The effects of supra-normal protein C levels on markers of coagulation, fibrinolysis and inflammation in a human model of endotoxemia

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Summary
The protein C pathway serves as a modulating system with both anti-inflammatory and anticoagulant properties and is intimately involved in the pathophysiology of inflammation and sepsis. Treatment with recombinant human activated protein C (rhAPC) can reduce the mortality of severe sepsis. We investigated whether an elevation of plasma protein C levels by infusion of a protein C zymogen concentrate has an effect on coagulation, protein C activation or inflammation in a human endotoxemia model. Eleven healthy male volunteers were enrolled in a double-blind, placebo-controlled two-way cross-over trial. Ten minutes after infusion of 2ng/kg endotoxin each volunteer received either placebo or a plasma-derived protein C zymogen concentrate (Ceprotin®, Baxter) (150 U/kg as a slow bolus infusion followed by 30 U/kg/h continuous infusion until 4 hours after LPS-infusion). Protein C antigen and activity increased 4–5-fold after infusion of the concentrate. APC was generated during endotoxin-induced inflammation in the placebo (1.6 fold increase) and the protein C period (4.0-fold increase). The increase of APC levels correlated with the TNF-α and IL-6 release in both periods (r=0.65–0.68; p<0.05) and paralleled the protein C antigen and activity levels in the period with supranormal protein C levels. Supra normal protein C levels resulted in slightly, although non-significant, lower tissue factor mRNA expression and thrombin generation (TAT, F1+2). Systemic inflammation (TNF-α, IL-6) was not influenced by protein C zymogen concentrate administration. Infusion of protein C zymogen was safe and no adverse effects occurred. The increase of protein C levels several fold above the normal range resulted in a proportional increase of the APC levels, but had no major anticoagulant, anti-inflammatory or profibrinolytic effects. Low grade endotoxemia itself induces significant protein C activation, which correlates with the TNF release.

Keywords
Endotoxin, protein C, coagulation, inflammation

Introduction
The haemostatic and fibrinolytic systems are intimately involved in the pathophysiology of sepsis. A pronounced activation of the procoagulatory mechanisms, together with a shut-down of fibrinolysis, leads to the formation of fibrin microthrombi in the microvasculature. The consumption of coagulation factors and platelets enhances the bleeding tendency – the result is the clinical picture of disseminated intravascular coagulation (DIC) (1, 2). The resulting hypoperfusion causes tissue damage: septic patients are considerably sick, need intensive treatment and have, in part, severely compromised organ dysfunction (MODS), associated with a high mortality.

Inflammatory and procoagulant host responses are closely related (3, 4). Infectious agents and inflammatory cytokines activate coagulation by stimulating the expression of tissue factor (TF) on endothelial cells and monocytes. The protein C pathway serves as a regulatory pathway by controlling the conversion of prothrombin to thrombin through a feedback inhibition mechanism. Reduced protein C levels are strongly correlated with mortality and septic shock (5–8). Recently, activated recombinant human protein C (rhAPC; drotrecogina lpha [activated]) has been shown to reduce mortality and morbidity of patients with severe sepsis in a large multi-center trial. (9).

A distinct manifestation of sepsis-associated DIC is purpura fulminans with its typical cutaneous morphology. The coagulo-
pathic processes in the microcirculation manifest as micro thrombosis and necrosis, and often acral gangrene and necrosis may develop. A dysfunction of the protein C pathway is always present in purpura fulminans and contributes to the development of coagulopathy and necrosis. Data from several case reports, case series, and from one open label study, lead to the hypothesis that early substitution of protein C zymogen could have a positive influence on the progression of DIC in sepsis induced purpura fulminans (7, 10–13).

The aim of the following study was to investigate whether an elevation of plasma protein C to levels beyond the upper normal range has a suppressive effect on endotoxin induced inflammation, coagulation activation, or fibrinolysis.

Methods

Study design
The trial was approved by the Ethics Committee of the University of Vienna and all participants gave written informed consent. The trial was performed at a single center (Department of Clinical Pharmacology).

