HHS Public Access

Author manuscript

Thromb Res. Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

Thromb Res. 2023 October ; 230: 84–93. doi:10.1016/j.thromres.2023.08.012.

Activated Protein C, Protein S, and Tissue Factor Pathway Inhibitor Cooperate to Inhibit Thrombin Activation

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Abstract

Introduction: Thrombin, the enzyme which converts fibrinogen into a fibrin clot, is produced by the prothrombinase complex, composed of factor Xa (FXa) and factor Va (FVa). Down-regulation of this process is critical, as excess thrombin can lead to life-threatening thrombotic events. FXa and FVa are inhibited by the anticoagulants tissue factor pathway inhibitor alpha (TFPIα) and activated protein C (APC), respectively, and their common cofactor protein S (PS). However, prothrombinase is resistant to either of these inhibitory systems in isolation.

Materials and Methods: We hypothesized that these anticoagulants function best together, and tested this hypothesis using purified proteins and plasma-based systems.

Results: In plasma, TFPIα had greater anticoagulant activity in the presence of APC and PS, maximum PS activity required both TFPIα and APC, and antibodies against TFPI and APC had an additive procoagulant effect, which was mimicked by an antibody against PS alone. In purified protein systems, TFPIα dose-dependently inhibited thrombin activation by prothrombinase, but only in the presence of APC, and this activity was enhanced by PS. Conversely, FXa protected FVa from cleavage by APC, even in the presence of PS, and TFPIα reversed this protection. However, prothrombinase assembled on platelets was still protected from inhibition, even in the presence of TFPIα, APC, and PS.

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Author Contributions. X. Li, X. Song, D.F. D Mahmood, M.M.S. Sim, and S.J. Bidarian performed experiments, analyzed data, and revised the manuscript. J.P. Wood designed the study, analyzed data, and wrote the manuscript. All authors reviewed the final version of the manuscript and gave permission for its submission.

Conflicts of Interest. J.P. Wood has an investigator-initiated grant through Pfizer, Inc., which funded part of this study. X. Li is now employed by GenScript Biotech Corp. but was not at the time of his participation in this study. All other authors have no conflicts of interest to declare.

Conclusions: We propose a model of prothrombinase inhibition through combined targeting of both FXa and FVa, and that this mechanism enables down-regulation of thrombin activation outside of a platelet clot. Platelets protect prothrombinase from inhibition, however, supporting a procoagulant environment within the clot.

Keywords

Prothrombinase; Thrombin; TFPI; Protein C; Protein S; Platelet

Introduction

The intrinsic and extrinsic pathways of coagulation converge on the formation of prothrombinase, a membrane-bound complex consisting of the serine protease factor Xa (FXa) and its non-enzymatic cofactor factor Va (FVa) [1]. Prothrombinase converts prothrombin into the serine protease thrombin, the enzyme which turns soluble fibrinogen into an insoluble fibrin clot [2]. Thrombin has multiple other prothrombotic functions, including activation of FVa [3, 4] and activation of platelets [5], which serve as a surface on which prothrombinase may assemble [6]. Excess thrombin generation is associated with thrombotic events, while insufficient thrombin generation is associated with bleeding. Given its central role in the process, the regulation of thrombin is critical. However, the mechanisms by which prothrombinase is inhibited, or down-regulated, are poorly understood.

Blood contains multiple anticoagulants, which target different components of the coagulation system. FXa is inhibited by serine protease inhibitors, including tissue factor pathway inhibitor (TFPI) [7]. TFPI is a Kunitz-type protease inhibitor that exists in two isoforms in humans, TFPIα and TFPIβ. TFPIα contains three Kunitz domains (K1, K2, K3) [8]. K2 binds the FXa active site, and this inhibitory interaction is promoted by the binding of K3 to protein S (PS) [9, 10]. PS has a membrane-binding domain that is thought to localize TFPI to cell surfaces and promote its interaction with surface-associated FXa. In support of this, PS does not promote the activity of membrane-tethered TFPIα [11]. TFPIβ lacks the K3 domain, and so does not bind PS [11], but has a glycosylphosphatidylinositol anchor and efficiently inhibits FXa in the absence of PS [12].

FVa is proteolytically degraded by activated protein C (APC), which is itself activated through a feedback mechanism [13, 14]. Thrombin binds the endothelial receptor thrombomodulin, which accelerates protein C activation [15]. APC degrades FVa through cleavages following Arg306, Arg506, and Arg679 in the FVa heavy chain [13, 16]. As with TFPI, PS acts as a cofactor for APC, enhancing the degradation of FVa [17, 18].

The above-described anticoagulants are effective in isolated systems. However, neither TFPIα/PS nor APC/PS efficiently inhibits the activation of thrombin by the prothrombinase complex. FXa protects FVa from degradation by APC, even in the presence of PS [17, 19]. In addition to FXa, thrombin-activated platelets also protect FVa from cleavage by APC [20]. Conversely, the effect of FVa on FXa inhibition by TFPIα has been less clear. While some have reported that thrombin-activated FVa does not directly protect FXa from inhibition by TFPIα [21], others have observed protection [22]. Either way, TFPIα

only effectively inhibits thrombin activation during the initiation of coagulation [23]. This inhibition requires TFPIα binding to a regulatory region in FVa, which is removed by thrombin [23, 24]. Thus, once the initial thrombin is generated, TFPIα loses this inhibitory activity [21, 23, 25]. Work of Orfeo and colleagues [26] showed that prothrombinase remains functional for hours *in vitro* in a reconstituted coagulation system, consisting of purified proteins used at normal plasma concentrations. This work led to the question of how prothrombinase can be shut down *in vivo* to limit thrombus growth.

A limitation of these previous studies is that they all assessed inhibitors of *either FVa or FXa*, not both at the same time. We hypothesized that the *combination* of FVa and FXa inhibition allows for down-regulation of thrombin activation. In support of this, PS, APC, and TFPIα have an additive effect when supplemented into PS/TFPI-depleted plasma [27], and the procoagulant phenotype of FV Leiden, a congenital form of APC resistance [28–31], involves TFPIα resistance [24, 32] and is amplified by reducing TFPIα concentration [33]. Here, we show that TFPIα, APC, and PS anticoagulant activities are cooperative in purified protein and plasma-based systems, that the presence of APC and PS enhances the apparent activity of TFPIα, and that the presence of TFPIα enhances the degradation of FVa by APC and PS, reversing the protection bestowed by FXa.

Materials and Methods

Materials

FXa, APC, and PS were from Enzyme Research Laboratories (South Bend, IN, USA). Hirudin and recombinant full-length TFPIα were from Sigma-Aldrich (St. Louis, MO, USA). Thrombin, thrombin-activated FVa, and a mouse monoclonal antibody that recognizes an epitope between Arg306 and Arg506 in human FVa were from Haematologic Technologies (now called Prolytix, Essex Junction, VT, USA). Sheep anti-human protein C (PC) and sheep anti-human PS polyclonal antibodies were from Cedarlane Labs (Burlington, NC, USA). Rabbit anti-TFPI polyclonal antibody was from ProteinTech (Rosemont, IL, USA). Bovine Type I Collagen was from Advanced BioMatrix (Carlsbad, CA, USA). Tissue factor (TF; Dade Innovin) was from Siemens Healthineers (Erlangen, Germany). Peroxidase-conjugated horse anti-mouse IgG was from Vector Laboratories (Newark, CA, USA). Spectrozyme TH, a chromogenic substrate for thrombin, was from Biomedica Diagnostics (Windsor, NS, Canada), and FluCa, a fluorogenic substrate for thrombin, was from Diagnostica Stago (Parsippany, NJ, USA). Phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were from Avanti Polar Lipids (Alabaster, AL, USA) and were used to make phospholipid vesicles containing 60% phosphatidylcholine, 20% phosphatidylserine, and 20% phosphatidylethanolamine, following the protocol of Morrissey [34].

