ORIGINAL ARTICLE

Recombinant factor VIIa reverses the *in vitro* and *ex vivo* anticoagulant and profibrinolytic effects of fondaparinux

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Summary. *Background*: Fondaparinux is a synthetic pentasaccharide, which selectively inhibits coagulation factor (F) Xa, and is registered for prevention of venous thromboembolism following hip fracture, hip replacement, and knee replacement surgery. Recently, it was shown that recombinant FVIIa (rFVIIa) reverses anticoagulant effects of fondaparinux in healthy volunteers. *Objectives*: In this study, we have explored the *in vitro* and *ex vivo* effects of rFVIIa on clot formation and thrombin-activatable fibrinolysis inhibitor (TAFI)-mediated down-regulation of fibrinolysis after fondaparinux administration. *Methods*: *In vitro* clot lysis assays were performed in pooled normal plasma from healthy volunteers to which fondaparinux was added, and in serial samples from healthy volunteers who received a single bolus dose of fondaparinux, a single bolus dose of rFVIIa, or both. *Results and conclusions*: Fondaparinux significantly delayed clot formation, and clot lysis was significantly increased due to decreased activation of TAFI. Addition of recombinant FVIIa corrected the inhibited clot formation induced by fondaparinux, and the acceleration of clot lysis was partially reversed. In vivo administration of fondaparinux (10 mg) to healthy volunteers similarly resulted in accelerated plasma clot lysis. Subsequent administration of rFVIIa ($90 \mu g \, kg^{-1}$) normalized the clot lysis time up to 6 h postadministration. rFVIIa might be a good therapeutic option in patients treated with fondaparinux who develop bleeding complications, since both clot formation as well as fibrinolytic resistance are improved.

Keywords: fibrinolysis, fondaparinux, rFVIIa, TAFI.

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Introduction

The synthetic pentasaccharide Org31540/SR90107A (fondaparinux sodium, Arixtra®) is a novel, chemically synthesized antithrombotic drug, which exerts its effect by selective inhibition of coagulation factor (F) Xa activity in an antithrombin-dependent manner [1]. Clinical studies showed superiority of fondaparinux over low-molecular-weight heparin in preventing venous thromboembolism after hip replacement surgery [2,3], hip-fracture surgery [4], and elective major knee surgery [5], and fondaparinux has been registered in Europe and the USA for these indications. Also, fondaparinux was shown to be effective for treatment of proximal vein thrombosis [6], as adjunctive therapy in acute myocardial infarction [7], and to prevent abrupt vessel closure during percutaneous transluminal coronary angioplasty [8]. Currently, two world-wide phase III trials (OASIS 5 and 6) explore the use of fondaparinux in acute coronary syndromes.

Adverse events associated with the administration of anticoagulants are mainly bleeding complications [9]. A meta-analysis of four randomized double-blind phase III clinical trials in more than 7000 patients in orthopedic surgery demonstrated a similar low risk in clinically important bleeding with fondaparinux and enoxaparin [10]. Therapeutic options in patients treated with fondaparinux who develop bleeding complications are until now limited. A recent trial suggested that recombinant FVIIa (rFVIIa) could reverse the anticoagulant effect of fondaparinux in case of serious bleeding complications. rFVIIa given as a single bolus dose of $90 \mu g \, kg^{-1}$ was able to reverse coagulation times and *in vitro* thrombin generation after fondaparinux administration (10 mg) in healthy volunteers [11].

rFVIIa has been originally developed for patients with hemophilia A or B with inhibitory antibodies [12]. Recently, however, it has been suggested that rFVIIa could become a general hemostatic agent as it has shown to be effective in arresting bleedings or limit blood loss in various clinical settings [13–17]. In a previous study we have shown that rFVIIa, in addition to its procoagulant properties, can function as an antifibrinolytic agent when added to plasma from patients with

severe hemophilia A by means of enhancing the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) [18]. As TAFI is activated by high concentrations of thrombin [19], we speculated that fondaparinux might not only function as an anticoagulant but also as a profibrinolytic agent by attenuating TAFI activation.

In this study, we investigated the effect of rFVIIa on clot formation and on TAFI mediated down-regulation of fibrinolysis in plasma from healthy individuals to which fondaparinux was added. Also, *in vitro* TAFI-mediated down-regulation was studied in serial plasma samples obtained from the above mentioned rFVIIa/fondaparinux interaction trial.

