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Cellular expression and biological activities of alternatively spliced forms of tissue factor pathway inhibitor

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Abstract

Purpose of review—Tissue factor pathway inhibitor (TFPI) is an anticoagulant protein that inhibits tissue factor–factor VIIa (TF–fVIIa) and factor Xa (fXa). Recent studies revealed distinct cellular expression patterns for TFPI and TFPI and spurred additional experiments to define unique functions for these alternatively spliced TFPI isoforms.

Recent findings—TFPI is produced by endothelial cells, localizes to an intracellular granule, and is released following cellular stimulation with thrombin or heparin. TFPI also is produced by megakaryocytes and released from activated platelets. Platelet TFPI limits clot growth following vessel injury and alters bleeding in hemophilia, suggesting that its primary physiological role is modulation of clot development. TFPI is made by endothelial cells, localizes to the endothelium surface, and is not in platelets. TFPI is an effective inhibitor of TF-mediated cellular migration and may act to dampen the adverse effects of intravascular TF expressed during inflammation.

Summary—Knowledge of TFPI isoform expression and activity provides new insights into the biochemical regulation of TF-mediated thrombotic and inflammatory disease. Recent findings have therapeutic implications for use of recombinant TFPI to treat severe sepsis in community-acquired pneumonia or to achieve improved engraftment of hematopoietic stem cells, and for development of TFPI-blocking pharmaceuticals to treat hemophilia.

Keywords

coagulation; hemophilia; tissue factor; tissue factor pathway inhibitor

INTRODUCTION

Tissue factor pathway inhibitor (TFPI) is the major physiological inhibitor of the tissue factor–factor VIIa (TF–fVIIa) complex that initiates blood coagulation [1]. TFPI dampens the onset of intravascular coagulation by providing hemostatic balance for TF expressed within the vasculature, thereby preventing vessel occlusion, disseminated intravascular coagulation, and other vascular-related diseases. The key role of TFPI in the regulation of TF-initiated blood coagulation is demonstrated in studies of genetically altered mice. Deletion of TFPI function results in embryonic lethality [2], but the lethality is subverted by breeding TFPI deficiency into mice with decreased TF activity [3]. Recent advances in our

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Conflicts of interest

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understanding of the cellular expression and physiological functions of the two major TFPI alternatively spliced isoforms, TFPI₁ and TFPI₂, are reviewed in this article.

REGULATION OF TISSUE FACTOR PATHWAY INHIBITOR EXPRESSION

TFPI is constitutively expressed in the endothelium. Although TF expression is readily upregulated by a variety of inflammatory mediators [4–6], alteration in TFPI₁ or TFPI₂ expression has not been consistently demonstrated [7,8]. A recent study using gene expression profiling to compare normal fibroblasts and fibroblasts lacking p21-activated kinase 1 (PAK1) found that the absence of PAK1 produces 3.46-fold upregulation of TFPI mRNA and 2.53-fold downregulation of TF mRNA [9]. These studies suggest inflammatory signaling pathways utilizing PAK1 may regulate intravascular expression of TF and TFPI, however further studies are needed to define these pathways and their physiological relevance. At present, available data suggest that endothelial TFPI inhibits small amounts of TF expressed within the vasculature, but strong inflammatory stimuli lead to expression of TF sufficient to overwhelm the TFPI present.

In contrast to inflammatory mediators that demonstrate variable effects on TFPI expression [10,11], estrogen consistently decreases plasma TFPI. Women on hormone replacement therapy or oral contraceptives have an approximately 25% decrease in plasma TFPI [12]. Use of estrogen therapies is strongly associated with increased risk for venous thromboembolism [13], but it is unclear how decreased plasma TFPI relates to this risk. Several heritable thrombophilias increase a woman's risk for thrombosis when combined with estrogen therapy. Of significance, estrogen use by women with the factor V Leiden (FVL) mutation, an altered form of factor V resistant to degradation by activated protein C, have thrombotic risk about five times that of those with isolated FVL or estrogen use [14]. Additionally, studies of genetically altered mice have demonstrated that combined FVL and heterozygous TFPI deficiency produce severe perinatal thrombosis [15]. Thus, it is tempting to speculate that the estrogen-mediated decrease in plasma TFPI synergizes with FVL to produce the greatly increased risk for thrombosis present in women with FVL using estrogen. Although the interactions between estrogen therapies, TFPI, and thrombotic risk are medically important, the biological regulation of TFPI by estrogen likely involves maintenance of hemostatic balance between TF and TFPI during embryonic development. TF and TFPI are key regulators of hemostasis in embryogenesis, angiogenesis, placental implantation, and pregnancy [16]. Interestingly, placental trophoblasts are directly exposed to blood and constitutively express both TF and TFPI [16,17]. Further studies are needed to correlate the biochemical regulation of TFPI expression by estrogen and pregnancy outcomes.

