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Comparison of the inhibitory activity of human TFPI α and TFPI β

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Summary

Background—Tissue factor pathway inhibitor (TFPI) is an alternatively spliced protein with two isoforms, TFPI α and TFPI β , which differ in their carboxy-terminal structure and cellular localization. Detailed characterization of their inhibitory activity is needed to define potentially unique inhibitory roles in tissue factor (TF) mediated thrombotic and inflammatory disease and to understand how pharmaceuticals targeted to different structural regions of the TFPI isoforms alter hemostasis in hemophilia patients.

Methods—The TF inhibitory activity of TFPI β localized to the surface of CHO cells was compared to soluble TFPI α using *in vitro* and *in vivo* assays.

Results—In TF-FVIIa-mediated FXa generation assays, TFPI β was a slightly better inhibitor than TFPI α , which was ~3-fold better than TFPI-160, a soluble, altered form of TFPI similar to TFPI β . In direct FXa inhibitory assays, TFPI β had an IC₅₀ 2.5-fold lower than TFPI α and 56-fold lower than TFPI-160. TFPI β inhibited TF-mediated CHO cell migration through Matrigel, while TFPI α or TFPI-160 were poor inhibitors, demonstrating that TFPI β effectively blocks TF-initiated signaling events during cellular migration through matrices not permeable to soluble forms of TFPI. Further, TFPI β inhibited TF-dependent CHO cell infiltration into lung tissue following tail vein injection into SCID mice and blocked development of consumptive coagulopathy.

Conclusions—When compared to TFPI α , TFPI β is a slightly better inhibitor of TF procoagulant activity. As a surface associated protein, TFPI β is a much better inhibitor of TF-mediated cellular migration than soluble TFPI α and may distinctly act in the inhibition of TF-mediated signaling events on inflamed endothelium and/or monocytes.

Keywords

alternative splicing; hemophilia; migration; tissue factor; TFPI

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Addendum

S. A. Maroney planned and performed experiments, analyzed data and contributed to writing the manuscript. P. E. Ellery analyzed data and contributed to writing the manuscript. J. P. Wood performed experiments, analyzed data and contributed to writing the manuscript. J. P. Ferrel and N. D. Martinez performed experiments. A. E. Mast planned experiments, analyzed data and contributed to writing the paper.

Disclosure of conflict of interests

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Introduction

Tissue factor (TF) is located within extravascular tissues and initiates blood coagulation following vascular injury by binding to factor (F) VIIa present in the blood [1]. The TF-FVIIa complex activates FIX and FX [2], which initiate hemostatic cascades that restore vascular integrity through formation of a meshwork of fibrin and platelets. In addition to preventing hemorrhage, TF-FVIIa can promote various cellular activities, including cellular migration through the extracellular matrix [3,4].

The primary physiological inhibitor of TF-FVIIa is tissue factor pathway inhibitor (TFPI) [5,6]. TFPI is a multivalent Kunitz-type serine protease inhibitor that simultaneously inactivates FXa and FVIIa, immediately following activation of FX by TF-FVIIa [7]. Alternative splicing results in the production of two isoforms in humans, TFPI α and TFPI β [8]. Both isoforms have the same N-terminal sequence, containing two Kunitz-type inhibitor domains that bind and inhibit FVIIa and FXa, but differ at their C-termini. The C-terminus of TFPI α has a third Kunitz domain (K3) followed by a stretch of amino acids rich in arginine and lysine residues, while the C-terminus of TFPI β encodes a glycosylphosphatidylinositol (GPI)-anchor attachment site [9].

TFPI α protein has been identified in human plasma [10], placenta [11], and platelets [12]. In human plasma TFPI α is 50–80% truncated at its carboxy-terminus [13], but increases 2- to 4-fold upon heparin infusion [10,14] suggesting the presence of an *in vivo* pool of full-length TFPI α that is non-specifically bound to endothelial glycosaminoglycans. However, heparin-releasable TFPI α is not present on the surface of cultured endothelial cells [15,16] but is localized within an intracellular compartment and released following treatment with heparin or thrombin [15–17]. TFPI present on the surface of cultured endothelial cells is removed with phosphatidylinositol phospholipase C (PIPLC), indicating that it has a GPI-anchor [11,18]. Consistent with this finding, TFPI β protein has been identified as the isoform present in all major vascular beds of adult mice [19] and in cultured human endothelial cells and human placental microsomes [20].

