BRIEF REPORT

Limited factor VIIa surface localization requirement of the factor VIIa–induced overall thrombin generation in platelet-rich hemophilia A plasma

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Abstract

Background: Thrombin generation assay (TGA) and thrombelastography (TEG) are increasingly employed, global, in vitro methods for assessment of the procoagulant potential of plasma/blood and possibly ideally suited tools to monitor, for example, therapy with recombinant factor VIIa (FVIIa). It remains controversial to what extent results obtained with spiked and postinfusion samples reflect the outcome in patients.

Objective: To characterize the TGA response to FVIIa in hemophilic plasma and compare with TEG data.

Methods: Hemophilia A (HA) was induced in platelet-rich plasma (PRP) from healthy volunteers, followed by spiking with FVIIa, γ-carboxyglutamic acid (Gla)-domainless FVIIa or V158D/E296V/M298Q-FVIIa (FVIIa$_{DVQ}$). Samples were triggered with tissue factor and analyzed by TGA and TEG in parallel.

Results: Addition of 25 nmol L^{-1} FVIIa to HA PRP normalized TEG parameters angle and R time, as well as TGA lag time, but had poor effects on the thrombin peak height and velocity index. All parameters (at least) returned to normal levels either upon adding a much higher concentration of FVIIa (~1500 nmol L^{-1}) or by using the superactive variant FVIIa$_{DVQ}$. Surprisingly, Gla-domainless derivatives of FVIIa and FVIIa$_{DVQ}$ also yielded considerable effects in HA PRP.

Conclusions: The good general responses to clinically effective concentrations of FVIIa (25 and 75 nmol L^{-1}) seen in TEG analyses, as well as for TGA lag time, were accompanied by far-from-normal thrombin peaks. A near-normal thrombin peak response required the presence of considerably higher FVIIa activity but, intriguingly, relied only marginally on a functional Gla domain (ie, on platelet surface localization).

KEYWORDS
factor VIIa, gamma-carboxyglutamic acid domain, platelets, thrombelastography, thrombin peak

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Essentials

- Factor VIIa (FVIIa; NovoSeven®) is a bypassing agent for treatment of hemophilia with inhibitors.
- Clinically effective concentrations of NovoSeven® result in a subnormal thrombin peak in vitro.
- FVIIa concentration needed for normal thrombin peak is largely independent of platelet binding.
- Cautious interpretation of drug effect based on thrombin peak in static assay is warranted.

1 | INTRODUCTION

Hemophilia A (HA) patients who develop inhibitory antibodies against factor VIII cannot be treated with conventional replacement therapy. Bleeds occurring in this group, constituting about one third of all HA patients, are treated with bypassing therapy using, for example, recombinant factor VIIa (FVIIa; NovoSeven®). The effects of bypassing drugs are more complex to measure in vitro, and global assays under more in vivo–like conditions have been suggested for optimal dose prediction and postinfusion monitoring.[1,2] Such assays include thrombin generation assay (TGA) and measurement of blood viscosity such as thromb-elastography (TEG). Increased understanding of the response to FVIIa and its mechanism in these assays is warranted.

There are large discrepancies regarding the usefulness of global assays. Some parameters have been suggested to reflect the clinical effect of FVIIa in HA patients with inhibitors,[1,3–5] the thrombin peak not being one of them. The thrombin peaks in HA plasma spiked with 25 or 75 nmol L^{-1} FVIIa, corresponding to the plasma levels reached in a subject after administration of 90 and 270 μg kg^{-1} body weight, respectively, are much smaller than that seen in normal plasma. Although the vast majority of thrombin is formed after the clot forms, it may have pivotal clot-stabilizing functions.[6]

The present study was conducted to quantify and characterize the effects of FVIIa in TGA, especially to gain insight about the mechanistic credibility of TGA and factors determining the height of the generated thrombin peak. The gathered data might help guide a decision on how best to monitor bypassing therapies in the clinic. In the course of this work, we made observations that raise concerns about the suitability of TGA for mimicking the physiological setting at which FVIIa exerts its pharmacologic effect and for studying some aspects of FVIIa's mode of action.

