The extrinsic coagulation pathway in coronary artery disease and endotoxemia
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Recombinant nematode anticoagulant protein c2, a novel inhibitor of tissue factor-factor VIIa activity, abrogates endotoxin-induced coagulation in chimpanzees

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Abstract

Background
Systemic activation of coagulation leading to disseminated intravascular coagulation (DIC) is an important feature in patients with severe sepsis. Tissue factor has been shown to play a primary role in this pathological response, as revealed by the use of specific inhibitors and antagonists of the tissue factor/factor VIIa pathway. This class of agents has been demonstrated to attenuate the coagulation response in human volunteers with induced low-grade endotoxemia and to reduce mortality in primate models of Gram-negative sepsis. The efficacy of these agents in attenuating the activation of coagulation and formation of microvascular thrombosis in sepsis may depend on the mechanism of inhibition. Here we demonstrate the efficacy of recombinant nematode anticoagulant protein c2 (rNAPc2) that specifically inhibits the tissue factor/factor VIIa complex by a novel mechanism, in a model of endotoxin-induced coagulation activation in chimpanzees.

Methods and Results

Administration of a low dose of Gram-negative endotoxin induced marked increases of thrombin generation as measured by plasma levels of prothrombin activation fragment F1+2, and thrombin-antithrombin complexes, which were completely blocked by rNAPc2. In chimpanzees receiving rNAPc2 alone, there was a significant reduction in the activation of factor X but not factor IX, compared to animals receiving placebo. In contrast to the effect of rNAPc2 on thrombin generation, there was no effect of this inhibitor on the well known enhanced systemic fibrinolytic response induced by endotoxin.

Conclusion
The recombinant peptide rNAPc2 is an effective inhibitor of tissue factor-driven thrombin generation during low grade endotoxemia. These results suggest that rNAPc2 may be a promising therapeutic option to inhibit coagulation activation in patients with sepsis.
Introduction
Systemic activation of coagulation, in its most extreme form known as disseminated intravascular coagulation (DIC), is a frequent complication in patients with sepsicaemia. DIC may contribute to multiple organ failure and is associated with an increased risk of mortality. Besides impairment of physiological anticoagulant pathways and dysfunctional fibrinolysis, tissue factor-dependent generation of thrombin is a predominant feature of this coagulopathy.

Tissue factor (TF) is a 263-residue transmembrane glycoprotein that is expressed under normal conditions by smooth muscle cells in the tunica media and predominantly by fibroblasts in the adventitia surrounding the vessels, consistent with the hypothesis that expression of TF is anatomically separated from blood. However, in response to a variety of stimuli including endotoxin and pro-inflammatory cytokines such as tumor necrosis factor and interleukin-1 and 6, TF expression can be induced by mononuclear cells and vascular endothelial cells. Indeed, significant TF expression by peripheral blood monocytes from endotoxemic patients has been demonstrated.

The key role of TF-mediated coagulation in patients with DIC has been demonstrated in studies of experimental bacteremia or endotoxemia in primates or healthy humans in which the coagulation response was blocked by monoclonal antibodies against TF and factor VII/VIIa, bolus infusions of active site inhibited factor VIIa (DEGR VIIa), and tissue factor pathway inhibitor (TFPI) infusion. To date recombinant TFPI (rTFPI) is the only agent in this class of compounds that has been studied clinically in patients diagnosed with bacterial sepsis. In this study, there was a trend toward an improved clinical outcome that appeared to be correlated with improvements in the DIC response in rTFPI treated patients although no dose-response effect was observed. However, a trend toward an increase in hemorrhagic complications limited the evaluation of only two doses of rTFPI which may have precluded a full appreciation of the effects of this agent in attenuating the DIC response. Therefore, the exact role of TFPI in the pathogenesis of sepsis-related coagulation disorders remains to be established.

Recently, a specific inhibitor of the complex TF/factor VIIa has been undergoing clinical evaluation as a novel injectable anticoagulant. This protein, termed recombinant Nematode Anticoagulant Protein c2 (rNAPc2), was originally isolated from extracts of the hookworm Ancylostoma caninum and potentially inhibits the TF/factor VIIa complex via a unique mechanism of action.
which sequentially binds to factor Xa at the catalytic center followed by formation of the quaternary TFPI/factor Xa – TF/factor VIIa complex, rNAPc2 binds with high affinity initially to zymogen factor X or factor Xa prior to formation of the quaternary inhibitory complex with TF/factor VIIa. The utilization of zymogen factor X as an inhibitory scaffold by rNAPc2 obviates the need for formation of factor Xa before the inhibition of TF/factor VIIa complex, which implies that rNAPc2 can form a complex with circulating factor X and rapidly inhibit TF/factor VIIa following a thrombogenic challenge such as vascular damage or systemic exposure to bacterial endotoxin. This mechanism of action may result in an in vivo anticoagulant profile for rNAPc2 that is distinguishable from TFPI and other inhibitors of TF/factor VIIa. The effectiveness of rNAPc2 in attenuating intravascular thrombosis has been confirmed in several pre-clinical studies as well as a recent phase II clinical study where there was an impressive reduction in the incidence of acute deep vein thrombosis in patients undergoing total unilateral arthroplasty compared to the best current prophylactic regimens²⁵. In addition, the potent antithrombotic efficacy of rNAPc2 in this study was not associated with an increase in hemorrhagic complications.