EA.hy926 Cell Culture

The endothelial cell line, EA.hy926 (CRL-2922), was from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Billings, MT), supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich). Cells were passaged

using TrypLE express trypsin (Gibco) and used between passages 1–5 for experiments. Cells were harvested for experiments as described [11]. Briefly, cells were harvested by treatment with phosphate-buffered saline (PBS) containing 5mM EDTA, washed with PBS, and resuspended in HEPES-buffered saline (HBS; 25mM HEPES, 150mM NaCl, pH 7.4). An aliquot was used to determine protein concentration by Bicinchoninic acid assay (Pierce), and cells were normalized to 50μg/mL total protein for experiments. As previously described [11], we have found normalizing by total protein to be more reproducible than cell count.

Plasma thrombin generation

All experiments using human samples were approved by the University of Kentucky Institutional Review Board. Citrated blood was collected from consenting, healthy, adult volunteers. Blood was processed to platelet-rich plasma (PRP) and platelet-poor plasma (plasma), and washed platelets were isolated as described [23]. PS-immunodepleted plasma was from Affinity Biologicals (Ancaster, ON, Canada). Thrombin generation was measured in plasma or PRP using calibrated automated thrombography, as described [23]. Briefly, 40 μL of plasma or PRP was incubated with PS, TFPIα, or antibodies against TFPI (50nM), PC (133nM), or PS (6.7μM) (antibody concentrations based on initial titration experiments) and added to wells containing 10μL of a mixture of TF (1pM final concentration) and phospholipids (4μM) or TF, phospholipids, and thrombomodulin (20nM). Experiments using PRP included collagen (10μg/mL), instead of phospholipids. Thrombin generation was initiated by addition of 10μL of a mixture of calcium and FluCa fluorogenic thrombin substrate and measured using a Fluoroskan Ascent microplate reader and Thrombinoscope software (Diagnostica Stago).

Continuous prothrombinase activity

Continuous prothrombinase activity assays were performed as described [11], with modifications. FVa (5nM) was incubated (15min, 25°C) with phospholipid vesicles (20μM) in HBS, containing 5mM $CaCl_2$ and 0.1% PEG-8000, in the presence or absence of APC (1nM), PS (150nM), and/or TFPIα (indicated concentrations). Reactions were initiated by the rapid, sequential addition of prothrombin (1.4μM), Spectrozyme TH (500μM), and FXa (0.5nM). Cleavage of the substrate was monitored at 405nm, at 25°C, using a SpectraMax Plus 384 microplate reader (Molecular Devices, San Jose, CA, USA). Assays using EA.hy926 cells were performed as above, with some modifications: (1) EA.hy926 cells (50μg/mL) were used in place of phospholipid vesicles. (2) 0.1% bovine serum albumin was used in place of PEG-8000.

Platelet prothrombinase assays were performed similarly, with the following modifications. Washed platelets were diluted to 1×10^9/mL in HEPES-buffered Tyrode's solution (HT; 5mM HEPES, 137mM NaCl, 53.6mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 8.4mM Na_2HPO_4, pH 7.4), containing Arg-Gly-Asp-Ser peptide (5mM; GenScript Biotech Corp., Piscataway, NJ) to block fibrin(ogen)-mediated platelet aggregation [35] and Gly-Pro-Arg-Pro (2mM; GenScript) to prevent fibrin polymerization [36], and were activated by incubation with thrombin (50nM, 2min, 37°C), followed by addition of hirudin (75nM) to quench the thrombin. HT was used as the reaction buffer for the prothrombinase activity assays.

Activated platelets (2×10^8/mL) were included in the incubation mixture, in place of phospholipid vesicles. In addition, no exogenous FVa was added, so that the reactions relied on platelet-released FVa. Reactions were incubated, initiated with the sequential addition of prothrombin, substrate, and FXa (5nM), and monitored as described above.

FVa cleavage by APC

FVa was incubated with phospholipid vesicles (20μM) in HBS containing 2mM $CaCl_2$, 1mM $MgCl_2$, and 0.1% bovine serum albumin. Incubations (15min, room temperature) were performed in the presence or absence of PS (150nM), TFPIα (1nM), and/or FXa (5nM). Reactions were initiated by the addition of APC (1nM). Aliquots were removed at timed intervals and quenched with 50mM EDTA and 1X EZBlock protease inhibitor cocktail (BioVision, Milpitas, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added (final concentrations: 80mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1.5mM bromophenol blue), and samples were incubated for 10min at 80°C.

For experiments using activated platelets, HT was used as the buffer, and the samples were centrifuged (10min, 700×g, room temperature) after quenching in EDTA and protease inhibitors to remove the platelets, prior to addition of SDS-PAGE sample buffer.

Immunoblotting

Samples were reduced with 5% β-mercaptoethanol, separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting, as described [23]. The membrane was incubated with a mouse anti-human FV heavy chain antibody (5μg/mL, 1hr), followed by a peroxidase-conjugated horse anti-mouse IgG secondary (0.2μg/mL, 1hr), and developed using SuperSignal West Pico chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA). Densitometry was performed using Image Lab v.6.0.1 (Bio-Rad, Hercules, CA, USA). The density of the 307–506 fragment was normalized to the total lane density, as a loading control.

Enzyme-linked immunosorbent assay (ELISA)

Plasma TFPI was measured using a TFPI Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), which recognizes free, full-length TFPI and a small fraction of lipoprotein-bound TFPI. For clarity, we interpret the results as measurement of TFPIα.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism v.8.0.2 (Graphpad Software, Inc.). Multiple t-test comparisons were performed using the method of Benjamini, Krieger, and Yakutieri [37].

Results

TFPI, PS, and APC cooperate in plasma

We recently reported that TF-initiated plasma thrombin generation is insensitive to PS anticoagulant activity, due to a lack of APC activation, and that supplementation of thrombomodulin into plasma increases PS sensitivity [38]. In those studies, we utilized

PS-immunodepleted plasma. Others have reported that immunodepletion of PS may result in loss of TFPIα [39], which binds PS in plasma. Indeed, TFPIα is almost undetectable in the PS immunodepleted plasma that we used in our study (Table 1). Thus, we have now assessed PS activity in PS-immunodepleted plasma reconstituted with 1nM TFPIα. In the absence of thrombomodulin, TFPIα significantly increased the lag time of thrombin generation, which was further prolonged by the addition of PS (Figure 1A–B, E), consistent with inhibition of TF/factor VIIa [9, 40] and early prothrombinase [23] by TFPIα and PS. However, the net effect of PS and TFPIα was still limited, as the endogenous thrombin potential (ETP; the area under the curve) was unchanged (Figure 1F), and peak thrombin and maximum velocity were not significantly reduced (Figure 1G–H). In the presence of thrombomodulin, ETP, peak thrombin, and velocity were all drastically reduced, consistent with our previous report (Figure 1C–D). TFPIα appeared to alter the kinetics of this reduction. At lower PS concentrations, these parameters were reduced to a greater extent in the presence of TFPIα than its absence, while at 150nM PS, there was no effect of TFPIα. Interestingly, there was no effect of TFPIα on lag time in the presence of thrombomodulin. In addition, 150nM PS induced a slight, but significant decrease in lag time in the presence of thrombomodulin. This may be an artefact of the way that lag time is calculated, as the total amount of thrombin produced was reduced in the presence of thrombomodulin. These data suggest that PS anticoagulant activity requires APC activation, and is enhanced, at least at low concentrations, by the presence of TFPIα.

We next assessed the function of PS, TFPIα, and APC in normal plasma. First, we observed an additive effect of antibody-mediated inhibition of TFPI and APC on thrombin generation (Figure 2). In the presence of thrombomodulin, inhibition of either TFPI or PC partially restored thrombin generation, while the combination of antibodies against TFPI and PC completely restored thrombin generation to the level seen in the absence of thrombomodulin. Inhibiting PS alone mimicked the effect of combined TFPI and PC inhibition.

