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Abstract—The extrinsic coagulation pathway is activated when circulating factor VII (FVII) gains access to tissue factor (TF) exposed as a consequence of vascular injury. Increasing evidence indicates that this TF-dependent activation of the coagulation plays an important role in the pathophysiology of intravascular thrombus formation. In the present study, we tested the effects of recombinant human, active site-blocked activated FVII (FVIIai) in a rabbit model of carotid artery thrombosis. Cyclic flow variations (CFVs), due to recurrent thrombus formation, were obtained in stenotic rabbit carotid arteries with endothelial injury. Carotid blood flow velocity was measured by a Doppler flow probe. After 30 minutes of CFVs, the animals received FVIIai ($100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ intracarotid infusion for 10 minutes, $n=9$). If CFVs were abolished, animals were followed for 30 additional minutes, after which recombinant human activated FVII (FVIIa) was infused into the carotid artery ($100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes) to determine whether FVIIai could be displaced from TF by FVIIa, thus restoring CFVs. To establish the duration of action of FVIIai, an additional group of animals received FVIIai at the same dose as above, and after CFVs were inhibited, they were followed until CFVs were restored or for up to 6 hours. To determine whether CFVs could be restored by epinephrine after their abolition with FVIIai, increasing doses of epinephrine were administered to a third group of 6 animals. FVIIai abolished CFVs in 8 of 9 rabbits ($P<.01$). This effect was reversible, as FVIIa administration restored CFVs in all animals. Prothrombin times and activated partial thromboplastin times did not change significantly throughout the study. One single 10-minute infusion exerted complete antithrombotic effects for at least 6 hours, despite the fact that at this time point, plasma FVIIai levels were well below threshold concentrations. Epinephrine restored CFVs in 3 of 6 animals in which CFVs were inhibited by FVIIai. FVIIai exerts potent antithrombotic effects in this model; these effects were prolonged even after FVIIai was almost completely cleared from the circulation, probably as a result of the tight binding of FVIIai to TF. Thus, FVIIai might represent an antithrombotic substance of potential interest. (*Circ Res.* 1998;82:39-46.)

Key Words: thrombosis ■ tissue factor ■ factor VII ■ active site-blocked activated factor VII

There is general agreement that intracoronary thrombus formation at sites of complicated atherosclerotic plaques plays an important role in the conversion from chronic to acute coronary artery disease syndromes.¹ This hypothesis is also supported by the observation that some chemical mediators released on platelet activation, such as thromboxane A₂ and serotonin, are found at increased concentrations in the coronary sinus of patients with unstable angina.^{2,3} Furthermore, FPA plasma levels, an index of thrombin activity, have been found to be elevated in patients with unstable angina compared with patients with chronic, stable, effort angina.⁴

Recent evidence indicates that activation of the extrinsic coagulation pathway may play an important role in the pathophysiology of intravascular thrombus formation follow-

ing arterial injury.⁵ The extrinsic coagulation pathway is initiated when TF, a 47-kD membrane-bound glycoprotein, is exposed to flowing blood as a consequence of vascular damage.⁶ TF complexes with FVII and FVIIa, permitting enzymatic activation of factors X and IX, the substrates for factor VIIa, ultimately leading to the generation of thrombin.⁶ Normally, endothelial cells, being in contact with circulating blood, do not express significant TF activity. However, TF is found across the arterial wall, with its activity increasing from the subendothelium to the adventitia.⁷ Significant TF activity has also been localized in human atherosclerotic plaques⁸ and, recently, in atherectomy specimens obtained from patients with unstable angina.⁹ In addition, we have recently shown that a monoclonal antibody against TF not only inhibits

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Selected Abbreviations and Acronyms

aPTT	= activated partial thromboplastin time
CFV	= cyclic flow variation
FFRcmk	= Phe-Phe-Arg chloromethyl ketone
FPA	= fibrinopeptide A
FVII	= factor VII
FVIIa	= activated FVII
FVIIai	= active site–blocked FVIIa
PPP	= platelet-poor plasma
PRP	= platelet-rich plasma
PT	= prothrombin time
t-PA	= tissue plasminogen activator
TF	= tissue factor
TFPI	= TF pathway inhibitor

intravascular thrombus formation in a rabbit model of recurrent arterial thrombosis⁷ but also enhances thrombolysis by t-PA and prevents reocclusion following t-PA discontinuation.¹⁰ Taken together, these data support the hypothesis that TF exposure following arterial damage plays a role in the pathogenesis of acute ischemic coronary syndromes by initiating intravascular thrombus formation.

Recently, the availability of recombinant human FVIIa with the active site blocked has permitted further investigation on the role of TF exposure/activation of the extrinsic coagulation cascade in the pathophysiology of intravascular thrombus formation. Recombinant human FVIIai possesses the same affinity for TF as does native FVIIa but, having the active site blocked, is not capable of converting factors IX and X to their activated forms, thus inhibiting the activation of the coagulation cascade at an early step. The purpose of the present study was, therefore, to determine the antithrombotic effects of FVIIai in a rabbit model of carotid artery thrombosis. This model was designed to provide conditions for thrombosis that closely resemble the acute coronary syndromes seen in humans. The results obtained demonstrate that FVIIai exerts potent antithrombotic effects, without incurring potentially harmful systemic effects.

