Dabigatran increases thrombin generation by inhibiting protein S and FVa degradation

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Research Article

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Abstract

Background: Low dose of dabigatran paradoxically increased thrombin generation (TG) through inhibition of protein C (PC) activation. Protein S (PS) is a co-factor in the activation of PC. However, the role of PS in the enhancement of TG has not been addressed.

Methods: Firstly, we measured TG by calibrated automated thrombinography (CAT) and prothrombin fragments 1+2 (F_{1+2}) assays. Secondly, we assessed the coagulation and anticoagulation factors activity in normal plasma spiking with dabigatran. Then, free PS and PC activation were measured. Finally, heavy chain of FVa and its degradation products were detected by WB.

Results: CAT assay showed that 70-141ng/mL dabigatran paradoxically increased TG in normal plasma. However, dabigatran suppressed TG in a concentration-dependent manner. F_{1+2} assay showed the similar results. ELISA assay was not affected by clot methods. Interestingly, results from ELISA showed that dabigatran (2-566ng/mL) suppressed free PS level in normal plasma. Combined with WB results, dabigatran inhibited PS and subsequently suppressed FVa degradation.

Conclusions: PS participated in the paradoxical enhancement of TG in normal plasma spiking with low concentrations of dabigatran.

1. Introduction

New oral anticoagulants are widely used to prevent thrombotic events in clinical practice [1]. Thrombin plays a central role in the clotting process by regulating blood coagulation cascade and platelet activation [2]. Hence, thrombin acts as an attractive target for anticoagulation. Dabigatran is a direct thrombin inhibitor, and it could overcome the limitations of vitamin K antagonists [3]. Dabigatran inhibits both thrombin and fibrin-bound thrombin through binding to the active site of thrombin [4].

Previous studies demonstrated that dabigatran and melagatran paradoxically increased thrombin generation (TG) [5, 6]. In rat model, melagatran significantly increased TG leading to rebound hypercoagulability [7]. Kamisato et al.[8] demonstrated that paradoxical enhancement of TG might be due to the reduced degradation of FVa and FVIIIa. PC serves as an native serine protease zymogen under normal physiological conditions and can be activated by the thrombin-thrombomodulin (TM) complex [9, 10]. Activated Protein C (APC) cleaves factor Va (FVa) at Arg306, Arg506, and Arg679 residues. The Arg306 cleavage requires the presence of protein S [11]. The fragment of $FVa^{307-506}$ indicates the complete loss of FVa activity [12].

Protein S (PS) is a vitamin K-dependent glycoprotein which plays a vital role in the blood coagulation. PS exists both in a free form (≈ 140nM) and in an inactive form [13]. PS functions as cofactor to activated protein C in the regulation of FVa and FVIIIa. Furthermore, previous study showed that PS inhibited FVa and FVIIIa activity in APC-independent manner [14]. Heterozygous PS deficiency increased the risk of

thrombosis. PS acts as an important factor of PC system. However, the role of PS in the paradoxical enhancement of TG has not been well described.

2. Methods And Materials

2.1 Agents

Dabigatran and dabigatran-d3 were synthesized at Toronto Research Chemicals (Toronto, Canada). Normal plasma was obtained from Boatman Biotech (Shanghai, China). PS- and PC-deficient plasmas and free PS ELISA kit were prepared by HYPHEN BioMed (Oise, France). Recombinant human soluble TM (rhs-TM) was from R&D Systems (North America) and dissolved in 0.9%NaCl. Polyclonal rabbit anti-human protein S antibody was obtained from Dako Cytomation (Carpinteria, CA, USA). Monoclonal mouse anti-human FV antibody and human protein S were from Haematologic Technologies (Essex Junction, VT). Monoclonal mouse anti-human IgG and D-Phe-Pro-Arg-chloromethylketone (PPACK) were purchased from Santa Cruz Biotechnology (Dallas, TX). PPP regent, FluCa, and thrombin calibrator were purchased from Thrombinoscope BV (Maastricht, The Netherlands). Pefabloc FG was from Pentapharm (Basel, Switzerland). Prothrombin fragment 1 + 2 (F_{1+2}) ELISA kit was from Dade Behring (Marburg, Germany). Activated PC-PC inhibitor complex (aPC-PCI) ELISA kit was from Affinity Biologicals.

