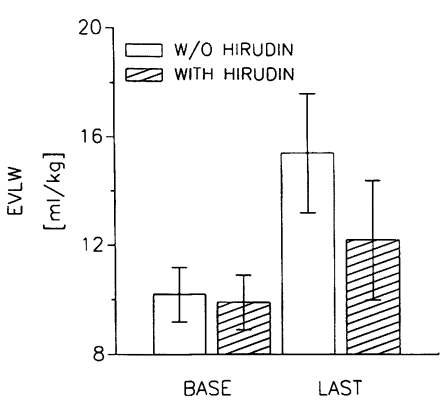

Fig. 4. Extravascular lung water (EVLW) before (base) and after (last) endotoxin infusion in the presence (with hirudin, n = 18) and absence of hirudin (without hirudin, n = 18) in pigs. EVLW was determined by the single-indicator thermal dilution technique. The increase in EVLW was significantly smaller in the presence of hirudin (p = 0.0299). Data are mean and 95% confidence interval (factorial ANOVA).

Hematologic Data

The peripheral leukocyte count (WBC) in the absence and in the presence of hirudin plummeted within 2 h after the start of the LPS infusion and remained low. The leukopenic response was not significantly different in the absence or presence of hirudin. However, since there was a significant difference in baseline WBC counts in the absence and in the presence of hirudin, interpretation of these results is limited (table 3). Circulating platelets decreased similarly in the absence and in the presence of hirudin to approximately 50% of baseline at the last measurement; hirudin had no significant effect (table 3). Saline and hirudin control animals did not show significant changes in WBC or platelets.

PMN Activation

We measured the concentrations of the cytosolic leukocyte neutral proteinase inhibitor (LNPI) and of LNPI-bound lysosomal PMN elastase in plasma to assess PMN activation and lysosomal discharge during LPS infusion. The LNPI concentration was 150 (138 to 162) ng/ml at baseline and increased to approximately three times baseline level during LPS infusion. LNPI by the last measurement was 450 (367 to 552) ng/ml in the absence of hirudin and 395 (320 to 487) ng/ml in the presence of hirudin; this difference was not significant (p = 0.0798). PMN elastase concentrations in plasma increased from values below the detection limit (< 1 ng/ml) at baseline to 37 (27 to 47) ng/ml in the absence of hirudin, whereas in the presence of hirudin this increase was significantly reduced to 23 (13 to 33) ng/ml (p = 0.0137; figure 6). In saline and hirudin controls, the LNPI concentration in plasma did not change and the PMN elastase concentration in

TABLE 3

LEUKOCYTE COUNTS (WBC) AND PLATELET COUNTS IN PERIPHERAL BLOOD DURING ENDOTOXIN INFUSION IN PIGS*

		Absence of Hirudin (*n* = 18)	Presence of Hirudin (*n* = 18)	p
WBC, 1,000/mm^3	Base	12.1 (10.4–13.7)	9.5 (7.8–11.2)	0.0340
	2 h	3.2 (2.5–4.0)	3.4 (2.6–4.1)	0.7926
	Last	2.0 (1.6–2.5)	2.1 (1.6–2.5)	0.8521
Platelets, 1,000/mm^3	Base	410 (342–478)	380 (314–446)	0.5325
	2 h	265 (213–316)	271 (221–321)	0.8711
	Last	176 (131–221)	203 (159–247)	0.3991

* Values are mean (95% confidence interval); p = factorial ANOVA.

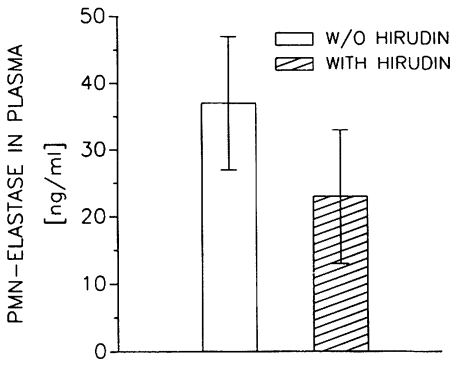

Fig. 6. Leukocyte neutral proteinase inhibitor (LNPI)-bound PMN elastase concentrations in porcine plasma during endotoxin infusion in the presence (with hirudin, n = 18) and absence of hirudin (without hirudin, n = 18) in pigs. Mean and 95% confidence interval of the last valid measurements of each animal are presented. At baseline, PMN elastase concentrations in plasma were below the detection limit (< 1 ng/ml) in all animals. The endotoxin-induced increase in PMN elastase in plasma was significantly smaller in the presence of hirudin ($p = 0.0137$; factorial ANOVA).

plasma was below the detection limit during the 6-h observation period.