Previous studies comparing the inhibitory activities of TFPI α and soluble forms of TFPI that mimic TFPI β , such as TFPI-160 (which contains the K1 and K2 domains), have demonstrated that TFPI α is the more effective inhibitor of FXa in amidolytic assays [21–23]. However, unlike TFPI-160, TFPI β is linked to the cell surface through a GPI-anchor, which may significantly alter its activity compared to soluble forms of TFPI [24]. Studies examining the inhibitory activity of TFPI β using small-interfering RNA (siRNA) techniques to limit TFPI α expression have suggested that it effectively inhibits TF-FVIIa-mediated generation of FXa on the surface of ECV304 cells [25], and the TF-mediated migration of MDA-MB-231 cells [26]. However, these inhibitory studies are limited by residual TFPI α produced by the cells and potential off-target effects of the siRNA, both of which complicate the identification of specific TFPI β inhibitory functions.

The inhibitory activity of cell-associated TFPI β , and how it compares to soluble TFPI α , is not well understood. A CHO cell model system in which human TF and human TFPI β are expressed on the cell surface was developed to further define the biological activities of cell-associated TFPI β and compare these activities to soluble TFPI α and TFPI-160 in a series of *in vitro* and *in vivo* assays. This model system has a distinct advantage in that the cells do not produce TFPI α , allowing for accurate determination of the amount of TFPI β on the cell surface and quantitative comparisons of TFPI α and TFPI β inhibitory activities. TFPI β is shown to be the more potent inhibitor of several TF-mediated physiological processes, particularly TF-mediated cellular migration.

Materials and methods

Production and characterization of CHO cells expressing TF and TFPI β

CHO (K1) cells were transfected with a hygromycin-resistant plasmid containing human full-length TF (gift of Dr. Wolfram Ruf, Scripps Research Institute, La Jolla, CA) to produce CHO-TF cells. CHO-TF cells were then transfected with a neomycin-resistant plasmid containing human TFPI β to produce CHO-TF/TFPI β cells. Cells were prepared for flow cytometry as previously described [27]. To verify the presence of a GPI-anchor, transfected CHO-TF/TFPI β cells were treated with 1 U/ml PIPLC for 1 hour at 37°C [27] and analyzed by flow cytometry.

Standardization of cell preparations

Cells were washed, harvested, pelleted by centrifugation (180 x *g*, 15 minutes), and resuspended in 1 mL of PBS. A 100 μ l aliquot was pelleted and lysed in 100 μ l CHAPS buffer for protein determination of each individual sample. Measurement of total protein was used to standardize cell concentration for all assays. This is a more reliable and reproducible method than cell counts.

TF-initiated amidolytic assay

Initially, the CHO-TF and CHO-TF/TFPI β cells were examined to confirm equal surface TF activity. Intact cells (30 μ g/well), untreated or treated with PIPLC or anti-TFPI polyclonal antibody, were added to FVIIa (10 pM) and 0.5 mM factor Xa substrate (MeO-CO-D-CHD-Cly-Arg-pNA.AcOH (American Diagnostica, Stamford, CT)). Reactions were initiated with FX (20 nM). Maximum FXa generation was determined by comparison of the peak slope obtained to a standard curve of FXa generation. In assays to determine IC₅₀ values CHO-TF/TFPI β cells or CHO-TF cells, alone or with indicated concentrations TFPI α (either glycosylated from HEK expression or non-glycosylated from *E.coli* expression) or TFPI-160 [21], were incubated with FX (20nM) and reactions initiated with 10 pM FVIIa. The total cellular protein concentration (CHO-TF and/or CHO-TF/TFPI β) was 90 μ g/ml in all reactions to ensure equal amounts of TF. Aliquots were removed at timed intervals over 6 minutes and quenched in 33 mM EDTA. FXa present at time points was determined by comparison to the standard curve. IC₅₀ values were determined using a variable slope dose-response curve (GraphPad Prism V5, La Jolla, CA).