Materials and Methods

Preparation of FVIIa and FVIIai

Recombinant human FVIIa was purified from culture media of a transfected baby hamster kidney cell line as previously described.¹¹ The active site of recombinant FVIIa was blocked by the addition of 2-fold molar excess of FFRcmk. The solution was allowed to incubate for 1 hour at 4°C. Unreacted FFRcmk was separated from FVIIai by Q-Sepharose fast-flow ion-exchange chromatography. FVIIai was eluted with 10 mmol/L CaCl₂, and the profile of the eluted peak was narrow and symmetric. The FVIIai solution was adjusted to a protein concentration of ≈2 mg/mL in 10 mmol/L glycylglycine, 150 mmol/L NaCl, and 10 mmol/L CaCl₂, pH 7.4, sterile-filtered, and stored at -80°C. The residual FVIIa activity in the FVIIai solution was <0.1% when measured in an FVIIa-specific amidolytic assay.¹²

Experimental Preparation

This study was performed using a rabbit model of recurrent carotid artery thrombosis as described in detail elsewhere.^{13,14} Briefly, New Zealand White rabbits of either sex were anesthetized with a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. Anesthesia was maintained during the course of the experiment by an intravenous infusion of ketamine sufficient to

abolish the corneal reflex. Through a median incision of the neck, the left or right common carotid artery was exposed and carefully isolated from the surrounding tissue. Polyethylene catheters were inserted into a jugular vein and a femoral artery for both drug administration and blood pressure monitoring. A segment of the exposed vessel was injured by gently squeezing the artery between a pair of rubber-covered forceps. An external plastic constrictor was placed around the damaged site. Carotid blood flow velocity was continuously measured by a Doppler flow probe positioned proximal to the constrictor. A small polyethylene catheter (25-gauge outer diameter) was placed into the carotid artery for the local infusion of drugs. After instrumentation, the animals developed cyclic fluctuations of carotid blood flow (CFVs) characterized by gradual decreases of flow to almost zero values followed by spontaneous or induced restorations of flow. Previous studies have shown that CFVs are due to recurrent cycles of thrombus formation and subsequent dislodgment.^{13–15}

Study Protocol

The present study comprises three different arms. In the first arm, the antithrombotic effects of FVIIai, its effects on systemic blood coagulation parameters (PTs, aPTTs, and FPA plasma levels), and platelet aggregation were determined. In addition, in these animals the ability of recombinant human FVIIa to revert the antithrombotic effects of FVIIai was also tested. The second arm of the study was included to determine the pharmacokinetics of FVIIai and to correlate its plasma levels with the antithrombotic effects. Finally, the third arm of the study was conducted to determine whether the antithrombotic effects of inhibition of the extrinsic coagulation pathway can be overridden by stimulating other activating pathways, namely, epinephrine-induced platelet activation.

CFV frequency (cycles per hour) and severity (carotid blood flow at its nadir, as a percentage of baseline), heart rate, and arterial blood pressure were continuously measured throughout the experiment. CFVs were monitored for 30 minutes, after which rabbits were assigned to the following groups.

Group 1: Antithrombotic Effects of FVIIai

The experimental protocol followed in this arm of the study is summarized in Fig 1A. A first group of 9 animals received an intracarotid infusion of FVIIai at a dose of 100 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes. A pilot study evidenced that an intracarotid infusion of FVIIai was necessary in order to achieve local concentrations sufficiently high to displace the tight binding of native FVII/VIIa from TF. If CFVs were inhibited at the end of the infusion, the animals were followed for additional 30 minutes to ensure that the antithrombotic effects were persistent. Thereafter, to show that the binding of FVIIai to TF was competitive, all animals that showed inhibition of CFVs received an intracarotid infusion of recombinant human FVIIa (100 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes). If CFVs returned during FVIIa infusion, they were monitored for an additional 30 minutes. Blood samples for the measurements of PT and aPTT were obtained at the end of the 30-minute CFV period and after FVIIai and FVIIa administration. In addition, blood samples were also obtained before and after FVIIai administration to measure ex vivo platelet aggregation (see below). Finally, blood samples for the measurements of FPA plasma levels were obtained before induction of CFVs (baseline), during CFVs, and after FVIIai and FVIIa administration.

