Thrombin Activatable Fibrinolysis Inhibitor and an Antifibrinolytic Pathway

Laszlo Bajzar

Abstract—Coagulation and fibrinolysis are processes that form and dissolve fibrin, respectively. These processes are exquisitely regulated and protect the organism from excessive blood loss or excessive fibrin deposition. Regulation of these cascades is accomplished by a variety of mechanisms involving cellular responses, flow, and protein-protein interactions. With respect to regulation mediated by protein-protein interaction, the coagulation cascade appears to be more complex than the fibrinolytic cascade because it has more components. Yet each cascade is regulated by initiators, cofactors, feedback reactions, and inhibitors. Coagulation is also controlled by an anticoagulant pathway composed of (minimally) thrombin, thrombomodulin, and protein C.1 Protein C is converted by the thrombin/thrombomodulin complex to activated protein C (APC), which catalyzes the proteolytic inactivation of the essential cofactors required for thrombin formation, factors Va and VIIIa. An analogous antifibrinolytic pathway has been identified recently. This pathway provides an apparent symmetry between coagulation and fibrinolysis and is also composed of thrombin, thrombomodulin, and a zymogen that is activated to an enzyme. The enzyme proteolytically inactivates a cofactor to attenuate fibrinolysis. However, unlike APC, which is a serine protease, the antifibrinolytic enzyme is a metalloprotease that exhibits carboxypeptidase B–like activity. Within a few years of each other, 5 groups independently described a molecule that accounts for this antifibrinolytic activity. We refer to this molecule as thrombin activatable fibrinolysis inhibitor (TAFI), a name that is based on functional properties by which it was identified, assayed, and purified. (Because of the preferences of some journals “activatable” is occasionally referred to as “activable.”) This review will encompass a historical account of efforts to isolate TAFI and characterize it with respect to its activation, activity, regulation, and potential function in vivo. (Arterioscler Thromb Vasc Biol. 2000;20:2511-2518.)

Key Words: coagulation ■ fibrinolysis ■ thrombosis ■ thrombolysis ■ risk factors

Discovery of TAFI

Our initial efforts were directed toward understanding the apparent profibrinolytic effect of the anticoagulant APC, which had been reported previously.2,5 In the work of Taylor and Lockhart,2 the modest acceleration of fibrinolysis evoked by APC in a cell-free system was more pronounced in clots formed from either whole blood or platelet-poor plasma supplemented with leukocytes. Other investigators showed that APC can consume plasminogen activator inhibitor-1 (PAI-1), thereby sparing tissue plasminogen activator (tPA), and this was offered as an explanation for the apparent profibrinolytic properties of APC.4,6 Consumption of PAI-1 by APC, however, is a kinetically unfavorable reaction when it is compared with inhibition of tPA by PAI-1.7 Alternative explanations included APC inhibition of thrombin production, which would otherwise inhibit fibrinolysis either by the stimulation of PAI-1 release from platelets8 or by some unknown mechanism.9

We investigated the profibrinolytic effect of APC by use of a clot lysis assay that was based on a decrease in turbidity that occurs during fibrin dissolution. The time required to attain the transition midpoint in the reduction of turbidity, associated with fibrin degradation, was defined as “lysis time.” In this assay, inclusion of APC decreased the lysis time of cell-free plasma clotted in the presence of tPA, thrombin, and Ca²⁺ ion,10 consistent with previous studies.2,4 However, the profibrinolytic effect of APC was observed in the absence of platelets and leukocytes, indicating that these cells are not obligatory, and the existence of a mechanism independent of cells was inferred. This mechanism was found to be dependent on the ability to generate thrombin during fibrinolysis. If thrombin formation was facilitated by prothrombinase, lysis time was prolonged in a manner in which APC was able to potentiate fibrinolysis.10 Activation of prothrombin with a protease from Echis carinatus venom, which is not inhibited by APC, also prolonged lysis time but was unaffected by APC.11 Activation of prothrombin generates a variety of products in addition to thrombin, including fragments 1, 2, 1.2, Pre1, Pre2, and meizothrombin, any or all of which could mediate the inhibition of fibrinolysis. Thrombin and meizothrombin, an enzymatically active transient intermediate, were subsequently identified as the products of prothrombin