Factor Xa Inhibition Assay

Reaction mixtures were prepared containing CHO-TF cells (90 μ g/mL), varying concentrations of glycosylated or non-glycosylated TFPI α , TFPI-160, or CHO-TF/TFPI β cells, and FXa chromogenic substrate (0.5 mM). The reaction was initiated by addition of FXa (0.1 nM), and monitored for 45 min at 405 nm. The steady-state concentration of free FXa was determined by comparison to a FXa standard curve. IC₅₀ values were determined as described for the TF-FVIIa inhibition assays.

TFPI ELISA

A total TFPI ELISA used a mouse monoclonal anti-K2 antibody [28] for capture and rabbit polyclonal TFPI antibody [11] for detection. Secondary anti-rabbit HRP antibody was detected using QuantaRed Enhanced Chemifluorescent HRP Substrate. TFPI released from the cells was determined by comparison to a TFPI α standard curve.

TF-dependent Matrigel cellular migration assays

Transwell inserts (8.0 μ m) were coated with Matrigel (1 mg/mL), a protein mixture from a murine tumor containing over 1851 proteins including FII, FVII and FX [29]. After coating,

inserts were incubated at 37°C, washed with warm serum-free media and seeded with CHO, CHO-TF, or CHO-TF/TFPI β cells (2.5×10^4 /well). After 36 hours non-migrating cells were removed, the insert membranes fixed with 1.5% formaldehyde and stained with crystal violet. In some experiments, cells were incubated with 10 μ M argatroban, SCH79797, recombinant tick anticoagulant peptide (rTAP), or recombinant nematode anticoagulant protein C2 (rNAPC2) (rTAP and rNAPC2 gifts from Dr. Sriram Krishnaswamy, University of Pennsylvania), or 100 nM soluble TFPI α or TFPI-160, prior to seeding. Migrating cells were counted using a Nikon Eclipse E600 light microscope at 20X field magnification.

In vivo TF-dependent cellular migration assays

All animal experiments were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. CHO-TF or CHO-TF/TFPI β cells were harvested and re-suspended in sterile PBS (35 μ g/100 μ L) and injected into the tail vein of 6–12 week old CB.17 SCID mice (C.B-*Igh-I^b/IhcrTac-Prkdc^{scid}*). On day 10, mice were anesthetized, whole blood was collected for complete blood count as described [30], and lungs were harvested for Trichrome staining and examination of cellular burden in a blinded fashion using the Nikon light microscope.

Statistical Analyses

To test for statistical significance, the students' *t*-test or one-way ANOVA were performed using GraphPad Prism. Bonferroni's Multiple Comparison test was performed to test for differences between groups when the one-way ANOVA was significant. A *p*-value less than 0.05 was considered statistically significant.

Results

Production and characterization of CHO-TF and CHO-TF/TFPI β cells

Human TFPI β was co-expressed with human TF in CHO cells, providing a model system that allowed testing and quantitative comparisons of TFPI α and TFPI β inhibitory activities. Flow cytometry experiments demonstrated presence of similar TF expression on CHO-TF and CHO-TF/TFPI β cell lines (Fig. 1A). TF-FVIIa-mediated FXa generation experiments demonstrate equal TF activity on the cell lines following neutralization of TFPI β activity with an anti-TFPI antibody TFPI β or its removal with PIPLC (Fig. 1B). TFPI ELISA assay of PIPLC treated cellular supernatant quantified 92.8 pg TFPI β /μg total cellular protein on the CHO-TF/TFPI β cell surface.