Mortality Rate

All control animals (n = 6) survived the 6-h observation period. Of 36 animals given LPS, 5 died between 2 and 4 h after the start of the LPS infusion. A total of 12 animals died between 4 and 6 h, resulting in an overall mortality rate of 17 of 36 (47%). In the absence of hirudin, the mortality rate was 10 of 18 (56%) and in the presence of hirudin 7 of 18 (39%), respectively. This difference was not statistically significant (Mantel-Haenszel test).

Discussion

The results of the present study show that hirudin reduces the consumption of plasma fibrinogen and the increase in soluble fibrin in plasma in a porcine model of endotoxemia. The administration of hirudin also attenuates the increase in pulmonary vascular resistance, the increase in extravascular lung water, and the loss of protein-rich fluid from the vasculature following LPS infusion. Furthermore, hirudin reduces the LPS-induced increase in PMN elastase concentration in plasma.

The LPS-induced consumption of coagulation factors in the present study was similar to that observed in septic patients (2, 30). The decrease in plasma prothrombin and plasma fibrinogen and the increase in soluble fibrin in plasma are evidence of the generation of thrombin. As expected, hirudin had no effect on the plasma prothrombin levels but prevented the consumption of plasma fibrinogen and the increase in soluble fibrin in plasma; this indicates that hirudin effectively inhibits thrombin generated during LPS infusion. However, AT III in plasma decreased similarly in the absence and presence of hirudin. This may not be surprising, since the decrease in AT III during endotoxemia does not necessarily reflect intravascular coagulation (38). The consumption of AT III in endotoxemia may be more the result of unspecific proteolysis by, for example, PMN elastase (4). Furthermore, hirudin did not seem to have an inhibitory effect on the activation of plasmin since antiplasmin activities in plasma were not significantly different in the absence or presence of hirudin.

It is conceivable that the observed hirudin effects result from the inhibition of thrombin by hirudin. Hirudin is a highly specific inhibitor of thrombin and does not inhibit other serine proteases such as trypsin, plasmin, factors IXa, Xa, and XIIa, or plasma and tissue kallikreins (26, 39). The dose of hirudin we administered resulted in plasma levels in the range of 70 to 100 nmol/L, which equal 5 to 9 antithrombin units/ml. Since hirudin is a high-affinity inhibitor of thrombin that forms enzyme-inhibitor complexes in an equimolar ratio (25), this dosage ensures sufficient molar inhibitor concentrations if the thrombin concentration in plasma during activation of the coagulation cascade is assumed to be approximately 1 to 2 U/ml (40).

We determined the total intravascular protein as a product of the plasma volume and the plasma protein concentration to assess the loss of protein-rich fluid from the vasculature during LPS infusion. Hirudin reduced the loss of protein-rich fluid by approximately 36%, which suggests that thrombin inhibition by hirudin may reduce LPS-induced capillary leakage. Furthermore, hirudin attenuated the LPS-induced acute lung injury as evidenced by a smaller increase in PVR and in extravascular lung water. All animals showed the typical biphasic response in PVR to endotoxin (7, 8). Hirudin inhibited the increase in the later phase, in agreement with previous studies using native hirudin from the leech in endotoxemic pigs (5). Hirudin also blunted the increase in EVLW that occurred during LPS infusion. This may be due to the reduced pulmonary pressor response or to less increase in permeability of the lung vasculature to protein; the results of the present study do not allow us to discriminate between these two possibilities. Despite reduced EVLW increases in the presence of hirudin, $aaPo_2$ decreased to the same degree in the absence and in the presence of hirudin; this suggests that pulmonary edema was not of major importance in the deterioration of pulmonary gas exchange. These findings are in accordance with observations in other animal models of LPS-induced acute lung injury (7, 41) and indicate that arterial hypoxemia following LPS infusion is primarily a consequence of ventilation/perfusion inequalities.