2 | MATERIALS AND METHODS

2.1 | Proteins

Recombinant FVIIa and V158D/E296V/M298Q-FVIIa (FVIIa$_{DVQ}$) were produced in house as described.[7,8] γ-Carboxyglutamic acid (Gla)-domainless (des[1-44])-FVIIa and -FVIIa$_{DVQ}$ were obtained by cleaving the full-length proteins with cathepsin G in the absence of free divalent metal ions.[9] Lipidated tissue factor (TF; Innovin) was from Siemens Dade (Deerfield, IL), sheep antihuman factor VIII (FVIII) antibodies from Haematologic Technologies (Essex Junction, VT), and blocking monoclonal rat antihuman endothelial cell protein C receptor (EPCR) antibody (RCR-252) from Sigma-Aldrich (Saint Louis, MO).

2.2 | Amidolytic activity assay

FVIIa, Gla-domainless FVIIa, FVIIa$_{DVQ}$ and Gla-domainless FVIIa$_{DVQ}$ were diluted to 150 nmol L^{-1} in 20 mmol L^{-1} HEPES, pH 7.4, containing 140 mmol L^{-1} NaCl, 5 mmol L^{-1} $CaCl_2$ and 2% (w/v) bovine serum albumin; 100 μL of this solution was mixed with 50 μL 3 mmol L^{-1} S-2288 (Chromogenix, Milan, Italy) in the same buffer. The absorbance at 405 nm was measured for 10 minutes using a SpectraMax 340PC spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

2.3 | Human material

Human blood was obtained from healthy individuals who were members of the Danish National Corps of Voluntary Blood Donors. Novo Nordisk A/S has approval from the Danish National Committee on Biomedical Research Ethics (journal no. H-D-2007-0055) to use donor blood for internal research purposes.

2.4 | TGA and TEG

To prepare platelet-rich plasma (PRP), citrate-stabilized blood was centrifuged at 220 g for 20 minutes, after which the platelets were transferred to a new tube and counted (range, 197 000-332 000 μL^{-1}). PRP was used without adjusting the platelet density. For some experiments, platelet-poor plasma (PPP) was prepared by centrifugation at 2500 g for 10 minutes, resulting in a residual platelet count <50 μL^{-1}. A plasma volume was kept in the normal (non-HA) state, whereas HA was induced in the remainder by adding antihuman FVIII antibodies to a final concentration of 0.1 mg mL^{-1} and incubating for 30 minutes. The FVIIa proteins and Innovin trigger were diluted in 20 mmol L^{-1} HEPES, pH 7.4, containing 140 mmol L^{-1} NaCl and 2% (w/v) bovine serum albumin, to a 34-fold higher concentration than that in the final sample mixture. The common, premixed samples for TGA and TEG were prepared in duplicate by mixing 16 μL FVIIa variant, 16 μL Innovin (final concentration 0.1 pmol L^{-1} TF [final dilution 1:60 000]) and 512 μL plasma. In TGA, the reaction was set off by adding 20 μL FluCa (Thrombinoscope, Maastricht, The Netherlands) to 100 μL sample. In TEG, 340 μL sample was initiated with 20 μL 20 mmol L^{-1} HEPES, pH 7.4, containing 0.2 mol L^{-1} $CaCl_2$. TGA[10] and TEG[11] were run essentially as previously described.

In some experiments, Innovin was diluted differently than described above to give final assay concentrations of 0.02 or 0.5 pmol L^{-1}

TF. In other experiments, instead of PRP, PPP was used together with phospholipids (135 μmol L^{-1}; 80% PC:20% PS [Haematologic Technologies], added to the Innovin) at a final concentration of 4 μmol L^{-1}. In yet another experiment, anti-EPCR antibody was added to PRP to a concentration of 340 nmol L^{-1}.

2.5 | Statistics

Mean values and standard deviations were calculated by the software Prism 7 (GraphPad Software, La Jolla, CA).

