The Intrinsic Threshold of the Fibrinolytic System Is Modulated by Basic Carboxypeptidases, but the Magnitude of the Antifibrinolytic Effect of Activated Thrombin-activable Fibrinolysis Inhibitor Is Masked by Its Instability*

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Activated thrombin-activable fibrinolysis inhibitor (TAFIa) is intrinsically unstable, a property that complicates the study of its role in regulating fibrinolysis. To investigate the effect of basic carboxypeptidases on fibrinolysis under conditions of constant carboxypeptidase activity, we employed pancreatic carboxypeptidase B (CPB), a homologous, stable basic carboxypeptidase, as a surrogate for TAFIa. Clots formed from TAFI-depleted plasma or from purified components were supplemented with tissue-type plasminogen activator and either CPB or TAFIa. The clot lysis data indicate that the down-regulation of fibrinolysis mediated by basic carboxypeptidases involves a threshold mechanism. At carboxypeptidase concentrations above the threshold, plasminogen activation is maintained in a fully down-regulated state; experiments in plasma showed that fibrinolysis is essentially halted by saturating concentrations of TAFIa and that fibrinolysis of TAFIa is provided. The threshold effect has potentially important implications regarding the role of TAFIa and the regulation of clot lysis in vivo.

The fibrinolytic cascade is regulated on several levels by the plasmin-catalyzed formation of C-terminal lysine and arginine residues on the surface of fibrin (1–9). High affinity binding of plasminogen to the degraded fibrin surface (1–4) increases the catalytic efficiency of tissue-type plasminogen activator (tPA)1-mediated plasminogen activation by 12-fold (5). The conversion of plasminogen from the Glu to the Lys form, which increases its affinity for intact fibrin, thereby increasing the catalytic efficiency of the reaction by nearly 20-fold (6), requires partial degradation of the fibrinogen molecule (7). In addition, plasmin bound to the fibrin surface is protected from inactivation by antiplasmin and α2-macroglobulin (8–10). Given the central role that C-terminal basic amino acids play in the regulation of fibrinolysis, it is not surprising that their removal from the degraded fibrin surface is also enzymatically controlled. This regulation is likely mediated by the activated form of a basic carboxypeptidase present in plasma, thrombin-activable fibrinolysis inhibitor (TAFI) (11–15). Because TAFI was independently isolated by several groups, it is known by several names in the literature, including procarboxypeptidase U (16), procarboxypeptidase R (17), and plasma procarboxypeptidase B (18).

TAFI and pancreatic carboxypeptidase B (CPB) are highly homologous members of the carboxypeptidase A family, sharing 40% amino acid identity (18, 19). Whereas CPB is constitutively active, TAFI requires activation to form the active enzyme, TAFIa. This activation occurs principally via the thrombin-thrombomodulin complex (12, 20) but may also occur more slowly by plasmin (21), trypsin (18, 22), or thrombin alone (11). Of particular importance, CPB is a stable enzyme, whereas TAFIa displays temperature-dependent instability; essentially stable at 4 °C, the half-life of TAFIa activity ranges from 1.4 to 15 min at 37 °C, depending on the particular isoform studied (23–25). Measurements of the most prevalent natural TAFIa isoforms showed that they decayed with either an 8-min (Ala147/Thr225, Thr147/Thr225) or 15-min (Ala147/Ile225, Thr147/Ile225) half-life at 37 °C, with the difference attributable to the variation at residue 325 (25). This apparently small difference in the half-life of TAFIa translated into a 50% increase in the maximal prolongation of tPA-mediated fibrinolysis; whereas the Thr225 variants prolonged lysis 2-fold, Ile225 variants prolonged lysis 3-fold (25). Recently, Marx et al. (26) generated a highly stable chimera from human TAFI and porcine CPB, TAFI-CBP282–401. Once activated, the chimera displayed a half-life of activity toward small substrates of ~1.5 h. Unfortunately, the activated TAFI-CBP282–401 chimera lost its antifibrinolytic potential, most likely due to a loss of substrate specificity for plasmin-degraded fibrin.

1 The abbreviations used are: tPA, tissue-type plasminogen activator; TAFI, thrombin-activable fibrinolysis inhibitor; TAFIa, activated TAFI; CPB, carboxypeptidase B; PC, t-α-phosphatidylycholine; PE, t-α-phosphatidylethanolamine; PS, t-α-phosphatidylserine; TdP, TAFI-deficient plasma; Solulin, soluble thrombomodulin; LT, lysis time.