Here we demonstrate the efficacy of rNAPc2 in a model of endotoxin-induced coagulation activation in chimpanzees as the first step in the evaluation of this agent in the control of coagulopathy observed during endotoxemia and sepsis.

**Methods**

**Chimpanzees**

Ten adult chimpanzees (Pan troglodytes) from the TNO-ITRI Primate Center, Rijswijk, The Netherlands, were used. All animals were clinically healthy, exhibited no laboratory evidence of kidney and liver disease, and had normal routine coagulation tests (platelet count, activated partial thromboplastin time and prothrombin time). The study protocols were approved by the animal health and welfare committees of the primate center and were conducted according to the guidelines of the Dutch Law for Animal Experiments.

**Experimental protocols**

After sedation with ketamine chloride, the chimpanzees were intubated and maintained under general anesthesia with nitrous oxide and halothane throughout the experiment. Arterial blood pressure and heart rate were continuously recorded with an automated
blood pressure device and rectal temperature was measured in 15-minutes interval.
An intravenous bolus injection of purified endotoxin (lot EC-5 from *Escherichia coli* 0113, FDA standard preparation) at a dose of 4 ng/kg bodyweight was administered to 5 chimpanzees. Three animals received only the bolus injection of purified endotoxin and in two other chimpanzees, the bolus injection of purified endotoxin was immediately preceded by the administration of rNAPc2 (Corvas International, Inc, San Diego, CA), administered as a subcutaneous bolus injection of 10 µg/kg bodyweight. Of the remaining 5 chimpanzees, two received a bolus of rNAPc2 alone at a dose of 50 µg/kg bodyweight, and three control animals received only a subcutaneous bolus injection of saline.
Recombinant NAPc2 was produced as described using the yeast *Pichia Pastoris* and provided as a sterile solution in phosphate buffered saline with a pH of 7.0 at a concentration of 11.7 or 3.0 mg/ml.

**Blood collection and processing**
Venous blood samples were obtained by separated venipunctures with a 21-gauge butterfly needle from the antecubital vein before the injection of study agents and at 30, 60, 90, 120, 180, 240, and 300 minutes.
Blood for measurements of prothrombin activation fragment 1+2 (F1+2), thrombin-antithrombin III (TAT) complexes, tissue-type plasminogen activator (tPA) antigen, and plasminogen activator inhibitor type 1 (PAI-1) antigen were collected in plastic syringes containing 3.2% sodium citrate; the ratio of anticoagulant to blood was 1:9 (vol/vol). For assays of factor X activation peptide (fXp) and factor IX activation peptide (fIXp), blood was collected into syringes containing 38 mmol/liter citric acid, 75 mmol/liter sodium citrate, 136 mmol/liter dextrose, 6 mmol/liter EDTA, 6 mmol/liter adenosine, and 25 U/ml heparin; the ratio of anticoagulant to blood was 1:5.
All blood samples were immediately placed in melting ice and centrifuged at 4°C for 20 minutes at 1,600 g. Plasma samples were stored at -70°C until assayed.

**Assays**
Plasma concentrations of F1+2, TAT complexes, tPA and PAI-1 antigen were measured by enzyme-linked immunosorbent assay (F1+2 and TAT complexes: Behringwerke AG, Marburg, Germany; tPA and PAI-1: Innogenetics, Nijmegen, The Netherlands). fIXp and fXp were determined by double-antibody radioimmunoassay as previously described26-27.
Statistical analysis
All values are given as mean ± standard deviation (SD). A P-value <0.05 was considered statistically significant. Repeated measurements analyses were performed. In the presence of interaction between time and treatment, the treatment groups were compared for each time point separately. For parameters measured in 4 treatment groups, we subsequently performed one-way analyses of variance followed by Scheffé’s contrast analyses. For parameters measured in two treatment groups we subsequently performed unpaired Student’s t tests.