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From the Hamilton Civic Hospitals Research Centre and McMaster University, Hamilton, Ontario, Canada.
Correspondence to Laszlo Bajzar, PhD, HCHRRC, 711 Concession St, Hamilton, ON, Canada L8V 1C3. E-mail lbajzar@thrombosis.hhscr.org
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The gene for TAFI maps to 13q14.1116 and contains 11 exons within 48 kb of DNA.17 Similarity of the intron/exon boundaries, compared with rat carboxypeptidases and human mast cell carboxypeptidase A, suggests that they originate from a common ancestral gene.17 The 5′-flanking region contains no TATA sequence but includes an ∼70-base sequence that confers liver-specific transcription. Transcripts may be initiated at multiple sites and polyadenylated at any of 3 sites.17 The transcript encodes a gene product of 423 amino acids (45 kDa); however, the protein migrates at 58 kDa in SDS-PAGE. The disparity in molecular mass can be accounted for by loss of a 22–amino acid signal peptide and potential glycosylation at any of 4 potential sites present in the protein. The disparity in molecular mass can be accounted for by loss of a 22–amino acid signal peptide and potential glycosylation at any of 4 potential sites present in the protein.18,19 At least 2 distinct isoforms exist: a silent base change (C to T) at 678 and a base change (A to G), which results in the substitution of alanine for threonine at residue 147; however, no functional differences between these isoforms have been described.20