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While investigating the effect of competitive inhibitors on the antifibrinolytic activity of TAFIa, we (27) and others (28) discovered the seemingly paradoxical situation that, under certain conditions, TAFIa could prolong fibrinolysis in plasma much more effectively in the presence of a competitive inhibitor (20-fold prolongation) than could saturating concentrations of TAFIa alone (3-fold). Because the inhibition of TAFIa activity by competitive inhibitors concomitantly prevents the decay of TAFIa activity (22–24, 27, 29), we proposed that the increase in the antifibrinolytic capacity of the inhibitor-stabilized TAFIa could be explained in terms of the concentration of TAFIa required to maximally inhibit plasminogen activation (TAFIa_{Max}), i.e., in terms of the threshold concentration of TAFIa (27). In addition, we were able to show that the fibrinolytic threshold varied with the rate of plasminogen activation in experiments in which the concentration of tPA was varied. Because the stability of TAFIa plays such a critical role in its ability to prolong lysis (23–25, 27, 29) and because a stable form of TAFIa retaining antifibrinolytic fidelity is unavailable, we employed CPB as a stable surrogate for TAFIa to investigate the threshold effect of basic carboxypeptidase-mediated down-regulation of fibrinolysis under conditions of constant carboxypeptidase activity and compared these results to those seen with TAFIa. Interim work by our group (30) and others (31) on the characterization of the fibrinolytic threshold are now complete and are presented here and in a recently published paper (32).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human fibrinogen and human protein C were obtained from Enzyme Research Laboratories (South Bend, IN). Porcine pancreatic CPB was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Human thrombin was a generous gift from Dr. Michael Nesheim (Queen’s University, Kingston, ON, Canada). Activase, the recombinant tPA, was a kind gift from Dr. Gordon Vehar at Genentech (South San Francisco, CA). Soluble thrombomodulin (Solulin) was the generous gift of Dr. John Morser at Berlex Biosciences (Richmond, CA). Pooled normal human plasma and protein C-deficient human plasma were obtained from Affinity Biologicals (Ancaster, ON, Canada). TAFI and TAFI-deficient human plasma (TdP) were simultaneously prepared from normal human plasma by immunodepletion as previously described (33). Human plasminogen was purified from normal human plasma on lysine-Sepharose as previously described (34). Human antiplasmin was purified from plasminogen-depleted normal human plasma using affinity chromatography over plasminogen-Sepharose and a single affinity-Sepharose as previously described (35). The purified plasminogen and antiplasmin preparations were depleted of contaminating TAFIa by chromatography over anti-TAFI-Sepharose as per TdP. Ancrod, the thrombin-like enzyme from the venom of the malayan pit viper Agkistrodon rhodostoma was from Abbott Laboratories (Montreal, PQ, Canada) and was obtained from the Henderson General Hospital pharmacy (Hamilton, ON, Canada) as a 70 IU/ml isotonic saline solution (Viprinex®). L-α-Phosphatidylcholine (PC), l-α-phosphatidylethanolamine (PE), and l-α-phosphatidyserine (PS) were obtained in chloroform from Avanti Polar Lipids (Alabaster, AL). PC:PE:PS (60:20:20) phospholipid vesicles were made according to the procedure of Barenholz et al. (36).

**Clot Lysis Experiments Using Either TAFIa-deficient Plasma or Purified Components**—Clots were formed in the wells of a microtiter plate from 1/3-diluted TdP or from purified fibrinogen (3 μg), plasminogen (0.7 μg), and antiplasmin (0–1 μg) and were supplemented with tPA (0.1–0.56 nM) and either TAFIa (0–250 nM) or CPB (0–25 nM). Clot lysis was monitored at 405 nm using a SpectraMax Plus PlateReader (Molecular Devices, Sunnyvale, CA) thermostatted at 37 °C. The lysis time, the time at which the turbidity of the clot equals one-half of the full-scale value, was determined for each clot. The maximum time elapsed between TAFIa dilution and the start of any experiment was less than 20 min. In each case, clotting was essentially complete by the start of the experiment.

**Clot Lysis Experiments in Protein C-deficient Plasma**—Clots were formed in the wells of a microtiter plate from 1/3-diluted protein C-deficient human plasma supplemented with tPA (0.6 nM), PC:PE:PS vesicles (10 μM), and Solulin (50 nM) in the absence or presence of protein C (125 nM). Clotting was initiated by the addition of Ancrod (1 IU/ml) and CaCl₂ (10 mM) in HBST. The turbidity of each clot was monitored at 37 °C as above. The concentrations for all components listed above are given as the final concentration in the clot.