Groups 2a and 2b: FVIIai Pharmacokinetic Studies

To determine the pharmacokinetics of FVIIai, as well as the duration of its antithrombotic effects, additional rabbits were included in the study. The protocol followed in this arm of the study is summarized in Fig 1B. CFVs were initiated as described above, and they were monitored for 30 minutes. Then, FVIIai was administered as an intracarotid infusion of 100 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, as previously described. The infusion was maintained until CFVs were inhibited (at which time the infusion was immediately discontinued) or for a maximum of 10 minutes. During FVIIai infusion, blood samples were obtained every 2 minutes to measure plasma FVIIai levels. When CFVs were

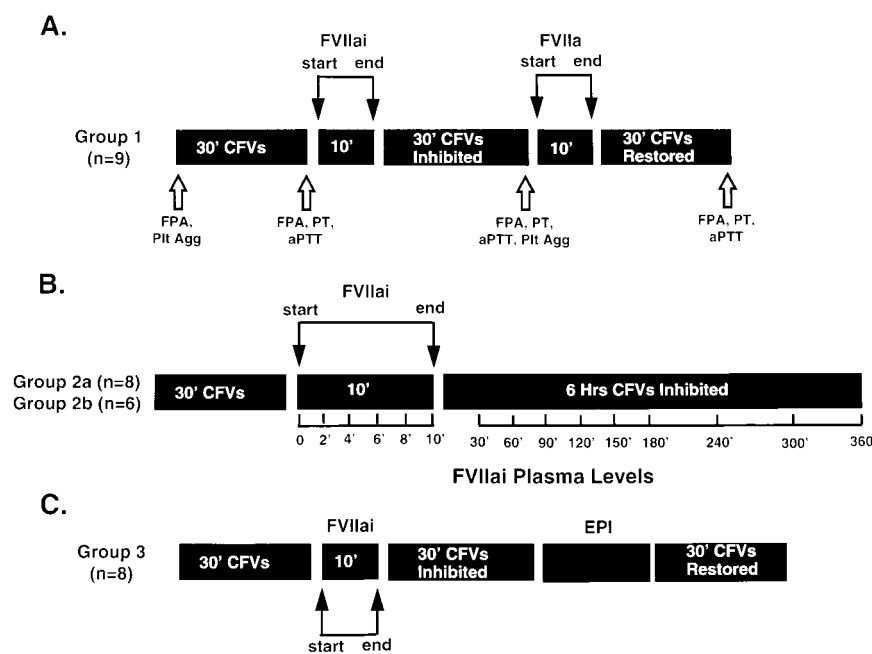


Figure 1. Schematic representation of the experimental protocols followed in the present study. **A,** Antithrombotic effects of FVIIai. In a group of 9 rabbits, CFVs were initiated as described in “Materials and Methods.” Thirty minutes later, FVIIai was infused into the carotid artery at a dose of $100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes (solid arrows). Once CFVs were inhibited, the animals were followed for 30 minutes, after which FVIIa was infused into the carotid artery at a dose of $100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes to determine whether CFVs could be restored. Once restored, CFVs were followed for an additional 30 minutes. Open arrows indicate the time points at which blood samples were obtained for the measurements of PT, aPTT, plasma levels of FPA, and ex vivo platelet aggregation (Plt Agg). **B,** Pharmacokinetic studies. Group 2a (n=8) and 2b (n=6) animals were included in this arm of the study. In these rabbits, CFVs were initiated as described in “Materials and Methods.” After observing CFVs for 30 minutes, FVIIai was infused into the carotid artery at a dose of $100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes (solid arrows). During

the infusion, blood samples for the measurements of FVIIai plasma levels were obtained every 2 minutes. Once CFVs were inhibited, the animals were followed for 3 hours (group 2a) or for 6 hours (group 2b). FVIIai plasma levels were measured every 30 minutes for the first 3 hours and every hour thereafter. **C,** Epinephrine (EPI)-induced restoration of CFVs. Eight animals were included. After CFVs were obtained, they were inhibited by FVIIai infusion. Thirty minutes later, an intracarotid infusion of EPI was started. EPI concentration was progressively increased until CFVs were restored or until systemic effects on hemodynamics appeared.

inhibited, these animals were followed for 3 hours (group 2a, n=8) or for 6 hours (group 2b, n=6). Blood samples to measure FVIIai plasma levels were obtained every 30 minutes for the first 3 hours and every hour thereafter.

Group 3: Epinephrine-Induced Restoration of CFVs

To determine whether FVIIai can also protect against epinephrine-induced restoration of CFVs, 8 additional rabbits were included in the study. The protocol followed in this arm of the study is summarized in Fig 1C. CFVs were initiated as described above, and they were monitored for 30 minutes. Then, FVIIai was administered as an intracarotid infusion of $100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes, as previously described. When CFVs were inhibited, these animals were followed for 30 minutes, after which an intracarotid infusion of epinephrine was started. This route of administration was chosen to obtain a high degree of platelet stimulation in the absence of significant systemic hemodynamic effects, which, by themselves, may alter CFVs. Epinephrine was given at an initial dose of $0.01 \mu\text{g}/\text{min}$ and progressively increased until CFVs were restored or until systemic effects appeared, ie, when blood pressure started to increase. Once CFVs were restored, they were observed for 30 minutes.