We enrolled 11 healthy male volunteers with an average age of 26 years (range: 23–34 years) and a mean body mass index of 22.0 kg/m² (range: 20.5–23.2) in a randomized, double-blind, placebo-controlled two-way cross-over trial using a standardized model of endotoxin-induced systemic inflammation. Medical screening included medical history, physical examination, laboratory parameters, and virologic and standard drug screening. In addition, study subjects were tested for hereditary thrombophilia i.e. Factor V Leiden, Protein C and S deficiency, to minimize potential risks of endotoxin-induced coagulation activation (14). Exclusion criteria were regular or recent intake of any drug.

Our human endotoxemia model is a well-standardized model of systemic inflammation and TF-induced coagulation activation. Detailed study procedures of the lipopolysaccharide (LPS) model have been outlined in other trials previously (15, 16). All volunteers reported to the study ward at 08.00 hours, after an overnight fast. Throughout the entire study period all volunteers had to lie in bed and were kept fasting for 2 hours after endotoxin infusion. Vital variables (ECG, heart rate and oxygen saturation, blood pressure) were monitored on an automated monitoring system (Care View System, Hewlett Packard, Böblingen, Germany). The study subjects received 2 ng/kg of endotoxin (National Reference Endotoxin, Escherichia coli, United States Pharmacopeial Convention Inc., Rockville, MD, USA) as an intravenous bolus infusion for 1 to 2 min. A continuous infusion of 200 m/h saline was given during 8 hours. All volunteers were requested to attend the clinic in the morning of the following day for blood sampling and to report of any adverse events.

Each participant was studied at 2 occasions: during the first study period, five volunteers received 150 U/kg (1U = ~4µg) of a plasma-derived human protein C zymogen concentrate (Ceprotin®, Baxter, Austria) as a slow bolus infusion (2mL/min i.e. 200U/min) starting 10 min. after the LPS infusion, followed by a continuous infusion with 30 U/kg/h until 4 hours after LPS-infusion. Six volunteers received identical volumes of saline as a placebo. After a washout period of 6 weeks a second study period was conducted according to the cross-over design, where placebo and protein C infusions were exchanged.

Blood sampling
Blood samples were collected into citrated or EDTA–anticoagulated tubes (Vacutainer; Becton Dickinson, Vienna, Austria) by venipuncture shortly before and 1, 2, 3, 4, 5, 6, 8, and 24 hours after LPS-infusion. For the determination of the circulating APC, 1/20 of blood volume of 0.2M Hepes and 0.6M benzamidine were additionally added to prevent the inactivation of APC during the plasma preparation procedure. Plasma was obtained by centrifugation at 2000g (15 minutes at 4°C) and stored in 0.5 mL aliquots at −80°C until batch analysis.

Laboratory analysis
Protein C antigen (PC:Ag) was determined by a commercial sandwich ELISA (Asserachrom Protein C, Boehringer-Mannheim, Mannheim, Germany) and protein C activity (PC:act) was measured with a chromogenic assay (ACL 3000 plus; Instrumentation Laboratory, Lexington, MA).

Circulating APC activity (APC) was measured with an ELISA Capture Assay slightly modified from Gruber and Griffin (17) as follows: Blood samples were taken in benzamidine-containing anticoagulant, which irreversibly inactivates APC, and protects it from the natural inhibitors during the plasma preparation and the sample handling process. Protein C and APC is captured from the plasma to the surface of ELISA plates

Table 1: Baseline levels of variables before administration of any drug.