**RESULTS**

The CPB-mediated Prolongation of Plasma Clot Lysis Exhibits a Threshold Effect That Is Not Apparent with TAFIa.—Fig. 1 shows a comparison of the effects of TAFIa and CPB on plasma clot lysis. Clots formed from 1/3-diluted TdP were supplemented with various concentrations of either TAFIa or CPB. Lysis, initiated with tPA, was followed by turbidity. The figure shows that the addition of an unstable carboxypeptidase, TAFIa (A), yielded a markedly different effect on clot lysis time than did the addition of a stable carboxypeptidase, CPB (B). TAFIa achieved a 2-fold prolongation of lysis by 6.25 nM TAFIa, and this was extended to a 3-fold prolongation by 200 nM TAFIa. This stands in stark contrast to the effect seen with CPB; lysis was prolonged 2-fold by 3 nM CPB, 28-fold by 10 nM CPB, and by more than 45-fold (lysis time, >70 h) at 20 nM CPB. The dose-response curves of the two carboxypeptidases are shown in Fig. 2. As expected, the effect of TAFIa was saturable and
The presence of various concentrations of TAFIa (formed in TdP with various concentrations of tPA in the absence of carboxypeptidase) showed that TAFIa was the more effective down-regulator of fibrinolysis at lower concentrations.

The lysis time, the time at which the turbidity equals one-half of the full scale value, was determined for each profile in Fig. 1, and the values were plotted against the carboxypeptidase concentration. The figure shows the relative lysis time, the lysis time observed in the presence of carboxypeptidase divided by the lysis time observed in the absence of carboxypeptidase. The effect of TAFIa (triangles) appeared saturable and yielded a 3-fold prolongation. In contrast, the effect of CPB (circles) exhibited threshold behavior, i.e., a point was reached after which the effect of CPB became disproportional to the concentration. This is highlighted in the inset, which also shows that TAFIa was the more effective enzyme at the lower carboxypeptidase concentrations.

Displayed a maximal 3-fold prolongation of lysis. This was not the case with CPB. The figure shows that the prolongation of lysis mediated by CPB exhibited a threshold effect; the effect of CPB on lysis was modest up to ~3 nM, after which the increase in clot lysis time became disproportional to the change in CPB concentration. This effect is highlighted in the inset, which also shows that TAFIa was the more effective down-regulator of fibrinolysis at lower concentrations.

The Effect of CPB on the Relative Clot Lysis Time Is Highly Dependent on tPA Concentration, Whereas the Effect of TAFIa Is Not—To further investigate the threshold effect observed with CPB, we varied the concentration of tPA in the lysis experiments. Fig. 3 shows the relative lysis times of clots formed in TdP with various concentrations of tPA in the absence and presence of various concentrations of TAFIa (A) or CPB (B). The figure shows that the threshold effect observed with CPB was dependent on the concentration of tPA. As the concentration of tPA decreased, the threshold concentration of CPB also decreased. The threshold concentrations of CPB were approximately >10, 10, 3, 1, and 0.75 nM at tPA concentrations of 0.40, 0.30 0.20, 0.15, and 0.10 nM, respectively. The lysis of clots formed in the presence of TAFIa did not display the threshold effect observed with CPB at any of the tPA concentrations tested. However, the magnitude of the apparent saturation in relative lysis time appeared to increase with increasing tPA concentration.

The Effect of CPB on the Lysis of Clots Formed from Purified Components Is Highly Dependent on the Antiplasmin Concentration, Whereas the Effect of TAFIa Is Not—Because plasma is an extremely complex milieu, we continued our investigation of the effects of carboxypeptidase stability on clot lysis in a simpler system consisting of purified fibrinogen, plasminogen, antiplasmin, tPA, thrombin, and carboxypeptidase. To ensure the effects of carboxypeptidase stability on clot lysis in a simpler system consisting of purified fibrinogen, plasminogen, antiplasmin, tPA, thrombin, and carboxypeptidase. To ensure that the series of increasing curves indicates that the prolongation of clot lysis was initiated with 1/3-diluted TdP supplemented with either TAFIa or CPB. The clots were incubated at 37 °C, and the turbidity was monitored at 405 nm. A, lysis was initiated with 0.14 (circles), 0.21 (triangles), 0.40 (squares), 0.42 (inverse triangles), or 0.56 nM (diamonds) tPA in the presence of 0, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.62, 31.25, 62.5, 125, or 250 nM TAFIa. B, lysis was initiated with 0.10 (circles), 0.15 (triangles), 0.20 (squares), 0.30 (inverse triangles), or 0.40 nM (diamonds) tPA in the presence of 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 7.5, 10, or 20 nM CPB. The curves in B show data only for the clots that lysed; the “missing points” (i.e., CPB 3 nM at 0.2 nM tPA, CPB >1 nM at 0.15 nM tPA, CPB >0.75 nM at 0.10 nM tPA) indicate that lysis had not occurred within 70 h. Note the different scales for both concentration and relative lysis time in A and B. The figure shows that the threshold effect mediated by CPB is dependent on the concentration of tPA. In addition, the figure indicates that the magnitude of the apparent saturation reached with TAFIa is also dependent on tPA concentration.

