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Tissue Factor Pathway Inhibitor: Multiple Anticoagulant Activities for a Single Protein

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Abstract

Tissue Factor Pathway Inhibitor (TFPI) is an anticoagulant protein that inhibits early phases of the procoagulant response. Alternatively spliced isoforms of TFPI are differentially expressed by endothelial cells and human platelets and plasma. The TFPI β isoform localizes to the endothelium surface where it is a potent inhibitor of tissue factor-factor VIIa complexes that initiate blood coagulation. The TFPI α isoform is present in platelets. TFPI α contains a stretch of 9 amino acids nearly identical to those found in the B-domain of factor V (fV) that are well conserved in mammals. These amino acids provide exosite binding to activated fV, which allows for TFPI α to inhibit prothrombinase during the initiation phase of blood coagulation. Endogenous inhibition at this point in the coagulation cascade was only recently recognized and has provided a biochemical rationale to explain the pathophysiological mechanisms underlying several clinical disorders. These include the east Texas bleeding disorder that is caused by production of an altered form of factor V with high affinity for TFPI, and a paradoxical procoagulant effect of heparins. In addition, these findings have led to ideas for pharmacological targeting of TFPI that may reduce bleeding in hemophilia patients.

Keywords

TFPI; tissue factor; factor VII; factor V; prothrombinase; hemophilia

Blood coagulation proceeds as a series of proteolytic activation reactions of factors that sequentially amplify activities of each other and culminate with a final burst of thrombin.^{1, 2} Thrombin then activates platelets and generates fibrin to produce a blood clot (Figure 1A). The proteins of the blood clotting pathways include three major cofactors, tissue factor (TF), factor Va (fVa) and factor VIIIa (fVIIIa), that each combine with a target serine protease in a manner that greatly increases the speed and specificity of the protease for its biological substrate (Figure 1A). These cofactor proteins are essential for normal hemostasis and absence of any one causes severe bleeding. TF is expressed by cells within the tissues surrounding the vasculature (within the tissue), but is not typically present within the blood.³ It tightly binds to the plasma protease factor VIIa (fVIIa). The TF-fVIIa catalytic complex initiates blood coagulation by activating fX and factor IX (fIX).⁴ fVa is a cofactor protein

for fXa.⁵ These two proteins combine in the presence of calcium ions on a phospholipid surface to form the prothrombinase complex, which rapidly converts prothrombin to thrombin.⁶ fVIIIa is a cofactor protein for fIXa. fVIIIa and fIXa combine form an enzymatic complex that rapidly converts fX to fXa.⁷ Deficiency of fVIII or fIX produces hemophilia A or hemophilia B, respectively. Since these two proteins act together in one enzymatic complex, deficiency of either one causes very similar symptoms such that laboratory testing is required to determine the type of hemophilia present in an individual patient.

TFPI inhibits TF-fVIIa and prothrombinase

Tissue factor pathway inhibitor (TFPI) is a multivalent, Kunitz-type, serine protease inhibitor that was originally recognized as a plasma component that inhibited TF-fVIIa in a manner that required fX and calcium ions.⁸⁻¹⁰ Subsequent studies demonstrated its pathophysiological importance by showing that immunodepletion of TFPI in rabbit models increased susceptibility for development of disseminated intravascular coagulation following injection of TF or endotoxin.^{11, 12} It is now known that TFPI dampens the blood coagulation pathway by blocking the activity of two of the major protease-cofactor complexes, TF-fVIIa¹³ and prothrombinase (Figure 1A).¹⁴ TFPI is the only endogenous protein that effectively inhibits these enzymatic complexes under physiological conditions. In doing so, it modulates the amount of procoagulant activity needed by the third major protease-cofactor complex, fVIIIa-fIXa, to produce a hemostatic blood clot (Figure 1B).¹⁵ This ability of TFPI to alter how the three major protease-cofactor complexes contribute to thrombin generation can be exploited to treat bleeding disorders, and pharmacological inhibitors of TFPI activity restore hemostasis in animal models of hemophilia.¹⁵⁻¹⁸ The biochemistry and physiology underlying TFPI inhibition of TF-fVIIa and prothrombinase, and the impact of TFPI on hemophilia bleeding, will be reviewed.

