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A Study of the Mechanism of Inhibition of Fibrinolysis by Activated Thrombin-activable Fibrinolysis Inhibitor*

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TAFI (thrombin-activable fibrinolysis inhibitor) is a recently described plasma zymogen that, when exposed to the thrombin-thrombomodulin complex, is converted by proteolysis at Arg^{92} to a basic carboxypeptidase that inhibits fibrinolysis (TAFIa). The studies described here were undertaken to elucidate the molecular basis for the inhibition of fibrinolysis. When TAFIa is included in a clot undergoing fibrinolysis induced by tissue plasminogen activator and plasminogen, the time to achieve lysis is prolonged, and free arginine and lysine are released over time. In addition, TAFIa prevents a 2.5-fold increase in the rate constant for plasminogen activation which occurs when fibrin is modified by plasmin in the early course of fibrin degradation. The effect is specific for the Glu- form of plasminogen. TAFIa prevents or at least attenuates positive feedback expressed through Lys-plasminogen formation during the process of fibrinolysis initiated by tissue plasminogen activator and plasminogen. TAFIa also inhibits plasmin activity in a clot and prolongs fibrinolysis initiated with plasmin. We conclude that TAFIa suppresses fibrinolysis by removing COOH-terminal lysine and arginine residues from fibrin, thereby reducing its cofactor functions in both plasminogen activation and the positive feedback conversion of Glu-plasminogen to Lys-plasminogen. At relatively elevated concentrations, it also directly inhibits plasmin.

TAFI (thrombin-activable fibrinolysis inhibitor)[1] is a recently discovered 60-kDa single-chain plasma protein that can be activated by thrombin-catalyzed proteolysis to a carboxypeptidase B-like enzyme that inhibits fibrinolysis (1, 2). It is present in plasma at a concentration of about 75 nM (3). TAFI was discovered independently in several different laboratories and consequently has acquired several aliases. It was described initially as an unstable carboxypeptidase in human serum and was named carboxypeptidase U (4, 5). It also was named plasma carboxypeptidase B by Eaton *et al.* (6) and subsequently plasma procarboxypeptidase B (pro-plasma carboxypeptidase B) by Tan and Eaton (7). In addition, on the basis of its instability in serum, it probably can be identified as carboxypeptidase R described by Campbell and Okada (8).

Recent studies show that the thrombin-thrombomodulin complex, rather than free thrombin, is probably the physiologic activator of TAFI (2). In addition, activated TAFI (TAFIa) down-regulates tissue plasminogen activator (t-PA)-induced fibrinolysis half-maximally at a concentration of 1.0 nM (2). Because this is only about 1.3% of the level of the zymogen in plasma, ample TAFIa could be generated to modulate fibrinolysis very significantly *in vivo* (2). Bajzar *et al.* (3) showed that the apparent profibrinolytic effect of activated protein C is absent in TAFI-deficient plasma or when plasma is supplemented with an anti-TAFI monoclonal antibody. They also showed in plasma systems supplemented with soluble thrombomodulin or in systems utilizing cultured endothelial cells as a source of thrombomodulin, that fibrinolysis is inhibited when and only when TAFI is activated (9). In addition, Redlitz *et al.* (10) showed that activated pro-plasma carboxypeptidase B and plasma carboxypeptidase N diminish the binding of plasminogen to U937 cells and that fibrinolysis occurs more rapidly in pro-plasma carboxypeptidase B (TAFI)-deficient compared with normal plasma. These observations suggest that the coagulation and fibrinolytic cascades are linked through TAFI and that TAFIa might participate fundamentally in the regulation of the fibrinolytic response. As a consequence, the following studies were carried out to elucidate the mechanism(s) by which TAFIa suppresses fibrinolysis.