Interestingly, neither TAFIa nor CPB appreciably prolonged the lysis of clots in the absence of antiplasmin. The series of offset but essentially identical curves (A) shows that the effect of TAFIa was saturable at all antiplasmin concentrations. The CPB-mediated prolongation of lysis was also shown to be saturable (B), with the maximal prolongation dependent on the concentration of antiplasmin present in the clot. The semi-log plot showing the log-linear relationship between lysis time and TAFIa (C) demonstrates that an exponential increase in TAFIa concentration yields only a linear increase in lysis time, as was observed in plasma (Fig. 1). The semi-log plot in D illustrates the threshold behavior of the antifibrinolytic effect in the presence of a stable carboxypeptidase, CPB. Finally panels E and F show the absolute clot lysis times at various carboxypeptidase concentrations versus antiplasmin concentrations. In the absence of carboxypeptidase, clot lysis time was essentially linear with respect to antiplasmin concentration up to 1.0 μM antiplasmin. With TAFIa (E) the plot yields a series of essentially parallel lines, indicating that the prolongation mediated by a given concentration of TAFIa was the same regardless of the antiplasmin concentration. This was not the case with CPB (F), where the series of increasing curves indicates that the prolong-
gation of lysis was highly dependent on the concentration of antiplasmin.

The Effect of TAFIa on Fibrinolysis Displays Threshold Behavior over the Picomolar Concentration Range—The relationship between TAFIa concentration and lysis time in Fig. 4 was found to be log-linear from 0.27 to 200 nM TAFIa. However, whether or not the relationship holds true at very low TAFIa concentrations has not been determined. In other words the question remains, what is the minimum TAFIa concentration required to exhibit an effect on lysis? Although this question may seem trivial, it has bearing on the potential physiological relevance of TAFIa, particularly under conditions in which TAFI activation may be ongoing. To address this, we investigated the effect of TAFIa on clot lysis over the picomolar concentration range in the absence and presence of various concentrations of antiplasmin. Fig. 5 shows the lysis time (A) and the absolute prolongation of lysis time (B) versus log([TAFIa]) in the absence and presence of various concentrations of antiplasmin. The figure shows that the effect of TAFIa on fibrinolysis over the picomolar concentration range was biphasic; clot
clots were formed in fibrinogen (3 μM), antiplasmin (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, or 0.6 μM), tPA (0.3 μM), and TAFIa (0, 6, 10, 17, 28, 47, 78, 130, 216, 360, 600, or 1000 μM). The figure shows the lysis time (A) and the absolute prolongation of lysis time (B) by TAFIa at various antiplasmin concentrations: ●, 0 μM; ▲, 0.05 μM; ■, 0.1 μM; ▽, 0.2 μM; ○, 0.3 μM; ◆, 0.4 μM; △, 0.5 μM; □, 0.6 μM. The lines show the fit of the data to Equations 14 and 15, as described under “Results.” The biphasic, log-linear nature of the curves is indicative of the threshold behavior of TAFIa. The figure also shows that the threshold effect is dependent on the antiplasmin concentration, as it was with CPB.

**Fig. 5. The threshold behavior of TAFIa is apparent over the picomolar concentration range.** Clots were formed with fibrinogen (3 μM), plasminogen (0.7 μM), antiplasmin (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, or 0.6 μM), tPA (0.3 μM), and TAFIa (0, 6, 10, 17, 28, 47, 78, 130, 216, 360, 600, or 1000 μM). The figure shows the lysis time (A) and the absolute prolongation of lysis time (B) by TAFIa at various antiplasmin concentrations: ●, 0 μM; ▲, 0.05 μM; ■, 0.1 μM; ▽, 0.2 μM; ○, 0.3 μM; ◆, 0.4 μM; △, 0.5 μM; □, 0.6 μM. The lines show the fit of the data to Equations 14 and 15, as described under “Results.”

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**Fig. 6. The activation of TAFI halts the progression of fibrinolysis in plasma.** Clots were formed in protein C-deficient plasma supplemented with tPA (0.6 nM), thrombomodulin (50 nM), and PC; PE:PS vesicles (10 μM) in the presence (dotted line) or absence (solid line) of protein C (125 nM). Clotting was initiated with Ancrod (1 U/ml) in the presence of CaCl₂ (10 mM). Under these conditions, the burst phase of thrombin generation and, therefore, TAFI activation occurs only in the clot lacking protein C. The figure shows that TAFIa can essentially halt fibrinolysis even when 50% of the clot has been solubilized and the rate of lysis is maximal.