<table>
<thead>
<tr>
<th></th>
<th>Placebo mean (295 CI); n = 11</th>
<th>Ceprotin® mean (95 CI); n = 11</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>activated protein C (ng/mL)</td>
<td>1.07 (0.87–1.26)</td>
<td>1.09 (0.94–1.26)</td>
<td>ns.</td>
</tr>
<tr>
<td>protein C activity (U/mL)</td>
<td>1.08 (0.93–1.22)</td>
<td>1.07 (0.93–1.21)</td>
<td>ns.</td>
</tr>
<tr>
<td>tissue factor activity (nM)</td>
<td>0.38 (0.30–0.46)</td>
<td>0.40 (0.28–0.51)</td>
<td>ns.</td>
</tr>
<tr>
<td>thrombin-antithrombin complex (μg/L)</td>
<td>2.11 (1.91–2.31)</td>
<td>2.24 (1.73–2.74)</td>
<td>ns.</td>
</tr>
<tr>
<td>plasmin-antiplasmin complex (mg/L)</td>
<td>0.37 (0.28–0.47)</td>
<td>0.37 (0.3–0.44)</td>
<td>ns.</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2.68 (1.01–4.33)</td>
<td>2.61 (1.15–4.08)</td>
<td>ns.</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.01 (0.73–1.29)</td>
<td>2.08 (0.21–3.94)</td>
<td>ns.</td>
</tr>
<tr>
<td>Tissue factor mRNA, cycles</td>
<td>35.1 (34.4–35.7)</td>
<td>34.6 (33.9–35.2)</td>
<td>ns.</td>
</tr>
<tr>
<td>Hemoglobin, (g/dL)</td>
<td>14.6 (13.6–15.5)</td>
<td>14.4 (13.5–15.3)</td>
<td>ns.</td>
</tr>
<tr>
<td>Platelet count (GL)</td>
<td>219 (189–249)</td>
<td>214 (185–243)</td>
<td>ns.</td>
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<tr>
<td>Neutrophil count (GL)</td>
<td>2.96 (2.23–3.69)</td>
<td>2.90 (2.36–3.34)</td>
<td>ns.</td>
</tr>
<tr>
<td>Monocyte count (GL)</td>
<td>0.58 (0.43–0.73)</td>
<td>0.53 (0.43–0.63)</td>
<td>ns.</td>
</tr>
<tr>
<td>Lymphocyte count (GL)</td>
<td>2.33 (1.80–2.85)</td>
<td>2.32 (1.88–2.76)</td>
<td>ns.</td>
</tr>
<tr>
<td>Heart rate, (beats per min)</td>
<td>66 (60–72)</td>
<td>63 (54–71)</td>
<td>ns.</td>
</tr>
<tr>
<td>Systolic blood pressure, (mmHg)</td>
<td>120 (113–128)</td>
<td>122 (116–127)</td>
<td>ns.</td>
</tr>
<tr>
<td>Diastolic blood pressure, (mmHg)</td>
<td>60 (55–65)</td>
<td>64 (58–70)</td>
<td>ns.</td>
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coated with a non-inhibitory antibody against protein C. After a defined wash-procedure, where the benzamidine is eliminated, the activity of bound APC is determined by incubation with an APC-specific chromogenic substrate. The results are calculated from a reference curve, using purified APC instead of the plasma sample. The circulating human APC activity was expressed in ng/mL, compared to a purified plasma-derived APC preparation (Baxter, Vienna, Austria). The detection limit was 0.5 ng/mL, which was the lowest concentration used for creating the reference curves.

Most coagulation and inflammatory parameters were measured by enzyme immunoassays. Plasma levels of prothrombin fragment (F1+2) (Enzygnost® F1 + 2 micro; Dade Behring, Marburg, Germany) and thrombin-antithrombin complexes (TAT) (TATs, Enzygnost® TAT micro; Dade Behring) were used as markers of in vivo thrombin generation and plasmin-antiplasmin complexes (PAP) (PAPs, Enzygnost® PAP micro; Dade Behring) as marker for endogenous fibrinolytic capacity.

Tumor necrosis factor- (TNF-) and IL-6 (high-sensitivity TNF- and IL-6; R&D Systems, Minneapolis, MN), soluble E-selectin and P-selectin (R&D Systems), elastase (PMN-Elastase; Immundiagnostik, Bengsheim; Germany) were measured to determine inflammatory responses, activation of endothelium and platelets, and degranulation of neutrophils, respectively.

Differential blood counts were quantified with a cell counter (XE-2100; Sysmex, Milton Keynes, United Kingdom).

For RT-PCR analysis, all blood samples were immediately processed to avoid storage-induced alterations of mRNA levels. After isolating total RNA from total blood with the QuiAmp RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, mRNA was directly transcribed into cDNA using the RT-Reagent kit (Applied Biosystems, Foster City, CA) and stored at –80°C until analysis.

