Influence of Antithrombin III on Coagulation and Inflammation in Porcine Septic Shock

Gerhard Dickneite, Boris Leithäuser

Abstract—The physiological inhibitor of thrombin, antithrombin III (ATIII, Kybernin P) was investigated for its antiinflammatory and anticoagulant effects in a pig model of septic shock. Pigs were infused with a dose of 0.25 μg · kg⁻¹ · h⁻¹ of lipopolysaccharide (LPS) over a period of 3 hours. Animals developed systemic inflammation, disseminated intravascular coagulation (DIC), organ failure and cardiovascular abnormalities, namely pulmonary hypertension and systemic hypotension. Twenty septic pigs were allocated to 2 study groups, treated either with ATIII (n=10) or placebo (n=10). ATIII was administered as a 250-U/kg IV bolus infusion for 30 minutes (~60 to ~30 minutes) followed by a single IV bolus of 125 U/kg (t=0) and a second 30-minute infusion of 250 U/kg (120 to 150 minutes). ATIII significantly prevented the development of a DIC; the increase in fibrin monomers (placebo, 11.4±9.1 reciprocal titers, at 6 hours) was completely overcome by ATIII (P<0.05). ATIII significantly prevented the increase in thromboxane (TXB₂) levels, which were 809±287 pg/mL in the placebo and 420±174 pg/mL in the verum group after 6 hours (P<0.02). On the other hand, ATIII had no influence on TNF levels. In a lethal study with an increased dose of LPS (0.5 μg · kg⁻¹ · h⁻¹). A significant reduction in mortality was observed in the ATIII group (0 of 7) compared with the placebo group (4 of 6) (P<0.05, χ² test) a significant reduction of pulmonary hypertension (placebo, 42.0±11.1 mm Hg; ATIII, 23.6±7.5 mm Hg, P<0.05), but no effect on systemic hypotension, was noted in the ATIII group. It was thus concluded that modulation of the procoagulatory state by substitution of ATIII results in a late beneficial antiinflammatory effect in this model of septic shock. (Arterioscler Thromb Vasc Biol. 1999;19:1566-1572.)

Key Words: lipopolysaccharide ■ disseminated intravascular coagulation ■ pulmonary hypertension ■ soluble fibrin monomers ■ thromboxane

Sepsis as a severe complication of infection is characterized by systemic inflammation, activation of proteolytic cascades, coagulation abnormalities (DIC) and a gradually developing hypodynamic status with impaired organ perfusion, and finally death in a septic shock. Lung circulation in septic shock is characterized by hypertension and increased pulmonary resistance, resulting in low cardiac output, and, frequently, in lung failure. Mortality in septic shock is usually high, ie, in the range of 40% to 50%. If associated with multi-organ dysfunction such as respiratory or renal failure, mortality might exceed 90%. The initiating event for the development of sepsis is the activation of macrophages by lipopolysaccharide (LPS) liberated from Gram-negative bacteria via binding to its surface receptor CD14. However, sepsis might be induced by other agents, such as Gram-positive bacteria, fungi or viruses. The initial excessive secretion of cytokine mediators such as interleukin 1 (IL-1) and tumor necrosis factor (TNF-α) is followed by the activation of biological cascades including the coagulation, the complement, the fibrinolytic and the kallikrein-kinin systems, which contribute to the maintenance of the inflammatory reaction. Arachidonic acid metabolites are thought to play an important role in hemodynamic alterations, thus thromboxane A₂ (TXA₂) appears to be associated with pulmonary arterial hypertension while prostacyclin (PGI₂) might contribute to the lowering of lung blood pressure. In particular the activation of the coagulation cascade induces the uncontrolled generation of thrombin from its precursor molecule prothrombin, and leads to DIC, which is associated with consumption of coagulation factors and microthrombosis. Under the conditions of hemostatic balance the activity of thrombin is controlled by its physiological antagonist antithrombin III (ATIII). ATIII is a single-chain glycoprotein with a Mr of 58 000 Da, which is a progressive inhibitor of serine proteases. The inhibitor has a binding site for heparin; its activity is increased dramatically by heparin through accelerating the binding rate to its target protease. ATIII interacts with several proteases of the plasma; besides thrombin, it inhibits kallikrein, factors IXa, Xa, XI, and XIIa.