Methods

Plasma samples

Pooled normal plasma obtained by combining plasma from 40 healthy volunteers, and individual plasma samples from 35 healthy laboratory volunteers were used in this study.

Additionally, plasma samples from healthy volunteers who participated in fondaparinux/rFVIIa interaction trial were included in this study. Details of this study have been published elsewhere [11]. In short, eight volunteers received a single bolus dose of 10 mg fondaparinux at $t = 0$, and a single bolus dose (90 μg kg^{-1} bodyweight) of rFVIIa at $t = 2$ h. Four volunteers received placebo at $t = 0$ and rFVIIa at $t = 2$ h, and three volunteers received fondaparinux at $t = 0$ and placebo at $t = 3$ h. Blood samples were taken at timepoints 0, 1.5, 2.5, 3, 3.5, 4, 5, 6, 8, and 24 h.

Blood samples were collected into 3.2% sodium citrate (9 : 1, v/v). To obtain platelet poor plasma, the samples were centrifuged twice at $2000 \times g$ for 15 min. Plasma samples were stored at $-80\,^{\circ}$C until use.

Materials

Recombinant FVIIa (NovoSeven®) was a generous gift from Novo Nordisk A/S (Bagsvaerd, Denmark), fondaparinux sodium was from NV Organon (Oss, the Netherlands), and tissue type plasminogen activator (t-PA) was from Chromogenix (Mölndal, Sweden). Recombinant human tissue factor (Innovin) was from Dade Behring GmbH (Marburg, Germany), and carboxypeptidase inhibitor from potato (CPI) was purchased from Calbiochem (La Jolla, CA, USA). Phospholipid vesicles consisting of 40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine (all from Sigma, St. Louis, MO, USA) were prepared according to van Wijnen *et al.* [20]. Total phospholipid content of the vesicles was determined by phosphate analysis [21].

Clot lysis assay

Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis essentially as described previously

[22], except for a change in tissue factor concentration. Fifty microliters of plasma was pipetted into a microtiter plate, after which 50 μL of a mixture containing tissue factor (diluted Innovin, final dilution 1000 times), CaCl$_2$ (final concentration 17 mM), t-PA (final concentration 30 U mL^{-1}; 56 ng mL^{-1}), and phospholipid vesicles (final concentration 10 μM), diluted in Hepes buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl$_2$, 0.1% BSA, pH 7.4) was added. After thorough mixing, turbidity at 405 nm was measured in time at 37 °C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA, USA). Coagulation time was defined as the time to reach the midpoint of clear to maximal turbid transition. Clot lysis time was defined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis. The contribution of TAFI activation to clot lysis time was determined by adding CPI (25 μg mL^{-1}), a specific inhibitor of activated TAFI [23], to the plasma.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (San Diego, USA) software package. Differences in clotting time and clot lysis time in pooled normal plasma was determined by standard *t*-test. Differences in clotting time and clot lysis time in the 35 individual samples were assayed by repeated measures one-way analysis of variance (ANOVA) using the Tukey-Kramer post-test. *P*-values < 0.05 were considered statistically significant.

Results

Effects of fondaparinux and rFVIIa on clot formation and clot lysis in pooled normal plasma

Figure 1 shows the effects of fondaparinux in a plasma system, in which coagulation was initiated with a diluted prothrombin time reagent as tissue factor source. Fondaparinux dose-dependently increased coagulation time in pooled normal plasma (Fig 1A, open circles). Addition of rFVIIa (40 nM) reversed the increase in coagulation time induced by fondaparinux over the whole concentration range tested (Fig 1A, closed circles).

The effect of fondaparinux on clot lysis induced by exogenous t-PA is presented in Fig. 1(B). Fondaparinux dose-dependently decreased clot lysis time (open circles), which was partially reversed by rFVIIa (closed circles). When CPI, a specific inhibitor of activated TAFI, was added to the plasma (Fig. 1B, squares), fondaparinux and rFVIIa showed no effects on clot lysis time.