3 | RESULTS AND DISCUSSION

Our aim was to characterize the general TGA response to FVIIa in HA plasma, with emphasis on the generated thrombin peak, and compare with TEG findings. Moreover, FVIIa was compared to Gla-domainless FVIIa and to full-length and Gla-domainless forms of the enzymatically superactive variant FVIIa$_{DVQ}$. TGA and TEG measurements were performed on identical samples, and parallel, directly comparable analyses were made feasible by spiking FVIIa derivatives into PRP and initiating the reactions with a common trigger (0.1 pmol L^{-1} TF).
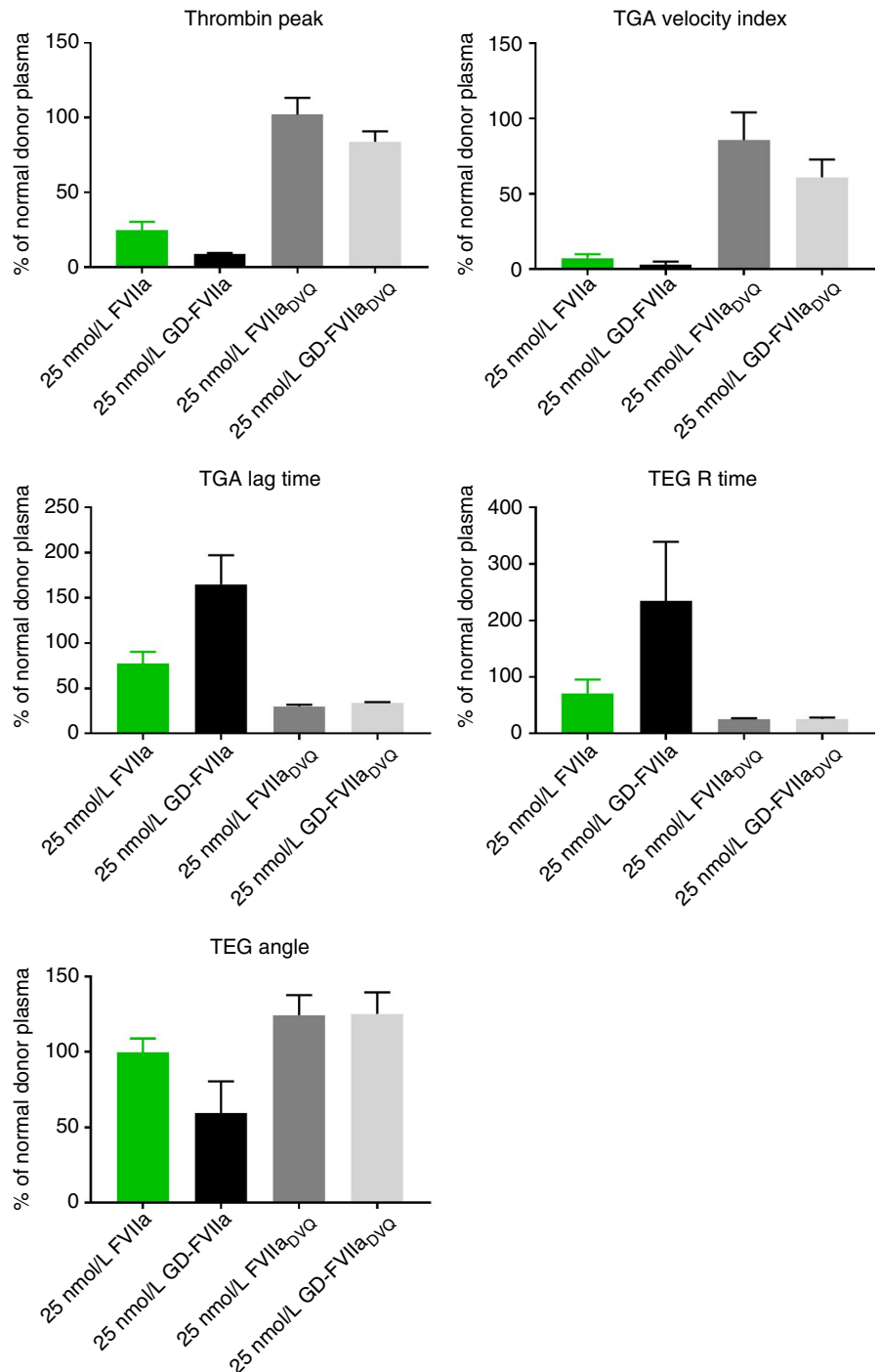

FIGURE 1 Response to 25 nmol L^{-1} FVIIa, Gla-domainless (GD-) FVIIa, FVIIa$_{DVQ}$, and GD-FVIIa$_{DVQ}$ in TGA and TEG. The indicated parameters were measured using PRP from healthy donors (n = 4) following antibody-induced HA and triggering by TF (0.1 pmol L^{-1}). Data are shown as mean ± SD. In HA PRP without added FVIIa, the thrombin peak was 1% to 4% of normal PRP, velocity index 0.5% to 1%, lag time 2.8- to 5.8-fold longer than in normal PRP, R time 3.0- to 5.8-fold longer (if clotting occurred, 2 of the HA PRP samples produced no fibrin clot), and angle 0% to 30%. FVIIa, factor VIIa; FVIIa$_{DVQ}$, V158D/E296V/M298Q-FVIIa; HA, hemophilia A; PRP, platelet-rich plasma; TEG, thromboelastography; TF, tissue factor; TGA, thrombin generation assay.

