Chapter 3

Endotoxemia induces resistance to activated protein C in healthy humans

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Abstract

Background
Systemic inflammation promotes tissue factor expression on monocytes and endothelial cells, leading to activation of the tissue factor/factor VIIa complex (TF/FVIIa). This leads to a procoagulant state, which may be enhanced by impairment of physiological anticoagulant pathways, such as the protein C system. Besides impaired protein C activation, resistance to activated protein C (APC) may occur. Aims of the current study were to evaluate the effect of endotoxemia on APC resistance, to analyze its determinants and to evaluate the effect of TF/FVIIa inhibition on endotoxin-induced APC resistance.

Methods and Results
Sixteen healthy male volunteers participated in the study: 8 subjects received endotoxin alone and 8 subjects received the combination of endotoxin and recombinant Nematode Anticoagulant Protein c2 (rNAPc2), a potent inhibitor of TF/FVIIa. Parameters of coagulation were subsequently studied. The sensitivity to APC was determined by two different tests: a test based on the endogenous thrombin potential (ETP) and a test based on the activated partial thromboplastin time (APTT). In response to endotoxemia, a transient APC resistance was detected by both tests. This transient APC resistance was predominantly mediated by an increase in factor VIII and was not influenced by TF/FVIIa inhibition. In vitro tests confirmed that an increase in factor VIII is able to induce APC resistance as measured by both tests.

Conclusions
Endotoxemia induces a transient APC resistance, which is predominantly mediated by an increase in factor VIII. This finding suggests that APC resistance might play a role in the procoagulant state occurring during human endotoxemia.
Introduction

In recent years, it has become clear that sepsis and endotoxemia entail a procoagulant response. A variety of inflammatory stimuli, including bacterial cell products and cytokines, promote the expression of tissue factor (TF) on the surface of monocytes and endothelial cells. This expression activates the extrinsic coagulation pathway, leading to thrombin generation [1]. The procoagulant response is regulated under normal conditions by various endogenous anticoagulant systems, involving antithrombin, protein C and tissue factor pathway inhibitor [2].

The protein C pathway is uniquely poised to regulate thrombin formation. The pathway is initiated when thrombin binds to the endothelial surface protein thrombomodulin, leading to rapid activation of protein C [3]. Once activated protein C (APC) is generated, it binds to protein S and this complex inactivates factors Va and Vila, thus inhibiting thrombin generation. It has been shown that the protein C pathway plays a critical role in antagonizing the procoagulant response during sepsis [4]. Treatment of septic patients with recombinant human APC resulted in a 19% reduction in the relative risk of death [5]. The efficacy of this treatment is explained by the fact that protein C activity is downregulated during sepsis. This downregulation is usually ascribed to several facts: first, protein C is degraded by neutrophil elastase released during sepsis; second, the conversion of protein C to APC is impaired because of endothelial dysfunction (leading to downregulation of thrombomodulin and the endothelial protein C receptor); third, the biosynthesis of protein C is inadequate [6]. In addition, the level of free protein S, the cofactor of APC, may be decreased due to the acute phase increase in C4b-binding protein, although the relevance of this mechanism has been contested [7]. An additional phenomenon that could lead to impairment of the protein C system is acquired resistance to APC. Although APC resistance is a well-known risk factor for hypercoagulability [8,9], it has never been investigated in human endotoxemia. To evaluate the importance of APC resistance in endotoxemia, we measured APC sensitivity ratios in a human endotoxemia model. In addition, since activation of protein C is dependent on thrombin generation initiated by tissue factor, we have also assessed the effect of specific tissue factor inhibition by recombinant Nematode Anticoagulant Protein c2 (rNAPc2) on activated protein C resistance during endotoxemia.

Hence, the current study had three aims: 1) to study the effect of endotoxemia on APC resistance in healthy humans, 2) to elucidate the mechanism by which this occurs and 3) to study the effect of a single intravenous dose of rNAPc2 on endotoxin-induced APC resistance.
Methods

Study design
The effect of rNAPc2 on parameters of coagulation and inflammation has been published separately [10]. The study was approved by the institutional scientific and ethics committee. Sixteen healthy men (age 18-35 years) volunteered to participate in the study. Written informed consent was obtained from each subject before the start of the study. None of the subjects had abnormalities on physical examination or routine laboratory investigation. The subjects did not take any medication and did not smoke or use illicit drugs. Eight subjects received endotoxin alone and eight subjects received the combination of endotoxin and rNAPc2. All subjects fasted overnight before endotoxin administration. Endotoxin (Escherichia coli lipopolysaccharide, lot G-1, US Pharmacopeia, Rockville, MD) was administered as a single i.v. dose of 4 ng/kg bodyweight. The combined treatment group received rNAPc2 as a single i.v. dose of 7.5 µg/kg bodyweight, immediately followed by endotoxin. Oral temperature, blood pressure, heart rate and oxygen saturation were measured at hourly intervals (Dinamap 1846 SX; Criticon, Tampa FL). Clinical symptoms such as headache, shivering, nausea, vomiting, tiredness and malaise were recorded throughout the study period using a graded scale (0=absent, 1=weak, 2=moderate, 3=severe).