Differences in the effects of fondaparinux and rFVIIa on fibrinolysis between individuals

The effect of addition of fondaparinux (3.5 μg mL^{-1}), rFVIIa (40 nM), or both on clot formation and clot lysis time was
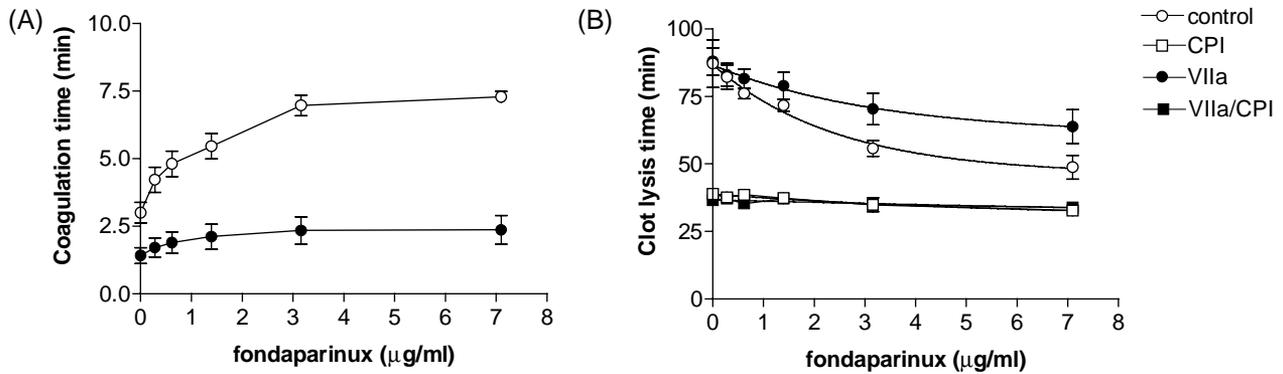
Fig. 1. Defective clot formation and TAFI-mediated down-regulation of fibrinolysis induced by fondaparinux can be improved by rFVIIa. (A) shows the effect of fondaparinux on clotting time in the absence (open symbols) or presence (closed symbols) of rFVIIa (40 nM). (B) shows the effect of fondaparinux on clot lysis time in the absence (circles) or presence (squares) of CPI, and in the absence (open symbols) or presence (closed symbols) of rFVIIa (40 nM). The mean ± standard deviation of 3 independent experiments is shown.

investigated in individual plasma samples from 35 healthy volunteers. We observed that addition of a fixed concentration of fondaparinux reduced clot lysis time in some, but not all plasma samples. Therefore, we separately analyzed samples that gave more than 5% reduction of clot lysis time on addition of 3.5 µg mL^{-1} fondaparinux (high responders; Fig. 2A,C), and samples that gave less than 5% reduction of clot lysis time on addition of 3.5 µg mL^{-1} fondaparinux (low responders; Fig. 2 B,D).

Figure 2A shows clot lysis times from the high responders. The decrease in clot lysis time on addition of fondaparinux was statistically significant (control 80 ± 13 min, fondaparinux 65 ± 15 min; mean ± SD, $P < 0.001$). On addition of rFVIIa

to fondaparinux-anticoagulated plasma in this group, a significant increase in clot lysis time was seen (from 65 ± 15 min to 72 ± 13 min, $P < 0.01$). Addition of rFVIIa in the absence of fondaparinux had no effect on clot lysis time. On addition of CPI, clot lysis times decreased to 38 ± 7 min, indicating that fondaparinux only partially decreased TAFI activation.