ATIII has been shown to be efficacious in several experimental models of sepsis and septic shock, regardless of the species investigated, as shown in baboons, dogs, sheep, rabbits, rats and chicken embryos. ATIII in these models proved to be effective after inducing a sepsis or
septic shock with different agents including live bacteria (Escherichia coli, Klebsiella pneumoniae), bacterial lipopolysaccharide and lactic acid. In a guinea pig model Kessler et al demonstrated that ATIII could prevent DIC and organ hemorrhage and improve mortality after infection with the Gram-positive bacterium Staphylococcus aureus. Clinical efficacy in patients with sepsis or septic shock has been demonstrated by several authors (for a review see Reference 22), the rationale for the therapy being the prevention of DIC. A recently published paper on the meta-analysis of ATIII in seve sepsis demonstrated a clear trend towards increased survival. However, some authors suggest that ATIII might have antiinflammatory as well as anti-DIC activity.

In the present study we induced a sepsis by the infusion of Salmonella abortus equi lipopolysaccharide into pigs; the development of systemic inflammation, coagulation activation and hemodynamic changes was followed over time. The influence of ATIII administration on the outcome of the sepsis was investigated.

Materials and Methods

Substances

Salmonella abortus equi lipopolysaccharide (S equi LPS) and Indomethacin were purchased from Sigma (Deisenhofen, Germany). Antithrombin III (Kybernin P) and human serum albumin (Human Albumin 25% Behring) were provided by Centeon Pharma GmbH. Pentobarbital was delivered by Sanofi-Ceva. Ketamine-HCI was from Parke-Davis and Xylazin-HCI from Bayer. Ringer-lactate solution (DAB7) was purchased from Braun-Melsungen.

Endotoxic Pig Model and Treatment Protocol

Male juvenile castrated pigs (German domestic pig, 19 to 32 kg, 3 to 4 months) were purchased from a local supplier. Animals were housed in conventional stables at an ambient temperature of 15°C to 21°C with straw bedding. They were fed a Deuka V pig chow (Deuka) and tap water ad libitum.

Animals were subjected to a veterinarian health inspection before use. Animals showing unusual coagulation, hyper- or hypotension, elevated temperature, leukopenia or leucocytosis were excluded from the study.

For the maintenance anesthesia, pentobarbital was given as a 7.5-mg/kg IV bolus followed by a 10-mg·kg⁻¹·h⁻¹ intravenous infusion, performed with a Havard infusion pump 22 (FMI). Pigs were ventilated mechanically via a tracheal tube by a RUS-130 respirator with room air and 150 L/min. Respiration rate was 16 breaths/min with 40% inspiration. CO₂ in the expiration air was monitored by a Normocap 200 CD2 to 02 (Hoyer).

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Test substances were infused via an abdominal vein by a polyethylene catheter (0.5 x 2.1 mm, Braun–Melsungen, Melsungen), and samples drawn from the vena jugularis externa. A volume substitution of 150 mL/h was performed with Ringer-lactate solution. A volume substitu-

Coagulation Assays

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined with Neothrombin or with Thromborel S (Behringwerke AG) in a Schnitter & Gross coagulometer.

Coagulation Factor VIII (F VIII)

The principle of the detection of pig factor VIII was to substitute factor VIII-deficient human plasma (Behringwerke AG) with the pig samples. The prolonged aPTT in the factor VIII-deficient human plasma was shortened by admixing defined amounts of standard human plasma (SHP) to obtain a calibration curve. Pig plasma factor VIII was expressed as percentage of SHP (defined as 100%).
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Figure 1. Course of endotoxemia in pig sepsis. Pigs were infused over a period of 3 hours with a dose of 0.25 μg·kg⁻¹·h⁻¹ of S equi LPS (●). Blood was withdrawn at the indicated time points, heparin-plasma was prepared and tested for endotoxin levels with a LAL assay. Values are given in European units (EU)/mL as means and standard deviations. A control animal receiving no LPS (n=1) generated only baseline values (<0.0125 EU/mL - ▲).

Tumor Necrosis Factor (TNF)

TNF was determined with the pig TNF-α ELISA test kit (Bioxol). The 2 antibodies used were a solid-phase polyclonal antibody against pig TNF-α and a POD-conjugated monoclonal antibody against pig TNF-α.

von Willebrand Factor (vWF)

With an Asserachrom vWF ELISA test kit the vWF concentration in plasma was determined (Boehringer). The 2 antibodies used were a solid-phase F(ab)₂, anti-human vWF antibody and an anti-vWF-peroxidase–conjugated antibody.

Detection of Endotoxin Plasma Levels

Endotoxin was measured with the LAL chromogenic QCL 1000 test kit (Bioproducts).

