Tissue Factor Pathway Inhibitor
Multiple Anticoagulant Activities for a Single Protein
Alan E. Mast

Abstract—Tissue factor (TF) 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 TFPIβ isoform localizes to the endothelium surface where it is a potent inhibitor of TF–factor VIIIa complexes that initiate blood coagulation. The TFPIα isoform is present in platelets. TFPIα contains a stretch of 9 amino acids nearly identical to those found in the B-domain of factor V that are well conserved in mammals. These amino acids provide exosite binding to activated factor V, which allows for TFPIα 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. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI:10.1161/ATVBAHA.115.305996)

Key Words: factor V ■ factor VII ■ hemophilia A ■ lipoprotein-associated coagulation inhibitor ■ thromboplastin

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 A). The proteins of the blood clotting pathways include 3 major cofactors, tissue factor (TF), factor Va (fVa), and factor VIIIa, that each combines with a target serine protease in a manner that greatly increases the speed and specificity of the protease for its biological substrate (Figure A). These cofactor proteins are essential for normal hemostasis, and the absence of any one causes severe bleeding. TF is expressed by cells within the tissues surrounding the vasculature (within the tissue), but it is not typically present within the blood.1 It tightly binds to the plasma protease factor VIIa (fVIIa). The TF–fVIIa catalytic complex initiates blood coagulation by activating factor X (fX) and factor IX (fIX).3 fVa is a cofactor protein for fXa.4 These 2 proteins combine in the presence of calcium ions on a phospholipid surface to form the prothrombinase complex that rapidly converts prothrombin to thrombin.6 Factor VIIIa is a cofactor protein for fIXa. Factor VIIIa and fIXa combine form an enzymatic complex that rapidly converts fIX to fXa.7 Deficiency of fVIII or fIX produces hemophilia A or hemophilia B, respectively. Because these 2 proteins act together in 1 enzymatic complex, deficiency of either one causes similar symptoms such that laboratory testing is required to determine the type of hemophilia present in an individual patient.

See Insight Into Alan E. Mast on page 6

Received on: September 24, 2015; final version accepted on: November 9, 2015.
From the Blood Research Institute, BloodCenter of Wisconsin, Milwaukee; and Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee.
Correspondence to Alan E. Mast, MD, PhD, Blood Research Institute, BloodCenter of Wisconsin, PO Box 2178, Milwaukee, WI 53201. E-mail alan.mast@bcw.edu
© 2015 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.115.305996
discussed here. TFPI is also produced in 2 minor isoforms, TFPIα20 and TFPIβ.21 Diagrams of the structures of all the TFPI isoforms are presented by Maroney et al.21 TFPIα contains an acidic N-terminal region followed by 3 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 TFPIα tightly bind some forms of fVa allowing it to inhibit prothrombinase.14 TFPIα is secreted by human endothelial cells.24 is present in human plasma, and binds human endothelium. Endothelial binding is likely mediated by electrostatic interactions with cell surface glycosaminoglycans because the plasma TFPIα concentration promptly increases 2- to 4-fold on heparin infusion.25,26 TFPIα is also produced by megakaryocytes27 and is present within quiescent platelets but not on their surface.28,29 Interestingly, platelet TFPIα is not localized within the platelet α-granules or lysosomes, and its intracellular location is not clear.30 After platelet activation, TFPI is externalized, with a portion released as a soluble protein and a portion associated with the surface of the activated platelet.27,28 Murine TFPIβ differs from its human counterpart; in that, it is not produced by endothelial cells and is not present in plasma.28 Murine platelet TFPIβ is similar to human platelet TFPIα in regard to the characteristics of its storage within the platelet and release after platelet activation.31 TFPIβ contains the first 2 identical Kunitz domains present in TFPIα but has an alternatively spliced C-terminus that replaces K3 and basic C-terminal region present in TFPIα with a different amino acid sequence that encodes for attachment of a glycosylphosphatidylinositol anchor.31,32 This anchor binds TFPIβ to the surface of endothelial cells.30,33 TFPIβ 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 TFPIβ 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 an 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 seems that TFPI simultaneously inhibits fXa and TF–fVIIa immediately after fX is activated by TF–fVIIa.33 TFPIα and TFPIβ are both able to inhibit TF–fVIIa because both have the first 2 Kunitz domains that mediate the inhibitory activity. However, the differences in the C-terminal structures of the 2 proteins modify their individual inhibitory activity in unique ways. The basic C-terminal region of TFPIα enhances direct inhibition of fXa in amido-lytic assays, such that TFPIα inhibits fXa ≈50-fold better than TFPI-160, a soluble, altered form of TFPI similar to TFPIβ in that contains only K1 and K2.2637 TFPIα inhibits TF–fVIIa ≈3-fold better than TFPI-160 in fXa generation assays.37 These studies initially suggested that TFPIα is a more potent anticoagulant protein than TFPIβ. However, localization of TFPIβ to the cell surface via its glycosylphosphatidylinositol anchor greatly increases its inhibitory activity, such that it is a slightly better inhibitor of fXa and TF–fVIIa than TFPIα in