Results
Effect rNAPc2 on thrombin generation after endotoxin exposure
As shown in Figure 1, the bolus infusion of 4 ng/kg endotoxin in chimpanzees resulted in a sharp and significant increase in circulating markers of thrombin generation after 120 minutes, compared to control animals (P <0.05). Maximal levels were observed after 240 minutes, showing a 4.6 fold and 7.2 fold increase for F₁₋₂ and TAT complexes, respectively (peak level F₁₋₂: 5.4 ± 0.4 mmol/L, TAT complexes: 46.7 ± 4.5 ng/L). Chimpanzees receiving endotoxin in combination with rNAPc2, thrombin generation was completely abrogated, as evidenced by unchanged levels of F₁₋₂ and TAT complexes. Administration of rNAPc2 alone resulted in a trend toward lower levels of basal thrombin generation compared to control animals, which reached statistical significance for TAT complexes after 300 minutes (Figure 1).

Figure 1. Effect of rNAPc2 administration on thrombin generation as measured by Prothrombin Fragment 1–2 (F₁₋₂; panel A) and Thrombin-Antithrombin III complexes (TAT complexes; panel B). Chimpanzees received a bolus injection of 50 μg/kg rNAPc2 SC (open circle), or 4 ng/kg endotoxin IV (closed circle), or placebo (closed triangle). An additional group of chimpanzees received a bolus infusion of 4 ng/kg endotoxin IV in combination with the administration of 10 μg/kg rNAPc2 SC (closed square). Data represent mean ± SD. *Significant differences (P <0.05, Scheffé’s contrast analyses) between rNAPc2/endotoxin and endotoxin alone; #between rNAPc2 alone and placebo.
Plasma levels of factor X and IX after rNAPc2 administration
Chimpanzees receiving rNAPc2 alone showed gradually decreasing plasma levels of factor X activation peptide after 60 minutes, which was sustained to the end of the observation period (Figure 2, panel A) and reaching statistical significance at 180 minutes after the injection (P <0.05). Plasma levels in the rNAPc2 treated chimpanzees declined from 107.0 ± 7.1 to 76.0 ± 1.4 pmol/L, a decrease of 29% as compared to unchanged levels in the control group receiving saline. Plasma levels of factor IX activation peptide did not change both in the control and rNAPc2 treated animals (Figure 2, panel B).

![Figure 2. Effect of rNAPc2 administration on the basal activation peptides factor X (panel A) and factor IX (panel B) after the administration of 50 μg/kg rNAPc2 SC(open circle), or placebo (closed triangle). Data represent mean ± SD. *Significant differences (P <0.05, unpaired Student's t test).](image)

Effect of rNAPc2 on fibrinolysis after endotoxin exposure
Antigen plasma levels of the fibrinolytic system are shown in Table 1. Tissue-PA antigen levels sharply increased early after the administration of endotoxin and reached its peak level at 120 minutes after the injection. Tissue-type PA antigen levels increased from 100.0 ± 6.2 to 345.0 ± 16.5 mg/ml, hereafter, the tPA plasma levels rapidly declined to baseline values, which was paralleled by a rapid 6.2 fold increase of PAI-1 antigen levels. At 180 minutes after endotoxin exposure, PAI-1 antigen reached its maximal plasma concentrations of 49.3 ± 4.2 mg/ml, which continued at this level to the end of the experiment. Administration of rNAPc2 did not affect these responses.
Table 1. Antigen plasma levels of tissue-type plasminogen activator and plasminogen activator inhibitor type 1 in chimpanzees receiving endotoxin (4 ng/kg iv) alone or in combination with rNAPc2 (10 μg/kg sc).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>tPA (mg/ml)</th>
<th>PAI-1 (mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Endotoxin+rNAPc2</td>
</tr>
<tr>
<td>(n=3)</td>
<td>(n=2)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>30</td>
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<td>106.0 ± 9.9</td>
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</tr>
<tr>
<td>300</td>
<td>94.3 ± 6.7</td>
<td>115.5 ± 4.9*</td>
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Values are expressed as mean ± standard deviation. tPA=tissue-type Plasminogen Activator. PAI-1=Plasminogen Activator Inhibitor type 1. *Significant difference (P<0.05, unpaired Student’s t test) between endotoxin+rNAPc2 vs endotoxin.

Discussion

It is widely accepted that the initiation of the coagulation system during sepsis and endotoxemia is mediated via the extrinsic (TF dependent) pathway. Several specific inhibitors of this pathway have been demonstrated to attenuate the coagulopathic response in different models of both bacteremia and endotoxemia\(^{14-21}\). However, only TFPI has been studied in a controlled clinical study in patients with severe bacterial sepsis and this trial indicated that, although this agent may be effective, its use is limited by the induction of bleeding complications\(^{22}\). Recombinant NAPc2, a novel inhibitor of the same pathway, has been shown to be well tolerated and safe in human subjects and patients undergoing total knee replacement or coronary angioplasty\(^{25-28}\). Our present results demonstrate that rNAPc2 functions as a specific potent inhibitor of endotoxin-induced coagulation activation in chimpanzees. Administration of a single subcutaneous injection of rNAPc2 completely inhibited factor Xa-mediated thrombin generation in our model of low grade endotoxemia.