TFPI β is a more potent inhibitor of TF-FVIIa and FXa than either TFPI α or TFPI-160

TFPI β activity was quantified and compared to that of soluble glycosylated and non-glycosylated TFPI α and TFPI-160 in TF-FVIIa and FXa-initiated assays (Fig. 2). Activity of soluble forms of TFPI toward TF-FVIIa was determined using CHO-TF cells as the source of TF (Fig. 2A). Experiments with TFPI β contained mixtures of CHO-TF/TFPI β and CHO-TF cells to yield the indicated TFPI β concentrations while maintaining a constant total amount of TF. TFPI β inhibited TF-FVIIa to a greater extent than glycosylated or non-glycosylated TFPI α or TFPI-160 at all concentrations. Extrapolation of data points for TFPI β yielded an estimated IC₅₀ of 0.11 nM, slightly lower than IC₅₀ values for glycosylated TFPI α (IC₅₀=0.74 nM), non-glycosylated TFPI α (IC₅₀=0.53 nM), or TFPI-160 (IC₅₀=2.18 nM). This IC₅₀ value assumes that TFPI β equivalently inhibits co-expressed TF and TF expressed on a different cell, a scenario which may not be true. Rather, it is probable that TFPI β is a potent inhibitor of co-expressed TF and a relatively poorer inhibitor of TF expressed on an adjacent cell. Thus, the IC₅₀ of TFPI β towards co-expressed TF may be significantly lower than 0.11 nM.

Reactions examining direct FXa inhibition by TFPI α or TFPI-160 contained 90 μ g/ml CHO-TF cells, mimicking the cellular concentration in reactions with CHO-TF/TFPI β (Fig. 2B). Glycosylated (IC_{50} = 0.66 nM) and non-glycosylated (IC_{50} =0.59 nM) TFPI α were better inhibitors of FXa than TFPI-160 (IC_{50} =12.90 nM). TFPI β (extrapolated IC_{50} =0.23 nM) was a slightly better inhibitor of FXa than TFPI α (2- to 3-fold) and substantially better (56-fold) than TFPI-160.

TFPI β inhibits TF-dependent cellular migration through Matrigel

A transwell assay system utilizing Matrigel, a protein mixture derived from a murine tumor that contains FII, FVII and FX [29], was utilized to compare the abilities of TFPI β , glycosylated and non-glycosylated TFPI α , and TFPI-160 to inhibit TF-mediated cellular migration. CHO cells migrated poorly, while CHO-TF cells had a 15-fold increase in cellular migration, demonstrating TF-dependent migration in this assay. When compared to CHO-TF cells, glycosylated TFPI α , non-glycosylated TFPI α , and TFPI-160 (100 nM) inhibited migration by approximately 30% (p <0.01), while CHO-TF/TFPI β cells (expressing only approximately 21 pM TFPI β per well) inhibited migration by 83% (p <0.001) (Fig. 3A).

The PAR-1 antagonist SCH79797 greatly reduced cellular migration demonstrating that it is dependent on PAR-1 activation (p <0.001) (Fig. 3B). Next, specific inhibitors were used to determine the protease responsible for PAR-1 cleavage. Inhibitors of TF-FVIIa (rNAP-C2), FXa (rTAP), and thrombin (argatroban) all greatly reduced cellular migration, demonstrating that thrombin, generated via TF-mediated activation of coagulation, is the PAR-1 cleaving protease (p <0.001) (Fig. 3B).

