Factor VIIa-Catalyzed Activation of Factor X Independent of Tissue Factor: Its Possible Significance for Control of Hemophilic Bleeding by Infused Factor VIIa

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Infusing factor VIIa (FVIIa) has been reported to control bleeding in hemophilic patients with factor VIII (FVIII) inhibitors. This is difficult to attribute to an enhanced FVIIa/tissue factor (TF) activation of factor X, since in vitro studies suggest that infusion of FVIIa should neither increase substantially the rate of formation of FVIIa/TF complexes during hemostasis (Proc Natl Acad Sci USA 85:6687, 1988) nor bypass the dampening of TF-dependent coagulation by the extrinsic pathway inhibitor (EPI) (Blood 73:359, 1989). Partial thromboplastin times have also been reported to shorten after infusion of FVIIa. The experiments reported herein establish that shortening of partial thromboplastin times after adding FVIIa to hemophilic plasma in vitro stems from an FVIIa-catalyzed activation of factor X independent of possible trace contamination of reagents with TF. Experiments in purified systems confirmed that FVIIa can slow activation of factor X in a reaction mixture containing Ca^2+ and phospholipid but no source of TF. The rate of activation was sufficient to account for the shortening of partial thromboplastin times observed. EPI, which turned off continuing FVIIa/TF activation of factor X, was unable to prevent continuing FVIIa/phospholipid activation of factor X. Because circulating plasma contains only a trace, if any, free FVIIa, such a reaction could never occur physiologically. However, infusing FVIIa creates a nonphysiologic circumstance in which a continuing slow FVIIa/phospholipid catalyzed activation of factor X could conceivably proceed in vivo unimpeded by EPI. Such a mechanism of factor X activation might compensate for an impaired factor IXa/FVIIa/phospholipid activation of factor X during hemostasis, and therefore control bleeding in a hemophilic patient.

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MATERIALS AND METHODS

Plasmas. Hereditary factor IX-deficient plasma was obtained from a patient seen in this laboratory. Hereditary FVIII-deficient plasma was obtained from George King Biomedical (Overland Park, KS) or from a patient seen in this laboratory.

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**FVIIa.** FVIIa was prepared by incubating purified human FVII,\(^1\) final concentration of 150 to 200 \(\mu g/mL\) with factor Xa at a ratio of 200:1 wt/wt in the presence of 0.5 mg/mL rabbit brain cephalin (Sigma Diagnostics, St Louis, MO) and 5 mmol/L calcium chloride for 20 minutes at 37°C. The reaction was terminated with ethylenediamine-tetraacetic acid (EDTA), final concentration 10 mmol/L. This treatment increased FVII activity 25- to 40-fold as measured in a one-stage FVII clotting assay\(^1\) and yielded FVIIa with a specific activity of 53 to 67.5 U/\(\mu g\). The FVIIa preparation was stored at \(-70\)°C and used within 1 month. It is important to point out that the FVIIa preparation contained no measurable contaminating factor IX(a) or FVIII(a). Because the FVIIa preparation contained factor Xa, a preparation was also made that contained factor Xa, cephalin, and calcium chloride, with buffer in place of FVII (sham preparation) for use as a control.

**Factor X and factor Xa.** Purified human factor X, specific activity 105 U/mg, was prepared, activated to factor Xa with insolubilized Russell’s viper venom, and stored as described earlier.\(^1\) Factor Xa specific activity was 1,207 U/mg.

**Tissue factor (TF).** Human brain TF apoprotein was purified to homogeneity\(^5\) and incorporated into phospholipid vesicles containing 40% phosphatidyserine and 60% phosphatidylcholine as described previously.\(^4\)

**TF antibodies.** Specific polyclonal anti-rabbit TF immunoglobulin G (IgG)\(^2\) and specific polyclonal anti-human TF IgG\(^3\) were prepared as described earlier. The purified IgG fractions were devoid of measurable contamination of factors IX and VIII activities. A 10 \(\mu g/mL\) concentration of the anti-rabbit TF IgG neutralized about 90% of the coagulant activity of a 0.05 \(\mu g/mL\) preparation of reconstituted purified rabbit TF. A 4 \(\mu g/mL\) concentration of the anti-human TF IgG neutralized more than 95% of the coagulant activity of a 0.05 \(\mu g/mL\) preparation of reconstituted human TF.