ALTERNATIVE SPLICING OF TISSUE FACTOR PATHWAY INHIBITOR

TFPI is a Kunitz-type serine protease inhibitor with several isoforms produced by alternative splicing [18,19]. Humans produce three isoforms, TFPI₁, TFPI₂, and TFPI₃. Mice also produce three isoforms, TFPI₁, TFPI₂, and TFPI₃. Splicing events within the 3' region of the mRNA generate these isoforms, which differ in their C-terminal regions. Details of the RNA splicing patterns and the resulting proteins have been reviewed previously [20]. Production of TFPI₁ and TFPI₂ proteins has not been demonstrated *in vivo* and their physiological functions remain unclear. Therefore, this review focuses on TFPI₁ and TFPI₂, which are the known physiologically expressed proteins. TFPI₁ has three tandem Kunitz-type domains (K1, K2, and K3) followed by a basic C-terminus [21]. Identical K1 and K2 domains are found in TFPI₂, but are followed by a stretch of amino acids encoding a glycosylphosphatidylinositol (GPI)-anchor attachment sequence [18,22].

TISSUE FACTOR PATHWAY INHIBITOR α AND TISSUE FACTOR PATHWAY INHIBITOR β INHIBIT TISSUE FACTOR-INITIATED BLOOD COAGULATION

TFPI anticoagulant function is mediated by K1 and K2. K2 directly inhibits fXa, and in a fXa-dependent manner, K1 inhibits TF–fVIIa immediately following activation of fX [1,23]. Thus, TFPI is the major physiologically relevant direct inhibitor of TF-initiated blood coagulation [3]. As TFPI α and TFPI β have K1 and K2, both isoforms inhibit TF-initiated blood coagulation. However, differences in their C-terminal regions alter their respective inhibitory activity [24].

K3 and the C-terminal region of TFPI β do not have apparent direct protease inhibitor activity; however they are well conserved back to zebra fish suggesting an important biological function [25]. The fXa inhibitory activity of TFPI β increases approximately 20-fold in the presence of K3 and the C-terminus [24,26–28]. FXa inhibition is further enhanced by the binding of K3 to protein S [29], an interaction that requires Arg199 and Glu226 of K3 [30,31]. Additionally, plasma concentrations of these two proteins correlate in healthy individuals and fall in tandem in individuals treated with oral contraceptives [32,33,34]. TFPI β –protein S anticoagulant activity is observed in plasma clotting assays initiated with low TF (1 pmol/l) concentrations, but not at higher (14 pmol/l) concentrations [35,36]. However, it has been proposed that in the presence of activated protein C, sufficient dampening of clot propagation occurs to allow for inhibition of high concentrations of TF by TFPI β –protein S [36]. Additional biochemical studies are needed to define the complex interactions between TFPI β , protein S, and other anticoagulant proteins in plasma.

TFPI α lacks the K3 domain and does not bind protein S. Instead, TFPI α directly links to the cell surface through a GPI-anchor [22], which is critical for its inhibition of coagulation proteases. Soluble, altered forms of TFPI containing only K1 and K2 domains demonstrate significantly reduced fXa inhibitory activity [26–28], suggesting that TFPI α may have greatly reduced anticoagulant activity *in vivo* when compared with TFPI β . However, recent data indicate that cell surface TFPI α is an equal, or slightly more effective, inhibitor of TF–fVIIa and fXa than is TFPI β [24]. TFPI α localizes to lipid rafts and caveolae on the cell surface through its GPI-anchor [37,38]. This localization enhances TFPI α anticoagulant activity by increasing its ability to inhibit TF–fVIIa, while having no effect on its fXa inhibitory activity [39,40]. The expression and activity of TFPI α may be further regulated by a protein called androgen-dependent TFPI-regulating protein (ADTRP) [41]. ADTRP, identified through expression microarray studies, coexpresses with TFPI and caveolae under a variety of conditions. It will be important to define the biological impact of the interaction between TFPI and ADTRP using *in-vivo* studies.