Conversely, excess TFPIα (5nM) had greater anticoagulant activity when thrombomodulin was present in the assay (Figure 3), consistent with the results of Peraramelli et al. [27]. In the absence of thrombomodulin, we noted a differential effect of TFPIα depending on the thrombin generation activity of the plasma. Therefore, Figure 3 shows sample curves from donors with high (fast, high peak), medium (slower, low peak), and low (slowest, low peak) thrombin generation. As might be expected, exogenous TFPIα (5nM) had the greatest effect in the plasma sample with the slowest baseline thrombin generation. TFPIα prolonged the lag time by 66% and decreased the peak thrombin, ETP, and velocity by 64%, 45%, and 74%, respectively. By contrast, in the other two donor samples, TFPIα had a relatively mild effect, prolonging the lag time by 18.5±2.6%, and decreasing the peak thrombin and ETP by 1.3±4.2% and 8.7±2.1%, respectively. The velocity increased slightly in the presence of TFPIα (by 15.9±4.9%). The “low” thrombin generation sample produced no detectable thrombin in the presence of thrombomodulin. The other two samples produced low amounts of thrombin (proportional to their respective measurements in the absence of thrombomodulin), which was rendered virtually undetectable by the addition of TFPIα.

Purified prothrombinase is inhibited by the combination of TFPI, PS, and APC.

We and others have previously reported that prothrombinase is resistant to inhibition by either TFPIα or APC, in the presence or absence of PS. However, those previous studies assessed these anticoagulant proteins individually. Based on the plasma thrombin generation data, we hypothesized that the TFPIα and APC anticoagulant systems may function best together. We tested this hypothesis in two ways. First, we measured thrombin activation using a previously described continuous prothrombinase assay [11], performed with limiting FXa to be sensitive to TFPIα activity. Consistent with previous reports, plasma concentrations of TFPIα had little or no effect on thrombin activation in the absence of APC or PS (Figure 4A). In addition, PS did not inhibit thrombin activation by itself, nor did it promote the inhibition of prothrombinase by TFPIα (Figure 4B). In contrast, when FVa was pre-incubated for 15 min with APC (with or without TFPIα), thrombin activation was significantly slowed, and was further delayed by addition of increasing concentrations of TFPIα (Figure 4C). The effect of TFPIα was more pronounced in the presence of PS (Figure 4D).

Second, we directly assessed APC activity by western blotting for the FVa cleavage fragment containing residues 307–506 (Figure 5). Our data confirm previous findings that: (1) treatment of FVa with APC results in the time-dependent generation of a 30-kDa band, consistent with residues 307–506; (2) PS accelerates the rate of FVa cleavage by APC (significantly different from APC alone between 30s and 1.5min, after which the APC alone condition catches up); and (3) FXa protects FVa from cleavage by APC/PS (statistically reduced at all time points). There has been some debate in the field about the third observation [17, 19], but our data show an ~50–60% decrease in FVa cleavage by APC/PS when FXa is present. We next extended these findings by including TFPIα in the assay system. This resulted in a complete reversal of the protection provided by FXa (significant at all time points, except 20min, which had p=0.12). There was no difference between the APC/PS and the APC/PS/FXa/TFPIα conditions at any time.

Platelet prothrombinase remains protected from inhibition in the presence of TFPIα, APC, and PS.

Platelets have also been shown to protect FVa from inactivation by APC [20]. Therefore, we next assessed the function of TFPIα, APC, and PS in the context of the activated platelet surface. Isolated platelets were activated with high dose (50nM) thrombin to allow for maximal prothrombinase assembly, as described [41]. In purified continuous prothrombinase activity assays (Figure 6A), platelet prothrombinase was resistant to inactivation by these anticoagulants, either in isolation or combination. There is variability between donors in the amount of platelet FVa present [42, 43] and the prothrombinase binding sites on platelets [44]. In addition, platelets store and release TFPIα [45, 46] and PS [47, 48], both of which vary in the healthy blood donor population [43]. Thus, we are again showing data from donors with fast, medium, and slow thrombin activation. Unlike with the plasma thrombin generation experiments, there was no increase in apparent anticoagulant activity when the platelet prothrombinase function was reduced, suggesting that platelet-mediated FVa resistance occurs through a different mechanism than FXa-mediated resistance. Consistent with that, the cleavage of FVa by APC was greatly reduced on the platelet surface in

comparison to phospholipid vesicles (Figure 5) (i.e. prothrombinase remained functional in the presence of APC), was not further reduced by addition of FXa, and was not enhanced by TFPIα (Figure 6B–C). There was increased variability in the results from the donor with the least thrombin activation (Figure 6B–C, Donor 3), but there was no apparent correlation between anticoagulant activity and the variability.

Next, we utilized inhibitory antibodies to assess anticoagulant activity in PRP-based thrombin generation assays (Figure 7). Similar to plasma, thrombomodulin decreased TF-initiated thrombin generation to nearly undetectable levels, and this effect was reversed with antibodies against either TFPI or PC. However, unlike in plasma, these antibodies increased thrombin generation beyond what was seen with TF alone. For example, addition of thrombomodulin decreased the average ETP from 795±353nM·min to 160±96nM·min, and inclusion of antibodies against TFPI or PC increased the ETP to 1133±101nM·min or 1382±201nM·min, respectively. An additive effect of TFPI and PC antibodies was again observed, further increasing the ETP to 2093±184nM·min, and this effect was mimicked by an antibody against PS alone, which increased the ETP to 1691±309nM·min. The peak with anti-PS was taller and narrower, though, as reflected by a higher peak thrombin (105±31nM vs. 58±14nM with combined anti-TFPI and anti-PC) and a faster velocity (11±5.1nM/min vs. 4.1±1.3nM/min with combined anti-TFPI and anti-PC). This difference may reflect TFPI- and APC-independent function of PS, such as direct factor IXa inhibition [49].

APC, PS, and TFPI function synergistically on the endothelial surface

The difference in anticoagulant activity on the surface of platelets and synthetic phospholipid vesicles is striking, and clearly indicates that the vesicles do not effectively model platelets in this context. However, they may model other cells. Others have demonstrated that FVa is susceptible to proteolysis by APC on the surface of endothelial cells [50, 51], and that prothrombinase function is reduced by incubating endothelial cells with APC and PS [51]. We assessed the synergistic effect of APC, PS, and TFPIα on the endothelial surface, utilizing the EA.hy926 cell line, which behaved similarly to phospholipid vesicles (Figure 8). APC and PS-mediated degradation of FVa reduced thrombin activation. Anticoagulant activity was increased in the presence of TFPIα, and reversed by addition of an anti-TFPI antibody, included to block endothelial TFPIβ activity. Collectively, the data indicate that the anticoagulant system functions similarly on endothelial cells compared to synthetic phospholipid vesicles, and that FVa protection is unique to platelets, consistent with the report of Oliver et al. [51].

Discussion

Inhibition of thrombin activation by prothrombinase has been the subject of investigation since the discovery of APC. APC inactivates FVa through proteolytic cleavages following Arg residues 306, 506, and 679 [13, 16]. Initial cleavage occurs at 506, and subsequent cleavage at 306 results in loss of cofactor activity [13, 52]. However, FVa is protected from degradation by APC through at least two mechanisms: FXa binding [17, 19] and platelet binding [20]. This protection remains even in the presence of PS [19]. Similarly, TFPIα does not effectively inhibit thrombin activation by prothrombinase [21, 23, 25], nor does

antithrombin-III [53], and plasma concentrations of protein Z (PZ) and the PZ-dependent protease inhibitor (ZPI) only partially inhibit thrombin activation [54]. In 2013, we reported the first description of an endogenous prothrombinase inhibitor, TFPIα, but it was only effective during the initiation of coagulation [23]. We showed that a positively charged region in TFPIα bound a negatively charged region in partially activated forms of FVa. The negatively charged region is removed by thrombin, and thus prothrombinase inhibition is lost once thrombin generation has commenced.