**EPI.** The EPI preparation used in this study was partially purified from plasma by chromatography on Mono Q Sepharose (Pharmacia, Piscataway, NJ) as described earlier.

**Clotting assays.** A commercial activated partial thromboplastin time (APTT) reagent (Automated APTT, General Diagnostics, Raritan, NJ) was used for the APTT test, and rabbit brain cephalin (Sigma) was used for the partial thromboplastin time (PTT) test. One hundred microliters of citrated plasma containing different concentrations of FVIIa were added to hemophilia B plasma, the plasma was divided, and anti-human tissue factor antibody (1% vol/vol, 100 \(\mu g/mL\) final concentration) was added to one aliquot (\(\Delta\)) and control buffer to the other (\(\odot\)). After 20 minutes of incubation at room temperature, the APTT test was performed as previously described. (C) Varying concentrations of human TF were added to hemophilia B plasma. The plasma was incubated with anti-human tissue factor antibody (\(\odot\)) or control buffer (\(\bullet\)) as described in (B) and the APTT was determined as previously described. To ensure there was no rabbit TF in the APTT reagent used to test plasmas containing anti-human tissue factor IgG (B and C), the APTT reagent was also incubated for 20 minutes at room temperature with anti-rabbit tissue factor IgG (1% vol/vol, 100 \(\mu g/mL\)). (D) Varying concentrations of factor Xa were added to the APTT reagent. Hemophilia B plasma, 100 \(\mu L\) was incubated in a plastic tube at 37°C for 3 minutes, and then 100 \(\mu L\) of the APTT reagent was added. Immediately thereafter, 100 \(\mu L\) of 37°C CaCl\(_2\) was added and the clotting time noted.

**RESULTS**

**Effect of FVIIa on the clotting times of hemophilic plasmas.** Since the reported rFVIIa-induced shortening of the PTTs of hemophilic plasmas could have stemmed from contaminating traces of TF in test reagents, we determined whether FVIIa would shorten the APTT and PTT of hemophilic plasmas in the presence of anti-tissue factor antibodies. Panels A and B of Fig 1 illustrate the progressive shortening of the APTT repeatedly observed when increasing concentrations of FVIIa were added to hemophilia B plasma and the inability of anti-human tissue factor IgG to prevent the shortening. FVIIa at a final concentration in hemophilic plasmas of 5 \(\mu g/mL\) (10 times the concentration achievable if all FVII in plasma were activated to FVIIa) shortened the APTT of hemophilia B plasma from 160 seconds to about 50 seconds, and shortened the APTT of hemophilia A plasma from 120 seconds to about 45 seconds. (This concentration of FVIIa shortened the APTT of normal plasma from 33 seconds to 28 seconds.) Adding increasing concentrations of native FVII to hemophilia A or hemophilia B plasmas failed to shorten the APTT (data not shown).

As a positive control for the above experiment, the ability of anti-tissue factor IgG to abolish TF-induced shortening of the APTT of hemophilic plasmas was also tested. As apparent from the clotting times shown in Figs 1B and 1C, adding 0.5 ng/mL of TF to hemophilia B plasma shortened the APTT as much as adding a 10,000-fold higher concentration of FVIIa (5 \(\mu g/mL\)). In contrast to its inability to prevent FVIIa-induced shortening, anti-tissue factor IgG abolished TF-induced shortening of the APTT.

The PTT differs from the APTT in that the contact
activation reactions are only minimally activated. The PTT of hemophilia A and B plasmas consistently exceeded 10 minutes. Adding FVIIa in a final concentration of 5 μg/mL to these plasmas shortened the PTT to the 95 to 110 second range. As with the APTT, anti-tissue factor IgG had no significant effect on the shortening (Table 1).