Amidolytic activity measurements assured that equal added concentrations of the various FVIIa forms indeed represented the same amounts of enzymatically active molecules using FVIIa as the comparator. In practice, the nominal concentrations of the stock solutions were slightly adjusted so that the specific activity of Gla-domainless FVIIa equaled that of FVIIa[12] and the same applied to Gla-domainless FVIIa$_{DVQ}$ vs. FVIIa$_{DVQ}$. Furthermore, FVIIa$_{DVQ}$ was set to having 8-fold higher specific activity than FVIIa, as established previously.[8] After this calibration, the proteins were ready for use in TGA/TEG measurements.

Initially, we quantified the effects of adding 25 nmol L^{-1} FVIIa derivative to HA PRP. Without added FVIIa, the thrombin peak was 1% to 4% of that in the corresponding control (non-HA) PRP. FVIIa induced the formation of a thrombin peak corresponding to 20% to 25% of that obtained in normal PRP (Figure 1). Gla-domainless FVIIa generated 8% to 10% of the normal thrombin peak, indicating that the presence of the Gla domain and the concomitant ability to associate with the platelet surface somewhat increased, but was not a prerequisite for, the thrombin-generating potential. Full-length and Gla-domainless FVIIa$_{DVQ}$-induced thrombin peaks were around 100% and 80% to 90% of that of normal plasma, respectively. The TEG angle and R time, as well as the TGA lag time, were normalized by 25 nmol L^{-1} FVIIa,[13] whereas the TGA velocity index was only around 10% of normal (Figure 1). FVIIa$_{DVQ}$ at 25 nmol L^{-1} brought R and lag times well below normal plasma values, normalized the angle, and virtually normalized the velocity index. Interestingly, Gla-domainless FVIIa$_{DVQ}$ was capable of correcting all parameters almost as effectively as the full-length enzyme, strongly indicating that platelet surface localization means less in these static systems than the level of enzymatic activity.

Because superactive FVIIa$_{DVQ}$ was capable of normalizing all TGA parameters of HA PRP, we investigated whether this could also be achieved by increasing the concentration of FVIIa. Indeed, the presence of larger amounts of FVIIa activity in the TGA sample continuously increased the thrombin peak with normalization reached at 1500 nmol L^{-1} FVIIa (Figure 2). The extra FVIIa activity needed to boost the thrombin-generating potential above that seen with 25 nmol L^{-1} FVIIa apparently did not need to be platelet bound because it could largely be provided by FVIIa$_{DVQ}$ lacking the Gla domain (Figure 1). To substantiate this hypothesis further, increasing concentrations of Gla-domainless FVIIa were added to HA PRP and a similar, parallel improvement of the thrombin peak reaching 60-70% of that of normal, plasma at 900 nmol L^{-1} was observed (FVIIa: 80%-90%; Figure 2). The major part of the FVIIa effect thus appeared to be attributable to the sheer presence of large amounts of FVIIa enzymatic activity rather than to increased activity localization to the platelet surface.

To examine whether platelet activation was somehow responsible for the modest thrombin peak observed with FVIIa, PRP was replaced by PPP supplemented with procoagulant phospholipids. This did not alter the pattern of FVIIa response, but the thrombin peak variation in percentage of the normal response was strikingly greater than with PRP (mean ± standard deviation at 25 nmol L^{-1} FVIIa: 18 ± 12% vs. 20 ± 3%; at 1500 nmol L^{-1} FVIIa: 119 ± 61% vs. 100 ± 10%). Increased variation was observed for all recorded parameters. Apparently, even though PRP samples from a group of normal donors behaved homogeneously as measured by TGA
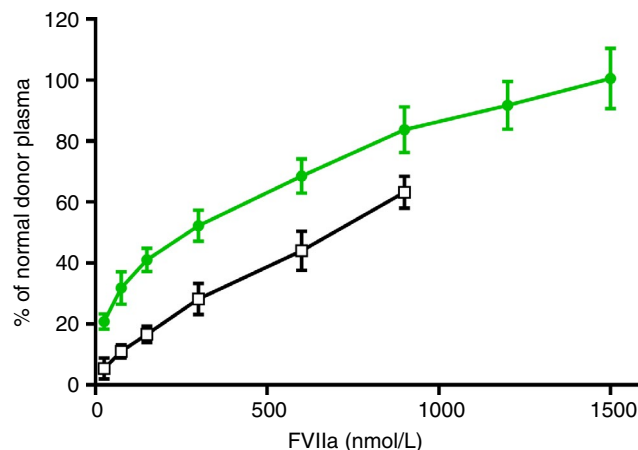
FIGURE 2 Thrombin peak height as a function of added FVIIa concentration. The results from titrations with FVIIa (solid green circles) and Gla-domainless FVIIa (open black squares) in PRP from healthy volunteers (n = 5) following induction of HA and triggering by. TF (0.1 pmol L^{-1}) are shown as mean ± standard deviation. FVIIa, factor VIIa; HA, hemophilia A; PRP, platelet-rich plasma; TF, tissue factor.