In plasma samples from the low responders, addition of rFVIIa to plasma containing 3.5 µg mL^{-1} fondaparinux did not change clot lysis time (Fig. 2B). The addition of 10.5 µg mL^{-1} fondaparinux to low responder plasma did decrease clot lysis time, which could be only partially corrected by the addition of rFVIIa (Fig 2B and 10.5 µg mL^{-1} fondaparinux 43.4 ± 7.8 min, 10.5 µg mL^{-1} fondaparinux + rFVIIa 47.2 ± 10.3, $P = 0.02$).
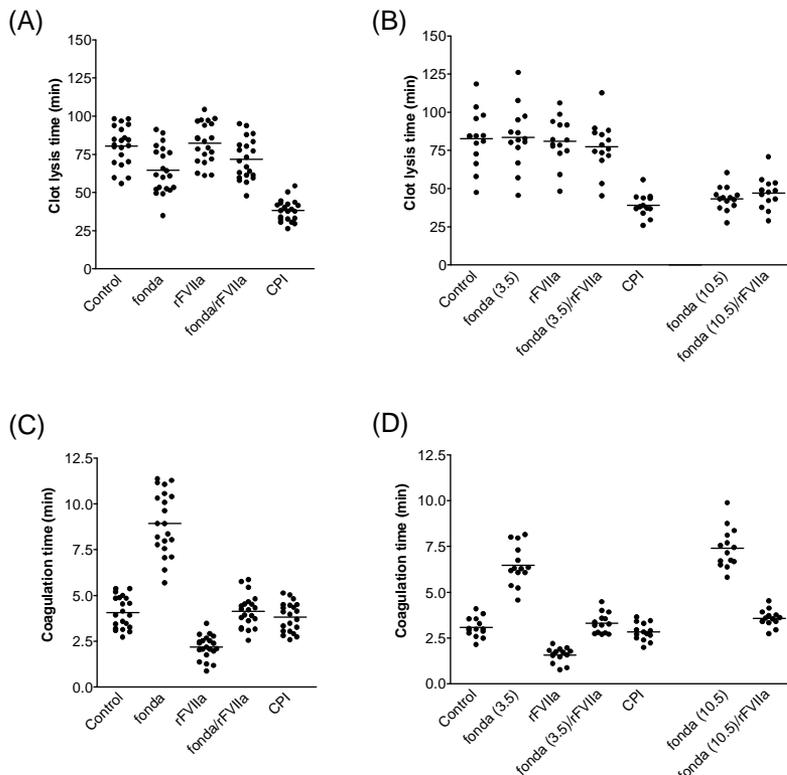

Fig. 2. Effect of fondaparinux (fonda, 3.5 µg mL^{-1}), rFVIIa (40 nM), a combination of fondaparinux and rFVIIa, and CPI (25 µg mL^{-1}) on clot lysis time and clotting time in plasma of 35 healthy volunteers. (A) and (C) show clot lysis times and clotting times, respectively, of plasma samples which gave a more than 5% reduction of clot lysis time on addition of fondaparinux. (B) and (D) show clot lysis times and clotting times, respectively, of plasma samples which showed a less than 5% reduction of clot lysis time on addition of fondaparinux. (B) and (D) also show the effect of a high concentration of fondaparinux (10.5 µg mL^{-1}), and of a combination of a high concentration of fondaparinux in combination with rFVIIa (40 nM) on clot lysis time and clotting time.
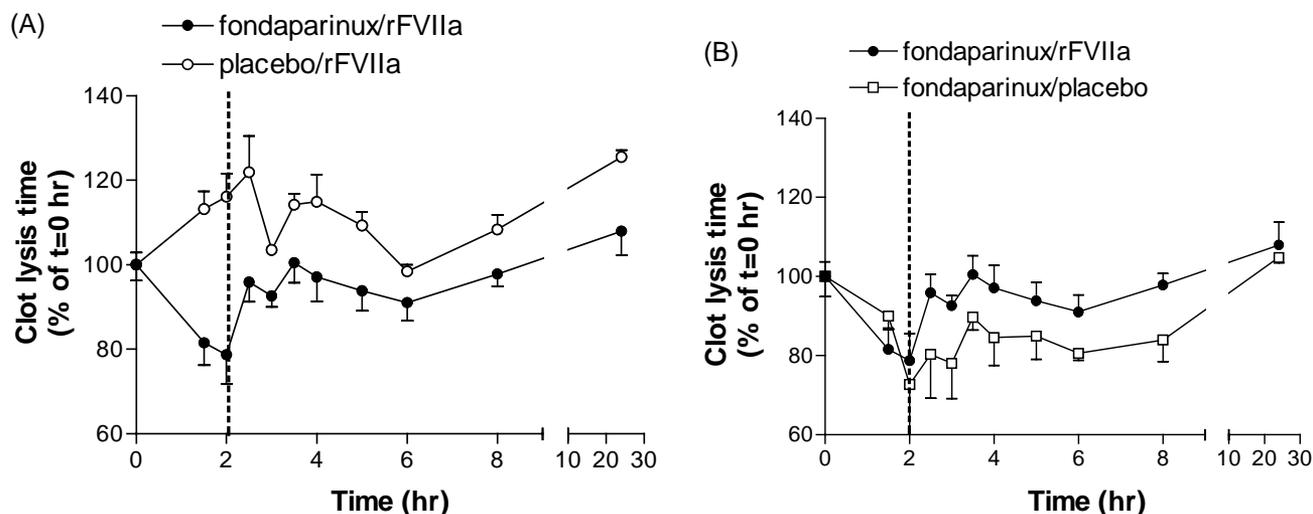
Fig. 3. (A) Progression of plasma clot lysis time in time in healthy volunteers receiving a single bolus dose of 10 mg fondaparinux at t = 0 and a single bolus dose of 90 μg kg^{-1} rFVIIa at t = 2 h (closed symbols) or placebo at t = 0 and rFVIIa at t = 2 h (open symbols). (B.)Progression of plasma clot lysis time in time in healthy volunteers receiving fondaparinux at t = 0 and rFVIIa at t = 2 h (closed symbols) or fondaparinux at t = 0 and placebo at t = 2 h (open symbols). Shown are mean values; values were normalized to clot lysis values at t = 0 h, which was set at 100%. Error bars indicate standard error of mean (SEM).