2.2 Preparation of dabigatran

Dabigatran was dissolved in 1N HCl and filled up to 1mL with distilled water. Different volumes of distilled water were added to make final concentrations of dabigatran (0.5, 2, 12, 35, 70, 141, 283 and 566nmol/mL).

2.3 Thrombin generation assay

Calibrated automated thrombogram (CAT) method (Thrombinoscope, Maastricht, The Netherlands) was used to detect TG according to Morishima's study [15]. Normal, PS-, and PC-deficient plasmas (76μL) were spiked with different concentrations (2μL) of dabigatran (n = 9) in the presence of rhs-TM (2μL). In control group, dabigatran was replaced with the same volume of solvent of dabigatran. Then, PPP reagent (20μL) was added to the plasma. After incubation at 37°C for 5min, plasma with dabigatran was added to the TG wells and plasma without dabigatran was added to the thrombin calibrator wells. Flu-buffer incubated at 37°C for 5min and Flu-substrate was mixed to generate FluCa. FluCa was automatically added to each well and results were read by a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific, Waltham, MA). The final concentrations of tissue factor, phospholipids, fluorogenic substrate, and $CaCl_2$ were 5pM, 4μM, 417μM, and 16.7mM. TG curves were generated by the Thrombinoscope software (Thrombinoscope BV).

2.4 Prothrombin fragment 1 + 2 assay

F_{1+2} is a marker of TG. TG was induced according to the method described above with minor modifications. Normal, PS-, and PC-deficient plasmas (76μL) were spiked with different concentrations

(2μL) of dabigatran (n = 6) in the presence of rhs-TM (2μL). The control group contained the appropriate vehicles. Plasma was defibrinated using 2μL PefablocFG (final concentration of 6mg/mL). The reaction was started by adding Fluo-buffer containing HEPES, calcium chloride and DMSO. PPACK was used to stop the coagulation reaction after 12min. The mixture (120μL) was transferred to microtiter plates and F_{1+2} assessed following the manufacturer's instructions. The intra-assay coefficient of variation (CV) was between 3.6–5.5% and the inter-CV was between 4.4–11.2%.

2.5 Free PS assay

Plasma free PS was detected by an ELISA kit. Free PS level was measured following the manufacturer's instructions. The intra-assay coefficient of variation (CV) was between 3–8% and the inter-CV was between 5–10%.

2.6 PC activation assay

PC activation was detected by an aPC-PCI ELISA kit. TG was induced as above and the reaction was stopped 15min after the time to peak in the TG assay by adding 13μL of 0.5M sodium citrate buffer (pH4.3). The assay was performed according to the manufacturer's instructions. The intra-assay coefficient of variation (CV) was between 3.6–5.5% and the inter-CV was between 4.4–11.2%.

2.7 FV, FVIII, PS, and PC activity

Normal and PS-deficient plasmas were spiked with different concentrations of dabigatran in the presence of rhs-TM. Plasma samples were directly loaded in the STA® Compact Max (Diagnostic Stago) using the same batches of reagents. Plasma samples (5μL) were automatically diluted with 45μL Owren-Koller buffer. Coagulation was induced. FV activity was detected by normalization of PT in FV-deficient plasma (50μL). FVIII activity was measured by normalization of APTT in FVIII-deficient plasma (50μL). PC activity in samples (50μL) was detected based on the prolongation of APTT in PC-deficient plasma (50μL). PS activity in samples (50μL) was measured based on the prolongation of clotting time in PS-deficient plasma (50μL).

2.8 Western blot analysis

TG was induced as described above in plasma without Fluca. Several reaction time points were set up in PS-deficient plasma and normal plasma spiked with or without 70ng/mL dabigatran according to CAT results. The reaction was stopped with 13μL of 0.5M sodium citrate buffer (pH4.3). FVa^{HC} and its degradation product $FVa^{307-506}$ were detected by FV antibody (2.5μg/mL). IgG (dilution 1:1000) was used as the loading control.

2.9 Statistics

Statistical analysis was completed with Stata 14.0. All data were expressed as mean ± standard deviation (SD). Unpaired parametric t test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used for data analysis. $P < 0.05$ indicated a significant difference.