Since factor Xa was used to activate the FVII, it was necessary to establish that this factor Xa did not contribute significantly to the shortening induced by the FVIIa preparation. This was done in three ways. First, the FVIIa preparation was shown to shorten neither the PTT (Table 1) or the APTT of factor X-deficient plasma (about 600 seconds). Second, adding the control sham FVIIa preparation (which contained factor Xa but no FVII) to hemophilic plasmas did not shorten the PTT (greater than 600 seconds), and shortened only minimally the APTT (Fig 1A). Third, an FVIIa preparation made with factor Xa insolubilized on Affi-Gel 15 beads (Bio-Rad, Richmond, CA) and removed after incubation by filtration through a 0.2 μm nylon membrane (West Coast Scientific, Inc, Hayward, CA) also shortened the APTT of hemophilic plasma (data not shown).

However, one should note (see legends of Fig 1 and Table 1) that the hemophilic plasmas were allowed to stand for 20 minutes after adding the FVIIa preparation to allow neutralization of added factor Xa before the APTT and PTT were performed. Indeed, when factor Xa was added in increasing concentrations to the APTT reagent and this reagent was added just before the calcium to hemophilic plasma, a final concentration of only 5 ng/mL of factor Xa in the APTT reagent shortened the APTT to 45 seconds (Fig 1D).

**Table 1. Failure of Anti-Tissue Factor IgG to Abolish FVIIa-Induced Shortening of the PTT**

<table>
<thead>
<tr>
<th>Type of Plasma</th>
<th>Clotting Time (Plasmas/Plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Anti-TF IgG</td>
</tr>
<tr>
<td>Hemophilia A</td>
<td>600</td>
</tr>
<tr>
<td>+ FVIIa</td>
<td>105</td>
</tr>
<tr>
<td>Hemophilia B</td>
<td>600</td>
</tr>
<tr>
<td>+ FVIIa</td>
<td>95</td>
</tr>
<tr>
<td>Factor X deficient plasma</td>
<td>600</td>
</tr>
<tr>
<td>+ FVIIa</td>
<td>600</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>300</td>
</tr>
<tr>
<td>+ FVIIa</td>
<td>84</td>
</tr>
</tbody>
</table>

FVIIa preparation (final FVIIa concentration 5 μg/mL) was added to one aliquot of plasma, and control sham FVIIa preparation was added to a second aliquot. Both plasma samples were divided and anti-human tissue factor IgG (1% vol/vol, 100 μg/mL, final concentration) was added to one half and control buffer to the other half. The test samples were then incubated for at least 20 minutes at room temperature. The cephalin reagent used to test plasmas containing anti-tissue factor IgG was also incubated for 20 minutes at room temperature with anti-rabbit tissue factor IgG (1% vol/vol, 100 μg/mL). The test was performed by incubating 100 μL of plasma with 100 μL of cephalin for 3 minutes at 37°C in a plastic tube and then adding 100 μL of 35 mmol/L CaCl₂ that had been warmed to 37°C.

**Fig 2.** FVIIa/phospholipid activation of factor X in a purified system. Fifty microliters of 20 μg/mL factor X containing 15 mmol/L CaCl₂ was incubated at 37°C in a microtiter well with 50 μL of varying concentrations of FVIIa and 50 μL of either 1 mg/mL cephalin (G) or buffer (Φ). Several wells were prepared for each concentration of FVIIa tested. For wells containing cephalin, 50 μL of 1.25 mg/mL of Chromozyme X (Boehringer Mannheim Biochemical, Indianapolis, IN) was added to a well after 10, 20, and 30 minutes of incubation, and absorbance was measured at 410 nm with a Dynatech elite reader (Dynatech Laboratories Inc, Alexandria, VA). For wells containing buffer, Chromozyme X was added after 30 minutes of incubation. Absorbance was converted to factor Xa concentration from a reference curve prepared with known concentrations of factor Xa.