EXPERIMENTAL PROCEDURES

Materials—The synthetic carboxypeptidase substrate hippuryl-L-arginine, and L-arginine, L-lysine, octopine dehydrogenase, and saccharopine dehydrogenase were obtained from Sigma. The chromogenic substrate S-2251 was purchased from Helena Laboratories (Beaumont, TX). The carboxypeptidase B inhibitor 2-guanidinoethylmercaptosuccinic acid and the plasmin inhibitor VFK-CMK were purchased from Calbiochem. NADH and carboxypeptidase B were purchased from Boehringer Mannheim. $Na^{125}I$ (100 mCi/ml) was purchased from ICN Biomedicals (Montreal, PQ), and IODO-BEADs were purchased from Pierce Chemical Company. DAPA, a specific thrombin inhibitor, was synthesized and isolated according to the method of Nesheim *et al.* (11). The human proteins fibrinogen, plasminogen, prothrombin, factor V, and antithrombin III were isolated from plasma; the enzymes thrombin, factor Xa, and plasmin and phospholipid vesicles containing 75% phosphatidylcholine and 25% phosphatidylserine were prepared as described previously (1). t-PA (Activase) was a generous gift of Dr. Gordon Vehar of Genentech (South San Francisco, CA). Recombinant soluble thrombomodulin (Solulin) was obtained as a generous gift from Dr. John Morser and colleagues at Berlex Biosciences (Richmond, CA). Recombinant human α_2-antiplasmin was isolated from culture supernatants of baby hamster kidney cells transfected with the human cDNA and grown in serum-free medium, as described before (1). TAFI was purified from human plasma according to the method of Bajzar *et al.* (1).

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[1] The abbreviations used are: TAFI, thrombin-activable fibrinolysis inhibitor; TAFIa, activated TAFI; t-PA, tissue plasminogen activator; VFK-CMK, D-Val-Phe-Lys chloromethyl ketone; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl) amide; Glu^1-Plg(S741C-fluorescein), recombinant human (S741C) plasminogen labeled at Cys^{741} with fluorescein; Lys^{78}-Plg(S741C-fluorescein), the Lys^{78} derivative of Glu^1-Plg(S741C-fluorescein).

Measurement of Inhibition of t-PA-mediated Fibrinolysis by TAFIa—TAFI was activated to the active species, TAFIa, by the thrombin-thrombomodulin complex according to the method of Bajzar *et al*. (2). The inhibition of t-PA-mediated fibrinolysis by TAFIa was then measured by lysis assays performed in a system of purified fibrinolytic components as described previously (1, 2).

Determination of the Release of Free Arginine and Lysine by TAFIa during Clot Lysis—Free arginine and lysine released from fibrin in the presence of TAFIa were measured during t-PA-mediated fibrinolysis. Clots were produced in the presence of various concentrations of TAFIa (0–63 nM), and lysis times were monitored. Once the clot lysed completely, the samples were deproteinated by perchloric acid (0.2 M, final) and subsequently neutralized by KOH. The insoluble potassium perchlorate was removed by centrifugation at 4 °C. The supernatants were used for determination of arginine and lysine. In separate experiments, the time courses of the release of arginine and lysine by TAFIa were measured by solubilizing fibrin clots with acetic acid (0.1 M, final), thereby quenching all reactions, at regular intervals over 3 h. The samples then were deproteinated, and free arginine and lysine in the supernatants were determined. Free arginine and lysine were determined by methods similar to those described by Gaede *et al*. (12) and Nakatani *et al*. (13). 100 μl of supernatant or a standard solution containing known concentrations of arginine or lysine was added to the wells of a microtiter plate containing 80 μl of a solution that included (final concentrations) 10 μM NADH and a 1 mM concentration of either pyruvate (in the case of arginine assay) or α-ketoglutaric acid (in the case of lysine assay) in 0.05 M HEPES buffer, pH 7.0. The reactions were initiated by the addition of 20 μl of a buffered solution of octopine dehydrogenase (0.5 unit) for the arginine assay or saccharopine dehydrogenase (0.1 unit) for the lysine assay. The diminution of fluorescence intensity was then monitored over the next 2 h in a fluorescence plate reader attached to a Perkin-Elmer model LS50B spectrofluorometer. The excitation and emission wavelengths were 340 and 450 nm, respectively, and a 430 nm cutoff filter was employed in the emission beam. The concentrations of arginine and lysine were determined according to standard curves that were constructed by plotting ΔEm_{450} of the standard samples *versus* the concentration of arginine or lysine.