A limitation of previous studies is that they assessed FVa or FXa inhibition in isolation, using either purified proteins or plasma systems in which protein C activation is limited (with the exception of the recent PZ/ZPI study, discussed further below). However, these anticoagulant systems do not naturally function in isolation. Our previous studies indicated that PC activation is minimal in TF-initiated plasma thrombin generation assays [24], and that TF-initiated thrombin generation is insensitive to PS in the absence of APC [38]. We extended those findings here by showing that TFPIα increases the anticoagulant activity of low concentrations of PS, but only when thrombomodulin is included in the assay to promote PC activation. These results are consistent with those of Peraramelli et al. [27], who directly added APC to plasma and observed an additive anticoagulant effect with TFPI and PS. The difference in the present study is that the APC was generated *in situ*, mimicking the *in vivo* situation. We also showed a synergistic effect of APC and TFPI in plasma using inhibitory antibodies, in which combined inhibition results in a much greater increase in thrombin generation than inhibition of either APC or TFPI alone. Interestingly, inhibiting PS had a similar effect as inhibiting both APC and TFPI, suggesting that the effect of PS in this assay is primarily as a cofactor for these two anticoagulant pathways. This may be due to the TF concentration that we utilized. PS directly inhibits factor IXa, independent of APC and TFPI, but that activity is most apparent at lower TF concentrations than we used, when coagulation is more dependent on factor IXa for FXa generation [49].

Based on the plasma results, we hypothesized that the APC/PS and TFPIα/PS systems cooperate to inhibit thrombin activation by prothrombinase, and this hypothesis was supported by our studies using purified proteins. In the absence of APC, TFPIα had little or no effect on thrombin activation by purified prothrombinase, either in the presence or absence of PS. However, in the presence of APC, TFPIα dose-dependently decreased prothrombinase activity. The lowest prothrombinase activity (greatest anticoagulant activity) was observed when all three anticoagulant proteins were present. These results are consistent with the report of Huang and colleagues [54], who showed that the plasma anticoagulant activity of PZ/ZPI is enhanced by the addition of thrombomodulin. We hypothesize that APC-mediated degradation of FVa reduces its binding to FXa and/or prothrombin and thus increases the susceptibility of FXa to inhibition by TFPIα and other plasma protease inhibitors.

As mentioned previously, FXa has also been shown to directly protect FVa from cleavage by APC/PS. We replicated those findings here and extended them by showing that TFPIα reverses this protection. There are multiple potential mechanisms for this. It is possible that FXa enzymatic activity is required for this protection. For example, inclusion of the substrate prothrombin has been shown to further reduce FVa cleavage by APC/PS [19].

In addition, FXa cuts FVa itself, following Arg1765 in the light chain [55–57], and the effect of this cleavage on APC-mediated FVa degradation is unknown. It is also possible that FXa binding to FVa sterically blocks APC. Our data are consistent with either of these explanations. TFPIα blocks the FXa active site [8] and interacts with a site on the FVa heavy chain near Arg506 and is thought to displace FXa from FVa [24].

In addition to FXa, platelets protect FVa from cleavage by APC [20]. Platelets are thought to be the primary *in vivo* hemostatic site of prothrombinase activity [6], contain their own pool of FVa which is phenotypically distinct from the plasma-derived FVa used in the purified protein studies [58], secrete TFPIα [45, 46] and PS [48, 59], and may serve as a site of PC activation [60]. Therefore, we assessed the anticoagulant function of APC, PS, and TFPIα at the activated platelet surface. Consistent with the reports described above, platelet-assembled prothrombinase was resistant to inactivation by APC or TFPIα. In contrast to the plasma and purified protein experiments, though, this resistance remained even when all three anticoagulants were present. The addition of TFPIα had no apparent effect on FVa cleavage by APC. However, when TF-initiated thrombin generation was measured in PRP, inhibitory antibodies against PC, TFPI, and PS behaved similarly to plasma experiments, suggesting a synergistic inhibitory activity in this system. The reason for the discrepancy between washed platelets and PRP is unclear but may be explained by the presence of other membrane surfaces in PRP, such as extracellular vesicles, which might behave more like synthetic phospholipid vesicles, or by the kinetics of platelet activation. In the thrombin generation assays, platelets were activated with collagen, a relatively slower agonist than thrombin used in the purified system [44]. Camire et al. [20] demonstrated that purified, platelet-derived FVa is not resistant to APC when incubated in the presence of phospholipid vesicles, and hypothesized that a component of the platelet surface confers resistance. If the FVa-protective activity of the platelets, whether that be a membrane protein or other component, is activation state-dependent, then it is possible that FVa susceptibility changes dynamically during the thrombin generation process.

We finally assessed anticoagulant function on the surface of endothelial cells, utilizing the EA.hy926 cell line, which we have previously shown supports TFPI-mediated FXa inhibition [11]. The anticoagulant system appears to function similarly on these cells as on phospholipid vesicles, suggesting that the protection mediated by platelets is due to a platelet component not present in endothelial cells. This is consistent with the results of Oliver et al., who observed that endothelial cells support more rapid degradation of FVa than do platelets [51].

In summary, on the surface of thrombin-activated platelets, prothrombinase is resistant to inactivation (Figure 9). While APC, TFPIα, and PS bind the platelet surface, FVa is inaccessible and maintains cofactor activity. In contrast, on other membrane surfaces, such as those provided by endothelial cells, the combination of the APC/PS and TFPIα/PS anticoagulant systems efficiently down-regulates prothrombinase activity. Our results suggest that maximal inhibition of prothrombinase activity can be achieved by targeting both FXa and FVa simultaneously, but that the protective properties of platelets and/or platelet-derived FVa promote localized procoagulant activity. We thus propose a model in

which these anticoagulants are optimized to down-regulate thrombin generation outside of a platelet clot, but to allow for procoagulant activity within the clot.

Funding.

This work was supported by National Heart, Lung and Blood Institute grant HL129193 (to J.P.Wood) and an investigator-initiated grant from Pfizer, Inc. (to J.P.Wood).

Abbreviations

FXa	Factor Xa
FVa	Factor Va
TFPI	Tissue factor pathway inhibitor
PS	Protein S
APC	Activated protein C
PC	Protein C
TF	Tissue factor
PBS	Phosphate-buffered saline
PRP	Platelet-rich plasma
HBS	HEPES-buffered saline
HT	HEPES Tyrode’s buffer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential
PZ	Protein Z
ZPI	Protein Z-dependent protease inhibitor

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Highlights

- Neither TFPIα nor APC individually inhibits thrombin activation by prothrombinase.
- TFPIα, APC, and their common cofactor PS combine to inhibit prothrombinase.
- TFPIα renders factor Va more susceptible to cleavage by APC.
- Factor Va degradation enhances the apparent inhibitory activity of TFPIα.
- Platelets protect FVa from APC, even in the presence of TFPIα and PS.