**Nomenclature**

Because TAFI was purified by several groups, it has several names. The first references to a novel carboxypeptidase with the ability to remove C-terminal basic amino acids found in serum appeared almost simultaneously by 2 groups.21,22 Both groups used a colorimetric assay, with hippuryl-L-arginine or hippuryl-L-lysine hydrolysis, to infer the existence of a novel carboxypeptidase B–like enzyme. Unlike carboxypeptidase N (CPN), a constitutively active and “stable” enzyme in plasma exhibiting a selectivity for C-terminal lysine, the “novel carboxypeptidase” activity was found in serum but not plasma; it was unstable, exhibiting a half-life of ∼15 minutes at 37°C, and it was more specific for arginine than lysine.16,21–23 Although the novel carboxypeptidases identified...
Thrombin is a relatively poor activator of TAFI. Concentrations of thrombin approaching 500 nmol/L are required to activate TAFI within 10 minutes, as assessed by hippuryl-Arg hydrolysis. Nevertheless, quantitative activation of prothrombin to thrombin in clots formed from plasma appears to be required to prolong lysis time. However, TAFI activation occurs simultaneously with the formation of fibrin in whole blood. Although the 35-kDa species correlates with activity, measurements must be corrected for temperature-dependent spontaneous decay. Thrombin activation of TAFI exhibits a $K_m$ of 2.14 μmol/L and $k_{cat}$ of 0.0021 s$^{-1}$ and is potently stimulated by thrombomodulin. TAFI and thrombomodulin were varied systematically in the presence of thrombin and Ca$^{2+}$ to assess the effect of thrombomodulin on TAFI activation. Activation of TAFI conforms to Michaelis-Menten kinetics, and interpretation of the data indicates that thrombomodulin enhances the catalytic efficacy of thrombin-dependent TAFI activation by 1250-fold, primarily through an increase in $k_{cat}$. These values are strikingly similar to potentiation of thrombin-mediated activation of protein C by thrombomodulin, although significant competitive inhibition between these substrates would not be expected because of their low plasma concentrations relative to the $K_m$ for the reaction. The similarity in activation kinetic constants cannot easily be rationalized on the basis of sequence surrounding the cleavage site within each substrate. Aspartic acid residues at the P3 and P3’ positions are thought to decrease the ability of thrombin to cleave protein C, which is countered by thrombomodulin. However, the residues at these positions in TAFI are valine and alanine, respectively, suggesting a different mechanism for the enhancement by thrombomodulin of TAFI activation. This is further supported by the difference in structural requirements for thrombomodulin and thrombin with respect to protein C and TAFI activation. Potentiation of protein C activation by thrombomodulin requires endothelial growth factor (EGF)-like domains 4 to 6, whereas activation of TAFI requires EGF-like domains 3 to 6. Further refinement indicates that the minimum primary sequence of thrombomodulin required for TAFI activation is composed of residues of the c-loop in EGF-3 through to EGF-6 and is 13 residues longer than the primary structure required for efficient protein C activation. Furthermore, oxidation of Met388, present in the connecting peptide between EGF-5 and EGF-6, significantly reduces the rate of protein C, but not TAFI, activation. These data suggest that different elements of thrombomodulin, in complex with thrombin, interact with protein C and TAFI, turning them into better substrates. This notion is supported by the recent solution of the crystal structure of a soluble form of thrombomodulin in complex with thrombin, which does not indicate a structural rearrangement of the active site of thrombin. Computer-simulated docking of protein C to the thrombin/thrombomodulin complex suggests that the cleavage site within the substrate is optimally presented to the enzyme when bound to the complex. The corresponding crystal structure of thrombin in complex with a longer form of thrombomodulin should provide an analogous concept for thrombomodulin-dependent activation of TAFI. In addition to enhancing the activation of TAFI by thrombin, thrombomodulin confers a relatively poor activator of TAFI. Concentrations of thrombin approaching 500 nmol/L are required to activate TAFI within 10 minutes, as assessed by hippuryl-Arg hydrolysis. Indeed, quantitative activation of prothrombin to thrombin in clots formed from plasma appears to be required to prolong lysis time. However, TAFI activation occurs simultaneously with the formation of fibrin in whole blood. Although the 35-kDa species correlates with activity, measurements must be corrected for temperature-dependent spontaneous decay. Thrombin activation of TAFI exhibits a $K_m$ of 2.14 μmol/L and $k_{cat}$ of 0.0021 s$^{-1}$ and is potently stimulated by thrombomodulin. TAFI and thrombomodulin were varied systematically in the presence of thrombin and Ca$^{2+}$ to assess the effect of thrombomodulin on TAFI activation. 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modulin also increases the catalytic efficiency of meizothrombin-dependent activation of TAFI, which approaches 10% of that achieved by thrombin/thrombomodulin. However, the precise mechanism and structural requirements of thrombomodulin for this rate enhancement are unknown. Thrombin also contains distinct domains required to mediate activation of protein C and TAFI. Residues of thrombin important for TAFI, but not protein C, activation are located above the active site cleft, constituting E25, D51, and R89/R93/E94, whereas residues R178/R180/D183, E229, and R233, which are required for protein C but not TAFI activation, reside below the active-site cleft.

Despite the differences in structural requirements of thrombin and thrombomodulin, the activation of TAFI and of protein C is consistent with an equilibrium, enzyme central, parallel-assembly model. Accordingly, the enzyme, either thrombin or meizothrombin, may form a complex with either the substrate or thrombomodulin first. Addition of the third component, either thrombomodulin or substrate, forms the ternary substrate/thrombin/thrombomodulin complex, which catalyzes the activation of the substrate. The magnitude of the enhanced rates of TAFI activation by thrombin/thrombomodulin suggests that this complex is the physiological activator. This reaction can occur on soluble and on cellular thrombomodulin. Although cellular thrombomodulin is certainly capable and may predominate as the cofactor for potentiating TAFI activation in vivo, the presence of soluble fragments of thrombomodulin present in plasma may also affect TAFI-dependent prolongation of thrombolysis. Furthermore, inclusion of soluble thrombomodulin in clot lysis experiments prolongs lysis time. Because quantitative activation of prothrombin can occur within a thrombus, high concentrations of thrombin may also play a role in its activation. This possibility may rationalize the factor XI-dependent prolongation of lysis time and explain the variable incidence of bleeding diathesis presented by individuals deficient in factor XI. Should high concentrations of thrombin be important in the regulation of TAFI activation, then a paradox seems to exist, inasmuch as thrombomodulin appears to potentiate fibrinolysis, indirectly through protein C activation, and inhibit fibrinolysis, by directly potentiating TAFI activation.