Ex Vivo Platelet Aggregation

To determine whether FVIIai affected platelet function per se, platelet aggregation was tested ex vivo both before and after FVIIai administration. Peripheral venous blood (14 mL) was collected in a syringe containing 1.5 mL of 3.8% sodium citrate, and PRP was obtained by centrifugation of blood at $120g$ for 20 minutes at room temperature. PRP was removed, and PPP was obtained by further centrifugation at $1000g$ for 5 minutes. Platelet aggregation was measured turbidimetrically on a Chronolog aggregometer and recorded on a linear recorder. The aggregometer was calibrated using PRP and PPP, and the test was performed on $250 \mu\text{L}$ PRP in a siliconized cuvette with continuous stirring. The platelet count in PRP was adjusted to $3 \times 10^5/\mu\text{L}$ by dilution with PPP as needed. Aggregation was induced in PRP in response to various concentrations of ADP and rabbit thromboplastin.

Coagulation Studies

To determine the effect of FVIIai administration on PT and aPTT, blood was collected in sodium citrate (3.8%) and centrifuged at $2000g$ for 10 minutes at 4°C to separate the plasma. PT and aPTT were measured in duplicate within 2 hours after blood collection.

To determine whether inhibition of the extrinsic coagulation pathway by FVIIai actually results in inhibition of thrombin formation in vivo, plasma FPA levels, an index of thrombin activity, were measured by a radioimmunoassay method using a commercially available kit (Byk-Sangtec). Blood samples were collected in pre-chilled tubes containing an anticoagulant supplied by the manufacturer, immediately placed on ice, and centrifuged at $3000g$ for 10 minutes at 4°C . The plasma was removed and stored at -70°C until the assay was performed. FPA levels were measured in triplicate according to the manufacturer's instructions.

Measurements of Plasma FVIIai Levels

The concentration of FVIIai in rabbit plasma was determined in triplicate by an ELISA method using the FVII EIA kit from Dako Corp according to the manufacturer instructions.

Statistical Analysis

All values are expressed as mean \pm SEM. The rate of inhibition of CFVs by FVIIai and the rate of CFV restoration by FVIIa were evaluated by Fisher's exact test. One-way and two-way ANOVAs with a design for repeated measurements were used to compare ex vivo platelet aggregation data, hemodynamic variables, FPA plasma concentrations, and PTs and aPTTs. When applicable, differences between groups were tested by a Student's *t* test for paired or unpaired samples with Bonferroni's correction. A value of $P < .05$ defined significant differences between populations.

Results

Antithrombotic Effects of FVIIai

After arterial injury and placement of the constrictor, CFVs developed in all group 1 rabbits (n=9). CFV frequency and

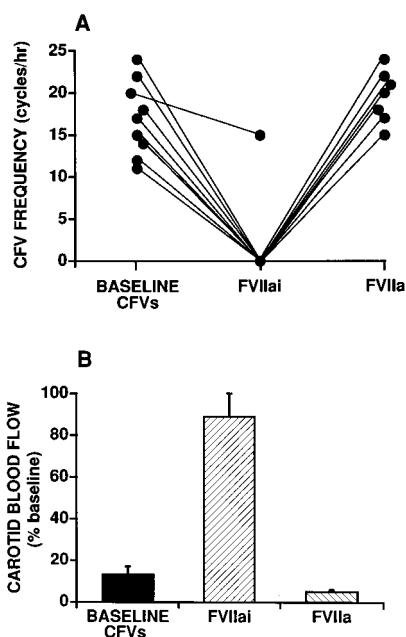


Figure 2. A, Graph showing the effects of FVIIai on CFV frequency expressed as cycles per hour. Baseline CFV frequency averaged 17 ± 2 cycles per hour and decreased to 0 cycles per hour after FVIIai administration in all but one animal ($P < .01$ by Fisher's exact test). CFVs were restored after FVIIa administration in all animals in which they were previously abolished ($P < .01$ by Fisher's exact test). B, CFV severity, expressed as carotid blood flow velocity (percentage of baseline value, ie, before inducing CFVs). Administration of FVIIai resulted in a significant increase in carotid blood flow compared with the value obtained during CFVs. Administration of FVIIa resulted in a significant decrease in carotid blood flow velocity similar to that observed during the initial CFVs.

severity (expressed as carotid blood flow velocity) averaged 17 ± 2 cycles per hour and $13 \pm 4\%$ of baseline values, respectively. After intracarotid infusion of FVIIai ($100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 minutes; total dose, 1 mg/kg), CFVs were completely inhibited in 8 of the initial 9 animals ($P < .01$), as CFV frequency decreased to 0 cycles per hour and carotid blood flow velocity increased to $89 \pm 11\%$ of baseline values (Fig 2). Thirty minutes after CFVs were inhibited, intracarotid infusion of FVIIa restored CFVs in all animals (CFV frequency, 19 ± 1 cycles per hour; carotid blood flow velocity, $5 \pm 1\%$ of baseline values; Fig 2), thus indicating that the binding of FVIIai to TF was reversible, as it could be displaced from TF by FVIIa. No significant changes in arterial blood pressure and heart rate were observed throughout the study (Table 1).