Thromboxane Plasma Levels

As TXA₂ is unstable in plasma the stable metabolite TXB₂ was measured with a competition ELISA (Amersham Life Science). Free TXB₂ competes with POD-labeled TXB₂ for the binding to the solid-phase anti-TXB₂.

Clinical Chemistry

Creatinine, urea and GOT (glutamate oxaloacetate transaminase) were measured in plasma by test strips (Reflotron, Boehringer).

Statistics

Differences between mortality rates were determined by the χ² test; differences between other parameters were detected with the Student’s t test.

Results

After infusion of 0.25 μg·kg⁻¹·h⁻¹ S equi LPS, pigs developed increasing endotoxin plasma levels off at 3 hours, the termination point of infusion (Figure 1). Endotoxin levels decreased thereafter to reach baseline levels 1 hour later. The inflammatory reaction was demonstrated by a rapid increase in TNF-α plasma levels, reaching a plateau between 1 and 2 hours to decrease again after 2 hours, ie, before the termination of the LPS infusion (Figure 2). The Table depicts the changes of parameters during sepsis in pigs (n=10, treated with HSA) at 6 hours after the start of the LPS infusion. As DIC developed, the levels of ATIII decreased to 70% of the baseline value; concomitantly the TAT levels increased about 10-fold. Uncontrolled thrombin activation led to an increase in soluble fibrin monomers (sFM); fibrinogen levels decreased slightly. Consumption of coagulation factors like F VIII resulted in a prolonged aPTT (nonsignificant) and PT. A decrease of platelets might indicate the formation of disseminated microthrombi. An increase in circulating vWF was demonstrated, which was thought to be secreted from the Weibel-Palade bodies of the disturbed endothelium. Marked leukocytopenia with a nadir at 2 hours was explained by the

Change of Physiological Parameters During Sublethal Porcine Sepsis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (t=0) Mean ± SD</th>
<th>6 Hours Post-LPS Mean ± SD</th>
<th>Significance, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mm Hg)</td>
<td>92.5 ± 17.6</td>
<td>31.1 ± 5.9</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>PCWP (mm Hg)</td>
<td>5.40 ± 2.21</td>
<td>7.2 ± 6.5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GPT (µL)</td>
<td>15.6 ± 5.1</td>
<td>32.8 ± 28.7</td>
<td>NS</td>
</tr>
<tr>
<td>Urea (mg/L)</td>
<td>214 ± 29</td>
<td>301 ± 42</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>6.0 ± 1.0</td>
<td>11.0 ± 3.0</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

n=10 pigs, treated with HSA. MAP indicates mean arterial pressure; PCWP, pulmonary capillary wedge pressure.

*% of standard human plasma; †% of standard porcine plasma; ‡median of WBC was 3.8 ± 1.4 at 120 minutes after LPS.
sticking of leukocytes to the activated endothelium. Cardiovascular changes were visualized by systemic hypotension and pulmonary hypertension as well as a slight increase in the pulmonary wedge pressure. Increased plasma levels of GOT, urea and creatinine were indicative of hepatic and renal failure.

To evaluate the mechanism by which ATIII interferes with sepsis and septic shock we allocated 20 septic pigs to treatment with ATIII (n = 10) or HSA (n = 10). ATIII plasma levels (activity) were up to about 500% to 600% of baseline level at the time when we started the administration of LPS (Figure 3). After the end of the third ATIII infusion plasma levels decreased again and the terminal half-life was calculated at ≈16 hours. We evaluated the influence of ATIII on the development of inflammatory cytokine levels. As shown in Figure 2, the placebo-treated group and the ATIII group depicted essentially the same plasma levels of TNF-α; in both groups maximal plasma levels were ≈700 pg/mL. TXB2 plasma levels increased rapidly in the placebo as well as in the ATIII group to reach a maximal value of ≈1100 pg/mL after 30 minutes (Figure 4). TXB2 levels in the placebo group stayed elevated until termination of the study, whereas in the ATIII group a significant decrease was obtained towards the end of the experiment. TXB2 levels at 6 hours were 809±287 pg/mL and 420±174 pg/mL in the placebo and ATIII groups, respectively (P<0.02, t test).