The structure and cellular expression of TFPI

TFPI is an alternatively spliced protein that is produced in two major isoforms, TFPI α and TFPI β ,¹⁹ which will be discussed here. TFPI is also produced in two minor isoforms, TFPI γ ²⁰ and TFPI δ .²¹ Diagrams of the structures of all the TFPI isoforms are presented in Maroney et al.²¹ TFPI α contains an acidic N-terminal region followed by three tandem Kunitz domains and a basic C-terminal region. The first Kunitz domain (K1) binds to the active site of fVIIa. The second Kunitz domain (K2) binds to the active site of fXa.¹³ The third Kunitz domain (K3) has not been found to inhibit any protease, but binds to protein S.²² The basic C-terminal region has a stretch of amino acids with sequence identity to a region within the FV B-domain.²³ These amino acids in TFPI α tightly bind some forms of fVa allowing it to inhibit prothrombinase.¹⁴ TFPI α is secreted by human endothelial cells,²⁴ is present in human plasma and binds human endothelium. Endothelium binding is likely mediated by electrostatic interactions with cell surface glycosaminoglycans, because the plasma TFPI α concentration promptly increases 2- to 4-fold upon heparin infusion.^{25, 26} TFPI α is also produced by megakaryocytes²⁷ and is present within quiescent platelets, but not on their surface.^{28, 29} Interestingly, platelet TFPI α is not localized within the platelet α -granules or lysosomes and its intracellular location is not clear.^{28, 29} Following platelet activation, TFPI is externalized, with a portion released as a soluble protein and a portion

associating with the surface of the activated platelet.^{28, 29} Murine TFPI α differs from its human counterpart in that it is not produced by endothelial cells and is not present in plasma.³⁰ Murine platelet TFPI α is very similar to human platelet TFPI α in regards to the characteristics of its storage within the platelet and release following platelet activation.³¹

TFPI β contains the identical first two Kunitz domains present in TFPI α , but has an alternatively spliced C-terminus that replaces K3 and basic C-terminal region present in TFPI α with a different amino acid sequence that encodes for attachment of a glycosylphosphatidyl inositol (GPI)-anchor.^{19, 32} This anchor binds TFPI β to the surface of endothelial cells.^{30, 33} TFPI β is not produced by megakaryocytes and is present neither within platelets nor on their surface.²⁷ The molecular events that control the cellular and tissue expression of the TFPI isoforms are not well understood. It is known that exon 2 within the TFPI 5' untranslated region undergoes alternative splicing and acts as a molecular switch that blocks TFPI β translation.³⁴ Further studies are needed to define how exon 2 expression contributes to temporal and tissue specific regulation of TFPI isoform production.

Inhibition of TF-fVIIa by TFPI

TFPI is a weak inhibitor of TF-fVIIa in the absence of fXa and, therefore, can be described as a fXa dependent inhibitor of TF-fVIIa. It is often stated that TFPI first binds fXa with K2, and then K1 within the TFPI-fXa complex, in a second inhibitory reaction, binds to TF-fVIIa. However, the rate limiting step in the reaction is the inhibition of fXa, not the inhibition of TF-fVIIa.³⁵ Therefore, it appears that TFPI simultaneously inhibits fXa and TF-fVIIa immediately after fX is activated by TF-fVIIa.³⁵ TFPI α and TFPI β are both able to inhibit TF-fVIIa, since both have the first two Kunitz domains that mediate the inhibitory activity. However, the differences in the C-terminal structures of the two proteins modify their individual inhibitory activity in unique ways. The basic C-terminal region of TFPI α enhances direct inhibition of fXa in amidolytic assays, such that TFPI α inhibits fXa approximately 50-fold better than TFPI-160, a soluble, altered form of TFPI similar to TFPI β in that contains only K1 and K2.^{36, 37} TFPI α inhibits TF-fVIIa approximately 3-fold better than TFPI-160 in fXa generation assays.³⁷ These studies initially suggested that TFPI α is a more potent anticoagulant protein than TFPI β . However, localization of TFPI β to the cell surface via its GPI-anchor greatly increases its inhibitory activity, such that it is a slightly better inhibitor of fXa and TF-fVIIa than TFPI α in these assays. Further *in vitro* studies of TFPI β have shown that it is a potent inhibitor of TF-mediated cellular migration through matrices that are not permeable to soluble forms of TFPI, while *in vivo* studies have shown that TFPI β inhibits TF-mediated cellular migration into murine lung following tail vein injection and prevents development of a consumptive coagulopathy.³⁷ Therefore, TFPI β is a highly effective inhibitor of TF-mediated coagulation and cell signaling events that produce cellular migration.