TABLE 1. Hemodynamic Variables in Rabbits With CFVs Receiving FVIIai (Group I)

	Heart Rate, bpm	Mean Arterial Pressure, mm Hg
Baseline	165 ± 6	73 ± 4
CFVs	159 ± 5	71 ± 5
FVIIai	169 ± 6	75 ± 5
FVIIa	163 ± 5	72 ± 4

Values are mean \pm SEM.

TABLE 2. Ex Vivo Platelet Aggregation in Response to ADP and TP Before and After FVIIai Administration

	Platelet Aggregation, % Max Aggregation	
	Before FVIIai	After FVIIai
ADP, mmol/L		
5	37 ± 5	39 ± 4
10	47 ± 8	50 ± 9
20	50 ± 7	53 ± 8
40	59 ± 5	62 ± 7
TP, $\mu\text{g/mL}$		
5	13 ± 5	13 ± 4
10	34 ± 6	39 ± 5
20	48 ± 8	46 ± 6
40	60 ± 7	56 ± 5

TP indicates thromboplastin. Values are mean \pm SEM.

Ex Vivo Platelet Aggregation and Coagulation Studies

Ex vivo platelet aggregation in response to ADP and thromboplastin was tested in blood samples obtained before (baseline) and after administration of FVIIai. No significant differences were observed after FVIIai administration in platelet aggregation in response to ADP and thromboplastin (Table 2). Thus, FVIIai at the doses used in the present study did not affect platelet function per se.

To study possible systemic effects of FVIIai, which may increase the risk of bleeding, PTs and aPTTs were measured in blood samples collected at 30 minutes of CFVs and after FVIIai and FVIIa administration. At the end of the 30-minute CFV period, PTs and aPTTs averaged 8.2 ± 0.6 and 25 ± 3 seconds, respectively. A slight increase in PTs to 10.1 ± 0.6 seconds was observed after FVIIai administration. This increase, however, did not reach statistical significance ($P = .09$ by ANOVA and Student's *t* test with Bonferroni's correction). aPTT did not change significantly after FVIIai administration. FVIIa administration resulted in a significant shortening in both PTs and aPTTs with respect only to the values obtained after FVIIai administration.

Plasma FPA levels averaged 6 ± 1 ng/mL before inducing CFVs and increased significantly to 25 ± 6 ng/mL during CFVs ($P < .01$ versus baseline, Fig 3), indicating that induction of recurrent thrombosis was associated with an increase in thrombin activity. In contrast, a decrease in plasma FPA levels to values similar to those obtained at baseline was observed when CFVs were inhibited after FVIIai administration ($P = \text{NS}$ versus baseline, Fig 3). This finding provides direct evidence that thrombin, generated through activation of the extrinsic coagulation pathway, is an important mediator of CFVs in this model. After administration of FVIIa, with CFVs restored, plasma FPA levels increased again to values similar to those observed during the initial CFVs (Fig 3).

FVIIai Pharmacokinetic Studies

Plasma FVIIai levels increased progressively during FVIIai infusion from undetectable levels before starting the infusion to $28.8 \pm 3.71 \mu\text{g/mL}$ at the end of the 10-minute infusion. A

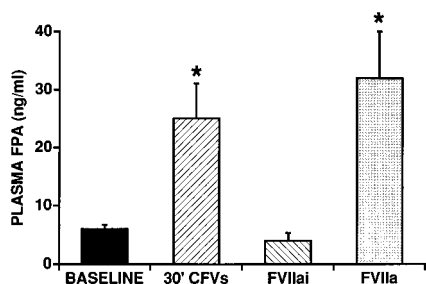


Figure 3. Plasma levels of FPA, an index of thrombin activity, in animals receiving FVIIai and FVIIa. FPA plasma levels increased significantly during CFVs compared with before CFVs (baseline). Administration of FVIIai, resulting in inhibition of CFVs, was associated with a decrease in FPA to baseline values. Restoration of CFVs by FVIIa was associated with a significant increase in plasma FPA levels. (* $P < .01$ vs baseline).

slow decrease in plasma FVIIai concentrations was observed over the 6-hour observation period. The apparent plasma half-life of FVIIai was ≈ 45 minutes (Fig 4). Fig 4 also shows the correlation between the antithrombotic effects of FVIIai and its plasma concentrations measured during the experiment. After 8 minutes of FVIIai infusion, CFVs were inhibited in only 1 of 8 animals in group 2a and none of group 2b, whereas at the end of the 10-minute infusion, CFVs were inhibited in a total of 11 of 14 animals (6 of 8 in group 2a, and 5 of 6 in group 2b). For the 6 rabbits in group 2a in which CFVs were inhibited, the experiment was terminated after 3 hours from the end of the infusion. At this time point, CFVs were still inhibited in all but 1 animal. In this animal, CFVs were spontaneously restored 167 minutes after the end of the infusion. Interestingly, all 5 animals in group 2b in which FVIIai successfully inhibited CFVs were still inhibited at 6 hours, despite the fact that at this time point FVIIai plasma levels were almost undetectable and largely below threshold concentrations (Fig 4).