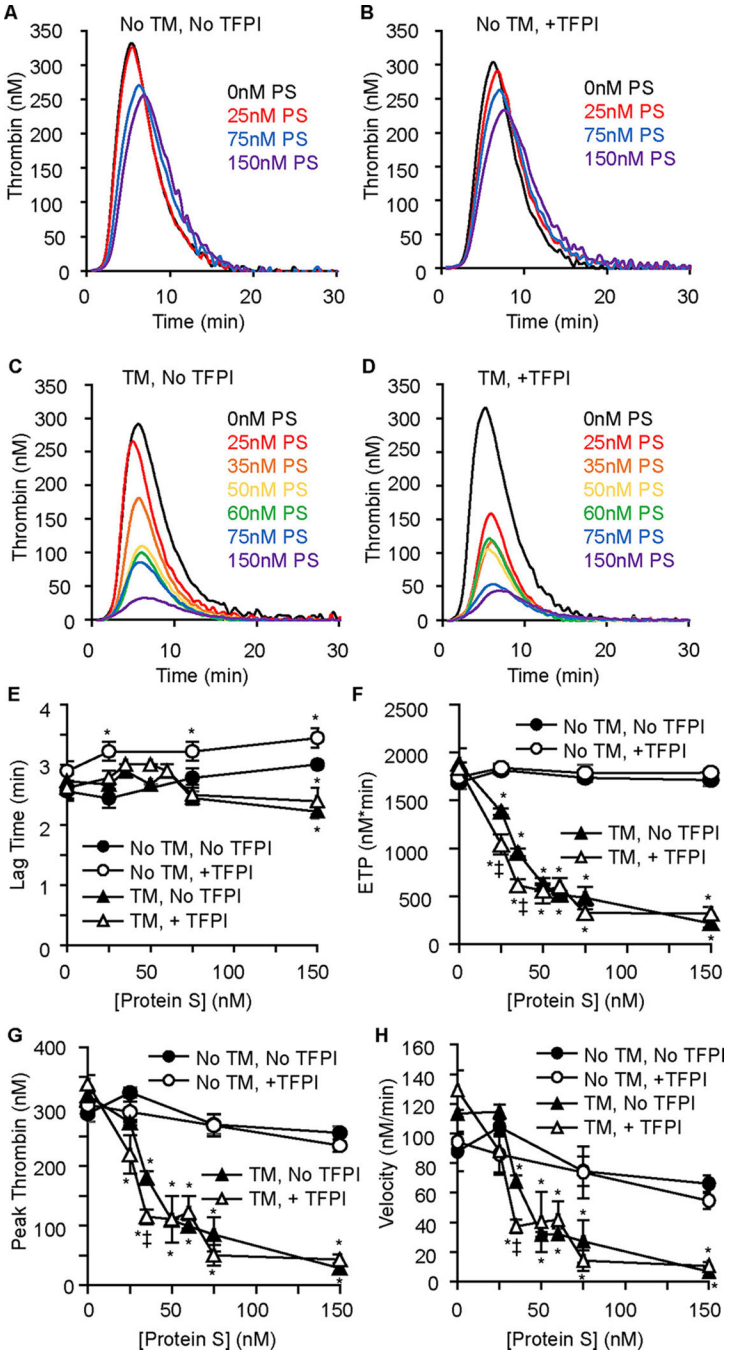

Figure 1. PS anticoagulant activity depends on both TFPI and APC.

Thrombin generation, initiated with 1pM TF, was measured in PS immunodepleted plasma in the absence (A-B) or presence (C-D) of thrombomodulin (20nM) and the absence (A, C) or presence (B, D) of exogenous TFPIα (1nM), and the indicated concentrations of PS. Lag time (E), endogenous thrombin potential (ETP) (F), peak thrombin (G), and maximum velocity (H) are presented as mean±SEM. All experiments were performed at least in triplicate. *p<0.05 compared to 0nM PS condition; ‡p<0.05 compared to no TFPI condition

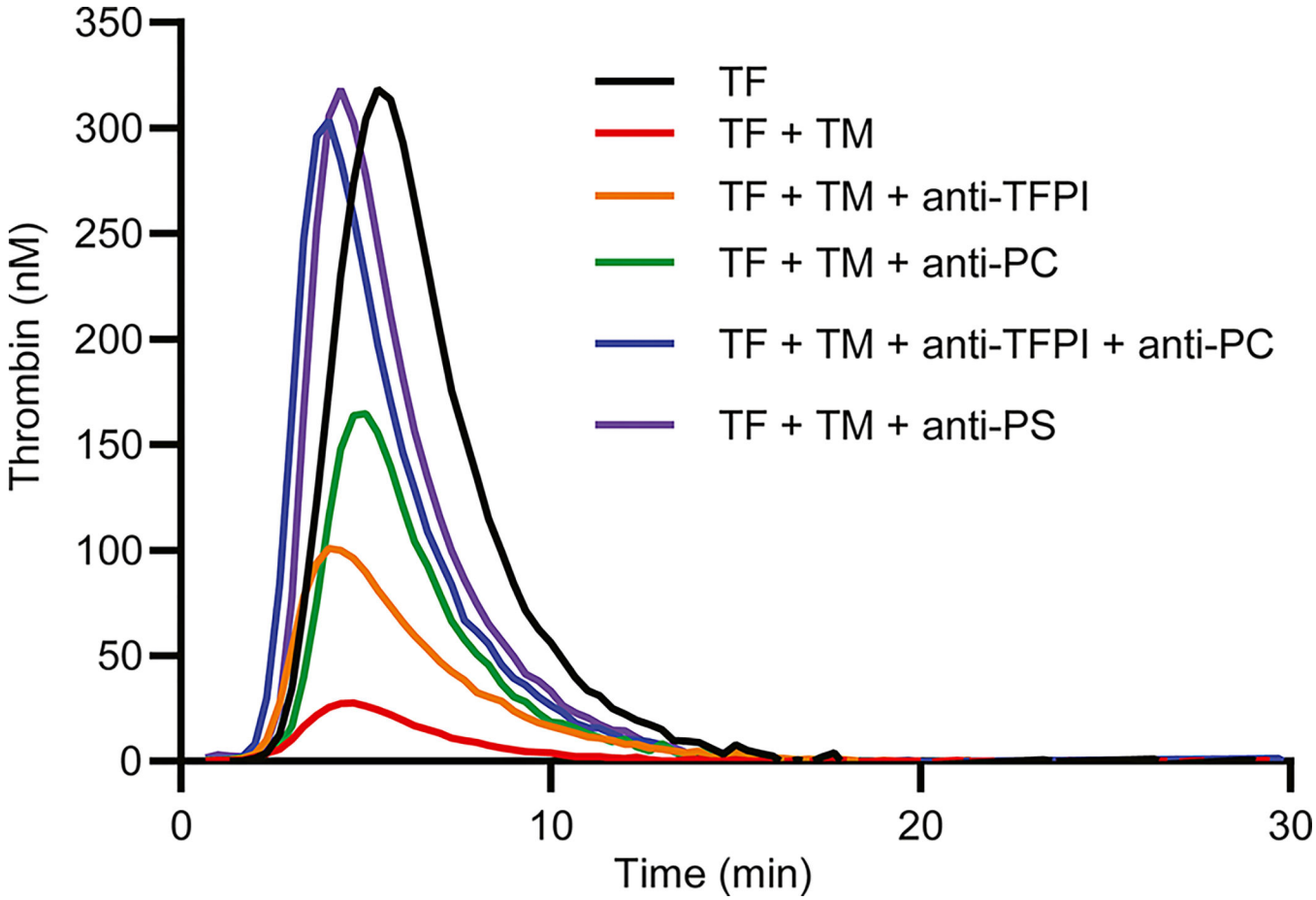

Figure 2. Inhibitory antibodies against TFPI and PC have an additive effect on plasma thrombin generation, mimicked by inhibiting PS alone.

Thrombin generation, initiated with 1pM TF (black line) or TF + 20nM thrombomodulin (all other lines), was measured in healthy donor plasma in the absence (red) or presence of antibodies against TFPI (50nM, orange), PC (133nM, green), TFPI and PC combined (blue), or PS (6.7μM, purple). Shown are the average curves from experiments performed in triplicate.