Activation of TAFI by plasmin may also occur physiologically. Plasmin catalyzes the activation of TAFI with a $k_w$ of 0.00044 s$^{-1}$ and $K_m$ of 55 nmol/L, such that the catalytic efficiency of plasmin is 8 times that of thrombin. However, unfractionated heparin, among other polysaccharides, increases the $k_w$ and decreases the $K_m$ for plasmin-mediated activation of TAFI, such that its catalytic efficiency is only 1/10 that of thrombin/thrombomodulin. Therefore, plasmin may also effect TAFI activation, physiologically, especially in the presence of glycosaminoglycans, such as those found in the extracellular matrix. Interestingly, these data would suggest that therapeutic use of heparin may be a double-edged sword, simultaneously preventing fibrin deposition, by stimulating inhibition of coagulation, yet preventing fibrin dissolution, by stimulating TAFI-dependent inhibition of fibrinolysis.

**Inhibition of Fibrinolysis by TAFIa**

The concentration dependence of TAFIa on lysis time was assessed by adding various concentrations of TAFIa to a system composed of purified components. Lysis time increased with TAFI concentration, exhibiting a 3-fold prolongation at saturation (10 nmol/L). Fifty percent of the maximal prolongation occurred by 1.0 nmol/L TAFIa. Because the concentration of TAFI in plasma is $\approx 75$ nmol/L, it is likely that sufficient TAFI is available to effectively inhibit fibrinolysis. However, the plasma concentration of TAFI varies dramatically between individuals. Mosnier et al demonstrated that the concentration ranges from 38% to 169% of normal, depending on the assay, with an average of 275 nmol/L and that TAFI concentration positively correlates with lysis time. This average concentration is $\approx 3.5$ times that initially reported. In part, this may be due to the different methods each group used to determine the concentration of the TAFI standard. Use of BSA as a standard for determination of the standard TAFI concentration leads to at least a 2-fold underestimation of the molar concentration (author’s unpublished data, 2000) compared with the use of $\mu$L of 26.4 or 20.6 and a molecular mass of 45 and 58 kDa, respectively. Others report an average concentration of 113±13 (mean±SD) nmol/L TAFI antigen normally distributed in 948 individuals. A concentration of 964±153 (mean±SD) U/L has also been reported with use of an assay based on function; however, molar concentrations were not provided. It is generally agreed that the use of oral contraceptives correlates with increased TAFI concentration; however, the effects of age on TAFI concentration appear to be dependent on how concentrations are determined. For example, TAFI concentration does not increase with age in men when it is determined by ELISA, but it does when a functional assay is used. Should thrombin/thrombomodulin be the predominant activator, with the $K_m$ being $\approx 10$ times the normal plasma concentration of TAFI and protein C, it would be predicted that the rate of activation would be directly proportional to the plasma concentration over the range found in the population, implicating a role for TAFI in bleeding or thrombosis at aberrantly low and high concentrations, respectively.