Inhibition of prothrombinase by TFPI

TFPI α inhibits early forms of prothrombinase, the coagulation complex of fXa and fVa that rapidly converts prothrombin to thrombin.¹⁴ The inhibitory mechanism requires the basic C-

terminal region of TFPI α and, therefore, prothrombinase inhibition is an isoform specific activity of TFPI that is not performed by TFPI β . fV has a large B-domain of more than 800 amino acids. When the B-domain is intact, fV remains in an inactive conformation that does not promote blood clotting. This inactive conformation is maintained through interactions between a basic region near the N-terminus of the B-domain and an acidic region near its C-terminus.³⁸ Proteolytic removal of either the basic or the acidic region of the B-domain produces a fully procoagulant form of fVa.³⁸ Proteases that cleave within the fV B-domain include fXa, which rapidly removes the basic region, but only slowly removes the acidic region, and thrombin, which rapidly removes the entire B-domain.³⁹ In addition, platelet α -granules contain forms of fVa that lack the basic region of the B-domain but retain the acidic region.⁴⁰ The C-terminus of TFPI α and the basic region of the fV B-domain both contain the amino acid sequence LIKT followed by five basic amino acids (TFPI: LIKTKRKRK; fV: LIKTRKRRK).²³ Early in the blood coagulation cascade, before thrombin is produced, prothrombinase assembles with forms of fVa that retain the acidic region of the B-domain. These early forms of prothrombinase tightly bind to the basic region of TFPI α allowing for rapid inhibition of prothrombinase that is directly mediated by binding of K2 to the active site of fXa.¹⁴ This inhibitory mechanism requires the presence of the acidic region of the fV B-domain that is rapidly removed by thrombin. Therefore, it is thought that TFPI α only inhibits prothrombinase during the initial stages of blood coagulation, perhaps preventing full procoagulant responses to sub-threshold stimuli that would otherwise occlude blood vessels.

Clinical relevance for TFPI inhibition of prothrombinase

TFPI α is the only endogenous protein recognized that inhibits prothrombinase at physiologically relevant rates and protein concentrations.¹⁴ The prothrombinase inhibitory activity of TFPI α is independent of its TF-fVIIa inhibitory activity.¹⁴ Identifying and characterizing the biochemical mechanism for inhibition of prothrombinase during the initiation of coagulation by TFPI α are directly relevant to understanding the pathogenesis of bleeding disorders and thrombotic disease. Three examples where the inhibition of prothrombinase by TFPI α may be relevant to patient care are as follows.

East Texas Bleeding Disorder

The east Texas bleeding disorder is an autosomal dominant condition associated with easy bruising, menorrhagia and life-threatening bleeding following trauma or surgery.⁴¹ Initial genetic studies identified an A2440G mutation encoding a glycine to serine amino acid substitution in the B-domain of fV that segregated with the disease phenotype. However, the presence of the serine residue did not alter clinical measurements of plasma fV antigen or activity. Thus, it was not initially thought to be related to the disease phenotype.⁴¹ Subsequent biochemical studies performed 10 years after identification of the genetic anomaly found that the A2440G mutation causes production of a fV splice variant, termed fV-short, that is missing 702 amino acids from the B-domain, including the basic region that is homologous with the basic region of TFPI α .⁴² The fV-short binds tightly to TFPI α . This stabilizes circulating TFPI α , such that patients with east Texas fV have an approximately 10-fold increase in plasma TFPI α .⁴² The TFPI α :fV-short complexes potently inhibit

coagulation in *in vitro* assays, probably by rapid inhibition of prothrombinase, which is the likely cause of the bleeding disorder in these patients. Another mutation at a different site in the fV B-domain has subsequently been identified that produces a similar form of fV and a similar bleeding disorder, confirming this interaction between TFPI and fV-short in a separate patient population.⁴³