TF-mRNA quantification was performed with the Abi Prism 7700 (Applied Biosystems) using primers designed by Primer Express Software (Applied Biosystems) and synthesized based on the human TF cDNA sequence as previously described (18). TF was normalized against the reference gene (18s) according to the 2-ΔΔct method (19) and data are expressed as fold increase over baseline values. Dilution curves of TF-mRNA obtained from LPS-incubated blood samples revealed linearity (r=0.999) of the assay up to 37.5 cycles, which was set as the limit of sensitivity.

**Data analysis**

The data of the two study periods were analyzed together. Therefore, 11 applications of protein C were compared with 11 place-
bo applications. Data are expressed as means and 95% confidence intervals (CI). All statistical comparisons of continuous variables were made with nonparametric tests. A repeated measurements analysis of variance (ANOVA) including treatment and period as independent variables, and outcome variables as dependent factor was used. A 2-tailed $P < 0.05$ was considered significant. The Spearman ranks correlation test was used for computations of associations. All statistical calculations were performed using commercially available statistical software (Statistica Vers. 5.0; Stat Soft, Tulsa, OK). Power calculation was done as previously described (20).

Results

The baseline characteristics of the volunteers at the beginning of the study periods are presented in Table 1. The parameters were obtained before any intervention was performed. No difference can be noted between periods.

Vital parameters

LPS induced a transient increase in heart rate and a transient decrease in mean arterial blood pressure (MAP) as previously reported (21, 22) (peak heart rate levels 88 (82–94) bpm after 4 hours; minimum MAP levels 74 (69–79) mmHg after 4 hours (means, 95%CI); all $P<0.05$ vs. time). Infusion of protein C zymogen did not have any significant influence on these endotoxin-induced transient changes. LPS infusion also induced a transient increase in body temperature which reached its maximum after 3 hours (37.5 (37.3–37.7) °C; $P<0.05$ vs. time) and was not significantly altered by infusion of protein C zymogen.
Pharmacokinetic/pharmacodynamic (PK/PD) effects of protein C zymogen infusion on APC, PC:Act, and PC:Ag

Infusion of protein C zymogen concentrate resulted in a 3.2-fold increase of PC:Ag from 1.24 (1.01–1.47) U/ml to 4.00 (3.65–4.36) U/ml within 1 hour after start of the infusion (P<0.001) (Fig. 1). The concentration increased further during continuous infusion up to 4.77 (4.39–5.15) U/ml after 4 hours. After the end of the protein C infusion the activity level decreased with a half-life of approximately 18 hours. In the placebo-treated volunteers PC:Ag remained unaffected throughout the whole study period.

A similar course was noted for the PC:Act; it increased 3.1 fold from 1.07 (0.98–1.17) U/ml to 3.31 (3.09–3.53) U/ml within 1 hour after start of the infusion (P<0.001), and increased further during infusion up to 4.18 (3.95–4.40) U/ml. The half-life of the decline after the end of infusion was the same as for PC:Ag. No changes were seen in the placebo group.

The increase of the APC levels was remarkably delayed in comparison with the PC:Ag and PC:Act levels: one hour after start of the infusion, when PC:Ag already was 3.2-fold increased, APC levels were only 240% of baseline. The maximum APC concentration was reached 3 hours after start of the infusion (4.37 (3.28–5.47) ng/ml), and began to decline thereafter despite ongoing protein C infusion. The half-life of this decline was approximately 12 hours.

Effects of supra-normal protein C levels on LPS-induced coagulation activation

TF-mRNA expression on circulating monocytes was at or below the detection limit of 37.5 amplification cycles in most of the subjects at baseline. Four hours after application of endotoxin TF-mRNA levels increased by a median 13.7 fold after placebo. The increase in the period with high protein C levels was less pronounced (10.5 fold), although not statistically different (Fig. 2).

Tissue factor expression resulted in coagulation activation and the formation of thrombin, as indicated by an increase of F1+2 and TAT. In the placebo group thrombin generation began 2 hours after LPS infusion and peaked after 3–4 hours. F1+2 increased 6.1 fold from 0.38 (0.30–0.46) to 2.34 (1.37–3.30) nmol/l, and TAT 11.0 fold from 2.11 (1.91–2.31) to 23.15 (13.78–32.51) µg/l (P<0.001) (Fig. 3). In subjects, who received...
infusion of protein C zymogen, this increase was less pronounced. F1+2 increased only 4.5 fold from 0.40 (0.28–0.52) to 1.81 (1.20–2.42) nmol/l, and TAT 8.2 fold from 2.24 (1.73–2.74) to 18.24 (9.63–26.86) µg/l (P<0.001). However, the increase in F1+2 and TAT was not statistically different between the protein C and the placebo period.