Figure. Blood coagulation pathway is a series of sequential proteolytic reactions that can be separated into intrinsic and extrinsic pathway. A, When tissue factor (TF) pathway inhibitor (TFPI) is present, it dampens activation of clotting through the extrinsic pathways by serving as a factor Xa (fXa)–dependent inhibitor of TF–factor VIIa (fVIIa) and of prothrombinase. Under these conditions, hemostasis occurs through activation of factor IX (fIX) by TF–fVIIa, which amplifies fXa generation such that TFPI inhibition of clotting is overcome. B, When TF activity is blocked, sufficient thrombin can be generated for hemostasis without requiring amplification by factor VIII/fIX, thereby providing the rationale for developing inhibitors of TFPI to treat hemophilia.
these assays. Further in vitro studies of TFPIβ have shown that it is a potent inhibitor of TF-mediated cellular migration through matrices that are not permeable to soluble forms of TFPI, although in vivo studies have shown that TFPIβ inhibits TF-mediated cellular migration into murine lung after tail vein injection and prevents development of a consumptive coagulopathy. Therefore, TFPIβ is a highly effective inhibitor of TF-mediated coagulation and cell signaling events that produce cellular migration.

Inhibition of Prothrombinase by TFPI

TFPIα inhibits early forms of prothrombinase, the coagulation complex of fXa and fVa that rapidly converts prothrombin to thrombin. The inhibitory mechanism requires the basic C-terminal region of TFPIα, and therefore, prothrombinase inhibition is an isofrom-specific activity of TFPI that is not performed by TFPIβ. fV has a large B-domain of >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. Proteolytic removal of either the basic or the acidic region of the B-domain produces a fully procoagulant form of fVα. Proteases that cleave within the IV B-domain include fXa that rapidly removes the basic region but only slowly removes the acidic region and thrombin that rapidly removes the entire B-domain. In addition, platelet α-granules contain forms of fVα that lack the basic region of the B-domain but retain the acidic region. The C-terminus of TFPIα and the basic region of the fV-B-domain both contain the amino acid sequence LIKT followed by 5 basic amino acids (TFPI: LIKTKRKRK; fV: LIKTRKKK). Early in the blood coagulation cascade, before thrombin is produced, prothrombinase assembles with forms of fVα that retain the acidic region of the B-domain. These early forms of prothrombinase tightly bind to the basic region of TFPI allowing for rapid inhibition of prothrombinase that is directly mediated by binding of K2 to the active site of fXa. 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 TFPIα only inhibits prothrombinase during the initial stages of blood coagulation, perhaps preventing full procoagulant responses to subthreshold stimuli that would otherwise occlude blood vessels.

Clinical Relevance for TFPI Inhibition of Prothrombinase

TFPIα is the only endogenous protein recognized that inhibits prothrombinase at physiologically relevant rates and protein concentrations. The prothrombinase inhibitory activity of TFPIα is independent of its TF–fVIIa inhibitory activity. Identifying and characterizing the biochemical mechanism for inhibition of prothrombinase during the initiation of coagulation by TFPIα are directly relevant to understanding the pathogenesis of bleeding disorders and thrombotic disease. Three examples where the inhibition of prothrombinase by TFPIα 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 after trauma or surgery. 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 initially thought that it might be related to the disease phenotype. Subsequent biochemical studies performed 10 years after identification of the genetic anomaly found that the A2440G mutation causes production of an 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 TFPIα. The fV-short binds tightly to TFPIα. This stabilizes circulating TFPIα, such that patients with east Texas fV have an ≈10-fold increase in plasma TFPIα. The TFPIα–fV-short complexes potently inhibit coagulation 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.

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. Heparin binding greatly increases the inhibitory activity of these proteins toward several blood coagulation proteases, including thrombin and fXa. However, in plasma lacking antithrombin and heparin cofactor II, heparin has paradoxical procoagulant activity. 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 TFPIα 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. Fucoidan is another large, negatively charged polysaccharide that also blocks the inhibition of prothrombinase by TFPIα, providing 1 plausible explanation for its therapeutic efficacy in a canine model of hemophilia A.