TFPI β inhibits TF-dependent cellular infiltration and consumptive coagulopathy

As TFPI β markedly inhibited the TF-mediated migration of cells *in vitro*, we directly tested its ability to inhibit tissue infiltration in a well-characterized murine model system [3]. In this assay CHO, CHO-TF, or CHO-TF/TFPI β cells were injected into the tail vein of SCID mice and cellular infiltration into lung tissue was assessed ten days later. Histological analyses demonstrated normal lung parenchyma with only rare cellular infiltrate detected in mice injected with CHO cells (Fig. 4A, arrowhead). In contrast, mice injected with CHO-TF cells often had large sheets of cells with very little remaining normal lung parenchyma (Fig. 4B). The lung parenchyma had alveoli with thickened walls and fibrin clots within the vasculature (Fig. 4B, arrow). Mice injected with CHO-TF cells had greatly decreased platelet counts compared to those injected with CHO cells ($400 \times 10^3/\text{mm}^3$ vs. $700 \times 10^3/\text{mm}^3$, p <0.0001), suggesting the presence of a consumptive coagulopathy (Fig. 4E). Mice injected with CHO-TF/TFPI β cells had reduced cellular infiltrate with a moderate amount of normal lung tissue present (Fig. 4C and 4D) and, importantly, had platelet counts similar to mice injected with CHO cells, suggesting that TFPI β completely prevented development of a consumptive coagulopathy.

Discussion

The expression of TFPI α in platelets and TFPI β on the surface of vascular endothelium suggests they may have distinct biological activities. As a first step to define these, our laboratory developed a CHO-cell model system to compare the inhibitory activities of different TFPI isoforms. This model system, in which CHO cells co-express human TF and human TFPI β , simulates disease states, such as infection or cancer, where TF and TFPI may be present on endothelium, monocytes or tumor cells [31–33]. The results demonstrate that while cell-surface associated TFPI β is only a slightly more effective inhibitor of TF-FVIIa and FXa than TFPI α in assays using purified proteins, it is a much more effective inhibitor

in cellular migration assays. Further, using a SCID mouse model it was demonstrated that TFPI β is a highly effective inhibitor of TF-mediated cellular migration and induction of disseminated coagulopathy. Thus, TFPI β may distinctly inhibit intravascular TF-mediated cell signaling events on inflamed endothelium or monocytes.

Consistent with the findings of Riewald and co-workers [34], the CHO cell system demonstrated that TFPI β is a potent inhibitor of TF-FVIIa-mediated FXa generation. TFPI β could inhibit either TF co-expressed on the same cell, which seems most likely, or on an adjacent cell. As these two scenarios cannot be differentiated, an accurate IC₅₀ value for inhibition of TF-FVIIa by TFPI β could not be calculated. However, at every concentration tested, TFPI β was a more potent inhibitor of TF-FVIIa-mediated FXa generation than TFPI α or TFPI-160. TFPI β also was a potent direct inhibitor of FXa. Based on IC₅₀ concentrations, it inhibits FXa two-fold better than TFPI α and 56-fold better than TFPI-160. The rapid FXa inhibition by TFPI β is somewhat unexpected. Since the third Kunitz domain and the C-terminal region are necessary for rapid FXa inhibition in solution phase assays [21–23], one may predict that TFPI β is a poor FXa inhibitor. However, the GPI-anchoring of TFPI β to the membrane compensates for the lack of these domains allowing rapid FXa inhibition. Finally, the inhibitory activity of the different forms of TFPI used in these experiments does not appear to depend on their glycosylation, since glycosylated and non-glycosylated TFPI α had similar inhibitory activity.

CHO-TF cells readily migrated through Matrigel, which contains FVII and FX [29]. This migration was mitigated by 83% with CHO-TF/TFPI β cells. Although the CHO-TF/TFPI β cells expressed more TF than TFPI β , it appears that in these assays the amount of FVIIa in Matrigel is limiting and that TFPI β is a very effective inhibitor of TF-mediated cell signaling required for migration. In contrast, high concentrations (100 nM) of soluble TFPI α (either glycosylated or non-glycosylated) or TFPI-160 inhibited CHO-TF cell migration only 30%. These results were somewhat surprising, since all forms of TFPI at 100 nM totally inhibit TF-FVIIa mediated FXa generation in amidolytic assays (Fig. 2A). Thus, TF-FVIIa may be inhibited by membrane associated forms of TFPI but protected from inhibition by soluble forms of TFPI during Matrigel migration. Alternatively, soluble forms of TFPI may be poorer inhibitors of TF-mediated cell signaling events than would be predicted from the TF-FVIIa inhibition assays. Consistent with this notion, Ahamed and colleagues have reported that 50- to 100-fold more TFPI α is required to inhibit TF-FVIIa mediated cellular signaling than is needed to inhibit TF-FVIIa mediated generation of FXa [35]. Thus, it appears that TFPI β , when localized to the same cellular surface as TF, as may occur on the surface of inflamed endothelium, monocytes or tumor cells [31–33], is a highly effective inhibitor of TF-mediated cellular migration, while circulating plasma TFPI α would be a relatively poor inhibitor. These findings have important implications for the use of TFPI α as a therapeutic agent, as is being considered for severe sepsis associated with community acquired pneumonia [36,37], and are consistent with the high concentration of human TFPI α (3049 ng mL⁻¹ or 9 μ M) necessary to observe therapeutic attenuation of coagulation, inflammation and bacterial growth in a murine model of pneumococcal pneumonia [38].