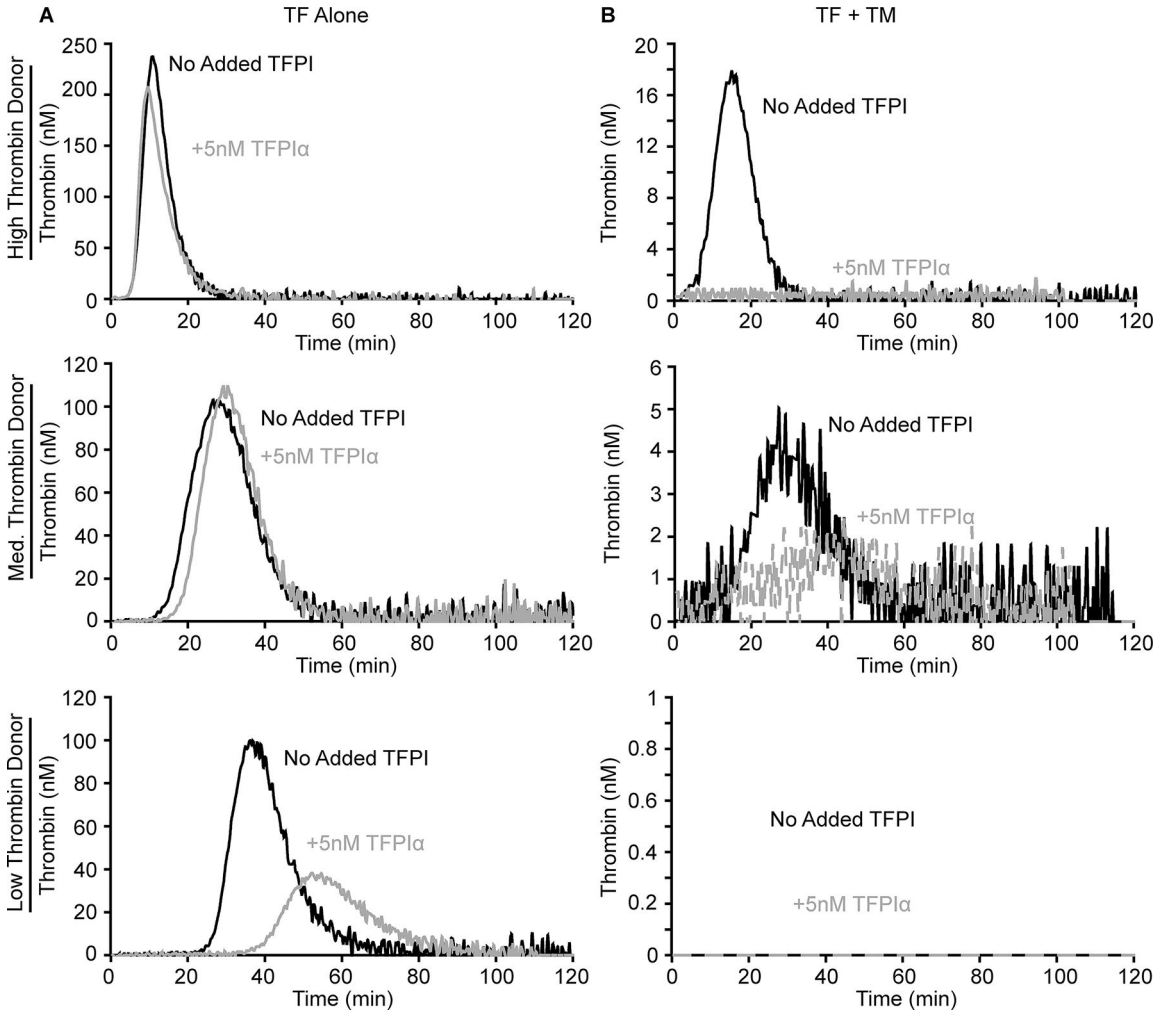

Figure 3. Exogenous TFPIα has greater anticoagulant activity in the presence of APC.

Thrombin generation, initiated with 1pM TF (A) or TF + 20nM thrombomodulin (B), was measured in healthy donor plasma in the absence (black) or presence of TFPIα (5nM, grey). Shown are the average curves from experiments performed in triplicate for three individual donors, one each with high, medium, and low thrombin generation. Flat lines indicate no detectable thrombin was produced.

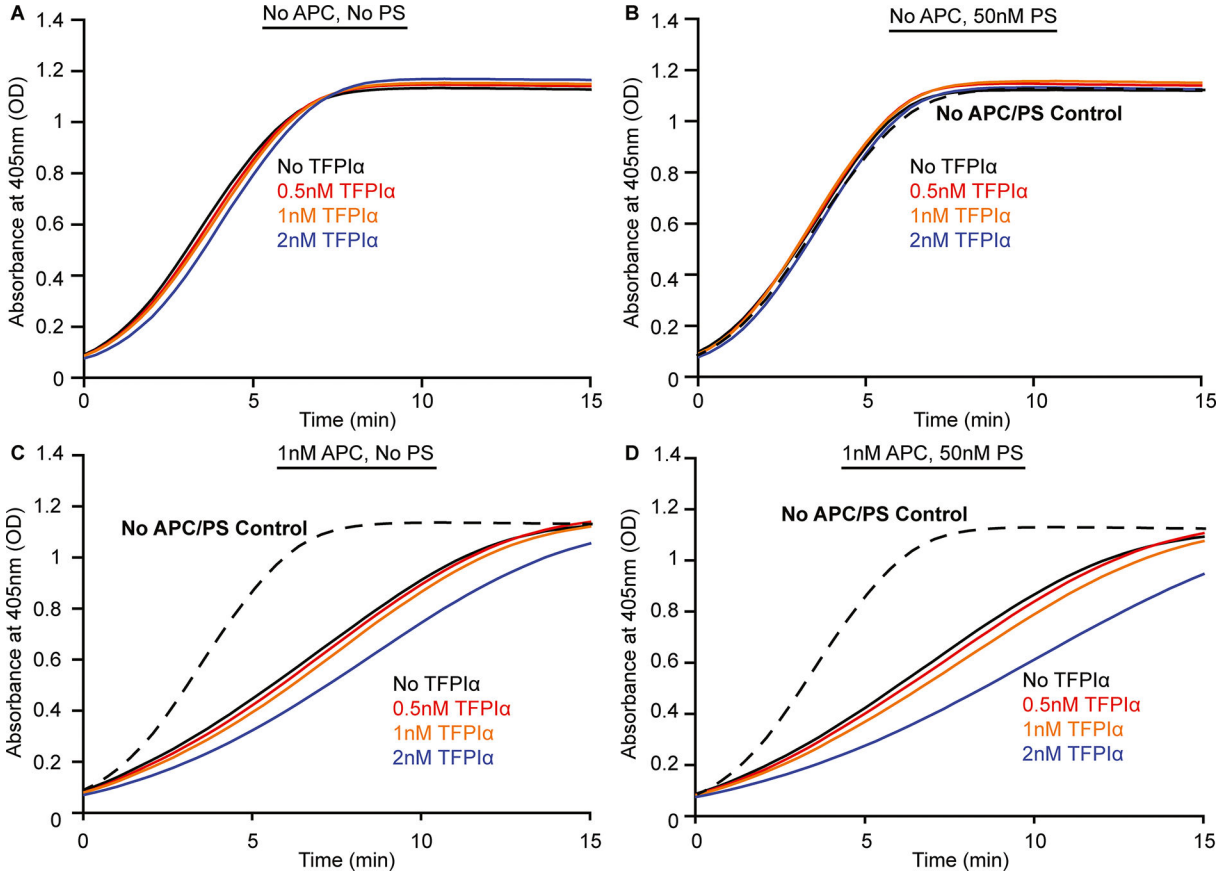

Figure 4. APC increases the apparent prothrombinase inhibitory activity of TFPIα.

FVa (5nM) was incubated for 15min in the absence (A-B) or presence (C-D) of APC (1nM) and the indicated concentrations of TFPIα. In B and D, PS (150nM) was included in the incubation. Prothrombinase activity assays were initiated by the rapid sequential addition of Spectrozyme TH substrate (500μM), prothrombin (1.4μM), and FXa (0.5nM), and cleavage of the substrate monitored at 405nm. Shown are the average progress curves (n=3). The "No TFPIα" line from A is reproduced in B-D as a dashed line for reference.