If the fibrinolytic threshold is intrinsic to the fibrinolytic cascade, then does one rationalize the differences in clot lysis observed with CPB versus TAFIa? In other words, why was the profound prolongation of fibrinolysis associated with the CPB threshold not observed with TAFIa? When the instability of TAFIa is taken into account, analysis of the data shows that the same threshold mechanism is present whether the carboxypeptidase is CPB or TAFIa, but the threshold effect is masked by the instability of TAFIa. To visualize why this is so, consider the lysis of a clot in the presence of TAFIa at an initial concentration, [TAFIa]₀, exactly equal to the threshold carboxypeptidase concentration, [TAFIa]ₜc. Due to intrinsic instability, the concentration of TAFIa will immediately decay to a level below the threshold concentration. The continuous loss of TAFIa activity results in an accelerating positive feedback loop of plasmin generation and a concurrent loss of plasmin inhibition, thereby hastening clot lysis. Let us call the time required for lysis under these conditions the lysis time at threshold carboxypeptidase concentration (LTₜc). Now consider the same experiment but with the TAFIa concentration doubled (i.e., [TAFIa]₀ = 2 × [TAFIa]ₜc). For exactly one half-life (tTAFIa), the TAFIa concentration will remain above the threshold concentration, thereby maintaining fibrinolysis in the maximally down-regulated state. After one half-life has passed, we arrive at the threshold TAFIa concentration, [TAFIa]ₜc, and clot lysis resumes. Therefore, the time required for lysis under these conditions equals LTₜc + tTAFIa. If we double the concentration of TAFIa n times (i.e., [TAFIa]₀ = 2ⁿ × [TAFIa]ₜc), we arrive at the threshold concentration after n half-lives have passed. Therefore, the lysis time under these conditions equals LTₜc + n > tTAFIa. Under this scheme, each doubling in TAFIa concentration extends clot lysis by one half-life of TAFIa. In support of this interpretation, Fig. 6 demonstrates that, to a good approximation, the progression of fibrinolysis is essentially halted after the activation of TAFI. Thus, the lysis time (LT) as a function of the initial TAFIa concentration can be expressed as follows.

\[
LT_{[TAFIa]₀} = LT_{T} + n \times t_{TAFIa} \times \log ([TAFIa]₀ / [TAFIa]ₜc) \quad \text{(Eq. 1)}
\]

Inspection of Equation 1 shows that it is not valid when [TAFIa]₀ < [TAFIa]ₜc, because \( \log ([TAFIa]₀ / [TAFIa]ₜc) < 0 \) over this interval. Therefore, another model is needed to explain the effect of TAFIa on lysis when the TAFIa concentration is below the threshold.

**Modeling the Threshold Effect of TAFIa**—In general, thresholds are intrinsic properties of systems as a whole rather than a specific property of a single component in the system (38, 39).
Basic Carboxypeptidases Modulate the Fibrinolytic Switch

By definition, the threshold concentration is the concentration of TAFIa at which the accumulation of plasmin-catalyzed C-terminal lysine and arginine residues on fibrin is prevented. At the threshold concentration, the fibrin surface is maintained in a fully down-regulated state. Because reaction rate is directly related to enzyme concentration, we can define the fractional catalytic potential of a given concentration of TAFIa (P) as the catalytic potential of TAFIa (P) relative to the catalytic potential required at the threshold TAFIa concentration (P_{TC}), which is simply the ratio of their concentrations,

\[ P = \frac{P}{P_{TC}} \] (Eq. 2)

TAFIa is intrinsically unstable, and its activity decays exponentially. Given an initial concentration, [TAFIa]_0, the concentration at any time t is given by,

\[ [TAFIa]_t = [TAFIa]_0 e^{-kt} \] (Eq. 3)

where k is the decay constant. Thus, the fractional catalytic potential at any time t is

\[ P(t) = \frac{[TAFIa]_t}{[TAFIa]_{TC}} = \frac{[TAFIa]_0 e^{-kt}}{[TAFIa]_{TC}} \] (Eq. 4)

When the initial concentration equals the threshold concentration, [TAFIa]_0 = [TAFIa]_TC, the fractional catalytic potential as a function of time becomes

\[ P(t) = e^{-kt} \] (Eq. 5)

Integrating with respect to time, we arrive at the relative catalytic “work” performed by TAFIa as it decays from the threshold concentration to zero, W_{TC},

\[ W_{TC} = \int P(t) \times dt = \int_0^\infty e^{-kt} \times dt \] (Eq. 6)

which yields

\[ W_{TC} = \frac{e^{-kt}}{k} - \left( \frac{1}{k} \right) \] (Eq. 7)

and becomes

\[ W_{TC} = \frac{1}{k} \] (Eq. 8)

Finally, using the relationship between the exponential decay constant and half-life,

\[ k = \frac{\ln(2)}{T_{1/2}} \] (Eq. 9)

we see that the work generated as TAFIa decays from the threshold concentration to zero is directly related to the half-life of TAFIa,

\[ W_{TC} = \frac{T_{1/2}}{\ln(2)} \times \text{TAFIa} \] (Eq. 10)