TISSUE FACTOR PATHWAY INHIBITOR AND OTHER ANTICOAGULANT PROTEINS

TFPI holds a unique position as an inhibitor of the initiation phase of the TF pathway during the clotting process, with endothelial and platelet TFPI apparently acting as very early inhibitors of clot formation. Other endogenous inhibitors appear to act further ‘downstream’ in the blood coagulation pathway. The serpins, antithrombin, heparin cofactor II and protein Z-dependent protease inhibitor, which circulate in plasma, and thrombomodulin, which is a transmembrane protein on endothelium that acts through activation of protein C, appear to inhibit the later amplification mechanisms of clot formation. In addition to acting at different stages of the coagulation process, tissue-specific expression of the endothelium-associated anticoagulant proteins, TFPI and thrombomodulin, may differentially regulate clotting in vascular beds. For example, mice with decreased thrombomodulin function do not produce

fibrin deposition within the brain even following treatment with lipopolysaccharide [42]. However, breeding heterozygous TFPI deficiency into these mice induces intravascular fibrin deposition in the brain suggesting that TFPI and thrombomodulin have distinct anticoagulant functions within the brain vasculature [43].

TISSUE FACTOR PATHWAY INHIBITOR α AND TISSUE FACTOR PATHWAY INHIBITOR β HAVE DISTINCT CELLULAR EXPRESSION PATTERNS

Intravascular TFPI is produced primarily by endothelial cells [44] and megakaryocytes [45], although monocytes also produce small amounts [46]. Recent studies found that TFPI and TFPI are distinctly expressed by platelets and endothelial cells, respectively [25,45,47]. TFPI is the only isoform made by megakaryocytes and is found within quiescent platelets but not on their surface [45]. The location of TFPI within platelets is not known, as it does not colocalize with resident proteins of α -granules or lysosomes [45]. Stimulation of platelets by thrombin releases soluble TFPI [48], while dual stimulation by thrombin and collagen results in localization of TFPI to the platelet surface as well as release in soluble form [45]. TFPI is the predominant isoform expressed in vascular beds of all major murine tissues and is expressed on the surface of cultured endothelial cells [25,47]. Cultured endothelial cells also produce TFPI, which is secreted into conditioned media [49]. In addition, TFPI localizes to a poorly defined intracellular granule in cultured endothelial cells and is released from the cells following stimulation with thrombin or heparin [49–51]. The plasma concentration of TFPI increases approximately four-fold following heparin infusion in humans [52,53]. This heparin-releasable TFPI has been attributed to a pool that is nonspecifically bound to endothelium glycosaminoglycans, however its relationship to the heparin-releasable pool of TFPI from granules of cultured endothelial cells is not known. Such distinct expression patterns suggest that TFPI and TFPI have unique anticoagulant functions, which our laboratory and others have sought to define using cellular and murine model systems.

PHYSIOLOGICAL FUNCTION OF TISSUE FACTOR PATHWAY INHIBITOR α

In contrast to humans, TFPI is not found in the plasma of mice [25]. Thus, murine models are optimal for studies to identify specific functions of platelet TFPI. Massberg *et al.* [54] demonstrated that degradation of platelet TFPI by neutrophil serine proteases promotes coagulation *in vitro*, suggesting that it is an important hemostatic regulator. This was confirmed using in-vivo studies of clot development in mice deficient in neutrophil elastase or cathepsin G, which demonstrated that neutrophil serine proteases degrade TFPI *in vivo*. Furthermore, TFPI degradation was enhanced by negatively charged extracellular nucleosomes released from neutrophils; this binding interaction likely requires the positively charged C-terminus of TFPI released from platelets or endothelial cells at the site of vascular injury, not occurring with mouse plasma TFPI, which lacks this region.