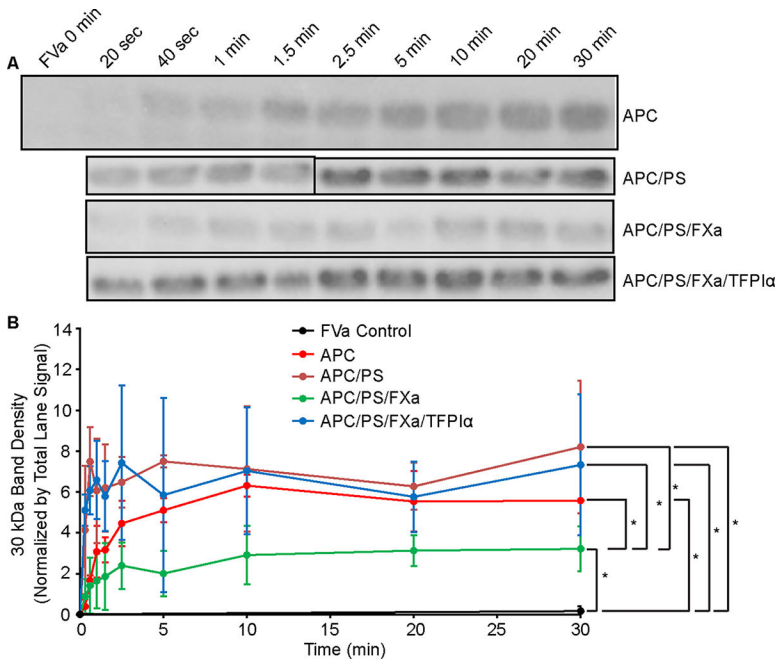

Figure 5. TFPIα reverses FXa-mediated protection of FVa from degradation by APC.

(A) FVa (0.5nM) was incubated for 15min in the absence or presence of APC (1nM), PS (150nM), FXa (0.5nM), and TFPIα (1nM), and generation of the 30kDa FVa fragment, containing residues 307–506, was monitored by immunoblotting. Shown are immunoblot images from a single experiment, which is representative of three independent experiments. Black boxes indicate separate immunoblots, which were run, probed, and developed simultaneously. (B) Densitometric analysis of the 30kDa FVa fragment, normalized to total lane density. Mean ± SEM (n=3). *p<0.05 at 30min

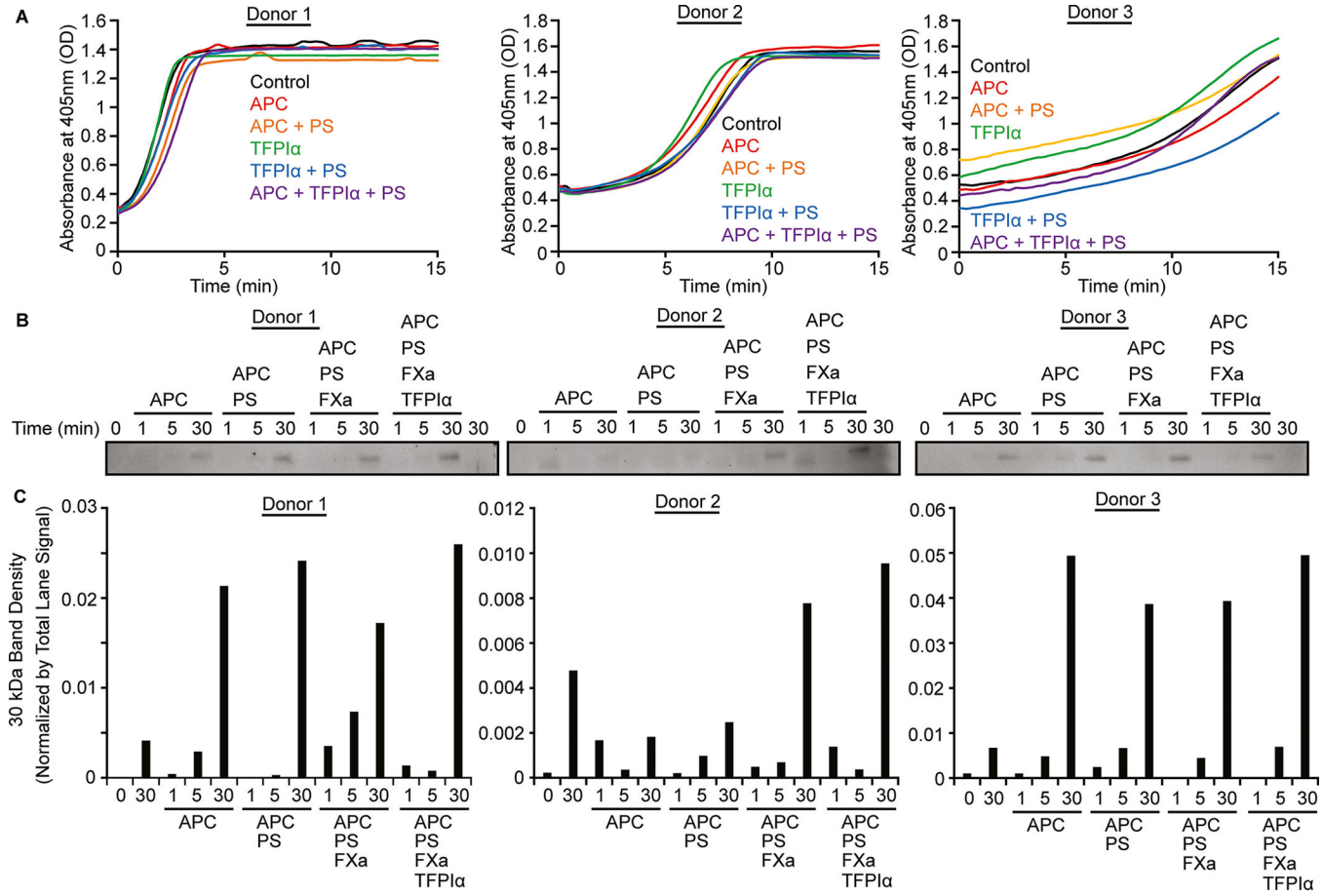

Figure 6. Platelets protect prothrombinase from inhibition.

(A) Platelets (2×10^8/mL) were activated with thrombin (50nM, 2min, followed by 75nM hirudin) and incubated in the absence or presence of APC (1nM), PS (150nM), and/or TFPIα (1nM), reactions initiated by the rapid sequential addition of Spectrozyme TH substrate (500μM), prothrombin (1.4μM), and FXa (0.5nM), and cleavage of the substrate monitored at 405nm. Shown are the progress curves (n=3) from three individual donors. (B-C) Activated platelets (2×10^8/mL) were incubated in the absence or presence of APC (1nM), PS (150nM), FXa (5nM), and TFPIα (1nM), reactions initiated by the addition of APC (1nM), and cleavage of platelet-derived FVa monitored by immunoblotting. Shown are immunoblots of the 307–506 fragment of the FVa heavy chain (B) and densitometry of this band normalized to total lane density (C), for three individual donors, each performed in triplicate.