The presence of TF on the CHO cell surface greatly enhanced their infiltration into SCID mouse lung tissue following tail vein injection [3] even though there is ample (44 nM) soluble TFPI in mouse plasma [30]. TF has been reported to support cell adhesion, migration, homing and extravasation [39,40], any or all of which may produce the cellular infiltration observed in this model. In various model systems TF has been shown to induce cellular infiltration through PAR-1 [41] or PAR-2 [4]. The Matrigel migration assay data suggests the underlying mechanism for CHO-TF cells is thrombin generation with activation of PAR-1. Importantly, the expression of relatively small amounts of TFPI β on the CHO-TF

cell surface dampened infiltration of CHO-TF cells into lung tissue and totally protected the mice from the decreased platelet count associated with a TF-induced consumptive coagulopathy, demonstrating that TFPI β effectively down-regulates TF-mediated procoagulant and cellular signaling events *in vivo*.

TFPI α , a soluble protein released from endothelial cells and platelets, is an effective anticoagulant that limits thrombus growth in murine vascular injury models [30] and is a physiological regulator of bleeding in murine hemophilia [42]. Further, TFPI α may have unique functions, not performed by TFPI β , that are mediated by its third Kunitz domain, which binds to protein S and enhances inhibition of FXa [43], and its basic C-terminal region. The data presented here suggest that expression of TFPI β on endothelial cells is optimized to dampen intravascular TF activity, where it is available at a high localized concentration to inhibit potentially detrimental TF-mediated coagulation and/or cell signaling events that occur during inflammation. The localization of TFPI β within lipid rafts/caveolae in endothelial cells has been shown to further enhance its TF inhibitory activity [27,44].

Continued characterization of the distinct inhibitory activities of TFPI α and TFPI β is needed to define their mechanism of action in TF mediated thrombotic and inflammatory disease and has increasing importance with the development of pharmaceutical agents for treatment of hemophilia that block TFPI anticoagulant activity[28,45,46]. These agents act by targeting different structural domains of TFPI; and the target selected may have important therapeutic implication. For example, depletion of hematopoietic cell TFPI, which is predominantly TFPI α within platelets, is sufficient for restoring hemostasis in FVIII deficient mice suggesting that compounds specifically targeting TFPI α may be effective hemostatic agents with decreased risk for inducing a generalized procoagulant state, since they would not alter the anticoagulant activity of TFPI β expressed on endothelium [42]. The data presented here suggest that TFPI α and TFPI β distinctly inhibit different TF functions and provide clues for targeting different regions of the protein that could limit off-target effects that may occur for TFPI blocking agents designed to treat hemophilia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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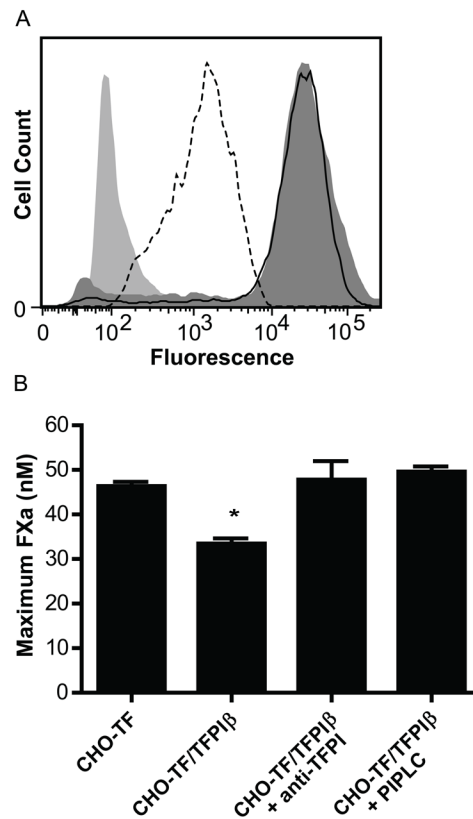