**Activation of factor X by FVIIa in purified systems.** Because the above APTT and PTT data were interpreted to mean that FVIIa could activate factor X in hemophilic plasma in the absence of TF, further experiments were performed in purified systems to confirm this. Purified factor X was incubated in microtiter wells with varying concentrations of FVIIa and Ca²⁺ in the presence or absence of phospholipid. In the presence of phospholipid, increasing concentrations of FVIIa yielded increasing rates of factor X activation (Fig 2). A 5 μg/mL concentration of FVIIa yielded a factor X activation rate of 27 ng/mL/min. In the absence of phospholipid, this concentration of FVIIa failed to activate measurable amounts of factor X.

When reaction mixtures were made with increasing concentrations of FVIIa and 2 ng/mL of purified, reconstituted TF, only 200 pg/mL of FVIIa was required to activate factor X at a rate comparable with that obtained with 5 μg/mL of FVIIa and phospholipid. When increasing concentrations of TF were added to reaction mixtures made with a 5 μg/mL concentration of FVIIa, 0.5 ng/mL of tissue factor yielded a factor X activation rate comparable with that observed with 5 μg/mL of FVIIa and phospholipid.

**Effect of EPI on factor X activation.** When FVIIa/TF catalyzes activation of factor X in reaction mixtures containing EPI, factor Xa can form an EPI/factor Xa complex that then shuts down further FVIIa/TF catalytic activity.12,13 Experiments were performed to test whether EPI could similarly shut down FVIIa/phospholipid catalyzed activa-
tion of factor X. Reaction mixtures were made to contain constant concentrations of factor X, FVIIa, and calcium ions, increasing concentrations of EPI, and either TF or phospholipid. Concentrations of tissue factor and phospholipid were used that yielded similar factor X activation in reaction mixtures containing buffer instead of EPI. Factor Xa activity was measured after 30 minutes of incubation. Data from 1 of 3 experiments giving similar results are summarized in Fig 3. As expected, EPI suppressed FVIIa/TF activation of factor X. In contrast, EPI failed to suppress FVIIa/phospholipid activation of factor X. (The minor reduction in factor Xa activity of reaction mixtures containing phospholipid is thought to reflect the known ability of EPI to neutralize factor Xa amidolytic activity.)

**DISCUSSION**

It is generally accepted that FVII must form a complex with TF to function in normal hemostasis. Nevertheless, the data reported herein support a hypothesis that a minimal but continuing FVIIa-catalyzed activation of factor X, requiring phospholipid but independent of TF, could be a mechanism whereby infusions of FVIIa control bleeding in patients and experimental animals with impaired factor IXa/FVIIIa/phospholipid catalyzed activation of factor X. Three types of indirect supporting evidence for this hypothesis were obtained. First, it was shown that a TF-independent, FVIIa-catalyzed activation of factor X can account for the previously unexplained shortening of PTTs reported after infusion of rFVIIa or adding FVIIa to hemophilic plasma in vitro. Second, it was confirmed in a purified system that FVIIa can activate factor X in the presence of Ca²⁺ and phospholipid. Third, it was shown that EPI, in contrast to its effectiveness in dampening FVIIa/TF activation of factor X, cannot inhibit FVIIa/phospholipid activation of factor X.

Shortening of APTT and PTT test results by FVIIa could have had two possible explanations: contamination of test reagents with traces of TF or FVIIa catalyzed activation of factor X independent of TF. The second explanation was proven correct. Adding anti-tissue factor IgG abolished shortening of the APTT induced by adding TF to hemophilic plasma mixtures (Fig 1C), whereas adding anti-tissue factor IgG had no effect on the shortening of the APTT and PTT induced by adding FVIIa to hemophilic plasma (Fig 1B and Table 1). The low concentration of factor Xa in the FVIIa preparation had no appreciable effect on the APTT (Fig 1A) or PTT, undoubtedly because the plasma was allowed to stand for 20 minutes to neutralize the factor Xa before the tests were performed. Therefore, the shortening of the APTT and PTT observed after adding the FVIIa preparation to hemophilic plasmas (Fig 1, A and B, and Table 1) had to stem from a TF-independent, FVIIa-catalyzed activation of factor X during the tests.