We investigated the course of DIC by measuring the formation of soluble fibrin monomers (sFM). Figure 5 demonstrates a steady increase of this marker fibrinogen activator in the placebo group. ATIII was able to totally prevent the increase in sFM (P<0.05, t test). Marked thrombocytopenia to about 70% of baseline level developed in the untreated animals (Figure 6). Although ATIII could not prevent platelet drop, there was a small but significant increase in platelet numbers when compared with control (P<0.05, t test). An increased inactivation of thrombin was demonstrated by the higher formation of TAT complexes in the ATIII group. Whereas in the placebo control TAT levels were 176.7±96.7 μg/L at the end of the experiment, the ATIII group TAT levels were 526±448.8 μg/L. Both the increase in aPTT and PT were less pronounced in the ATIII group, although the differences were not statistically significant (data not shown). None of the 20 animals in this study died during the observation period.

In a separate lethality study, with the LPS dose increased to 0.5 μg·kg⁻¹·h⁻¹, we included a total of 13 pigs. Seven animals were given the same ATIII regimen as in the sublethal study; HSA was administered to 6 septic pigs. Sixty-six percent (4 of 6) of the placebo animals were dead at 6 hours post-LPS, whereas none (0 of 7) of the pigs of the ATIII group died during the observation period of 6 hours (P<0.01, χ² test).

Pulmonary artery pressure in the lethal study showed the typical, 2-peaked increase after the induction of sepsis (Figure 7). In particular, the second increase in pressure, which had a peak value of 42.0±11.1 mm Hg in the placebo group.
At 3 hours, was prevented by ATIII treatment (23.6±7.5 mm Hg, P<0.05). In contrast, in the sublethal study the increase of the PAP was less pronounced and was not different in the ATIII and placebo groups (23.5±6.1 versus 22.3±5.0 at 180 minutes post-LPS).

No influence of ATIII on systemic arterial blood was detected in the lethal or in the sublethal study (data not shown).

Discussion

The aim of the present study was to provide data on the mechanism of action of ATIII on sepsis and septic shock. We selected the prophylactic ATIII regimen that had been introduced by Taylor et al for their studies in baboons, and which included 3 administrations of ATIII.11 For our studies we used the pig model because the hemodynamic situation in this species resembles the human one. The major data presented here were obtained from a sublethal pig sepsis study, and a smaller part concerning ATIII’s effect on mortality rate and pulmonary artery pressure came from a lethal study. In the sublethal study we investigated ATIII’s anticoagulant and anti-inflammatory mechanism. The scope of these investigations was to provide data for ATIII’s mechanism of action.

In the lethal study we demonstrated prevention of sepsis-related mortality in animals infused with ATIII in our endotoxic pig model. Whereas mortality was high in the placebo group (66%) and thus in a range observed in patients suffering from severe septic shock, all ATIII-treated animals survived. The results in our pig model are thus in line with the observations of other authors obtained in animal models or in clinical studies.27

However, the precise mechanism by which ATIII exerts its protective effect in sepsis and septic shock is still under discussion. ATIII is the principal inhibitor of thrombin and several other proteases of the coagulation system, thus being responsible for the hemostatic balance. As sepsis and septic shock are frequently associated with DIC, resulting in decreased ATIII levels,28,29 there is a clear rationale for a substitution therapy with ATIII in the treatment of sepsis and septic shock. The bulk of evidence in preclinical and clinical studies suggest that ATIII can modulate excess activation of the coagulation system and prevent the consumption of clotting factors and the decrease in platelets,11,14,12,21,30 although a limited impact on DIC has occasionally been reported.16 During sepsis the extrinsic pathway of coagulation is activated by the expression of tissue factor after stimulation of the endothelium with inflammatory cytokines such as TNF-α or IL-1β. Our data indicate that ATIII does not interfere with this early step in the inflammatory response. TNF levels were essentially the same in the ATIII treatment and placebo groups.