It is well documented that C-terminal lysines on cell-surface proteins and partially degraded fibrin define a potent mechanism that enhances fibrinolysis by providing binding sites for plasminogen, which, on binding, adopts a more activatable conformation. With respect to fibrin, plasmin-mediated proteolysis of fibrin constitutes a positive-feedback process that enhances plasminogen activation (Figure 2A). TAFIa catalyzes the release of arginine and lysine from fibrin during tPA-mediated fibrinolysis in a purified system. The magnitude of the initial increase in arginine concentration correlates with its removal from the C-term of fibrinopeptides A and B, which are released during fibrin formation, although only fibrinopeptide B is a substrate for TAFIa in clots formed from whole blood. As fibrinolysis continues, the concentration of released arginine and lysine increases. Because the concentration of free arginine and lysine markedly exceed the concentration of fibrinogen, the component present at the highest concentration in the system constituting purified components, it is likely that fibrin is a substrate for TAFIa. Clearly demonstrate that binding of plasminogen to fibrin during lysis is diminished in the
presence of TAFIa compared with its absence and that binding correlates with prolongation of lysis. Wang et al further demonstrate, with the use of plasminogen (S741C) labeled with fluorescein at the “active site,” that TAFIa inhibits tPA-mediated activation of plasminogen by 2.5-fold in the presence of fibrin modified by plasmin. This value closely approximates the maximal relative prolongation in lysis time effected by TAFIa at saturation. TAFIa may also directly inactivate plasmin, further impairing fibrinolysis, although the concentrations of TAFIa required to inhibit plasmin approach those achieved by quantitative activation of TAFI. In contrast, concentrations of TAFIa on the order of 1/10 the plasma concentration of TAFI are able to maximally prolong lysis time. Furthermore, TAFIa appears to inhibit tPA-induced lysis only when Glu-plasminogen, but not Lys-plasminogen, is present, suggesting that the antifibrinolytic effect of TAFIa is mediated primarily through the inhibition of Glu-plasminogen activation. Therefore, the antifibrinolytic effect of TAFIa is mediated primarily through the inhibition of Glu-plasminogen activation. Therefore, the profibrinolytic effect of TAFIa is likely rationalized by its ability to remove C-terminal lysine residues from fibrin, partially degraded by plasmin, thereby eliminating their feedback potential of Glu-plasminogen activation (Figure 2B).

Regulation of TAFIa

Inhibitors of carboxypeptidases, including TAFIa, are found in the potato and the medicinal leech; however, a physiological inhibitor of TAFIa has not been identified in plasma. TAFIa specifically binds to α1-macroglobulin in plasma from a variety of species; however, TAFIa is not inhibited when bound. Therefore, TAFIa is likely negatively regulated by another mechanism(s). Cleavage of TAFIa by its activators and its intrinsic instability were appreciated at the time of discovery. However, proteolysis of functional TAFIa by thrombin does not seem to occur. TAFIa decays rapidly at 37°C, which correlates with the loss of chromogenic activity and a concomitant decrease in fluorescence (Figure 1). The enthalpic, entropic, and intrinsic fluorescence changes associated with spontaneous decay indicate that TAFIa undergoes a considerable conformational change during this process. Only after the decay of TAFIa (TAFIa) does it appear to become a substrate for thrombin. Cleavage of TAFIa by thrombin has been mapped to Arg302, which is itself a substrate for TAFIa. The released carboxy-terminal peptide may be further proteolyzed to yield the N-terminal Arg330. However, spontaneous decay of TAFIa is attenuated in the presence of substrate. Furthermore, TAFI and TAFIa are both cross-linked to fibrin by factor XIIIa, possibly through the major amine acceptors Gln2, Gln5, and Gln292, which are located in the activation peptide and in the mature enzyme. This may serve to locate both TAFI and TAFIa to the fibrin clot and could regulate activation and activity. It is tempting to speculate that TAFIa activity is regulated predominantly by intrinsic instability (a condition in which inactivation due to a conformation change is reversible), which is influenced by the presence and absence of substrate. Subsequent proteolytic cleavage of TAFIa may prevent TAFIa from regaining activity and thereby provide a mechanism for permanent inactivation.