Effect of TAFIa on the Kinetics of the Fibrin-dependent Activation of Plasminogen by t-PA—These experiments were performed by measuring by fluorescence the time course of cleavage of the Glu^1 and Lys^{78} forms of recombinant (S741C-fluorescein) plasminogen described by Horrevoets *et al*. (14, 15). These are derivatives of plasminogen which show a 50% decrease in fluorescence intensity when cleaved and do not yield active plasmin, thereby allowing facile measurement of cleavage kinetics within fibrin and eliminating plasmin-catalyzed feedback cleavage of fibrin, t-PA, and plasminogen. To determine the effect of TAFIa on the kinetics of cleavage of the plasminogen derivatives within a clot, solutions (88 μl) of the derivatives (227 nM) and fibrinogen (3.4 μM), in 0.02 M HEPES, 0.15 M NaCl, pH 7.4, and 0.01% Tween 80 were pipetted into the wells of a microtiter plate. The temperature was 22 °C. To some samples 2.0 μl of a solution of 250 nM plasma plasminogen was added, and to the others 2.0 μl of buffer was added. The fluorescence intensities of the samples were then read several times to establish initial values. The reactions were started by pipetting into the wells a solution (10.0 μl) of t-PA (one- or two-chain), thrombin, $CaCl_2$, and, when present, TAFIa. The final concentrations of t-PA were 2.5 and 5.0 nM, respectively, with the Lys^{78} and Glu^1 forms of the substrate. The final concentrations of thrombin, Ca^{2+}, and TAFIa were 6.0, 5.0, and 0–5.0 nM, respectively. The time courses of the fluorescence decrements were measured in 1-min intervals over the next hour in a fluorescence plate reader linked to a Perkin-Elmer model LS50B spectrofluorometer. The excitation and emission wavelengths were 490 and 535 nm, with a 530 nm cutoff filter in the emission path. The concentrations of remaining substrate were calculated from the fluorescence data. Rate constants were calculated for successive 1-min intervals by the equation

$$k = \ln([P(\tau_1)]/[P(\tau_2)])/(\tau_2 - \tau_1), \quad \text{(Eq. 1)}$$

where $[P(\tau_1)]$ and $[P(\tau_2)]$ are the concentrations of plasminogen at the beginning and end times of each interval (τ_1 and τ_2). The two-chain t-PA was prepared by incubating one-chain t-PA (4 μM) with plasmin (10 nM) for 30 min at 37 °C and then inhibiting the plasmin with the plasmin inhibitor VFK-CMK (105 nM). SDS-polyacrylamide gel electrophoresis under reducing conditions was used to confirm quantitative conversion to the two-chain form. VFK-CMK was also added without plasmin to control for the presence of the inhibitor in one-chain t-PA.

Quantitation of the Activation of Plasminogen and the Conversion of Glu-plasminogen to Lys-plasminogen during Fibrinolysis—Glu-plasminogen was iodinated with ^{125}I using IODO-BEADS as described previously (1). The final concentration of ^{125}I-Glu-plasminogen was 95.7 μg/ml, and the specific radioactivity was 9.73×10^3 cpm/ng. The activation of plasminogen and the conversion of Glu-plasminogen to Lys-plasminogen in the absence and presence of TAFIa were quantitated during lysis of fibrin clots. A solution of fibrinogen (3.3 μM) containing plasminogen (0.77 μM), antithrombin III (0.98 μM), and α_2-antiplasmin (0.48 μM) was supplemented with ^{125}I-Glu-plasminogen (1.8 nM). Two series of identical clots were formed by adding 100-μl aliquots of this solution to microtiter wells containing small, separated aliquots of either TAFIa (40 nM, final) or buffer, t-PA (441 pM, final), thrombin (6 nM, final), and $CaCl_2$ (10 mM, final). The final volume was 110 μl. Each clot was incubated at 37 °C, and turbidity was monitored at 405 nm at 2.5-min intervals. At various times between 0 and 180 min the reaction in one clot from each series (plus and minus TAFIa) was quenched with acetic acid (0.1 M, final). All samples were immediately frozen at −70 °C. Subsequently, the samples were thawed, and 25 μl of each was brought to 15% sucrose, 0.9 M acetic acid, with basic fuschin as an electrophoresis indicator and were subjected to urea/acetic acid 7.5% polyacrylamide gel electrophoresis (16) at 120 V for 15 h at 22 °C in an LKB model 2001 vertical electrophoresis unit on 15-cm gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and destained. The gels were dried and exposed to x-ray film (Kodak XAR-5) at −70 °C. After development, the autoradiograph was aligned with the gel, and the locations of all radioactive species were determined. The bands were excised and counted for 10 min in an LKB model 1275 MiniGamma counter. The species that were excised included Glu-plasminogen, Lys-plasminogen, and plasmin-α_2-antiplasmin. These were the only species evident on the gels.