In both groups (Fig. 2C,D) clotting time increased significantly on addition of fondaparinux, which could be reversed by addition of rFVIIa.

Evaluation of clotting times of the two groups demonstrated that clotting times in the high responder group in the control situation were significantly higher compared to clotting times in the low responder group (4.1 ± 0.9 min vs. 3.1 ± 0.5 min, $P < 0.001$). The clotting times were significantly correlated with the percentage of reduction of clot lysis time on addition of fondaparinux ($r = 0.61$, $P = 0.0001$).

Effects of administration of fondaparinux and rFVIIa to healthy volunteers on plasma clot lysis time

Clot lysis assays were performed in plasma samples obtained from a fondaparinux/rFVIIa interaction trial in healthy volunteers [11]. Figure 3 shows the progression of clot lysis time in healthy volunteers receiving fondaparinux, rFVIIa, or both. Clot lysis times at t = 0 h were set at 100%. On administration of fondaparinux at t = 0, clot lysis time decreased to around 80% of control values (closed circles). Subsequent administration of rFVIIa at t = 2 h, increased clot lysis time to the baseline value, remaining at around 100% until t = 24 h. In contrast, in the fondaparinux alone group (Fig. 3B, open squares), the clot lysis time remained decreased (at around 80%) up to 8 h post-fondaparinux administration.

In the rFVIIa alone group (Fig. 3A, open circles), rFVIIa administration did not seem to affect clot lysis times, which remained relatively constant until t = 24 h.

To confirm that changes in clot lysis time on fondaparinux or rFVIIa infusion are linked to alterations in thrombin generating capacity and thus in changes in TAFI activation, we investigated the correlation between normalized clot lysis data and thrombin generation time (TGT) and endogenous thrombin potential

(ETP) values which were reported previously [11]. Both TGT and ETP values were significantly correlated with normalized clot lysis times ($r = 0.59$ and 0.36, respectively; $P < 0.0001$).

Discussion

Administration of fondaparinux to healthy volunteers leads to a delay of tissue factor-induced clot formation, and to acceleration of fibrinolysis due to diminished TAFI activation. This phenomenon was observed in pooled normal plasma to which fondaparinux was added, as well as in plasma from healthy volunteers to which a single bolus dose of fondaparinux was administered.

The observation that inhibition of coagulation FX results in inhibition of TAFI activation is in accordance with previous studies, which showed premature fibrinolysis in plasma deficient of either one of the intrinsic coagulation factors [24,25]. It was therefore postulated that hemophilia is not only a disorder of coagulation, but that defective TAFI activation might also cause part of the bleeding tendency observed in these patients. Likewise, part of the bleeding diathesis of patients anticoagulated with heparin derivatives, may be due to acceleration of fibrinolysis as a consequence of defective TAFI activation. On the other hand, induction of fibrinolysis by anticoagulants such as fondaparinux could facilitate clot dissolution. This hypothesis is supported by recent data showing that prolonged anticoagulation with fondaparinux after hip surgery (patients received fondaparinux at a dosage of 2.5 mg once daily for 28 instead of 7 days) resulted in a >95% reduction in the incidence of venographically detected venous thrombosis [26]. Interestingly, this study showed a trend towards increased major bleeding in the fondaparinux group ($P = 0.06$), which might be in part caused by prolonged induction of accelerated fibrinolysis.