The data demonstrated in our pig model show a modulation of DIC by ATIII as it prevents the increase in fibrin monomers and, in part, the decrease in platelet counts. Moreover, increased TAT levels in the ATIII group indicate that newly generated thrombin was effectively bound to and inhibited by ATIII. As thrombin is inhibited predominantly by ATIII, one can conclude that less free thrombin is present in the ATIII group resulting in reduced levels of fibrin monomers, the primary product of thrombin’s action on fibrinogen. It can be concluded that prevention of DIC by ATIII is an important, but not the only contribution of ATIII in overcoming septic shock. Our own previous data in a rat-Klebsiella sepsis model demonstrate only a limited effect of ATIII on DIC parameters18 but a significant prevention of sepsis-related death. On the other hand, the highly potent inhibitor of thrombin, recombinant hirudin, could very efficiently suppress DIC in the same sepsis model. However, no further improvement in mortality was observed with this treatment.31 Thus, the search for an alternative mechanism of action for ATIII appears to be a challenge. Our data are in line with those of Spannagl et al,32 who used a complex of ATIII and heparin in an LPS-pig sepsis model. The authors clearly showed a prevention of FM increase by ATIII/heparin. As heparin accelerates the binding of ATIII to thrombin, this was clearly attributed to ATIII’s anti-DIC effect. However, the ATIII/heparin complex failed to show a significant effect on survival. The failure of ATIII/heparin to be protective in that model might be explained by a series of experiments performed by Okajima and coworkers.25,33 This group suggested that heparin impairs the positive effect of ATIII by competing for the glycosaminoglycan binding sites at the endothelium. Direct binding of ATIII to endothelial cells has been demonstrated by Stern et al34 in bovine aortic segments. It has been
shown by other authors that ATIII, in vitro or in vivo, stimulates the production of PGI$_2$ from endothelial cells, which might be explained by its binding to the glycosaminoglycan structure. As PGI$_2$ inhibits platelet aggregation and promotes vasodilation in lung arteries, this might explain ATIII’s beneficial effects on sepsis. Additional evidence that ATIII binds to endothelial glycosaminoglycans came from studies other than sepsis studies. Pseudorabies viruses bind to endothelial cells via their heparin sulfate receptors. Voigt et al demonstrated that ATIII inhibits the binding of the virus to the endothelium. In a recent paper Ostrovsky et al describe that the leukocyte-endothelium interaction was inhibited by ATIII in a feline mesenterial ischemia/reperfusion injury.

Our data show that pulmonary hypertension in the pig sepsis model is decreased by ATIII, thus leading to improved lung function. On the other hand, ATIII has no effect on systemic blood pressure. Prevention of pulmonary hypertension by ATIII might be explained by the decrease in plasma TX$_B_2$. As thromboxane is secreted by platelets it might be speculated that the decrease in TX$_B_2$ is related to the inhibition of thrombin’s action on platelets by ATIII.

It must be taken into consideration, however, that this study was a prophylactic one and that these data must be confirmed by a therapeutic ATIII regimen. A series of experiments has begun in pigs with a therapeutic ATIII treatment and a dose regimen adopted from the ongoing phase III sepsis study in humans.

However, an alternative explanation for the prevention of pulmonary hypertension by ATIII has to be considered. Seeger et al have shown that fibrin monomers generated by excess thrombin activity might induce vasoconstrictor response in lungs via thromboxane. As ATIII effectively prevented fibrin monomer formation, improvement of lung function might be mediated via this mechanism.

ATIII does not inhibit the early inflammatory event in sepsis, the production of TNF-$\alpha$, but it reduces the plasma levels of intermediate or end products of the inflammatory mediator systems. Thus, it might be concluded that ATIII produces a late antiinflammatory effect through modulation of the procoagulatory reactions during the progression of septic shock.

The development of a septic shock is paralleled by the decrease in ATIII levels, and the mortality rate was shown to be high in patients with low ATIII levels. When ATIII was introduced for the treatment of sepsis several years ago it was suggested for treatment of patients with 20 to 40 U/kg ATIII, a dose leading to ATIII levels of maximally 100%. In recent years evidence accumulated that this dose might not be sufficient for the treatment of sepsis. In a clinical study in patients suffering from peritonitis, Jochum et al were able to demonstrate a clinical benefit when ATIII plasma levels were adjusted to values of 120% to 140%. Obertacke et al reported a shorter stay in the ICU in polytraumatised patients with ATIII plasma levels brought to $\sim 140\%$. In a placebo-controlled double-blind study conducted by Fourrier et al, ATIII levels were kept at 200% for $\geq$ 3 days in septic patients. The authors reported a reduction in mortality of $\sim 30\%$; however, due to the low number of patients, the difference was not significant. Our own data support the assumption that substantially higher levels than 100% ATIII are needed. In accordance with other animal studies ATIII plasma levels should reach a range of $\geq 400\%$ to 500%, 11,18,19 The question as to whether this high concentration really is necessary for a clinical study in humans cannot be answered conclusively, because one has to bear in mind that in all animal experimentation a human protease inhibitor (ATIII) has to interact with an animal protease (thrombin). It might thus be concluded that in humans lower levels of ATIII ($\sim 200\%$) could be sufficient, as suggested in a recent ATIII pharmacokinetic study in septic patients.74 Future clinical trials with supranormal ATIII plasma levels are thus mandatory to clarify this question.

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References
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