Effect of TAFIa on Plasmin-induced Clot Lysis—To study the effect of TAFIa on plasmin-induced clot lysis, clots were produced by the addition of 100 μl of a solution of fibrinogen (3.0 μM, final) to wells containing 2 μl of plasmin (2 nM, final), 2 μl of thrombin (6 nM, final) and $CaCl_2$ (10 mM, final), and 7 μl of TAFIa (0–120 nM, final). Turbidity at 405 nm was then recorded at 37 °C over 10 h.

Effect of TAFIa on Plasmin Activity during Fibrinolysis—Three series of 12 identical clots were formed with fibrinogen (3.0 μM, final), thrombin (6 nM, final), $CaCl_2$ (10 mM, final), and plasmin (4 nM, final) at 37 °C. One series was formed in the absence of TAFIa and the other two in the presence of 25 and 50 nM TAFIa, respectively. As described previously (1), clots were solubilized at various times and reactions quenched by adding acetic acid (0.1 M, final). Equal volumes of a solution of S-2251 (500 μM) in 437 mM HEPES, containing 10 mM EDTA, 2 mM ϵ-amino caproic acid, and 40 μM DAPA, pH 8.0, were added to the wells, and the time courses of the increase of absorbance at 405 nm were then monitored. The initial rates of S-2251 hydrolysis, relative to that at zero time, were determined to infer plasmin levels. Similar experiments were performed in the absence of fibrinogen.

RESULTS

Release of Arginine and Lysine from Fibrin by TAFIa during Fibrinolysis—Previous studies showed that the inhibitory effect of TAFIa on fibrinolysis correlates with carboxypeptidase activity measured with hippuryl-L-arginine (1), suggesting that the antifibrinolytic effect of TAFIa can most likely be attributed to the removal of COOH-terminal arginine and lysine residues from partially degraded fibrin in the clot. We therefore measured free arginine and lysine in completely lysed clots. Released arginine and lysine were found in all samples that contained TAFIa. The lysis times were 33 min in the absence of TAFIa and 92 min in the presence of 63 nM TAFIa. No free arginine or lysine was detected in the absence of TAFIa. We then determined the time course of the release of free arginine and lysine from fibrin by TAFIa during clot lysis (Fig. 1). The arginine level rose to about 9.8 μM immediately after clotting, most likely because of the release of fibrinopeptides A and B during the clotting of the 3.0 μM input fibrinogen (two fibrinopeptides A and B are released from each fibrinogen molecule). The arginine level then increased gradually to about 25 μM by 180 min. The lysine level rose smoothly to about 6 μM over the same interval. In separate experiments, the effects of free arginine and lysine on fibrinolysis, at the levels found with TAFIa, were tested by adding them to the lysis assay system, and no prolongation of lysis time was observed. Thus, the