Epinephrine-Induced Restoration of CFVs

Eight animals were included in this arm of the study. Two animals did not respond to FVIIai administration; therefore,

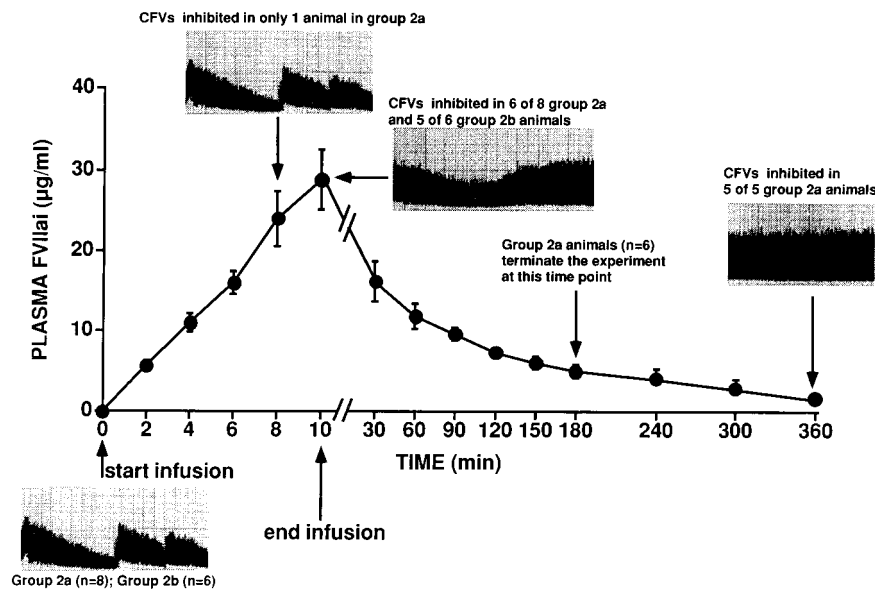


Figure 4. Graph showing the correlation between plasma levels of FVIIai and its antithrombotic effects after a single 10-minute FVIIai infusion. FVIIai plasma levels increased steadily during the infusion and slowly decreased during the observation period. Animals in group 2a ($n=8$) were followed for 3 hours and those in group 2b were followed for 6 hours after the administration of FVIIai. Eight minutes after starting the infusion, CFVs were inhibited in only 1 rabbit in group 2a and none in group 2b, whereas they were inhibited in 6 of 8 rabbits in group 2a and in 5 of 6 rabbits in group 2b at the end of the 10-minute infusion. At 3 and 6 hours, CFVs were still inhibited in all animals, despite the fact that the plasma levels of FVIIai decreased below the values necessary to inhibit CFVs. In particular, animals in group 2b, which were followed for 6 hours, showed very low plasma FVIIai levels at the end of the experiment, even though CFVs were still inhibited at the same time point.

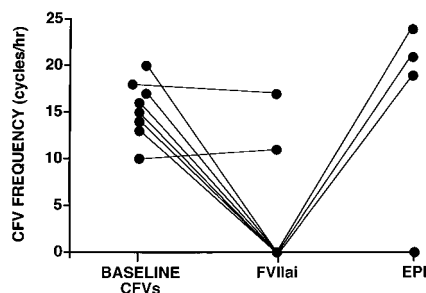


Figure 5. Effects of FVIIai inhibition of the coagulation cascade on epinephrine-induced restoration of CFVs. CFVs were inhibited by FVIIai in 6 of 8 animals; in these animals, epinephrine restored CFVs in 3 animals at a mean dose of 0.04 ± 0.01 $\mu\text{g}/\text{min}$. In the other 3 animals, epinephrine did not restore CFVs, even though a mean dose of 0.9 ± 0.1 $\mu\text{g}/\text{min}$ was reached.

the experiment was terminated for these animals. Of the remaining 6 animals, 3 animals experienced restored CFVs after administration of epinephrine at a mean dose of 0.04 ± 0.01 $\mu\text{g}/\text{min}$ (Fig 5). In the other 3 animals, epinephrine did not restore CFVs, despite the fact that a mean dose of 0.9 ± 0.1 $\mu\text{g}/\text{min}$ was reached (Fig 5). This dose increased the mean blood pressure by an average of 20% (data not shown).

Discussion

The main findings of the present study are that administration of recombinant human FVIIai has potent antithrombotic effects in this rabbit model of recurrent carotid artery thrombosis and that no significant changes in platelet aggregation in response to ADP and thromboplastin or changes in PTs and aPTTs were observed after VIIai administration. Furthermore, an interesting observation of the present study is that the antithrombotic effects of a single 10-minute infusion of FVIIai persisted for at least 6 hours, when the plasma FVIIai levels were largely below threshold concentrations. Taken together, these data outline the importance of TF exposure, with the consequent activation of the extrinsic coagulation pathway, in the pathophysiology of intravascular thrombus

formation and indicate that FVIIai might represent a useful antithrombotic intervention.