Fig. 1. CHO-TF and CHO-TF/TFPI β cells have similar TF expression and activity. (A) Flow cytometry for TF on CHO-TF (filled dark grey) and CHO-TF/TFPI β cells (solid line) and for TFPI on CHO-TF/TFPI β cells (dotted line). The isotype control is shaded light grey. The data are representative of 3 experiments. (B) TF-FVIIa-mediated FXa generation on CHO-TF cells and CHO-TF/TFPI β cells. TFPI β decreases the amount of FXa generated ($p < 0.001$) and can be totally reversed by anti-TFPI polyclonal antibody or by treatment of the cells with PIPLC (mean \pm SEM, $n=3$).

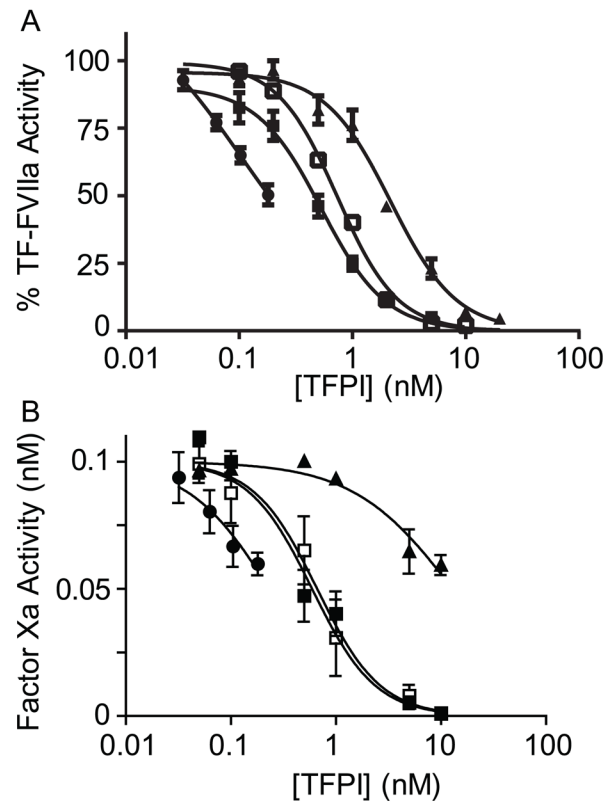
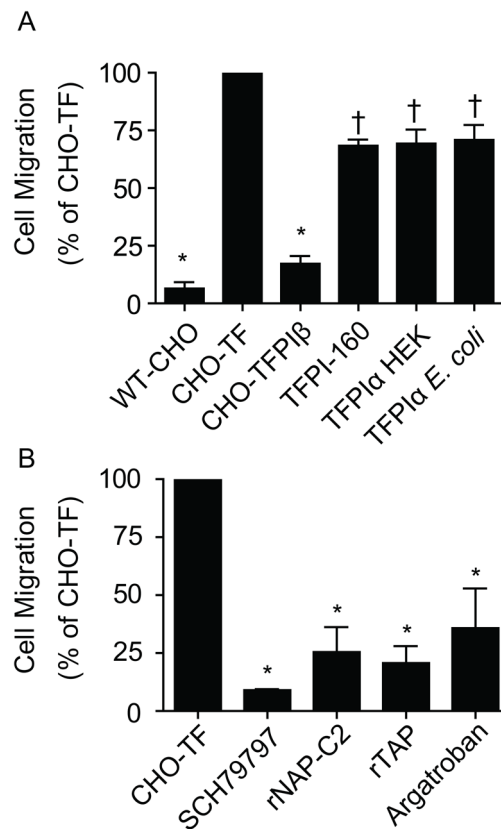