Blood collection
Blood was collected from an intravenous cannula at 10 min before endotoxin administration, at 5, 15 and 30 min and at 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h after endotoxin administration. Blood samples were collected in citrate vacutainer tubes. Plasma was prepared by centrifugation of blood at 1800 g for 20 min at 16°C, followed by storage at -80°C until assays were performed.

Assays
The APC sensitivity was determined in two different ways: a test based on the endogenous thrombin potential (ETP-based test) and a test based on the activated partial thromboplastin time (APTT-based test). The ETP-based test is defined as the ratio of time integrals of thrombin formation, determined in the presence and absence of APC, divided by the same ratio of normal plasma [11]. The APTT-based test is defined as the ratio between two APTTs, one in the presence and one in the absence of APC. The APC-mediated prolongation of clotting time was first described by Dahlbäck et al. [12]. Resistance to APC increases the result of the ETP-based test and decreases the result of the APTT-based test. The ETP (i.e. the time integral of free thrombin concentration in a thrombin generation test) and the ETP-based APC-sensitivity ratio were determined as described by Rosing
et al. [11]. The ETP was calculated from the amidolytic activity of the α2-macroglobulin-thrombin complex (α2M-Ⅱa). The ETP-based APC sensitivity ratio was defined as the ratio of α2M-Ⅱa determined in the presence and absence of APC, divided by the ratio determined in normal pool plasma. Normal levels of ETP-based APC sensitivity ratio vary from 0.65 to 1.28. An APTT-based APC sensitivity test (Protein C Global, Dade Behring, Marburg, Germany) was performed according to the instructions of the manufacturer. Normal levels for the APTT-based APC sensitivity ratio exceed 0.8. The activity of tissue factor pathway inhibitor (TFPI) was measured on the Behring Coagulation System (BCS) according to a method described by Sandset et al. [13]. Coagulation factors II, V, X, IX and VIII activity were determined in one-stage clotting assays in a BCS with reagents and protocols from the manufacturer (Dade Behring). Protein C was determined using the Coamatic protein C activity kit (Chromogenix, Milano, Italy). Total protein S antigen was assayed by ELISA using antibodies from Dakopatts (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant.

**In vitro experiments**

In order to clarify the influence of factor VIII on both APC-sensitivity tests, we spiked pooled plasma of healthy males with recombinant factor VIII (Baxter, Deerfield IL, USA). In each sample, the factor VIII level, the endogenous thrombin potential (ETP) and both the ETP-based and the APTT-based APC-sensitivity were measured. In addition, the influence of factor V on the ETP-based test was evaluated by spiking the pooled plasma with factor V (Haematologic Technologies Inc., Essex Junction VT, USA). In each sample, factor V level, ETP and ETP-based APC-sensitivity were measured.

**Statistical analysis**

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 11.0 (SPSS, Chicago IL, USA). Differences in coagulation parameters between the two treatment groups were tested by analysis of repeated measures, using mixed linear models. Changes of coagulation parameters from baseline to a certain time point within the same group were analyzed by a paired student’s t-test. Mixed linear models were also used to determine the impact of different coagulation factors on the APC sensitivity tests. Both a univariate and a multivariate analysis were performed. The influence of coagulation factors on the ETP-based APC sensitivity test was calculated in the endotoxin group only, as thrombin generation was completely blocked by rNAPc2. For the calculation of the influence of coagulation factors on the APTT-based test, the endotoxin group and the endotoxin + rNAPc2 group were combined. Values are given as means ± SD. Significance was defined as $p<0.05$. 

35
Chapter 3

Results

Administration of endotoxin induced a febrile response, together with a tachycardia and transient flu-like symptoms including headache, nausea, malaise and chills. In addition, endotoxin administration elicited activation of neutrophilic granulocytes, as described elsewhere [10].

As shown in figure 1, administration of endotoxin elicited a significant resistance to APC, as confirmed by both the ETP- and APTT-based tests. In the ETP-based test, the APC resistance increased to 2.8 times baseline, reaching a maximum after 6 h. In the APTT-based test, the APC sensitivity declined to 67% baseline, reaching a nadir after 3 h. After administration of rNAPc2, the APC sensitivity could not be determined by the ETP-based test, because of complete inhibition of the thrombin generation. The APC sensitivity as determined by the APTT-based test was not affected by the administration of rNAPc2.