Figure 2. Overview of mechanism of TAFIa-dependent antifibrinolytic pathway. A, Model depicting relative cofactor activity of fibrin. Rates of plasminogen activation by tPA are expressed relative to the rate observed in the absence of cofactor. Fibrin itself stimulates tPA-mediated plasminogen activation 100-fold compared with tPA alone or tPA in the presence of fibrin degradation products (FDPs). However, as fibrin is degraded by plasmin, C-terminal lysines (K) are exposed. C-terminal lysines enhance the rate of plasmin formation compared with nondigested fibrin. TAFIa is not an active site inhibitor of either the plasminogen activator or plasmin; rather, it inhibits activation of plasminogen by removing C-terminal lysine residues from plasmin-cleaved fibrin, thereby attenuating the cofactor activity. Data from References 56 and 76. B, Model illustrating potential mechanism of TAFIa-dependent inhibition of fibrinolysis. Plasmin (Fn) may cleave fibrin n times before complete inactivation of fibrin (Fn) as a cofactor, terminating in the formation of FDPs. Each cleavage event not only truncates fibrin but also exposes a new C-terminal lysine (Fn-K). Fn-K is a more potent cofactor for tPA-dependent activation of plasminogen (Pgn) than is Fn. TAFI is activated by thrombomodulin (TM) in complex with thrombin (IIa). Because TAFIa catalyzes the removal of C-terminal lysines from Fn-K, it attenuates the cofactor activity and therefore the rate of Fn formation. Consequently, the rate of fibrinolysis is reduced. The reverse arrow Fn-K to Fn plus free K (not shown) does not indicate a reversible reaction; Fn length is reduced with each cleavage, and this arrow indicates only that the Fn cofactor activity oscillates between the presence and absence of a C-terminal lysine. C, Model illustrating anticoagulant and antifibrinolytic pathways. The TM/IIa complex catalyzes the formation of APC and TAFIa, which inhibit IIa and Fn generation, respectively. IIa catalyzes the cleavage of fibrinogen (Fgn) to form Fn, whereas Fn degrades Fn, terminating in the formation of Fn degradation products.
(Patho)physiological Role for TAFI

The acronym TAFI implies that its physiological role is the regulation of fibrinolysis. The data tend to support the hypothesis. TAFI appears to be present in the plasma of a variety of mammals and, in all cases studied, can be activated by thrombin in a manner potentiated by thrombomodulin to yield a thermally unstable enzyme capable of inhibiting fibrinolysis. These data suggest that TAFI is part of a conserved pathway involved in the regulation of fibrinolysis. Redlitz et al provided the first indication that TAFI was involved in the regulation of fibrinolysis in vivo. They showed that TAFI activity, in plasma samples from dogs with electrically induced thrombosis, is increased during thrombosis and thrombolytic therapy. Furthermore, concentrations of TAFI activity were positively correlated with the time to restore blood flow. The influence of TAFI on thrombolysis is further evidenced by using an inhibitor of TAFI as a thrombolytic adjuvant in rabbits. Coinfusion of an inhibitor of TAFI with tPA consistently enhanced thrombolysis or markedly reduced the amount of tPA needed to achieve the same amount of lysis. Augmenting a thrombolytic agent with a TAFI inhibitor may enhance thrombolytic efficacy without a concomitant decrease in safety. Because TAFI is activated by thrombin, which is found at the site of a thrombus, inhibition of TAFI might provide enhanced thrombolysis with additional fibrin specificity. An inhibitor of TAFI also reduces the mortality associated with thrombin-induced thromboembolism by 50% in mice. Finally, in humans, TAFI concentrations exceeding 129 nmol/L (122 U/dL) are correlated with a 2-fold increase in risk for deep-vein thrombosis. Although TAFI is perhaps only a mild risk factor, the preponderance of evidence suggests the involvement of TAFI in the regulation of fibrinolysis. Although the aforementioned pertains specifically to the antifibrinolytic potential of TAFI, the role of TAFI may encompass a far greater breadth. C-terminal basic amino acids are present in a variety of proteins and may be important for their function. Therefore, TAFI may affect systems that are composed of these potential substrates, thereby connecting them with coagulation and fibrinolysis. For example, bradykinin is a substrate for TAFI, suggesting that TAFI may affect vascular tone. Certainly, molecules constituting the complement cascade are inhibited by CPN and, therefore, are also potential substrates for TAFI. Even with minimal speculation, it is not likely that the physiological role of TAFI is limited to the inhibition of fibrinolysis.

Conclusions

TAFI exhibits carboxypeptidase B–like specificity, which likely inhibits fibrinolysis by catalyzing the removal of C-terminal lysines of fibrin partially degraded by plasmin. Therefore, TAFI and its activation by thrombin-thrombomodulin and plasmin constitute an antifibrinolytic pathway analogous to the anticoagulant protein C pathway (Figure 2C). The physiological importance of TAFI has yet to be fully appreciated; however, initial data suggest that TAFI plays an important role in connecting the coagulation and fibrinolytic cascades. Elucidation of further interactions, mediated by TAFI, between other systems offers exciting opportunities for further investigation and therapeutic possibilities.

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