A procoagulant property of heparin

Heparin is an anticoagulant drug that produces its activity by binding to antithrombin, and to a lesser extent, heparin cofactor II (HCII). Heparin binding greatly increases the inhibitory activity of these proteins towards several blood coagulation proteases, including thrombin and fXa.⁴⁴ However, in plasma lacking antithrombin and HCII, heparin has paradoxical procoagulant activity.⁴⁵ The biochemical mechanism underlying this procoagulant activity of heparin is important to understand because it may be clinically relevant in patients with conditions that cause simultaneous bleeding and clotting and are associated with low antithrombin, such as sepsis or disseminated intravascular coagulation. Heparin is a large, negatively charged polysaccharide. Its procoagulant effect is mediated, at least in part, by blocking the charge-dependent exosite interaction between the basic C-terminal region of TFPI α and the acidic region of the fVa B-domain. Consistent with this biochemical mechanism, the procoagulant activity of heparin is abolished by addition of thrombin-activated fVa to the plasma.¹⁴ Fucoidan is another large, negatively charged polysaccharide that also blocks the inhibition of prothrombinase by TFPI α , providing one plausible explanation for its therapeutic efficacy in a canine model of hemophilia A.¹⁶

New treatments for hemophilia

Blocking TFPI activity restores thrombin generation through the extrinsic blood coagulation pathway and restores hemostasis in several animal models of hemophilia.¹⁵⁻¹⁸

Pharmacological agents that block TFPI activity are currently under development for treatment of hemophilia.⁴⁶ Blocking TFPI, or “inhibiting the inhibitor”, as a therapeutic strategy has several advantages over intravenous factor replacement therapy, which is currently used to treat patients with hemophilia. These include: the ability to treat hemophilia A and hemophilia B with the same drug; the ability to treat patients with antibody inhibitors directed at fVIII or fIX; the potential for subcutaneous, rather than intravenous, treatment options; and the potential for weekly or less frequent dosing. Characterization of the cellular expression patterns defining TFPI α as the TFPI isoform produced by platelets and definition of the biochemical mechanism for inhibition of prothrombinase by TFPI α has produced new insights into how therapeutic agents targeted against different structural regions of TFPI may produce hemostasis in patients with hemophilia. Studies performed in mice with hemophilia A found that total inhibition of plasma and endothelial pools of TFPI using an anti-TFPI polyclonal antibody dampened blood loss in a tail clip assay.⁴⁷ While this was an expected finding, these experiments had another very interesting result. The amount of tail blood loss continued to progressively decrease even after all the plasma and endothelial TFPI was inhibited. This suggested that the TFPI α sequestered within platelets and released at the site of the tail wound had to be inhibited in order to obtain an optimal hemostatic response from the antibody. This idea was tested by producing mice with hemophilia A that lacked platelet TFPI α , but had normal

plasma and endothelial TFPI, using fetal liver transplantation. When these mice were subjected to the tail clip assay, they had significantly less bleeding than mice containing normal amounts of platelet TFPI α , suggesting that platelet TFPI α modulates bleeding in hemophilia.⁴⁷ These data also suggest that anti-TFPI agents designed to specifically block platelet TFPI α would improve hemostasis in patients with hemophilia without altering the anticoagulant properties of TFPI β on endothelium.