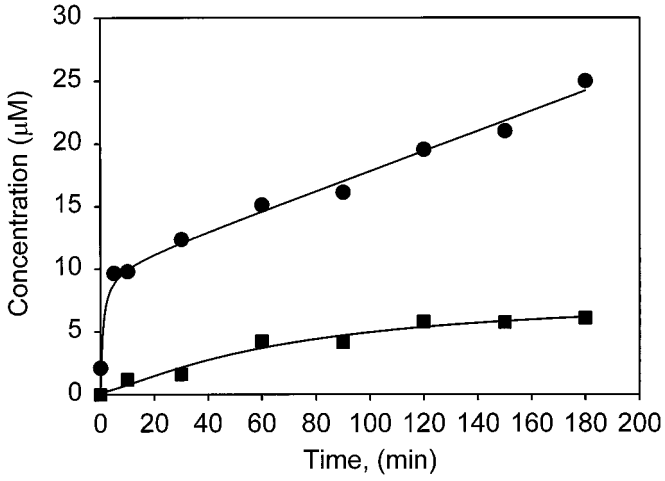

FIG. 1. **Release of arginine and lysine by TAFIa during clot lysis.** t-PA-mediated fibrinolysis was performed in the presence of TAFIa (63 nM) in a system of purified fibrinolytic components. The lysis time with TAFIa was 92 min; that without TAFIa was 33 min. At time intervals (0, 10, 30, 60, 90, 120, 150, and 180 min) clots were solubilized with acetic acid (0.1 M, final) thereby quenching all reactions. The samples were then deproteinated, and the concentrations of released arginine (●) and lysine (■) were determined. No free arginine or lysine was detected in the control experiment without TAFIa (not shown).

inhibition of lysis by TAFIa correlates with the release of, but is not caused by, free arginine and lysine in the clot.

Effects of TAFIa on Fibrin-dependent Plasminogen Activation—Plasminogen activation by t-PA is stimulated several hundredfold by fibrin, and this cofactor activity is augmented by plasmin-catalyzed feedback modification of fibrin, whereby carboxyl-terminal lysine and arginine residues are generated (14, 15, 17, 18). Because TAFIa is a carboxypeptidase B-like enzyme, it might possibly prevent or attenuate the effect of the plasmin-catalyzed feedback cleavages in fibrin. To explore this possibility the effects of TAFIa on the kinetics of cleavage of Glu^1 and Lys^{78}(S741C-fluorescein) plasminogen were investigated. These plasminogen derivatives do not generate plasmin; thus, plasmin feedback does not occur unless native plasminogen is included also. Results obtained when the time course of cleavage of the fluorescent plasminogen derivative, catalyzed by one-chain or two-chain t-PA within a clot, are shown in Fig. 2. The control results (no plasma plasminogen, no TAFIa) are shown by the *solid circles*. The results obtained when 5.0 nM plasma plasminogen was included are shown by the *solid squares*. These data show that initially, rates of fluorescent plasminogen consumption are the same. Later in the course of the reaction, however, the fluorescent plasminogen is consumed more rapidly when native plasminogen is present to generate plasmin, thereby modifying fibrin and accelerating the reaction. This effect is attenuated progressively when TAFIa is included at increasing concentrations. When the TAFIa concentration is 5.0 nM, the effect of included native plasminogen is eliminated completely. This occurs with both one- and two-chain t-PA. These effects are quantified in Fig. 3, where the rate constants for plasminogen activation are shown as a function of time over the first 1,600 s of the reaction. With both one- and two-chain t-PA the rate constant evaluates to 1×10^{-4}/s in the absence of added plasma plasminogen or TAFIa (*solid circles*). The near constancy of this value over time in the controls indicates that the reaction is approximately first order, which is consistent with the fairly high K_m (0.5 μM) for this reaction under these conditions (15). They also show that one- and two-chain t-PA are identically active under these conditions. When a trace of plasma plasminogen is included, the value of the rate constant increases up to about 2.5×10^{-4}/s