Figure 1. Influence of the administration of endotoxin and rNAPc2 on APC resistance in healthy humans. Results of the ETP-based test (left panel) and the APTT-based test (right panel) after administration of a single i.v. dose of 4 ng/kg endotoxin (o) or the combination of a single i.v. dose of 4 ng/kg endotoxin and a single i.v. dose of 7.5 μg/kg rNAPc2 (▲) to healthy male volunteers. Data represent mean ± SD. rNAPc2, recombinant Nematode Anticoagulant Protein c2; APC, activated protein C; ETP, endogenous thrombin potential; APTT, activated partial thromboplastin time; ETP-based test, ETP-based APC-sensitivity ratio; APTT-based test, APTT-based APC-sensitivity ratio.
Endotoxemia induces APC resistance

Figure 2. Influence of the administration of endotoxin and rNAPc2 on different coagulation parameters in healthy humans. Levels of TFPI, factors II, V, X, IX, VIII, protein C, total and free protein S after administration of a single i.v. dose of 4 ng/kg endotoxin (○) or the combination of a single i.v. dose of 4 ng/kg endotoxin and a single i.v. dose of 7.5 μg/kg rNAPc2 (▲) to healthy male volunteers. The level of free protein S is expressed as the percentage of the total protein S level. The levels of all other parameters are expressed as the percentage of the level present in pooled plasma of healthy hospital personnel. Due to the nature of the test, TFPI levels could not be determined in the presence of rNAPc2. Data represent mean ± SD. rNAPc2, recombinant Nematode Anticoagulant Protein c2; TFPI, tissue factor pathway inhibitor.

To elucidate by which mechanism the endotoxin-induced resistance to APC was elicited, the dynamics of TFPI, coagulation factors II, V, X, IX, VIII, protein C, and total and free
protein S were studied. Among all coagulation parameters studied, factor VIII showed the greatest change over time: after the administration of endotoxin, factor VIII increased to more than 2.5 times baseline, reaching a maximum after 3 h in both treatment groups (Figure 2).

![Graphs showing changes in ETP and ETP-based APC sensitivity ratio](image)

**Figure 3.** Influence of the in vitro addition of factor V and factor VIII on ETP and ETP-based APC sensitivity ratio. Influence of the addition of factor V (○, left panels) and factor VIII (▲, right panels) to normal pool plasma on ETP (lower panels) and the ETP-based test (upper panels). ETP, endogenous thrombin potential; APC, activated protein C; ETP-based test, ETP-based APC sensitivity ratio.
Endotoxemia induces APC resistance

Analysis of repeated measurements was performed to determine the impact of the different parameters on both APC sensitivity tests. The results of the univariate analysis showed that the increase in the ETP-based test was significantly dependent on factor V ($p=0.014$) and factor VIII ($p=0.008$). In the multivariate analysis, factor V ($p<0.001$) and factor VIII ($p=0.001$) remained the only two factors independently influencing the ETP-based APC sensitivity test. The increase in the ETP-based test was independent of the levels of factor II, free protein S and TFPI.

We also determined the impact of the different parameters on the APTT-based APC sensitivity test. Results of the univariate analysis of repeated measurements showed that the APTT-based test was significantly dependent on TFPI and coagulation factors II, X, IX and VIII. In the multivariate analysis, only factor VIII ($p<0.001$) and factor IX ($p=0.004$) independently influenced the APTT-based APC sensitivity test.

The influence of factor V and factor VIII on APC sensitivity as measured by the ETP-based test, was further investigated by spiking normal pool plasma with recombinant factors V and VIII. As shown in figure 3, addition of both factor V and factor VIII resulted in an increase in APC resistance as measured by the ETP-based test. Addition of factor V and factor VIII did not have an effect on thrombin formation, since the ETP remained stable. The influence of factor VIII on the APTT-based test was also evaluated. As shown in figure 4, addition of factor VIII to normal pool plasma resulted in APC resistance, as reflected by a decrease in the APTT-based APC sensitivity ratio.

![Figure 4. Influence of the in vitro addition of factor VIII on the APTT-based APC sensitivity ratio. Influence of the addition of factor VIII to factor VIII deficient plasma on the APTT-based test. APTT, activated partial thromboplastin time; APC, activated protein C; APTT-based test, APTT-based APC sensitivity ratio.](image-url)
Discussion

In this study, we demonstrated that endotoxemia elicits a transient APC resistance in healthy humans, as measured by two different tests. This transient APC resistance is predominantly mediated by an increase in factor VIII and is independent of TF/FVIIa inhibition.