Fig. 2. TFPI β is an effective inhibitor TF-FVIIa and FXa. (A) TF-FVIIa-mediated FXa generation (mean \pm SEM, n=3) and (B) FXa activity (mean \pm SEM, n=3) were measured in the presence of glycosylated TFPI α (□), non-glycosylated TFPI α (■), TFPI-160 (▲) or CHO-TF/TFPI β cells (●). Cellular TF was constant in all experiments. Data are shown as percent activity compared to the “no TFPI” control in (A) or as uninhibited FXa activity in (B).

**Fig. 3.**

TFPI β blocks CHO-TF cell migration through Matrigel by preventing thrombin generation and activation of PAR-1. Cellular migration was standardized to that of CHO-TF cells. (A) Migration of CHO-TF/TFPI β cells compared to CHO cells, CHO-TF cells and CHO-TF cells in the presence of 100nM/well recombinant TFPI-160, glycosylated TFPI α or non-glycosylated TFPI α (mean \pm SEM, n=3). (B) Migration of CHO-TF cells in the presence of SCH79797 (PAR-1 antagonist), rNAP-C2 (TF-FVIIa inhibitor), rTAP (FXa inhibitor), or argatroban (thrombin inhibitor), all at 10 μ M (*p<0.001, †p<0.01) (mean \pm SEM, n=3).

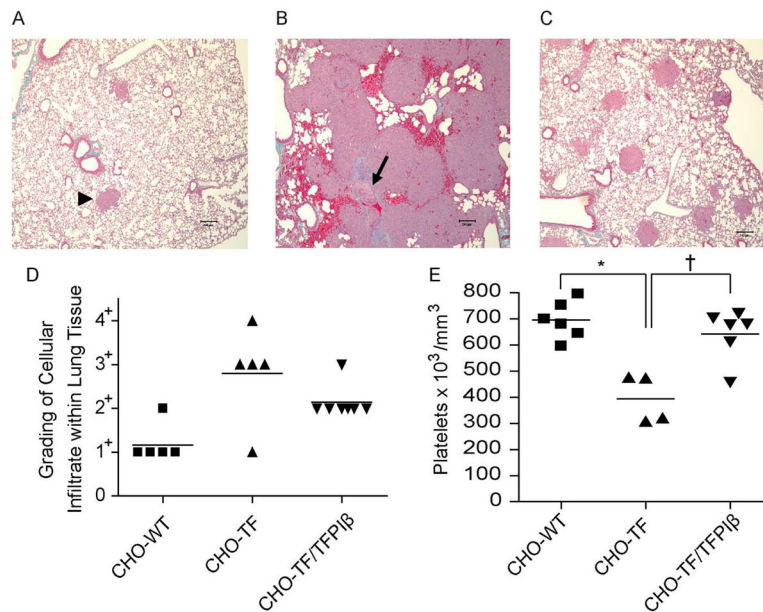


Fig. 4. TFPI β reduces TF-mediated tissue infiltration and consumptive coagulopathy. Histology of the lungs from SCID mice ten days following injection with (A) CHO; (B) CHO-TF; or (C) CHO-TF/TFPI β cells. (A) Arrowhead indicates a small cluster of CHO cells within the lung. (B) Arrow indicates a large intravascular thrombus. (D) Histopathological grading (1 $^{+}$ -4 $^{+}$) of lungs ten days following injection cells. (E) Platelet counts ten days following injection of cells (*p<0.001, † p<0.05).