Conclusions

TFPI is an anticoagulant protein that blocks the initiation of blood coagulation by inhibiting TF-fVIIa and early forms of prothrombinase. Alternative splicing of TFPI produces two primary isoforms, TFPI α and TFPI β . TFPI α is a soluble protein secreted by endothelial cells and activated platelets. TFPI β is a GPI-anchored protein expressed primarily on the surface of endothelium. Although TFPI α and TFPI β are both capable of inhibiting TF-fVIIa activity, TFPI β is a particularly effective inhibitor of TF-fVIIa present on cellular surfaces and may act *in vivo* to dampen intravascular procoagulant activity on the surface of inflamed vascular cells. TFPI α , but not TFPI β , inhibits prothrombinase. The inhibitory mechanism for prothrombinase inhibition by TFPI α has identified fXa activated fV and forms of platelet fVa that retain the acidic portion of the B-domain, as key proteins for physiological regulation of blood coagulation. This has produced new understanding of the pathophysiology of clinical disorders including the east Texas bleeding disorder, a potential procoagulant activity of heparin, and new ideas for how to best target inhibitors of TFPI activity for treatment of hemophilia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

fV	factor V
fVII	factor VII
fX	factor X
fIX	factor IX
TF	tissue factor
TFPI	tissue factor pathway inhibitor
GPI	glycosylphosphatidyl inositol
K1	Kunitz domain 1
K2	Kunitz domain 2
K3	Kunitz domain 3

Significance

TFPI dampens very early intravascular procoagulant activity thereby minimizing the development of occlusive thrombi and consumptive coagulopathy. TFPI is an alternatively spliced protein with two primary isoforms in humans, TFPI α and TFPI β . The TFPI isoforms block early events in blood coagulation through two separate mechanisms: 1) inhibition of TF-FVIIa by K1 and K2, which is mediated by TFPI β on endothelium; and 2) the inhibition of early forms of prothrombinase by K2 and the C-terminal region of TFPI α released from within platelets. The inhibition of prothrombinase by TFPI α is recently recognized. Further characterization of prothrombinase inhibition by TFPI α will generate important new knowledge of human bleeding and thrombotic disorders and potentially lead to targeted new therapies.

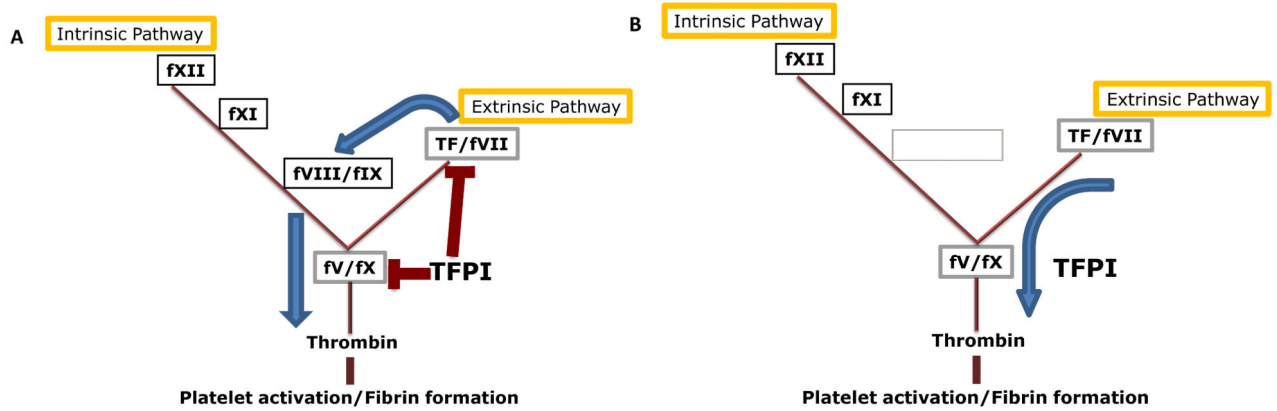


Figure 1.

The blood coagulation pathway is a series of sequential proteolytic reactions that can be separated into intrinsic and extrinsic pathway. A) When TFPI is present, it dampens activation of clotting through the extrinsic pathways by serving as a fXa-dependent inhibitor of TF-fVIIa and of prothrombinase. Under these conditions, hemostasis occurs through activation of fIX by TF-VIIa, which amplifies fXa generation such that TFPI inhibition of clotting is overcome. B) When TFPI activity is blocked, sufficient thrombin can be generated for hemostasis without requiring amplification by fVIII/fIX, thereby providing the rationale for developing inhibitors of TFPI to treat hemophilia.