Recombinant NAPc2 is an 85 amino acid containing protein, originally isolated from the hematophagous hookworm Ancylostoma caninum\(^{23}\). The anticoagulant activity of rNAPc2 results primarily from the inhibition of the catalytic complex of TF/factor VIIa by a mechanism that requires the initial binding of rNAPc2 to an inhibitory
rNAPc2 inhibiting endotoxin-induced coagulation in chimpanzees

scaffold that can be either factor X or factor Xa prior to the formation of an inhibitory complex with TF/factor VIIa\(^2\). Unlike TFPI, which binds to the catalytic site on activated factor X, rNAPc2 binds with high affinity to catalytically inactive zymogen factor X. This obviates the need to form factor Xa prior to binding and may result in an accelerated anticoagulant effect compared to TFPI. Consistent with this mechanism, rNAPc2 completely blocked endotoxin-induced activation of coagulation and also appeared to suppress basal thrombin generation. Indeed, basal thrombin generation is thought to be dependent on the TF pathway and it has been shown that factor VII deficient individuals have lower levels of prothrombin fragment F\(_{1+2}\)\(^2\). Chimpanzees receiving a bolus injection of rNAPc2 alone showed also reduced basal levels of factor X activation peptide at 60 minutes, which became significant after 180 minutes compared to control animals. In the same chimpanzees, no effect of rNAPc2 was observed on levels of factor IX activation peptide. These results suggest that the anticoagulant effect of rNAPc2 in low-grade endotoxemic chimpanzees is mainly mediated via a direct or indirect (after formation of the inhibitory complex TF/factor VIIa – rNAPc2/factor X\(_{a}\)) inhibition of factor X activation. Inhibition of factor IX, as part of the intrinsic route in the activation of the coagulation system, appears less important in our model. This is in accordance with studies showing substantial factor X-mediated generation of thrombin after endotoxin or tumor necrosis factor administration to healthy volunteers, whereas plasma levels of markers for intrinsic pathway activation (i.e. factor XIIa-C1 inhibitor complexes, kalikrein-C1 inhibitor complexes, and factor IX activation peptide) remained within the normal range\(^3\).

Consistent with earlier studies using the same model in chimpanzees or healthy subjects\(^4\), activation and subsequent inhibition of the fibrinolytic mechanism preceded endotoxin-induced activation of the clotting system, demonstrated by a rapid increase of tPA followed by increased levels of PAI-1 antigen. Like other specific inhibitors of the extrinsic pathway as well as hirudin\(^4\), a direct thrombin inhibitor, administration of rNAPc2 did not affect this response, which underscores that activation of fibrinolysis occurs independently from thrombin generation during low-grade endotoxemia. It has been suggested that tumor necrosis factor primarily initiates this fibrinolytic response\(^4\). Although thrombin has demonstrated to induce the release of tPA\(^4\), we did not observe an increase in tPA antigen levels after thrombin generation in chimpanzees receiving endotoxin alone. Probably this response was inhibited by sustained elevated levels of PAI-1.
The clinical applicability of rNAPc2 in other settings where tissue factor expression or exposure to the circulation is important, has been demonstrated in recent dose-ranging trials. A rNAPc2 dose of 3.0 μg/kg administered subcutaneously 1 hour after an elective, unilateral total knee replacement, showed to be very effective in the prevention of deep venous thrombosis and pulmonary embolism, without an increase in bleeding events. The drug also appears to be safe and well tolerated when added to standard therapy in patients undergoing elective percutaneous coronary angioplasty. A significant suppression of thrombin generation compared to patients receiving standard therapy alone, continuing for at least 36 hours after one subcutaneous injection administered 2-6 hours before the intervention, was observed across 4 doses of rNAPc2 up to 10 μg/kg, which was the dose used in the current study in conjunction with endotoxin. It should be noted that there were no observable overt bleeding in any of the animals receiving rNAPc2 in the present study.

In conclusion, our study confirms the pivotal role of the TF/factor VIIa pathway in endotoxin induced coagulation activation. Administration of a single subcutaneous injection of rNAPc2 completely inhibited factor Xa-mediated thrombin generation in our model of low grade endotoxemia in chimpanzees, without affecting the fibrinolytic response and without any bleeding consequences. Hence, rNAPc2 represent a clinically applicable inhibitor of TF, which may be relevant for TF directed treatment of patients with sepsis and DIC and support the further investigation of rNAPc2 during bacteremia and endotoxemia in humans.

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rNAPc2 inhibiting endotoxin-induced coagulation in chimpanzees

References