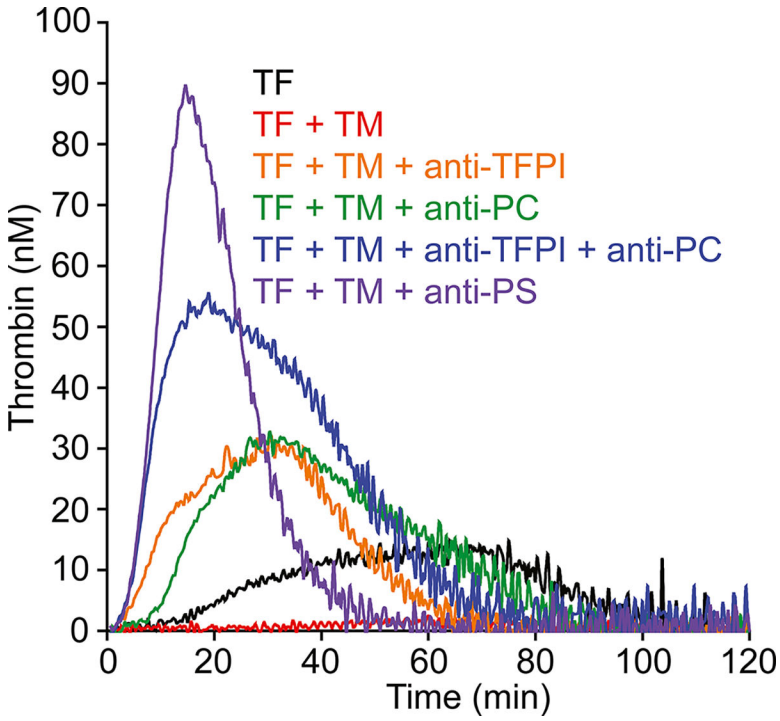

Figure 7. Inhibitory antibodies against TFPI and PC have additive effect on PRP thrombin generation, while inhibiting PS alone has even greater effect.

Thrombin generation, initiated with 1pM TF (black line) or TF + 20nM thrombomodulin (all other lines), was measured in healthy donor PRP in the absence (red) or presence of antibodies against TFPI (50nM, orange), PC (133nM, green), TFPI and PC combined (blue), or PS (6.7μM, purple). Shown are the average curves from experiments performed in triplicate.

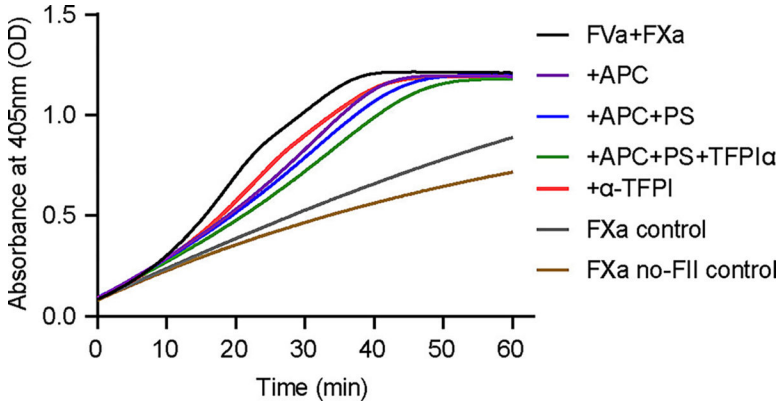

Figure 8. APC, PS, and TFPIα are synergistic anticoagulants on the endothelial surface.

EA.hy926 endothelial cells (50μg/mL) were incubated for 10min in the absence or presence of FVa (50nM), APC (1nM), PS (150nM), FXa (5nM), TFPIα (1nM), and anti-TFPI antibody (50nM). Prothrombinase activity assays were initiated by rapid sequential addition of Spectrozyme TH substrate (500μM), prothrombin (1.4μM), and FXa (0.5nM), and cleavage of the substrate monitored at 405nm. Shown are the averaged progress curves (n=3).

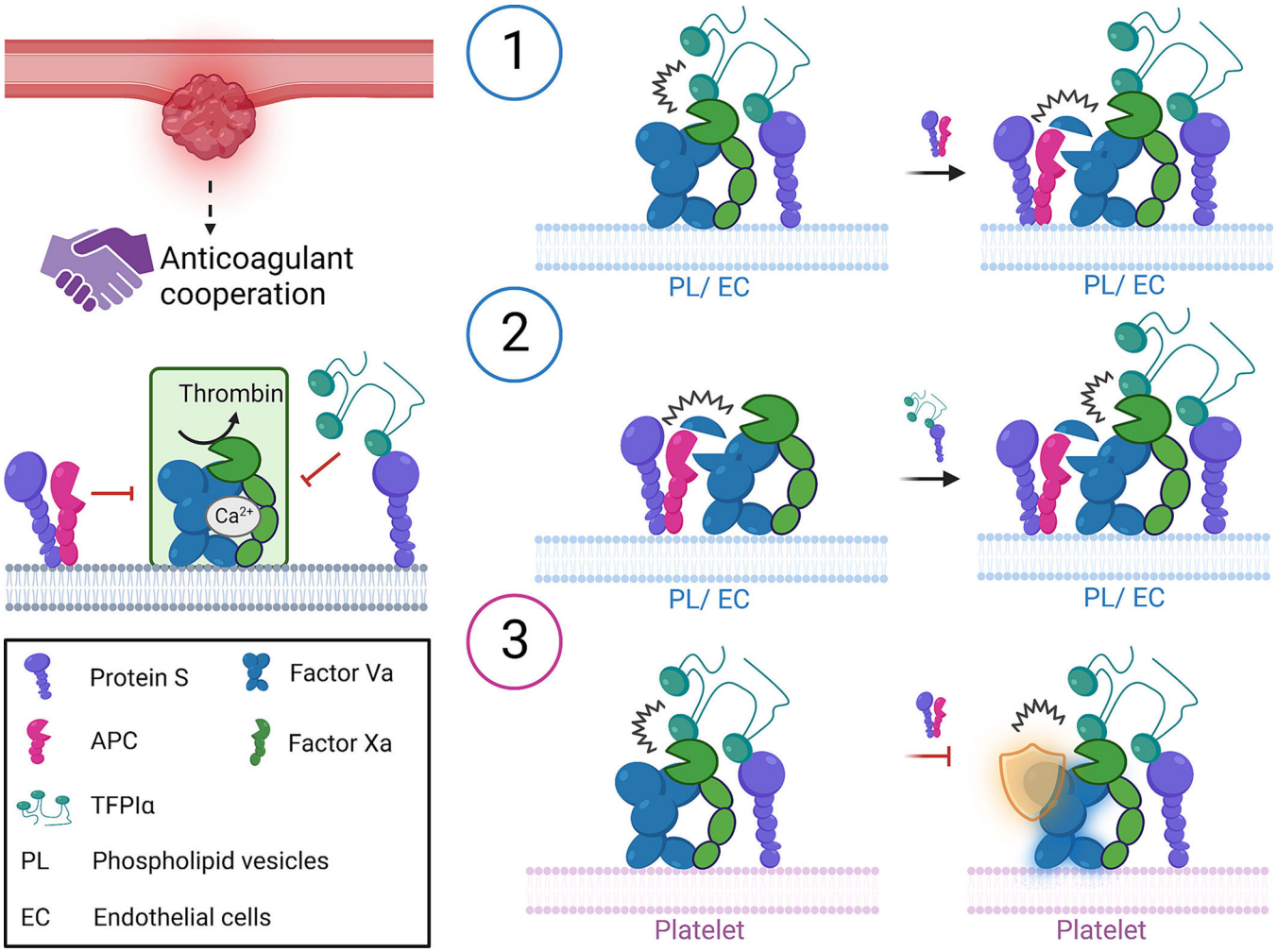

Figure 9. Model of prothrombinase inhibition.

Our data support a cooperative model of prothrombinase inhibition on the surface of phospholipid vesicles and endothelial cells, in which inhibition of FXa by TFPIα/PS promotes the degradation of FVa by APC/PS (1). Conversely, APC/PS-mediated FVa degradation promotes FXa inhibition (2). In contrast, FVa is protected from degradation on the platelet surface, either in the presence or absence of TFPIα and PS (3).

Table 1.

TFPIα Concentration in Normal and PS-Depleted Human Plasma.

Sample	TFPIα (ng/mL ± S.D.)
Pooled Plasma 1	3.59±0.35
Pooled Plasma 2	1.71±0.12
Individual Donor 1	4.99±0.38
Individual Donor 2	1.95±0.38
Individual Donor 3	2.23±0.34
Individual Donor 4	2.01±0.12
Individual Donor 5	2.6±0.081
PS-Depleted Plasma	<0.3 (below limit)