The extrinsic coagulation pathway is activated when circulating FVII gains access to TF in the vessel wall as a consequence of endothelial damage. Soluble FVIIa has a low affinity for its substrates, factors X and IX.⁶ However, when TF binds to FVII/VIIa, the resulting complex is capable of activating both factor X and factor IX 1000-fold more efficiently than soluble FVII/VIIa.⁶ After formation of the complex TF/FVII, blood coagulation proceeds through the “common” pathway, ultimately leading to the generation of thrombin.⁶ Accumulating evidence indicates that TF-dependent activation of the coagulation cascade is involved in the formation of intravascular thrombi. Coronary thrombosis generally occurs at stenotic sites, and it is often precipitated by disruption of an atherosclerotic plaque.¹⁶ Since atherosclerotic plaques are rich in TF-synthesizing cells, like monocytes, foam cells, and mesenchyma-like cells, plaque rupture may result in exposure of significant amounts of TF to circulating blood.⁸

The importance of TF exposure in triggering intravascular thrombus formation has also been suggested in a previous study from our laboratory.⁷ In the same experimental model used in the present study, we have shown that TF is normally present across the arterial wall, with its activity increasing from the intima to the adventitia.⁷ We have also shown that exposure of TF following endothelial injury plays an important role in triggering thrombus formation in this model via activation of the extrinsic coagulation pathway⁷ and that blocking TF activity by AP-1, a monoclonal antibody against TF, resulted in a complete inhibition of intravascular thrombus formation.⁷ Furthermore, inhibition of TF-procoagulant activity by AP-1 also resulted in enhancement of the thrombolytic properties of t-PA and in prevention of reocclusion after thrombolysis in an experimental model similar to the one used in the present study.¹⁰ In addition, Annex et al⁹ have recently shown significant TF activity in atherectomy specimens obtained from patients with unstable angina, suggesting that in these patients unstable angina may be precipitated by the activation of the extrinsic coagulation pathway caused by exposure of TF in the subendothelial tissue.

In the present study, recombinant human FVIIai was used as an antithrombotic intervention. The ability of FVIIai to inhibit the procoagulant activity of the complex TF/FVIIa was measured *in vitro* in a previous study.¹² It has been shown that the concentration of FVIIai needed to reduce the FVIIa-dependent factor X activation by 50% (IC₅₀ value) was 0.045±0.012 nmol/L.¹² In addition, binding studies carried out with J82 cells, a human bladder carcinoma cell line expressing TF, showed that the IC₅₀ value for FVIIai under these circumstances was 1.1±0.2 nmol/L.¹² Thus, the preparation of FVIIai used in the present study showed a potent anti-TF activity *in vitro*.

The antithrombotic effects of FVIIai were first described by Harker et al.¹⁷ These investigators have shown that FVIIai administration prevented thrombus formation in nonhuman primates at sites of carotid endarterectomy. These effects were obtained without significant changes in bleeding time.¹⁷ However, in the present study, several original observations can be found that differentiate it from Harker's study. Indeed, the

antithrombotic effects of FVIIai observed in the present study were obtained without potentially harmful systemic effects: PTs, aPTTs, and *ex vivo* platelet aggregation in response to ADP and thromboplastin did not change significantly after FVIIai administration. Of note is the observation that the antithrombotic effects of FVIIai could be promptly reversed by administration of recombinant human FVIIa. This finding indicates that the binding of FVIIai to the exposed vascular TF is reversible and makes this substance particularly attractive for clinical use, as the effects of FVIIai could be rapidly reverted in case of bleeding complications.