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. Pharmacological agents that block TFPI activity are currently under development for treatment of hemophilia. Blocking TFPI, or inhibiting the inhibitor, as a therapeutic strategy has several advantages compared with 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 F IX, 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 TFPIα as the TFPI isoform produced by platelets and definition of the biochemical mechanism for inhibition of prothrombinase by TFPIα 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. Although this was an expected finding, these experiments had another 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 TFPIα sequestered within platelets and released at the site of the tail wound had to be inhibited to obtain an optimal hemostatic response from the antibody. This idea was tested by producing mice with hemophilia A that lacked platelet TFPI 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 TFPIα, suggesting that platelet TFPIα modulates bleeding in hemophilia. These data also suggest that anti-TFPI agents designed to specifically block platelet TFPIα would improve hemostasis in patients with hemophilia without altering the anticoagulant properties of TFPIβ 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 2 primary isoforms, TFPIα and TFPIβ. TFPIα is a soluble protein secreted by endothelial cells and activated platelets. TFPIβ is a glycosphosphatidylinositol–anchored protein expressed primarily on the surface of endothelium. Although TFPIα and TFPIβ are both capable of inhibiting TF–fVIIa activity, TFPIβ 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. TFPIα but not TFPIβ inhibits prothrombinase. The inhibitory mechanism for prothrombinase inhibition by TFPIα 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.

Sources of Funding
This work was supported by grant R01 HL088353 from the National Heart, Lung, and Blood Institute of the National Institutes of Health.

Disclosures
A.E. Mast received research grant funding from Novo Nordisk.

References


**Significance**

Tissue factor (TF) pathway inhibitor (TFPI) dampens early intravascular procoagulant activity thereby minimizing the development of occlusive thrombus and consumptive coagulopathy. TFPI is an alternatively spliced protein with 2 primary isoforms in humans, TFPIα and TFPIβ. The TFPI isoforms block early events in blood coagulation through 2 separate mechanisms: (1) inhibition of TF–factor VIIa by Kunitz domains 1 and 2, which is mediated by TFPIβ on endothelium; and (2) the inhibition of early forms of prothrombinase by Kunitz domain 2 and the C-terminal region of TFPIα released from within platelets. The inhibition of prothrombinase by TFPIα is recently recognized. Further characterization of prothrombinase inhibition by TFPIα will generate important new knowledge of human bleeding and thrombotic disorders and potentially lead to targeted new therapies.
ATVB Named Lecture Reviews
—Insight Into Author

ATVB Named Lecture Reviews—2015 Jeffrey M. Hoeg
ATVB Award for Basic Science and Clinical Research

Insight Into the Author: Alan E. Mast, MD, PhD, Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI

Why did you choose the profession of scientific investigation?
I have always been curious. I enjoy figuring out new things.

Who have been your role model(s) in your scientific and professional life?
I have had many scientific mentors that I have learned from over the years. Guy Salvesen, my PhD mentor, taught me how to approach scientific questions and enjoy the wonder of working in the laboratory.

What have been important influences on your professional life?
My father was a professor of electrical engineering and taught me about a career in academics.

What are your scientific inspirations?
To ask and answer questions that help put the pieces of a scientific puzzle together.

How have mentors contributed to your professional development?
During my residency, Sam Santoro encouraged me to continue doing clinical as well as basic science projects during my career. This was really good advice and has allowed me to approach a wide diversity of scientific questions during my professional career.

If you knew then what you know now, would you do anything different?
I would have started doing triathlons when I was younger.

What wisdom do you impart on new investigators?
Patient hard work over time leads to success.

If you were not a scientist, which profession would you pick?
A National Park Ranger.

Which direction do you envisage your science taking?
I think we will begin to collaborate with more scientists due to the complex nature of the questions we are beginning to ask.

What are your nonscientific activities?
I enjoy exercising, hiking in the mountains, and have a tank with tropical fish.

What sports do you follow?
I have followed University of Illinois sports since I was 6 years old when my mother would let me have the radio next to my bed to listen to basketball games. I also have become a Green Bay Packer fan since moving to Wisconsin.

What are your favorite books, movies, music (pick one or all)?
I listen to country music; for many songs, I enjoy the silly lyrics more than the music. I enjoy reading about American history.

What are you favorite foods and are they heart healthy?
M&Ms. They are like vitamin pills, so they have to be heart healthy.
Tissue Factor Pathway Inhibitor: Multiple Anticoagulant Activities for a Single Protein
Alan E. Mast

Arterioscler Thromb Vasc Biol. published online November 24, 2015;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2015/11/24/ATVBAHA.115.305996

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/