The increase of APC levels correlated significantly with the formation of thrombin in the placebo period as well as in subjects infused with the protein C zymogen concentrate: Peak APC correlated with peak TAT levels in both periods (placebo: r=0.72, p<0.05; protein C zymogen: r=0.92, p<0.05) and with peak F1+2 levels only in the protein C period (placebo: r=0.55, p=0.08; protein C zymogen: r=0.70, p<0.05).

**Effects of supra-normal protein C levels on the fibrinolytic system after LPS-induced inflammation**

LPS-induced inflammation and coagulation activation resulted in an activation of fibrinolysis. Plasmin is generated two hours after endotoxin injection as indicated by the 5.1 fold increase of PAP (plasmin-antiplasmin) complexes from 0.37 (0.28–0.47) to 1.99 (1.2–2.7) mg/l (P<0.001). The elevation of protein C to supra-normal levels did not alter plasmin generation (Fig. 3).

**Effects of supra-normal protein C levels on LPS-induced inflammatory markers, and endothelial and platelet activation**

LPS-induced cytokine release occurred within 2 hours after LPS infusion. In the placebo period TNF-α levels increased about 70 fold to a maximum of 183 (113–253) pg/mL, and IL-6 levels increased to 512 (230–795) pg/mL (Fig. 4). The cytokine levels decreased quickly thereafter. In the subjects with supra-normal protein C levels the course of TNF-α and IL-6 was identical without any statistical difference (peak levels 184 (86–283) pg/mL and 436 (114–758) pg/mL, respectively).

Peak APC levels correlated with peak TNF-α levels (placebo: r=0.65, p<0.05; protein C zymogen: r=0.67, p<0.05) as well as with peak IL-6 levels (placebo: r=0.67, p<0.05; protein C zymogen: r=0.68, p<0.05) in both periods.

Consistently, protein C zymogen did not alter plasma levels of markers of endothelial activation (E-selectin), and leukocyte degranulation (elastase; Fig. 5). Soluble E-selectin increased on average 3.5 fold in either period (P=ns between period).

Similarly, infusion of protein C zymogen did not alter the LPS-induced changes in the platelet activation marker P-selectin (Fig. 5).

**Effects of protein C zymogen on endotoxin (LPS)-induced changes in blood cell counts**

Levels of platelet counts (Fig. 5), and differential blood counts (Fig. 6) were not different between the both periods during the time studied.

**Side effects**

No severe, serious, or unexpected adverse events were observed after LPS infusion. The infusion of the protein C zymogen concentrate produced no untoward effects.
cytes (25, 26). As already mentioned, we did not observe a shortened half-life, which is probably additionally due to the fact that in our model the systemic response occurs only within the first 4 hours after endotoxin challenge and is quickly attenuated thereafter.

Our data indicate that protein C zymogen is activated to APC during endotoxin-induced inflammation. In the placebo group the APC level increased 1.6 fold. This observation is in good agreement with our previous data in the placebo group of the rhAPC study (18). In contrast, Faust et al claimed that protein C activation may be decreased in patients with meningococcal sepsis, due to a lower expression of thrombomodulin or endothelial protein C receptor (EPCR) on injured endothelial cells (10). This hypothesis has already been refuted by the observations of DeKleijn et al (27), who demonstrated an enhanced and dose-dependent activation of protein C after infusion of protein C concentrate into children with meningococcal sepsis. We also observed a more pronounced protein C activation in the volunteers who received protein C zymogen concentrate.

