Thrombin generation response of rFVIIa in haemophilia A patient plasma when using different triggers and phospholipids

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Objective

- To investigate the influence of different triggers and phospholipids on the thrombin generation response when monitoring therapeutic relevant rFVIIa levels in plasma from haemophilia A (HA) patients.

Conclusions

- The dose-response of rFVIIa in thrombin generation assays is highly dependent on the trigger and phospholipid source used.
- High concentrations of phospholipids increased the analytical window.
- With nM concentrations of sTF an assay with a significantly improved analytical window and a sensitivity of 1 nM rFVIIa was obtained.

Introduction

- Treatment of bleedings in haemophilia inhibitor patients with rFVIIa show high efficacy.
- In the standard commercial thrombin generation assay CAT (Calibrated Automated Thrombogram) therapeutic relevant rFVIIa levels (< 25 nM) do, however, often show weak but variable dose-responses[1-2].
- Liposomes containing phosphatidic acid (PA) support higher proteolytic activity by FVIIa than phosphatidylserine (PS) containing liposomes[3] suggesting that the phospholipid source may be of relevance for rFVIIa in CAT measurements.
- It has previously been shown that soluble TF (sTF) variants improve thrombin generation assessments of pharmacological relevant rFVIIa levels (<25 nM)[5]
- Soluble tissue factor (sTF) is truncated tissue factor (TF) consisting only of the extra-cellular domain of TF and has a low affinity to FVII zymogen.

Methods

Thrombin generation measurements

- Haemophilia A patient plasma pool (George King Bio-Medical Inc., USA).
- Haemophilia A plasma (80 µL) spiked with rFVIIa (0-500 nM) was mixed with different combinations of trigger (tissue factor (TF) or activated FIX (FXIa)) and phospholipid (PL) preparations.
- Thrombin generation was initiated by addition of a fluorogenic thrombin substrate containing $CaCl_2$ and thrombograms for rFVIIa in combination with below described triggers and phospholipid sources were determined.
- Peak thrombin responses are used to represent the thrombin generation at the different conditions.
- Each data point is based on a single CAT determination.

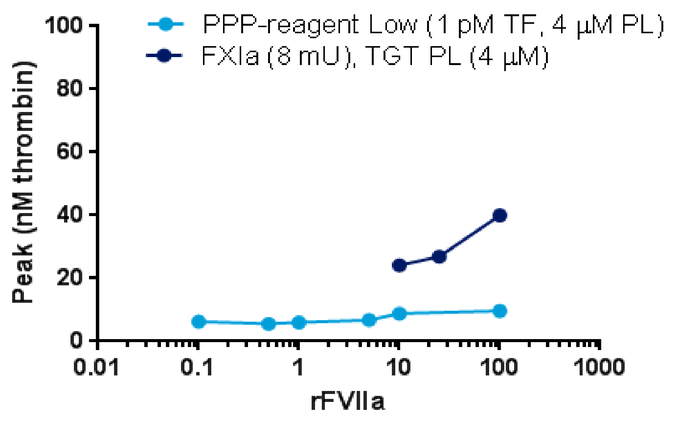
Trigger and phospholipid reagents

- The following triggers were used:
  - TF (full-length (1-263), Innovin®, Dade Behring, Germany)
  - sTF (1-219, Creative Biomart, USA)
  - sTF variant (D61A, produced in-house)
  - FXIa (Enzyme Research Laboratories, USA).
- The following phospholipid sources were used:
  - PS:PC (20:80) (in-house reagent)
  - PS:PC:PE (20:40:40) (in-house reagent)
  - PS:PA:PE (5:25:70) (in-house reagent)
  - PS:PC:PA:PE (2:40:28:30) (in-house reagent)
  - TGT PL (Rossix AB, Sweden)
  - PC:PS PL (HTI Diagnostics, USA)
  - MP reagent (Thrombinoscope, The Netherlands)
  - PPP-Reagent Low (Thrombinoscope, The Netherlands)
- The trigger and phospholipid concentrations used are indicated in result section and figures. The indicated concentrations are final concentration in the reaction mixture. For FXIa 8 mU/mL was used.