Our laboratory investigated the physiological functions of platelet TFPI through study of mice lacking hematopoietic cell TFPI. These adult mice were generated by lethal irradiation followed by rescue with transplantation of TFPI-null fetal liver cells [55,56], which contain hematopoietic precursor cells sufficient to repopulate the bone marrow and produce mice lacking TFPI in platelets and monocytes. Platelet TFPI deficiency was predicted to impact thrombosis and hemostasis as platelets actively accumulate at vascular injury sites where they secrete TFPI [45,48]. The lack of hematopoietic cell TFPI did not significantly alter the plasma TFPI concentration [55], suggesting that endothelial cells, or an unidentified cell [57], are sources of plasma TFPI in mice. The plasma concentration of thrombin–antithrombin complex also was unaltered, indicating these mice do not have a generalized prothrombotic state [55]. However, when subjected to large vessel venous or

arterial injury, significantly larger clots developed than in control mice transplanted with fetal liver cells from wild-type embryos. Interestingly, clot enlargement was due to platelet accumulation rather than increased fibrin formation, suggesting that platelet TFPI acts as an important modulator of platelet activation or aggregation within a growing thrombus [55] (Fig. 1).

TFPI₂, but not TFPI₁, binds to glypican-3, a protein that is not produced by endothelial cells [58]. Khurana *et al.* [59] recently found that TFPI₂ is an effective inhibitor of the ectopeptidase CD26 when bound to glypican-3. They further demonstrated that in-vitro treatment of hematopoietic stem cells with TFPI₂ improved homing and engraftment in bone marrow following transplantation through a mechanism that involves reduced inactivation of CXCL12 by CD26. Additional studies are needed to confirm and biochemically characterize the inhibition of CD26 by the TFPI₂-glypican-3 complex.

EFFECT OF TISSUE FACTOR PATHWAY INHIBITOR ON HEMOPHILIA BLEEDING

Several studies have demonstrated that therapeutic agents designed to block TFPI activity effectively attenuate bleeding in animal models of hemophilia [60–63]. These therapies hold promise for a novel treatment of both hemophilia A and B that will not require intravenous infusion of purified factor VIII (fVIII) or factor IX (fIX) to prevent spontaneous bleeding in patients with severe disease. The biochemical mechanism of anti-TFPI therapeutic agents is as follows [64]. TFPI exerts anticoagulant activity by inhibiting TF–fVIIa and fXa thereby promoting coagulation to proceed through activation of fIX. Therapeutically, blocking TFPI inhibitory activity, or ‘inhibiting the inhibitor’, allows for TF-initiated coagulation to proceed directly through activation of fX, bypassing the need for fVIII or fIX to obtain hemostasis [64]. Our laboratory, in collaboration with investigators at Novo Nordisk, has used murine model systems of TFPI and fVIII deficiency to investigate how targeting different structural regions of TFPI may alter bleeding in hemophilia [56]. In breeding studies, total deficiency of fVIII did not rescue the embryonic death of TFPI-null mice. Thus, it appears that unfettered activation of fX by TF–fVIIa, as occurs in the absence of TFPI, produces an extensive coagulopathy that does not require further amplification by the intrinsic coagulation pathway, and leads to embryonic death. Interestingly, in experiments using adult fVIII-deficient mice, infusion of anti-TFPI antibody at concentrations beyond that necessary to completely inhibit plasma TFPI continued to decrease tail-bleeding times, suggesting that platelet TFPI released at the injury site promotes bleeding. This was confirmed by demonstrating fVIII-deficient mice lacking hematopoietic cell TFPI, but with normal endothelial cell TFPI, had decreased tail-bleeding. These findings implicate platelet TFPI as a primary physiological regulator of bleeding in hemophilia. Thus, development of therapeutic agents specifically targeting platelet TFPI₂, but not TFPI₁ on endothelium, may provide hemostasis in hemophilia without increasing the risk for generalized thrombosis that may result from total inhibition of intravascular TFPI.