3. Results

3.1 Effect of dabigatran on TG

We measured TG by CAT assay in plasma spiking with different concentrations of dabigatran (n = 9). Figure 1A showed that there was a gradual dose-dependent increase of TG in normal plasma. Interestingly, the paradoxical enhancement of TG was absent in PS- or PC-deficient plasma (Fig. 1B, C). We analysed important indices including peak thrombin generation (C_{max}) and endogenous thrombin potential (ETP) from CAT assay. Low concentrations of dabigatran (35-141ng/mL) significantly increased C_{max} in normal plasma (Fig. 1D). In normal plasma spiking with 70-141ng/mL dabigatran, there was a gradual dose-dependent increase of ETP (Fig. 1E). Higher concentrations of dabigatran (283ng/mL) suppressed ETP in normal, PS- or PC-deficient plasma (104.28 ± 20.28, 175.86 ± 13.96, 178.6 ± 13.94mmol L^{-1} thrombin×min). C_{max} and ETP reached a maximum in the presence of 70ng/mL dabigatran.

3.2 Effect of dabigatran on F1 + 2 assay

F_{1+2} assay is another method to assess TG. F_{1+2} assay showed results similar to CAT assay (n = 6) (Fig. 2). In normal plasma, 70ng/mL dabigatran paradoxically significantly increased F_{1+2} from 289.4 ± 136.9 to 435.3 ± 115.8nmol/L. There was an enhancement in TG which decreased at higher concentrations. In PS- or PC-deficient plasma, the paradoxical enhancement of TG was absent. Higher concentrations of dabigatran significantly suppressed the generation of F_{1+2}. F_{1+2} decreased from 589.3 ± 101.6 to 290.5 ± 127.2nmol/L or from 600.7 ± 209.4 to 305.6 ± 98.1nmol/L in PS- or PC-deficient plasma in the presence of 141ng/mL dabigatran.

3.3 Effect of dabigatran on FV, FVIII, PS, PC activity, free PS, and APC levels

We used clot methods to evaluate the effect of dabigatran on FV, FVIII, PS and PC activity (n = 6) There was a gradual concentration-dependent decrease of FV and FVIII activity. FV and FVIII activity significantly decreased at 283 and 70ng/mL dabigatran in normal plasma. PS and PC activity increased with the enhancement of dabigatran concentrations. PS and PC activity increased above 15% at 12 and 141ng/mL dabigatran in normal plasma (Fig. 3A). In PS-deficient plasma, the activity of FV, FVIII and PC was similar to that in normal plasma. FV and FVIII activity significantly decreased at 283ng/mL and 141ng/mL dabigatran. PC activity increased at 141ng/mL dabigatran (Fig. 3B). Furthermore, we evaluated free PS and APC levels by ELISA kit. Interestingly, dabigatran could affect free PS levels. Free PS was initially suppressed by 2ng/mL dabigatran (Fig. 3C). APC was significantly suppressed at 70ng/mL dabigatran in both normal and PS-deficient plasmas (Fig. 3D-E). In normal plasma, the concentrations of dabigatran for inhibition of free PS and PC activation were almost the same as for

enhancement of TG. In PS-deficient plasma, free PS levels can't be detected. Dabigatran dose-dependently inhibited TG and APC.

3.4 Effect of dabigatran on the generation and degradation

FVa^{HC} and $FVa^{307–506}$ are indices of FVa generation and inactivation (n = 3). In normal plasma, FVa^{HC} increased with time, peaking around the lag time of CAT assay and then decreased. In contrast, $FVa^{307–506}$ increased along with the proteolysis of FVa^{HC} (Fig. 4A, D). Interestingly, $FVa^{307–506}$ was not observed in PS-deficient plasma. FVa^{HC} was sustained during this period (Fig. 4B, E). In normal plasma spiking with 70ng/mL dabigatran, the lag time was prolonged and FVa^{HC} increased around the lag time. Dabigatran significantly delayed the degradation of FVa (Fig. 4C, F).

3.5 Effect of anti-PS and PS on CAT assay

In normal plasma, CAT assay showed that anti-PS neutralizing antibody could increase TG (Fig. 5A). In normal plasma spiking 70ng/mL dabigatran, PS concentrations dependently reversed the enhancement of TG (Fig. 5B).