In additional experiments, low concentrations of factor Xa were added to the APTT reagent and the resultant mixture added to warmed hemophilia B plasma just before the calcium. Under these conditions, which minimized neutralization of factor Xa before the test, only 5 ng/mL of factor Xa in the APTT reagent shortened clotting times to those obtained in the standard APTT test after adding 5 μg/mL FVIIa to hemophilic plasma (compare clotting times of Figs 1A, B, and D). One should note that an FVIIa-catalyzed generation of 5 ng/mL of factor Xa in the standard APTT test would require activation of less than 0.1% of factor X in the test mixture (assuming a factor X concentration in the hemophilic plasma of 8 μg/mL).

The experiments in purified systems (Fig 2) confirm and extend Østergard’s earlier observation that factor X can be activated with FVIIa, Ca²⁺, and phospholipid. The rates of generation of factor Xa measured were sufficient to account for the shortening of the APTT induced by adding FVIIa to hemophilic plasmas (see Figs 1D and 2). Nevertheless, the enzymatic efficiency of FVIIa/phospholipid was minimal compared with that of FVIIa/TF, eg, 5 μg/mL FVIIa with phospholipid yielded a rate of activation equivalent to that obtained with 0.2 ng/mL of FVIIa in the presence of TF.

Consequently, one might assume that a slow FVIIa/phospholipid activation of factor X would be of no hemostatic significance. However, EPI could not inhibit generation of factor Xa in a reaction mixture made with factor X, FVIIa, and phospholipid, whereas EPI markedly diminished generation of factor Xa in a reaction mixture made with factor X, FVIIa, and a low concentration of tissue factor (Fig 3). Thus, if a limited number of FVIIa/TF complexes are formed at a wound site and subsequently inhibited by EPI/factor Xa, then FVIIa/phospholipid complexes formed at the wound site might well provide a slow but hemostatically meaningful mechanism for continuing the activation of factor X.

When normal human plasma was clotted in vitro with diluted crude brain tissue factor, barely measurable amounts of free plasma FVIIa were generated. Moreover, when rabbits were infused with a concentration of TF that caused mean fibrinogen levels to fall to about 10% of the level before infusion, no circulating FVIIa was demonstrable. Thus, it is unlikely that plasma normally ever contains more than a
trace of free FVIIa, so the free FVIIa needed for an FVIIa/phospholipid activation of factor X is not available. A factor IXa/FVIIa/phospholipid (or membrane equivalent) activation of factor X is the mechanism whereby EPI-damped FVIIa/TF activation of factor X is supplemented in normal human hemostasis. When this mechanism fails, as in hemophilic patients, serious bleeding results.

However, if one raises plasma FVIIa concentration by infusing FVIIa, then an FVIIa/phospholipid-catalyzed activation of factor X might be able to compensate for an impaired factor IXa/FVIIa/phospholipid activation of factor X. The known intravascular half-time of FVIIa is about 2½ hours and the presumed presence of the required phospholipid cofactor on aggregated, activated platelets and tissue cells at a wound site makes this a feasible possibility. The hypothesis is attractive to us because it permits one to reconcile the evidence that infused FVIIa controls hemophiliacs' bleeding with present concepts of the initiation and regulation of the tissue factor pathway of coagulation. It could explain why infusing FVIIa would have a hemostatic effect, despite a plasma concentration of FVII sufficient to saturate TF at an injury site, and the preferential, rapid activation to FVIIa of FVII in vivo. Most importantly, one can then understand why infusing FVIIa would bypass regulation by EPI of FVIIa/TF catalytic activity. However, we must emphasize that our in vitro data cannot extrapolate with certainty to explain the mode of action of high concentration of infused FVIIa in vivo. Our next step is to test this hypothesis further with in vivo experiments to elucidate the mechanism whereby the infusion of rFVIIa controls bleeding in hemophiliacs.

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REFERENCES