The kinetics of the increase of APC was quite different from that of PC:Ag in the protein C zymogen period. The initial increase in APC is likely due to the bolus infusion of APC with the protein C zymogen (0.98 ng APC/PC antigen), before thrombin and cytokine generation is measurable. From previous studies with protein C zymogen and APC we know, that the half life of APC is approximately 20 to 40 minutes (18, 28, 29) compared to an approximately 20 times longer half life of protein C zymogen, as measured by protein C antigen and protein C activity levels (29). The fact that the measured clearance rate of APC was similar to the clearance rate of protein C zymogen suggests, that a slow but continuous activation of protein C took place. If no activation had occurred, the APC levels would have declined to normal levels within approximately 2–3 hours (4–5 half lives) after end of protein C zymogen infusion as observed in the trial by Derhaschnig et al (18). This was clearly not the case (Figure 2). We assume that the delayed increase of APC reflects conversion of protein C zymogen to APC.

Peak APC levels reached after protein C infusion were approximately 20-fold lower than those levels reached upon rhAPC-application (18). On the one hand, the infused and partially also the generated APC might have been inactivated due to the study design (slow infusion rate) continuously by the circulating inhibitors (30). On the other hand, such as observed in meningococccemia (27), the velocity of protein C activation by thrombin/thrombomodulin appears to be slow and determined by the actual protein C levels.

Of interest, we could confirm our own previous observation that the increase of APC levels in vivo correlates with the degree of systemic inflammation, i.e. with TNF release in this model (18) (Figs. 1 and 2). The observed correlation between the increase of APC levels and cytokine release may partly explain the variance in the ability of severely septic patients to generate activated Protein C. Thus, it may be of interest to investigate in future trials whether an inhibition of TNF-generation decreases in vivo generation of APC in this model. Such a putative decrease in APC generation during TNF blockade could partially contribute to the failure of anti-TNF strategies in sepsis (31).

One of the most interesting results of this study was the fact that LPS induced thrombin generation and systemic inflammation were not influenced by the 4 to 5 times elevated protein C levels. The levels of TNFα and IL-6 were slightly, but not significantly, lower in the active period (Fig. 3). The minimal anticoagulant effect is in good agreement with our previous publication on the infusion of rhAPC (18), which also failed to blunt LPS-induced coagulation activation. TF-mRNA levels were also slightly lower in the subjects, who received protein C zymogen concentrate. However, the results were not statistically significant and thus it appears that supra-normal protein C levels have limited anticoagulant effects in this model of endotoxin-induced coagulation.

High protein C levels did not influence systemic inflammation as measured by TNFα or IL-6 release (both Fig. 4). This is in line with the results of Derhaschnig et al. (18) on the effects of rhAPC in this model. Furthermore, protein C zymogen did not blunt neutrophil elastase release (Fig. 5). Consistently, protein C infusion did not affect fibrinolysis as assessed by PAP complexes (Fig. 3) or endothelial activation as assessed by E-selectin (Fig. 5), which are both dependent on TNFα release (32). These results are also in good agreement with the rhAPC study (18). Finally, treatment with protein C after LPS challenge had no effect on differential peripheral blood counts, platelet counts and P-selectin levels as compared to placebo (Fig. 5), again consistent with no effect of rhAPC (18).

There are several limitations of this trial. Importantly, and as discussed previously, the human low grade endotoxemia model is certainly not a sepsis model (18, 33). Therefore, no extrapolation is possible whether a substitution with protein C zymogen may have beneficial effects in septic patients. Along similar lines, our results should not in any way be interpreted to contradict the well known beneficial effects in protein C deficiency states, either congenital or acquired such as in meningococcemia (34–36). Secondly, the small sample size of this trial may not be adequate to detect small differences between the periods, although their clinical relevance may be questionable. Nevertheless, the experimental model is excellent to test the potency of conventional anticoagulants (14, 16, 37, 38).

In conclusion, the infusion of the protein C zymogen concentrate was safe and not associated with relevant adverse effects in healthy human volunteers. Increasing protein C levels several fold above normal resulted in a proportional increase of APC levels, but had no major anticoagulant effects. Further, protein C zymogen lacks profibrinolytic or anti-inflammatory effects in this model. Low grade endotoxemia itself induces significant protein C activation, which correlates with TNF release.

Acknowledgement

Protein C zymogen concentrate (Ceprotin®) was a generous gift from Baxter BioScience, Vienna, Austria.
References