Note that the units of \( W_{TC} \) are time. Because \( W_{TC} \) derives from the fractional catalytic potential, it is defined in terms of equivalent minutes of work generated by the threshold concentration of TAFIa. In other words, \( W_{TC} \) represents the maximum number of minutes that lysis could be prolonged given the amount of work that the threshold concentration of TAFIa can generate as it decays to zero. Thus, \( W_{TC} \) sets an upper limit for the magnitude of the difference between the lysis time at the threshold TAFIa concentration, LT_{TC}, and the lysis time in the absence of carboxypeptidase (the intrinsic lysis time, LT_0),

\[ \Delta L_T = LT_{TC} - LT_0 \leq W_{TC} \] (Eq. 11)

Using logic similar to that underlying Equations 2–8, the work that can be generated by any given initial concentration of TAFIa as it decays to zero is represented by,

\[ W_{(TAFIa)_0} = \int_0^\infty [TAFIa]_0 e^{-kt} \times dt = \frac{[TAFIa]_0}{k} \] (Eq. 12)

and the amount of work relative to that performed by the threshold concentration of TAFIa is simply

\[ \frac{W_{(TAFIa)_0}}{W_{TC}} = \frac{[TAFIa]_0}{[TAFIa]_{TC}} \times \Delta L_T \] (Eq. 13)

Therefore, when the initial TAFIa concentration is at or below the threshold, the lysis time can be approximated as the intrinsic lysis time (LT_0) plus the fraction of the maximal prolongation (\( \Delta L_T \)) that is generated by the TAFIa as it decays to zero, which is simply the ratio of \([TAFIa]_0 /[TAFIa]_{TC}\)

\[ LT_{(TAFIa)_0} = LT_0 + \frac{[TAFIa]_0}{[TAFIa]_{TC}} \times \Delta L_T \] (Eq. 14)

For consistency, we can apply the relationship described in Equation 11 to Equation 1 to give

\[ LT_{(TAFIa)_0} = (LT_0 + \Delta L_T) + \frac{T_{1/2}}{\log([TAFIa]_0 / [TAFIa]_{TC})} \log(2) \] (Eq. 15)

Note that both Equations 14 and 15 give the same result when \([TAFIa]_0 = [TAFIa]_{TC}\). The lines in Fig. 5 show a global fit of the data to Equations 14 and 15. The data were fit to both equations simultaneously using the Solver function of Microsoft Excel (Redmond, WA). The fit was generated using a single global value for \(k_{TAFIa}\) and unique values of \(\Delta L_T\), LT_0, and [TAFIa]_TC for the data collected at each separate antiplasmin concentration. The model returned a global half-life for TAFIa of 10.4 min, in good agreement with published reports (25, 27, 29, 40). The values returned by regression of the model were: \(\Delta L_T = 4.0, 4.5, 12.4, 14.9, 15.0, 13.0, 15.0, 15.0\), and \(15.0\) min; \([TAFIa]_{TC} = 9444, 504, 382, 196, 137, 78, 73, and 59\) pm; LT_0 = 14.9, 22.2, 29.7, 48.5, 69.1, 88.5, 108.7, and 134.4 min at 0, 50, 100, 200, 300, 400, 500, and 600 ng antiplasmin, respectively. The \(\Delta L_T\) and \(T_{1/2}\) values (0.39, 0.44, 1.19, 1.44, 1.44, 1.25, and 1.44) are consistent with the maximum value predicted for the model, 1.44 (Equation 10). The predicted LT_0 values did not differ from the measured values by more than 3%.

DISCUSSION

The work described in this manuscript shows that the down-regulation of fibrinolysis mediated by both stable and unstable basic carboxypeptidases involves a threshold mechanism (for reviews on thresholds in biological systems, see Refs. 38 and 39). Like many systems with thresholds (or ultrasensitive systems (39)), the fibrinolytic system has a stimulus (plasminogen activation), an effector of positive feedback (plasmin, via degradation of fibrin, increases the rate of plasminogen activation, and decreases the rate of plasmin inhibition), a mechanism for eliminating the effector (antiplasmin), and a means of modulating the feedback response (basic carboxypeptidase-mediated removal of C-terminal lysine and arginine residues from the degraded fibrin surface). Thus, the state of the system with respect to the fibrinolytic threshold is a complex function, dependent on factors affecting both the rate of plasmin formation and the rate of plasmin inhibition.

Although we can properly refer to (and determine) the
threshold concentration” of a particular component in the in vitro clot lysis assay, it must be recognized that the threshold observed is an intrinsic property of the fibrinolytic system as a whole. For example, the threshold concentration of TAFIa is necessarily dependent on the concentrations of plasminogen activator, plasminogen, and antiplasmin; changing the concentrations of any of these factors will necessarily alter the threshold concentration of TAFIa. As such, threshold values are valid only under the defined conditions of the system. Furthermore, the state of the system with respect to the threshold can change with time, because significant consumption of the components shifts the fibrinolytic balance. Additional complexity is added to the threshold effect when one considers the flow of components into and out of the system as may occur in clot lysis in vivo. With these caveats in mind, however, we offer a simple interpretation of the fibrinolytic threshold with an eye toward explaining the modulation of fibrinolysis observed with basic carboxypeptidases.