It may be exceedingly difficult to determine the separate and specific mechanisms of the observed attenuation of acute lung injury in the presence of hirudin. Less consumption of plasma fibrinogen and a smaller increase in the soluble fibrin in plasma in the presence of hirudin may be one explanation. In isolated perfused rabbit lungs, soluble fibrin causes vasoconstriction and pulmonary hypertension (19). It is also conceivable, however, that less fibrin generation in the presence of hirudin resulted in less generation of fibrin degradation products (FDP). FDP released after the action of plasmin on fibrin are thought to contribute to the development of acute lung injury by increasing the permeability of the lung vasculature to protein (21, 42). We have previously shown that the infusion of a fibrin-derived peptide into pigs causes an increase in EVLW (43). However, there may be alternative explanations, other than inhibition of the proteolytic cleavage of fibrinogen by thrombin, for the observed hirudin effects. In this respect it is notable that in the absence of fibrinogen in isolated guinea pig lungs perfused with Ringer's albumin, hirudin also blocks the pulmonary pressure response and pulmonary edema formation after the injection of thrombin (44). In this and other models of thrombin-induced acute lung injury, as well as in models of LPS-induced acute lung injury, the pulmonary hemodynamic response has been shown to be mediated in part by the generation of cyclo-oxygenase-derived metabolites such as thromboxane (8, 44, 45). Thrombin is known to directly induce the *in vitro* generation of thromboxane from endothelial cells, vascular smooth muscle cells (16, 17), and PMN leukocytes and lymphocytes (18). Hirudin has been shown to abolish the thrombin-induced thromboxane generation from PMN leukocytes and lymphocytes *in vitro* (18). Likewise, hirudin inhibited the thrombin-induced contraction of vascular smooth muscles of isolated rabbit aorta and dog coronary

arteries (14). Therefore it also seems possible that hirudin may block the direct cellular effects of thrombin that are independent of the proteolytic cleavage of the fibrinogen molecule.

In animal models of thrombin infusion, PMN leukocytes appeared to be important in mediating thrombin-induced acute lung injury since the response was attenuated by PMN depletion (9). We observed a rapid and sustained decrease in the circulating leukocyte count following endotoxin infusion, but we did not detect a significant difference in leukopenia in the presence or absence of hirudin. One explanation may be that plasmin activation occurred similarly in the absence and in the presence of hirudin. Plasmin causes the cleavage of complement proteins and the formation of the complement-derived chemotactic peptides, C3a and C5a (22, 46). On the other hand, the thrombin molecule itself is a weak chemotactic agent and induces PMN aggregation (47); this effect is blocked when thrombin is complexed with hirudin (15). Although peripheral leukopenia does not seem to be affected by hirudin, we provide evidence that hirudin attenuated the lysosomal discharge of PMN leukocytes as determined by inhibitor-bound PMN elastase concentrations in plasma. Plasma levels of LNPI-bound PMN elastase were lower in the presence of hirudin than in the absence of hirudin. LNPI, an inhibitor of neutral proteinases, was recently isolated and characterized from porcine PMN leukocytes (34). The occurrence of this inhibitor and of the LNPI-PMN elastase complex in plasma has been identified as a marker of PMN leukocyte activation in porcine experimental septicemia (35). The data therefore demonstrate that hirudin reduces endotoxin-induced increases in PMN elastase in plasma, suggesting thrombin activation is an important factor in PMN activation after LPS infusion.

In conclusion, our data substantiate the role of thrombin in mediating acute lung injury following LPS infusion in pigs. Possible mechanisms involve the generation of soluble fibrin and FDP, a direct effect of thrombin, and the activation of PMN leukocytes. Administration of hirudin, a highly selective thrombin inhibitor, prevented the LPS-induced intravascular coagulation and attenuated the LPS-induced acute lung injury.

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