(and TEG), the properties of the corresponding PPP differed considerably among individuals. The great variations seen with PPP plus phospholipids would plausibly be reflected in similar variations if platelets isolated from various donors were mixed with the same PPP pool.

The precise TF concentration was not critical for the relative thrombin peak after spiking HA PRP with 25 nmol L^{-1} FVIIa. Compared with 0.1 pmol L^{-1} TF, a 5-fold decrease or increase had no significant effect on the thrombin peak height in absolute numbers nor as a percentage of normal plasma, whereas the times to thrombin (TGA lag time) and clot formation (TEG R time) were shortened with increasing TF concentration, all in agreement with existing data.[13] With 600 nmol L^{-1} FVIIa or Gla-domainless FVIIa present, lag and R times were already short and insensitive to changes in TF level.

FVIIa interacts with EPCR through the Gla domain,[14,15] and activated human platelets recently have been shown to express EPCR.[16] In our study, a blocking anti-EPCR antibody did not influence the TGA/TEG parameters, including the thrombin peak, after spiking FVIIa derivatives into HA PRP. This is in some disagreement with the findings of Fager et al,[16] who claimed a modest decrease of thrombin generation upon EPCR blockage. This might be explained by different modes of platelet activation and/or plasma environment (containing factor V and protein C) vs. a purified system (lacking protein C). Conceivably, the reason for the lacking effect of EPCR blockage in PRP is that a reduced formation of activated protein C balances a decreased FVIIa localization.

Our compiled data strongly suggest that the thrombin peak measured using TGA after adding FVIIa to HA PRP is governed by FVIIa enzymatic activity, not influenced by TF concentration (in the sub-pmol L^{-1} range used) or EPCR binding, and only modestly increased by platelet surface (phospholipid) localization. FVIIa in HA plasma at concentrations mimicking those reached and effective in HA patients with inhibitors treated with NovoSeven® does not produce a thrombin peak even closely resembling that seen in normal plasma.[13] Other TGA (and TEG) parameters are (almost) normalized and apparently more closely related to the clinical

efficacy. However, thrombin peak normalization is feasible with FVIIa but requires 20 to 50 times higher levels (or a superactive variant). A limited impact of platelet surface binding on the FVIIa-induced thrombin peak is supported by data with Gla-domainless FVIIa and FVIIa$_{DVQ}$ and is presumably an assay artifact owing to the static, contained conditions. In a voluminous flowing system, such as under in vivo conditions, the situation is quite different. Gla-domainless FVIIa would not stop a bleed because it, unlike FVIIa, fails to localize to the site of injury. Accordingly, if TGA were used to screen for hemostatic drugs based on their induced thrombin peak, false-positive compounds lacking platelet surface binding ability would be anticipated owing to confinement of the reaction cascade leading to thrombin generation to the assay well. Finally, FVIIa mode of action studies using TGA should be performed with caution; in sharp contrast to our TGA finding, the relatively poor factor X–activating ability of FVIIa in solution leaves little doubt in our minds that phospholipid association is utterly important for the pharmacologic effect of FVIIa,[17] and neither does the documented effect of FVIIa on platelet adhesion and activation under flow conditions.[18] Recent data strongly supporting a TF-independent mode of action of FVIIa, neither competing with zymogen for TF nor requiring TF for its own effect, support the necessity of a direct interaction with procoagulant membrane surfaces.[19–21]

ACKNOWLEDGMENTS

RELATIONSHIP DISCLOSURE

The authors are employees of Novo Nordisk A/S, the manufacturer of NovoSeven® (recombinant FVIIa).

AUTHOR CONTRIBUTIONS

EP designed the research, analyzed data, and wrote the manuscript. MW performed the experiments and analyzed data.

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