At its simplest, the threshold concept in fibrinolysis essentially describes the potential for the plasminogen activation system to sustain a self-reinforcing acceleration of plasmin generation. Although the plasminogen activator generates the plasmin, the force driving the acceleration of plasminogen activation in this system is plasmin itself, and its effects are mediated primarily through the degradation of fibrin. By increasing the concentration of the degraded fibrin cofactor, plasmin increases the rate of its own formation by up-regulating plasminogen activation (1–7) and concomitantly decreasing the rate of its own inhibition via the degraded fibrin-mediated protective effect (8–10). Factors that decrease the concentration of plasmin either directly (plasmin inhibitors) or by decreasing the concentrations of plasminogen activator (plasminogen activator inhibitors) will push the system toward the sub-threshold state. However, given a system with sufficient plasminogen activator, plasminogen, and antiplasmin, it is the basic carboxypeptidase that constitutes the primary force driving the system toward the down-regulated (sub-threshold) state, since the removal of the C-terminal lysine and arginine residues from the degraded fibrin surface strongly modulates both the rate of plasmin generation (decreases) and the rate of plasmin inhibition (increases). In this manner, basic carboxypeptidases negate the accelerating action of plasmin and maintain the system in a maximally down-regulated state.

How well do the experimental findings support this interpretation? First, we have shown that the fibrinolytic threshold is a function of the concentrations of plasminogen activator, plasmin inhibitor, and basic carboxypeptidase in both plasma and purified systems, indicating that the threshold is a property of the fibrinolytic system as a whole and is not specific to a single component. Second, the threshold concentration of basic carboxypeptidase was dependent on the rate of plasmin generation and the rate of plasmin inhibition, as shown by varying the concentrations of tPA in plasma (Fig. 3) and antiplasmin in a purified system (Figs. 4 and 5), respectively. In fact, the presence of a plasmin inhibitor was found to be an independent requirement for significant prolongation of lysis with both CBP and TAFIa (Fig. 4). Together, these results indicate that the basal steady-state concentration of plasmin determined the threshold carboxypeptidase concentration in these experiments. Third, the ability of TAFIa to halt the progression of fibrinolysis in a clot where 50% of the fibrin had already been solubilized and the rate of lysis was maximal shows that the threshold is a systemic property, present at all stages of fibrinolysis, and underscores the principal role of TAFIa in pushing the fibrinolytic system toward the sub-threshold state. This observation also highlights the potential for TAFIa to down-regulate fibrinolysis at any time during clot lysis, a property that will likely have important consequences for in vivo fibrinolysis.

Although the goodness of fit generated by non-linear regression of the data to the model supports our interpretation of the threshold mechanism, it is unfortunate that the computational engine does not return errors for the parameter estimates. However, we can derive additional predictions from the model regarding the effects of TAFIa on clot lysis, which can be used as further tests of validity. Note that the linear dependence of clot lysis time on the half-life of TAFIa and the logarithmic dependence on TAFIa concentration (Equation 15) can explain why the magnitude of the apparent saturation in lysis time varies according to the stability of the TAFIa isofrom (23, 25). This effect is shown in Fig. 7, which plots hypothetical curves drawn using Equations 14 and 15 and illustrates the consequences of TAFIa stability and changes in the threshold concentration on the relative lysis time. It is also worth noting that the model predicts that the apparent saturation in the relative prolongation of clot lysis times should increase as LT90 decreases, i.e. with increasing tPA or decreasing antiplasmin, since the prolongation conferred by TAFIa becomes a larger fraction of the intrinsic lysis time. This relationship was demonstrated by varying tPA in TdP (Fig. 3) and by varying antiplasmin in the purified system (Fig. 4A and 5A). This raises an important point that should be considered when evaluating the effect of TAFIa on fibrinolysis. Expressing the effect of TAFIa in terms of relative lysis time can lead to different conclusions regarding the efficacy of TAFIa as compared with the same data expressed in terms of absolute prolongation of lysis time. Compare the relative and absolute prolongation of lysis time mediated by 1 nM TAFIa in Fig. 5 at antiplasmin concentrations of 0.05 versus 0.6 µM: relative prolongation, 1.7 versus 1.4; absolute prolongation, 15 versus 57 min, respectively. Reporting relative prolongation may lead to the conclusion that TAFIa is more effective at lower antiplasmin concentrations, whereas the absolute prolongation values belie this interpretation. To our minds, the more informative of the two is the absolute prolongation value when comparing data from experiments where the intrinsic lysis times are not the same.
The threshold effect has important consequences regarding the potential roles of TAFIa in vivo when one considers situations where TAFI activation may be ongoing, even at very low rates, especially given the sub-nanomolar threshold TAFIa concentrations observed in our experiments. The potential relevance of the fibrinolytic threshold can be illustrated by considering the steady-state conditions, where the rate of activation of TAFIa is balanced by the rate of intrinsic decay of TAFIa.