Recently, we have shown that acceleration of fibrinolysis occurs not only with fondaparinux, but also with other anticoagulant drugs targeting FXa, such as low molecular weight heparin, unfractionated heparin, and tissue factor pathway inhibitor [27,28]. However, accelerated fibrinolysis was not observed when anticoagulant drugs targeting thrombin or tissue factor were used [27].

The concentration of fondaparinux used in some of the experiments [Fig. 2] exceeds the plasma peak levels of fondaparinux when administered as prophylaxis (2.5 mg once daily) or as a therapeutic dose (5–10 mg once daily), which are between 0.34 and 1.1 $\mu g\,mL^{-1}$ [11,29]. Although we appreciate that fondaparinux levels in some of our experiments exceed clinically relevant levels by one order of magnitude, we are convinced our findings may have clinical relevance. Firstly, we observe an antifibrinolytic effect of fondaparinux, albeit small, already at $0.28\,\mu g\,mL^{-1}$ (Fig. 1B), and furthermore an antifibrinolytic effect of fondaparinux is observed in plasma from healthy volunteers who received a single bolus dose of fondaparinux administered at a therapeutic dosage (10 mg, Fig. 3). Finally, the antifibrinolytic effect of a certain concentration of fondaparinux depends on the tissue factor concentration used to initiate the reaction. When a lower concentration of tissue factor is used, the amount of fondaparinux required to decrease clot lysis time diminishes as well (data not shown).

In this study, we have shown that addition of rFVIIa to plasma containing fondaparinux not only leads to a profound acceleration of clot formation, but also to an improvement of TAFI-mediated inhibition of fibrinolysis. Also, the acceleration of plasma clot lysis of healthy volunteers treated with a single bolus dose of fondaparinux could be corrected by administration of a single bolus dose of rFVIIa. Clot lysis times in the *ex vivo* study were significantly correlated with *in vitro* thrombin generation tests, indicating that the changes in clot lysis time are related to changes in thrombin generation and thus the capacity to activate TAFI.

In the *in vitro* study, rFVIIa was able to completely reverse the effects of fondaparinux on coagulation, but interestingly the profibrinolytic effect induced by fondaparinux was only partially reversed. Even at supratherapeutic levels, rFVIIa did not completely normalize clot lysis in fondaparinux containing plasma (data not shown). This is in contrast with the situation in FVIII deficient plasma, in which addition of sufficient rFVIIa normalized clot lysis time (i.e. clot lysis time in FVIII deficient plasma with high dose rFVIIa equalled clot lysis time in the same plasma with $1\,U\,mL^{-1}$ of purified FVIII) [18]. The reason why, in the presence of rFVIIa, fondaparinux is still able to (partially) inhibit generation of the large amount of thrombin required for TAFI activation ('secondary thrombin generation'), but not the generation of the small amount of thrombin required for clot formation ('primary thrombin generation') is unclear. Either the concentration of FXa required for secondary thrombin generation is much higher than that required for primary thrombin formation, or inhibition of FXa by fondaparinux is in the presence of rFVIIa simply not fast enough to delay clot formation.

Interestingly, the extent of reduction of clot lysis time induced by fondaparinux varied strongly between samples of healthy individuals. On addition of a fixed concentration of fondaparinux, accelerated fibrinolysis was seen in some (high responders), but not all (low responders) plasma samples from healthy volunteers, and an effect of rFVIIa on clot lysis time was only observed in those plasma samples in which fondaparinux accelerated clot lysis. This high responder/low responder phenomenon appeared to be correlated with clotting times in the absence of fondaparinux (i.e. basal thrombin generating capacity). A larger basal thrombin generating capacity indicated less inhibition of TAFI activation by the fixed concentration of fondaparinux. Thus, it seems that the capacity of a plasma sample to generate thrombin determines the extent of acceleration of fibrinolysis by fondaparinux.

In conclusion, rFVIIa may be a useful therapeutic option in those instances where patients treated with fondaparinux develop bleeding complications as both clot formation and fibrinolytic resistance of a clot are improved.

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