4. Discussion

This study showed that PS participated in the paradoxical enhancement of TG in plasma spiking with low concentrations of dabigatran. Dabigatran increased thrombin generation by inhibiting protein S and FVa degradation. Previous study demonstrated that low concentrations of dabigatran paradoxically increased TG in PC-dependent manner [8]. Dabigatran inhibited thrombomodulin-bound thrombin more aggressively than thrombin at low concentrations resulting in the decrease of PC activation and FVa degradation. Our previous study speculated that higher concentrations of dabigatran may sufficiently suppress thrombin and coagulation cascade [16]. PS is a cofactor to activated protein C. PS could cleave FVa and FVIIIa in PC dependent and independent manners [11]. The effect of PS on the paradoxical enhancement of TG has not been well addressed.

It is known that a transient hypercoagulable state occurs within the first 12-60h of warfarin treatment because of inhibition of PC and PS [17]. Previous study showed that low concentrations of dabigatran paradoxically increased TG. We aimed to disclosure whether the paradoxical enhancement of TG induced by low concentrations of dabigatran depends on PS. PS is a vitamin K-dependent glycoprotein that plays vital role in blood coagulation. PS circulates in plasma at a concentration of around 350nM which exists both in a free form (≈ 140nM) and in an inactive form (~ 60%) combined with the complement regulatory factor C4b-binding protein (C4BP) [13]. Only the free PS exerts co-factor activity. Hence, the detection of free PS but not total PS was applied in the study [18].

Firstly, TG was assessed by CAT assay in normal, PS- or PC-deficient plasma containing 10nM rhs-TM. It has been previously shown that 10nM rhs-TM added to the plasma was appropriate to mimic the

condition *in vivo* [19]. Blood coagulation cascade includes two pathways, one of which is contact factor-dependent intrinsic pathway and the other is TF-induced extrinsic pathway. In most cases, TG was assessed in coagulation reaction induced by extrinsic pathway. In our study, final concentration of tissue factor, phospholipids, fluorogenic substrate, and $CaCl_2$ were 5pM, 4μM, 417μM, and 16.7mM. Under these circumstances, coagulation cascade process could be induced completely. Wagenvoord et al. [20] reported that the paradoxical enhancement of TG induced by low concentrations of dabigatran may be caused by the presence of α_2-macroglobulin-thrombin (α_2MT) complex. CAT assay uses an algorithm to subtract α_2MT activity from the total amidolytic activity. The transient enhancement of α_2MT induced by dabigatran could not be subtracted which leads to a false increase of TG. However, there is study showing different opinion. Study reported α_2M may not participate in the enhancement of TG [21]. CAT assay showed that low concentrations of dabigatran paradoxically induced TG in normal plasma. Higher concentrations of dabigatran may sufficiently suppress thrombin and coagulation cascade.

Secondly, TG was assessed by F_{1+2} assay under the same circumstance. F_{1+2} is also a marker of TG. Our results showed that F_{1+2} increased in normal plasma spiking with 70ng/mL dabigatran. The paradoxical enhancement of F_{1+2} was absent in PS deficient plasma. The results are inconsistent between assays. However, the trend of CAT and F_{1+2} assays is same. Previous study showed that TG increased at 136-545ng/ml dabigatran. But only 273ng/ml dabigatran increased F_{1+2} [22]. Our results are similar to other study.

Furthermore, FV, FVIII, PS and PC activity was measured to assess the haemostatic situation in clotting blood using the same batches of reagents. Previous study showed different batches of reagents and different coagulometers could lead to highly variable results [23]. Dabigatran dose-dependent decreased FV and FVIII activity and increased PS and PC activity. It has been reported dabigatran would affect APTT more than PT. FV activity is measured by normalization of PT. Whereas FVIII and PC activity is associated with APTT. Hence, FVIII and PC activity would be more affected than FV. The activity of coagulation and anticoagulation factors using clot methods may be adversely affected by dabigatran [24]. To avoid the influence by dabigatran, free PS and aPC-PCI ELISA were used. It is well known that dabigatran is a direct thrombin inhibitor. Interestingly, dabigatran could inhibit free PS levels. In normal plasma, the concentrations of dabigatran for inhibition of free PS and PC activation were almost the same as for enhancement of TG. Except for PC, participates in the paradoxical enhancement of TG in plasma spiking with low concentrations of dabigatran.