Of even more interest is the observation that the antithrombotic effects of a single 10-minute infusion of FVIIai were prolonged, as they persisted for at least 6 hours after the infusion was discontinued. At this time point, plasma FVIIai levels, which averaged 3.1 µg/mL, were largely below threshold concentrations. Indeed, Fig 5 shows that after 8 minutes of infusion, FVIIai plasma levels averaged 23.4 µg/mL. However, at this time point, only 1 animal out of 14 showed inhibition of CFVs. A possible explanation for this phenomenon may reside in the high affinity of FVII for its cofactor, TF. TF is an integral membrane single-chain glycoprotein that is associated with phospholipids. It has an N-terminal extracellular domain, a membrane-spanning region, and a cytoplasmic region constituting the carboxyl-terminal end of the protein.^{18,19} Several studies using a series of site-directed mutants of recombinant TF, in which alanine residues have replaced selected amino acids in the TF sequence, have been used to identify a candidate region of TF for binding FVII.^{20,21} It has also been shown that a dansyl-Glu-Gly-Arg chloromethyl ketone-treated FVIIa (another type of active site-blocked FVIIa, different from the one used in the present study) possesses a very high affinity for TF (K_i, 0.7×10⁻⁷ mol/L).²² Other experiments, using ¹²⁵I-labeled FVIIa, have shown that a 50-fold molar excess of unlabeled FVIIa over an hour of incubation time was necessary to displace 80% of the ¹²⁵I-FVIIa previously bound to TF.²³ In addition, compared with native FVIIa, it has been recently shown that inactivation of the active site of FVIIa with FFRcmk (the same method used to obtain the FVIIai preparation used in the present study) resulted in a 5-fold higher affinity for TF and a 2-fold slower dissociation rate from TF.¹² Thus, it can be hypothesized that FVIIai, once bound to TF, dissociates very slowly from its cofactor, such that significant antithrombotic effects can be still achieved even when FVIIai is almost completely cleared from the circulation. We did not measure FPA plasma levels in group 2b animals at 6 hours of CFV inhibition. Thus, we cannot directly demonstrate that the long-lasting antithrombotic effects of FVIIai are indeed due to a prolonged inhibition of the extrinsic coagulation pathway. However, the observation recently reported¹² that FVIIai has a slower dissociation rate from its ligand, TF, than does native FVIIa, leading to a prolonged binding of FVIIai to TF, seems to support our hypothesis. Nevertheless, lack of FPA measurements in this group of rabbits represents a possible limitation of the present study.

An alternative explanation for the prolonged antithrombotic effects of FVIIai may be related to the mechanisms regulating the cell surface TF/FVIIa proteolytic activity. A recent work by Sevinsky et al²⁴ has evidenced that TF procoagulant activity

is downregulated in the cells expressing TF by a translocation of the complex TF/FVIIa into noncoated plasmalemma vesicles. Interestingly, this translocation of TF is mediated by cell-associated TFPI, the natural inhibitor of the extrinsic coagulation cascade,²⁵ indicating that formation of the quaternary complex TF/FVIIa/factor Xa/TFPI is necessary for the transport of TF in the cytoplasm.²⁴ Therefore, it can be speculated that the prolonged antithrombotic effects of FVIIai observed in the present study might be explained by an increased translocation of TF from the cell surface to the cytoplasm. However, this possibility seems unlikely, considering the strict requirement for the binding of TFPI to the TF/FVIIa complex. In fact, FVIIai, having the active site blocked, cannot bind TFPI; this should result in inhibition of translocation of the complex to the cytoplasm.

The pathophysiological mechanisms responsible for the formation of intravascular thrombi continue to be investigated. It has been shown that thrombin plays a central role in this phenomenon, as it not only represents the key enzyme of the coagulation cascade but also is a powerful platelet agonist. In addition to heparin, new direct thrombin inhibitors have been recently identified, including hirudin and hirulog.²⁶ These new thrombin inhibitors have the advantage over heparin in that they are antithrombin III independent, inactivate clot-bound thrombin, and prevent thrombin-induced platelet aggregation.²⁶ However, a potential limitation of these agents is that new formation of thrombin is not affected.²⁶ This may cause persistence of thrombin activity despite the presence of an inhibitor.^{26,27} In addition, these compounds may carry an increased risk of bleeding when administered in conjunction with thrombolytic therapy, which represents the most disturbing adverse effect.²⁷

In this regard, a potential advantage of FVIIai is that it inhibits an early step of the extrinsic coagulation pathway, involving binding of native FVII/VIIa to TF, which ultimately results in inhibition of new thrombin formation, interrupting the positive feedback loop that autoamplifies thrombin generation. In the present study, evidence of inhibition of thrombin formation by FVIIai is provided by the measurements of FPA levels. FPA is cleaved from fibrinogen by the action of thrombin and thus represents an index of thrombin activity. FPA markedly increased in rabbits during the development of CFVs, indicating the presence of high thrombin activity during recurrent thrombus formation in this model. In contrast, FPA levels decreased significantly after FVIIai administration to baseline values, demonstrating that FVIIai, under the experimental conditions of the present study, interrupts the formation of the thrombus and prevents activation of the extrinsic coagulation pathway, ultimately leading to a reduction of thrombin formation. Inhibition of the extrinsic coagulation pathway by FVIIai also offers an advantage over blocking later steps in the coagulation pathway in that this substance binds to TF only where arterial damage is present. As a consequence, FVIIai, at the doses used in the present study, did not exert significant effects on blood coagulation and platelet aggregation. This should ultimately translate into a lower risk of bleeding compared with other antithrombotic interventions.

Conclusions

In the present study, we have demonstrated that administration of human recombinant FVIIai exerts potent antithrombotic effects in this rabbit model of recurrent carotid artery thrombosis. Furthermore, the antithrombotic effects of FVIIai were prolonged and persisted after the plasma levels of FVIIai decreased almost to baseline values. Thus, a significant antithrombotic effect can be obtained with FVIIai without significant changes in coagulation parameters or platelet function. Further studies are warranted to elucidate the potential clinical applications of FVIIai as an antithrombotic agent.

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