\[
\frac{dTAFIa}{dt} = v_{\text{activation}} - v_{\text{decay}} = 0 \quad (\text{Eq. 16})
\]

Because the rate of decay is proportional to the TAFIa concentration, the rate of activation at the steady-state is,

\[
v_{\text{activation}} = v_{\text{decay}} = k \times [TAFIa]_{ss}
\]

where \( k \) is the decay constant, and \([TAFIa]_{ss}\) is the steady-state concentration of TAFIa. Using the identity for \( k \) from Equation 9, we see that,

\[
v_{\text{activation}} = \frac{\ln(2)}{t_{1/2}} \times [TAFIa]_{ss}
\]

which becomes

\[
\frac{v_{\text{activation}}}{[TAFIa]_{ss}} = \frac{\ln(2)}{t_{1/2}} \quad (\text{Eq. 19})
\]

Equation 19 indicates that the rate of activation of TAFIa required to maintain TAFIa at a given steady-state concentration is inversely proportional to the half-life of the TAFIa isofrom. In other words, the rate of activation required to maintain TAFIa at or above some threshold concentration is dependent on the stability of TAFIa. Thus, TAFIa isoforms with longer half-lives require lower rates of activation to maintain fibrinolysis in the maximally down-regulated state.

The model described by Equations 14 and 15 fits the data very well at the low TAFIa concentrations. The fit was not as good, however, when the model was applied to the data collected at the higher TAFIa concentrations (not shown). Inspection of the curves in Fig. 4C shows that they are bell-shaped at the lower concentrations of antiplasmin; as TAFIa concentration increased, a point was reached after which additional TAFIa resulted in a decrease in lysis time. This effect was dependent on the antiplasmin concentration and diminished as the antiplasmin concentration increased. We have also observed this effect in TdP supplemented with TAFIa, although the concentrations of TAFIa at which the effect became apparent varied with the source of plasma used in the experiments (data not shown). One hypothesis that may explain this profibrinolytic effect is that, like carboxypeptidase B (41), TAFIa may decrease the affinity of antiplasmin for plasmin by removal of the C-terminal lysine residue, Lys\(^{452}\). At high concentrations of TAFIa and low concentrations of antiplasmin, a significant fraction of the antiplasmin may be converted to the lower affinity form, thereby decreasing the rate of plasmin inhibition. In this fashion, TAFIa may limit its own ability to prolong lysis by decreasing the efficiency of the principle plasmin inhibitor. Although a detailed study of this effect falls outside the scope of this manuscript, confirmation of this hypothesis would aid in understanding the importance of Lys\(^{452}\) in mediating the interaction between antiplasmin and plasmin as mutation of Lys\(^{452}\) to Glu\(^{452}\) or Thr\(^{452}\) had no significant effect on the second order rate constant of inhibition. Determining the role of Lys\(^{452}\) has particular importance given the recent reports indicating the principle role of TAFIa in the inhibition of plasmin by antiplasmin in the presence of plasmin-degraded fibrin and fibrin degradation products (10, 44) and the observation in this study that the threshold concentrations of both TAFIa and CPB were dependent on the concentration of antiplasmin.

In summary, the work performed in this study demonstrates that fibrinolysis involves a threshold mechanism. We have shown that the discrepancy between CPB and TAFIa on the inhibition of clot lysis is explained by a system with both a threshold mechanism and an intrinsically unstable enzyme with a short half-life: exponential decay rapidly and inevitably decreases TAFIa to a concentration below the threshold regardless of the initial TAFIa concentration. We have derived equations describing such a system and have shown that they can explain the observation that the apparent saturation in the maximal relative lysis time observed with TAFIa is dependent on the half-life of the TAFIa isofrom. Thus, the stability of the basic carboxypeptidase is the principal determinant of overall antifibrinolytic potency in a static, in vitro clot lysis assay. However, we have also shown that TAFIa essentially halted the progression of fibrinolysis in a clot where 50% of the fibrin had already been solubilized and the rate of lysis was maximal, indicating that the threshold is both present and can be modulated by TAFIa at all stages of clot lysis. Taken together with the demonstration that the threshold for TAFIa occurs over the picomolar concentration range when TAFIa is present from the start, these results indicate that very low steady-state levels of TAFIa may effectively down-regulate fibrinolysis in vivo. These observations may be relevant to the resolution of thrombus-related pathologies under conditions in which TAFIa generation may be ongoing.

REFERENCES

Basic Carboxypeptidases Modulate the Fibrinolytic Switch

The Intrinsic Threshold of the Fibrinolytic System Is Modulated by Basic Carboxypeptidases, but the Magnitude of the Antifibrinolytic Effect of Activated Thrombin-activable Fibrinolysis Inhibitor Is Masked by Its Instability

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