Last but not least, we used WB assay to evaluate the generation and degradation of FVa. In normal plasma, $FVa^{307-506}$ increased in line with the decrease of FVa^{HC}. In PS-deficient plasma, the degradation of FVa was absolutely suppressed. PS plays an important role in the degradation of FVa. In normal plasma spiking with 70ng/mL dabigatran, the generation of FVa was not affected. However, the degradation of FVa was inhibited. We speculated low concentrations of dabigatran suppressed PS and APC more than coagulation cascade leading the paradoxical enhancement of TG.

This study still has some limitations. In one hand, most parts of our results are based on CAT assay. It has been reported α_2MT would affect the results. What is more, there may be some confounding factors affect the results. In the other hand, this study performed in *vitro*. Although the concentrations of dabigatran used *in vitro* are close to the condition *in vivo*, the results may not reflect the actually situation in *vivo*. It would be more interesting to perform the experiments in patients.

Declarations

Funding

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Competing Interests

The authors state that they have no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Chi Zhang and Mianxian Li performed the research; Bin Jiang designed the research study; Yue Zhang contributed essential reagents or tools; Weixiang Chen analysed the data；Chi Zhang wrote the paper.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

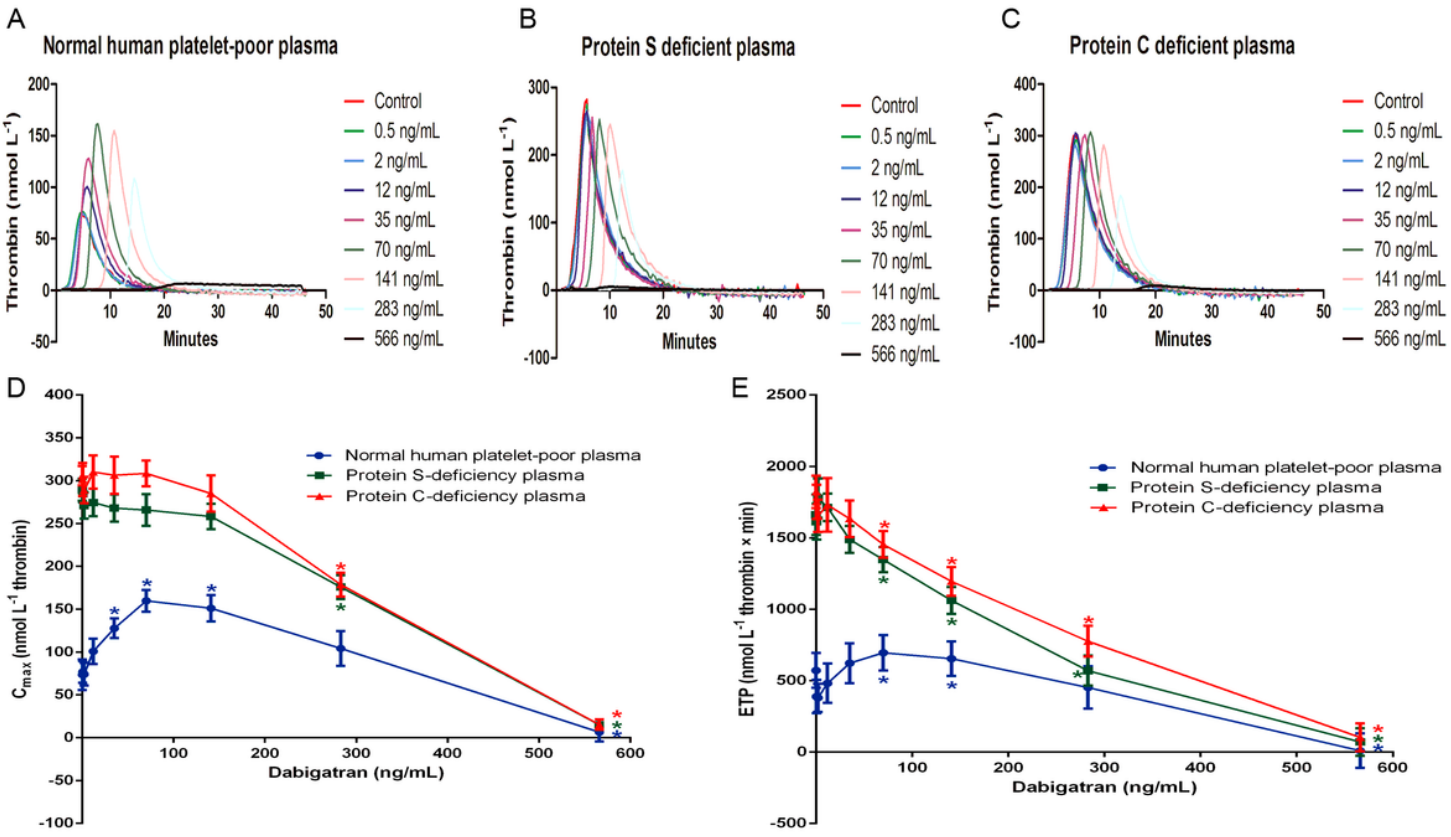

Figure 1

Effect of dabigatran on TG. Different concentrations of dabigatran (0-566ng/mL) affected TG in normal, PS- or PC deficient plasma (A-C). The expression of C_{max} and ETP in normal, PS- or PC deficient plasma spiking with different concentrations of dabigatran (D-E). * P<0.05 vs. respective controls

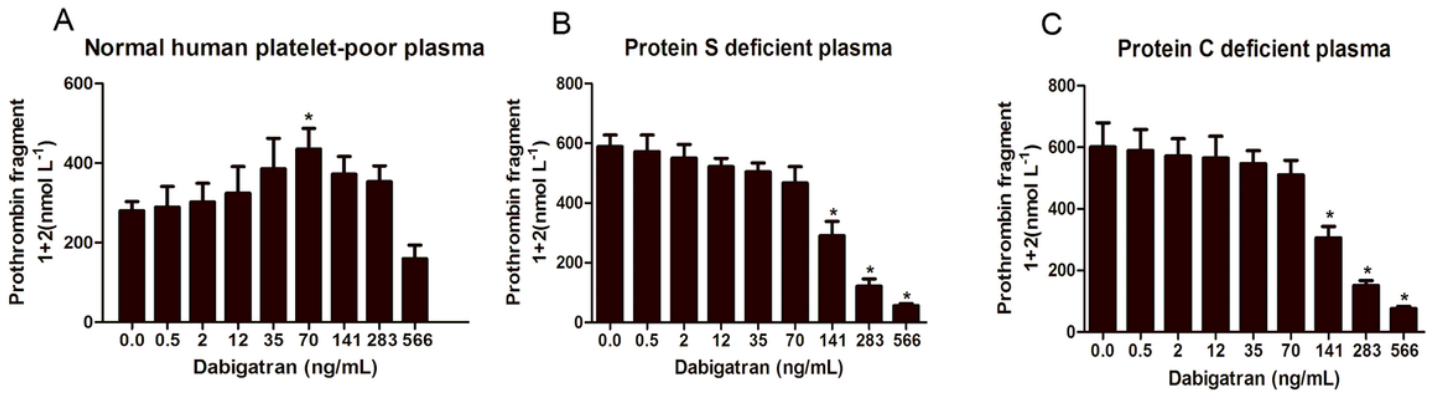

Figure 2

Effect of dabigatran on F_{1+2} assay. Low concentrations of dabigatran dose-dependent increased F_{1+2} in normal plasma. In PS- or PC-deficient plasma, the paradoxical enhancement of TG was absent. * *P*<0.05 vs. respective controls