To our knowledge, this is the first time endotoxemia has been demonstrated to induce APC resistance in humans. APC resistance is a well-known risk factor for venous thromboembolism [14,15]. It is often associated with a mutation in factor V (factor V_{Leiden}), which causes the replacement of an amino acid (Arg506→Gln) at a predominant APC cleavage site in factor Va. This results in impaired inactivation of factor Va by APC and in enhanced thrombin generation [16]. APC resistance can also be acquired during oral contraceptive use, pregnancy and cancer [15-19].

Endotoxin-induced APC resistance is predominantly mediated by an increase in factor VIII, as demonstrated by two different tests and in vitro spiking of normal pool plasma with recombinant factor VIII. A relationship between an elevated level of factor VIII and resistance to APC has been noticed before [20]. In the PLAT study, factor VIII was identified as an independent univariate predictor of vascular disease events [21]. However, this is the first time that a rise in factor VIII has been demonstrated to be a major determinant of endotoxin-induced APC resistance.

The ETP-based APC sensitivity test was originally described by Rosing in 1997. In this test, α2M-IIa was assayed and was shown to be proportional to the ETP [11]. The ETP-based test has previously been shown to be sensitive to levels of protein S and prothrombin and to the factor V_{Leiden} mutation [16,17], but not to other factors. In a large case-control study, de Visser et al. demonstrated sensitivity of the ETP-based test to free TFPI and free protein S [22]. However, in all these studies, blood was collected only once. This is the first time that the time course of APC resistance has been studied in relation to other coagulation factors in healthy individuals exposed to endotoxin. In our study, the ETP-based APC sensitivity test was only influenced by factor V and factor VIII. The endotoxin-induced decline in factor II and protein S was probably too small to influence the ETP-based test.

The influence of factor VIII on the APTT-based APC sensitivity ratio is well known. De Ronde and Bertina described the influence of protein S and coagulation factors II, X, IX and VIII on the APTT-based test [23]. In a study by Henkens et al., factor VIII was identified in a multiple regression model as one of the two independent factors influencing the APTT-based test [24]. In our study, the dependency of the APTT-based test on coagulation factors II, X, IX and VIII was confirmed. The multivariate analysis showed that the APTT-based test is only influenced by factor IX and factor VIII. The level of protein S did not influence the APTT based test. This can be explained by the fact
that in our study, protein S levels decreased by only 20%, whereas in the study by de Ronde et al., the APTT-based APC sensitivity ratio only declined when protein S levels decreased by 80% or more.

As APC is a natural inhibitor of factor VIII, it is conceivable that an increase in factor VIII leads to relative resistance to APC. The increase in factor VIII in response to endotoxin was recently described by Reitsma et al. [25]. The endotoxin-induced factor VIII response was more pronounced in their study, which may be due to the fact that factor VIII antigen levels were measured, whereas in our study, factor VIII activity was determined. A rise in factor VIII might induce APC resistance by inhibition of protein S function. Koppelman et al. demonstrated that factor VIII binds to protein S in a reversible and specific manner [26].

The ability of endotoxemia to increase factor VIII levels and induce APC resistance in humans, is an important clue in the elucidation of the mechanism of action of APC during human sepsis. As factor VIII is elevated and levels of protein C are severely reduced during sepsis [27], it is conceivable that administration of activated protein C is needed to overcome the APC resistance provoked in this manner. In a human endotoxemia model similar to the one used in this study, Derhaschnig et al. demonstrated that the endotoxin-induced thrombin generation was not blunted by the administration of APC [28]. As a possible explanation, Derhaschnig et al. mentioned the fact that healthy subjects have normal levels of protein C, whereas septic patients are usually protein C deficient. Moreover, normal levels of factor II and factor X can inhibit the anticoagulant potency of APC in healthy humans, whereas this inhibition will probably not occur with the low levels of factor II and factor X such as found during sepsis. Finally, the endothelial dysfunction during sepsis leads to downregulation of thrombomodulin and the endothelial protein C receptor, thus diminishing the activation of protein C [29]. We would like to add an alternative explanation: increased factor VIII levels during endotoxemia result in a transient APC resistant phenotype.

In summary, this study demonstrates that the administration of endotoxin to healthy humans can induce APC-resistance, which is predominantly mediated by an increase in factor VIII and is independent of TF/FVIIa inhibition. This indicates that APC resistance may play a role in the procoagulant state that occurs during human endotoxemia.

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