PHYSIOLOGICAL FUNCTION OF TISSUE FACTOR PATHWAY INHIBITOR β

A Chinese hamster ovary (CHO) cell model system in which TF and TFPI are expressed on the cell surface (CHO-TF/TFPI) was used to investigate the biological activity of TFPI [24]. As CHO cells do not produce TFPI₂, TFPI₁ on the CHO cell surface could be accurately measured, allowing for quantitative comparison of TFPI₁ and TFPI₂ inhibitory activities. The expression of TF on CHO cells greatly promoted their migration through Matrigel, a gelatinous protein mixture produced by a murine tumor containing over 1851 proteins, including coagulation factors II, VII and X. Coexpression of TFPI₁ (21 pmol/l per well) with TF on CHO cells inhibited cellular migration by 83%. In contrast, soluble TFPI

(100 nmol/l per well) inhibited the migration of CHO cells solely expressing TF (CHO-TF) by only 30%. Thus, TFPI is a highly effective inhibitor of the TF-mediated cell signaling events required for cellular migration through matrices impermeable to soluble forms of TFPI. A TF-mediated murine model of tissue infiltration was used to further investigate the biological inhibitory activity of TFPI. In this model CHO, CHO-TF, or CHO-TF/TFPI cells were injected into the tail vein of severe combined immunodeficient mice. Cellular infiltration into the lungs and circulating platelet counts were assessed 10 days later. The lung parenchyma and platelet count remained normal following injection of CHO cells. However, injection of CHO-TF cells produced large sheets of cells within the lungs, fibrin clots within the vasculature, and a large decrease in platelet count, indicating the presence of a consumptive coagulopathy. Injection of CHO-TF/TFPI cells significantly reduced cellular infiltration within the lungs and maintained normal platelet counts. Thus, TFPI effectively inhibits TF-mediated coagulation and cellular signaling events when both proteins are expressed on the same surface, as occurs on endothelial cells, monocytes or tumor cells within the vasculature, while soluble TFPI is a relatively poor inhibitor (Fig. 1). These findings have important implications for therapeutic use of TFPI, which is being considered for treatment of severe sepsis, associated with community-acquired pneumonia [65], and is consistent with the high TFPI concentration (9 μ mol/l) needed for therapeutic efficacy in a murine model of pneumococcal pneumonia [66].

CONCLUSION

Recent advances in our understanding of the cellular expression of TFPI and TFPI suggest the isoforms may function as two independent anticoagulant proteins with distinct biological activities. Currently, it appears that TFPI is released from endothelial cells and platelets and acts primarily to regulate clot development and hemostasis following vascular injury. TFPI is constitutively expressed on endothelium and serves primarily to dampen the procoagulant potential of intravascular TF expressed in inflammatory conditions. Additional biochemical and physiological studies of TFPI function are needed to further test and define these proposed roles.

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of special interest

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 478).

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KEY POINTS

- TFPI is the only physiological inhibitor of TF–fVIIa initiated coagulation, counterbalances TF-mediated cellular processes, and is required for viable gestation and birth.
- The two primary alternatively spliced isoforms of TFPI, TFPI₁ and TFPI₂, have unique cellular expression patterns and physiological roles.
- Endothelial cells and megakaryocytes release TFPI₂, which regulates clot growth and hemostasis.
- TFPI₁ is made in the endothelium and localizes to lipid rafts at the cell surface through a GPI-anchor, which enables it to optimally inhibit TF-mediated coagulation and cell migration.
- Due to its role in regulating localized hemostasis, platelet TFPI₂ is a potential therapeutic target for treatment of severe hemophilia.

FIGURE 1.

A simplified model describing potentially distinct functions for tissue factor pathway inhibitors TFPI α and TFPI β . TFPI α (left side of diagram) is released from activated platelets upon vascular injury and dampens thrombin generation, which minimizes further accumulation of platelets at the site of vascular injury. TFPI β (right side of diagram) is expressed constitutively on endothelium and inhibits tissue factor–factor VIIa (TF–fVIIa) activity produced following inflammatory stimuli at the endothelial surface. This then dampens thrombin generation and subsequent activation of protease-activated receptors (PAR).