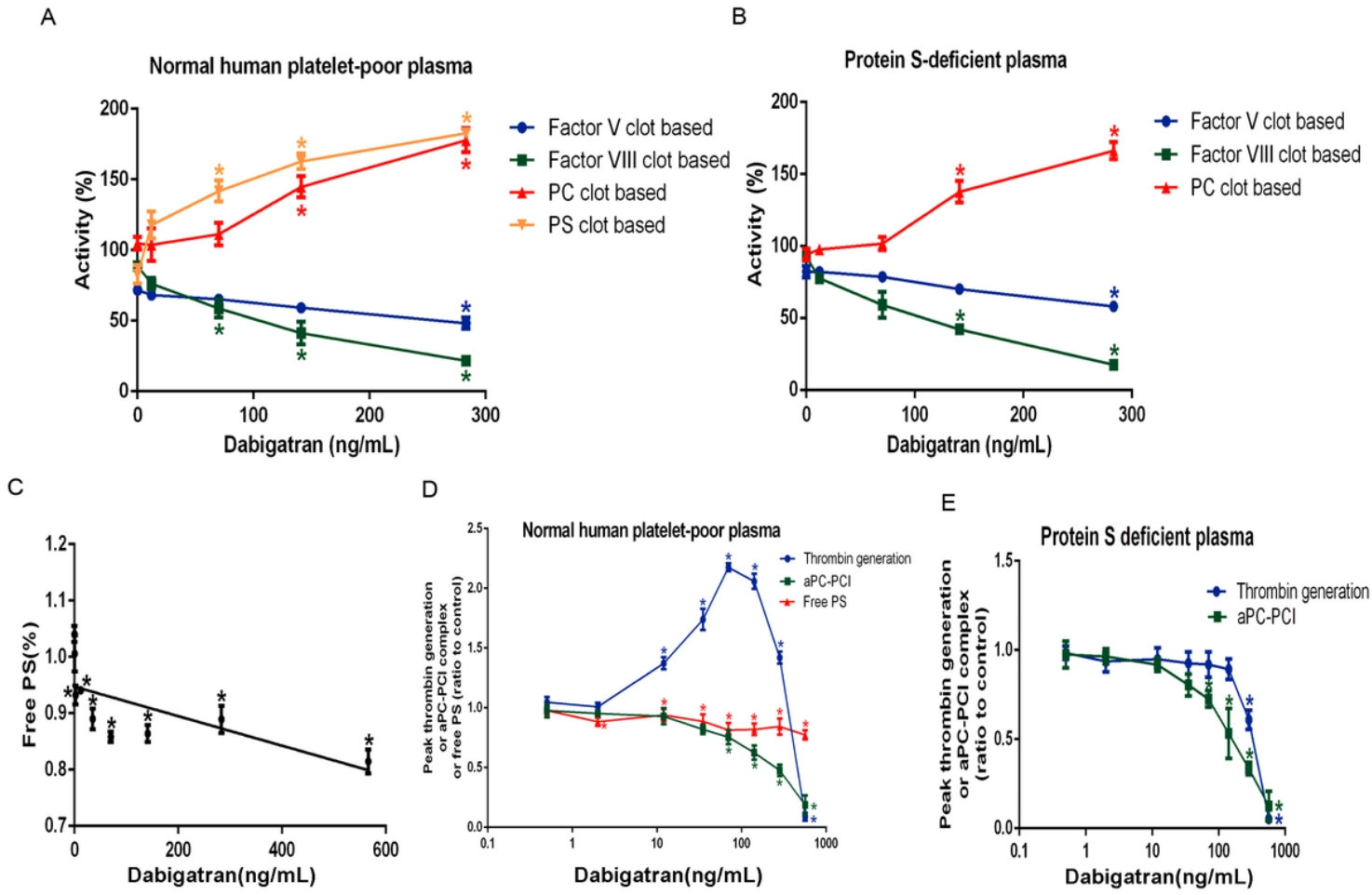

Figure 3

Effect of dabigatran on FV, FVIII, PS, PC activity, free PS, and APC levels. Dabigatran dose-dependent decreased FV and FVIII activity and increased PC and PS activity (A-B). ELISA assay showed dabigatran suppressed free PS and PC activation in normal The concentrations of dabigatran for inhibition of free PS and PC activation were almost the same as for enhancement of TG (C-E). * P<0.05 vs. respective controls

Figure 4

Effect of dabigatran on the generation and degradation. The expression of FVa^{HC} and $FVa^{307\text{-}506}$ levels by WB assay after the activation of the coagulation cascade in normal plasma (A, D), PS-deficient plasma (B, E), and normal plasma spiking with 70ng/mL dabigatran (C, F). * P<0.05 vs. respective controls

Figure 5

Effect of anti-PS and PS on CAT assay. Anti-PS neutralizing antibody could increase TG (A). In normal plasma spiking 70ng/mL dabigatran, PS concentrations dependently reversed the enhancement of TG (B)