Results

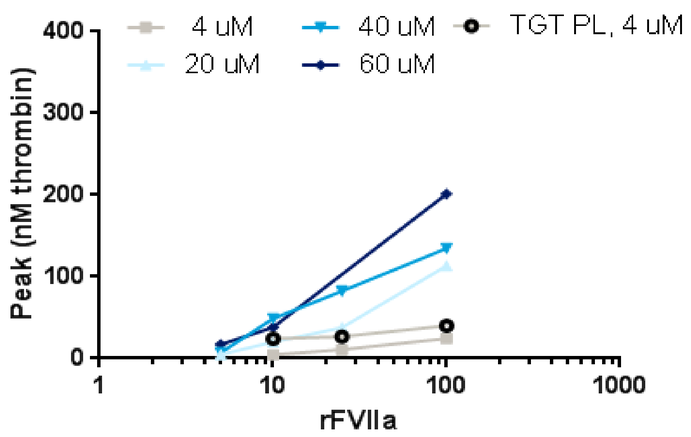
- As expected, the commercial PPP-Reagent Low (Thrombinoscope) containing 1 pM TF and 4 µM PL resulted in a weak dose-response of rFVIIa.
- The same was observed when using FXIa as trigger in combination with the commercial TGT PL reagent from Rossix Ab (4 µM PL) (Fig. 1).

Figure 1 Dose-response of rFVIIa with different standard triggers and phospholipids



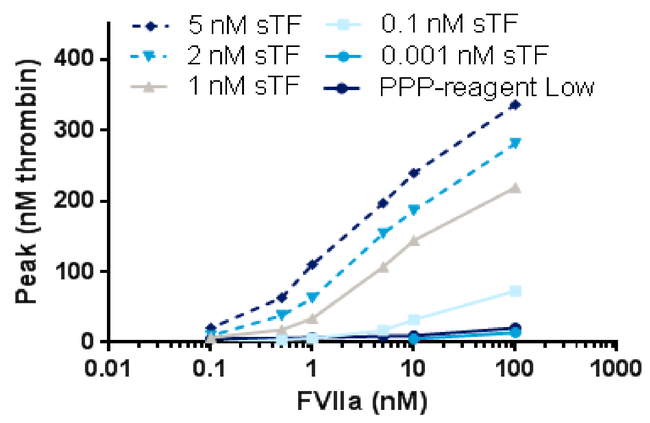
- Using different phospholipid sources results in different thrombin generation responses.
- In combination with FXIa the PS:PC:PE and PS:PC:PA:PE vesicles showed the strongest rFVIIa dose-responses.
- For PS:PC:PA:PE concentrations ≥ 20 µM the analytical window increase significantly, however only with a slight improvement of sensitivity (Fig. 2).

Figure 2 Dose-response of rFVIIa using FXIa as trigger in combination with PS:PC:PA:PE vesicles



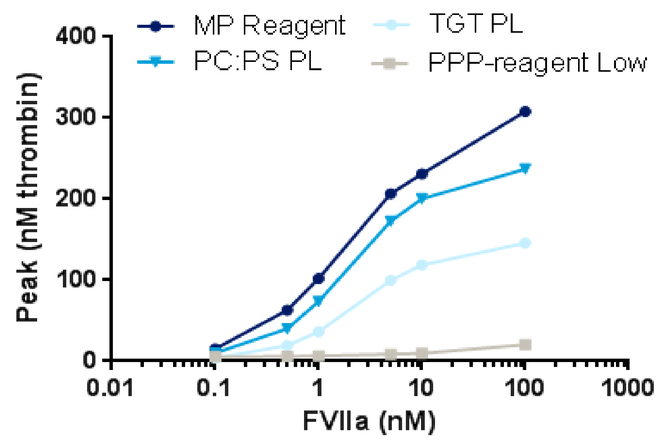
- Usually 1 pM of TF is used for triggering in thrombin generation assays. Increasing concentrations of full-length TF typically results in lack of analytical window (data not shown)
- Using high concentrations (nM range) of sTF (1-219) does, however, improve both the analytical window and the sensitivity significantly (Figure 3)

Figure 3 Dose-response of rFVIIa when using soluble TF as trigger; PL source is TGT PL (4 µM). PPP-reagent Low is used as comparator.



- Similar observations have been made with a sTF variant (D61A), (data not shown).
- sTF is known selectively to react with FVIIa and does not promote the conversion of FVII zymogen to FVIIa[5].
- Also with sTF the dose-response of rFVIIa is highly dependent on the phospholipid source used (Fig. 4)

Figure 4 Influence of different commercial phospholipid sources (4 µM); trigger is 1 nM sTF. PPP-reagent Low is used as comparator



References

1. Dargaud Y *et al*. *Blood* 2010;116:5734–5737
2. Mancuso ME *et al*. *Haemophilia* 2016;22:e292–e300
3. Tavoosi N *et al*. *Biochemistry* 2013;52:5545–5552
4. Shibeko AM *et al*. Poster 560, XXV ISTH Congress, 2015
5. Morrissey JH *et al*. *Blood* 1993;3:734–744.

Conflict of interest disclosure

All the authors are employed at Novo Nordisk A/S

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