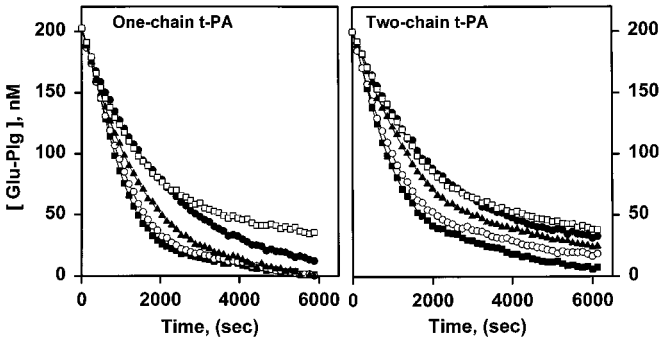

FIG. 2. **Effect of TAFIa on fibrin-dependent activation of plasminogen.** 88 μl of a solution of fibrinogen (3.4 μM) containing 227 nM Glu^1-Plg(S741C)fluorescein and 2 μl of 250 nM plasma plasminogen was pipetted into wells of a microtiter plate. Controls (●) lacked plasma plasminogen. The reactions were initiated by adding 10 μl of a solution of one- or two-chain t-PA (5.0 nM, final) containing IIa (6 nM, final), $CaCl_2$ (5 mM, final), and TAFIa (0 (■), 0.5 (○), 1.0 (▲), and 5.0 (□) nM). The diminution of emission at 535 nm of the samples was then monitored over the next 1.7 h with a microtiter plate reader coupled to a spectrofluorometer. The concentration of uncleaved substrate (*vertical axis*) is plotted against time.

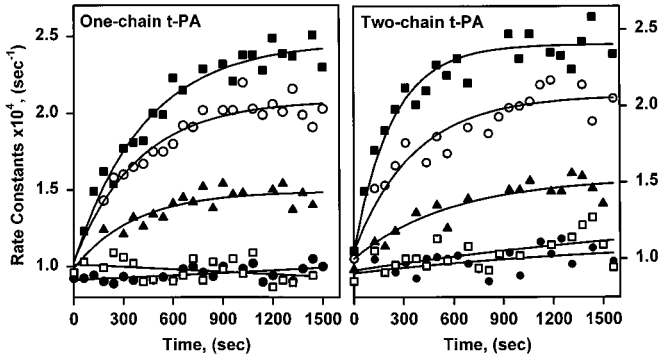

FIG. 3. **Effect of TAFIa on the rate constant for Glu1-Plg(S741C)fluorescein cleavage.** The point to point rate constants for the data of Fig. 2 are plotted *versus* time. *Symbols* are as in Fig. 2. The data indicate that TAFIa suppresses the up-regulation of the t-PA cofactor activity of fibrin during fibrinolysis.

(*solid squares*), presumably as a result of plasmin-catalyzed modification of fibrin by plasmin, with a concomitant enhancement of cofactor activity. This effect is attenuated progressively by TAFIa, with the half-maximal effect at approximately 1.0 nM TAFIa (*solid triangles*). At 5.0 nM TAFIa (*open squares*) no increase in the rate constant is observed. The effects with one- and two-chain t-PA were very similar. Although the data are not presented, the magnitude of the increase in rate constants was not increased by higher levels of plasminogen. These data thus confirm that plasmin formation in the early stages of fibrinolysis can promote the kinetics of t-PA-induced plasminogen activation as shown by others (17, 18) and that TAFIa eliminates this effect, presumably by removing newly formed carboxyl-terminal lysine and possibly arginine residues within fibrin. The inclusion of plasma plasminogen had no effect on the kinetics of cleavage of Lys^{78}-Plg(S741C-fluorescein) (not shown).

Effect of TAFIa on the Activation of Plasminogen and Conversion of Glu-plasminogen to Lys-plasminogen during Fibrinolysis—Previous work, in which the flow of plasminogen to plasmin during the fibrinolytic process within a clot was analyzed, showed that about 50% of the plasmin generated by the time the clot lysed was obtained through Lys-plasminogen (19). The following experiments were performed to determine whether TAFIa influences this process. With radiolabeled Glu-plasminogen, we monitored by urea/acetic acid-polyacrylamide

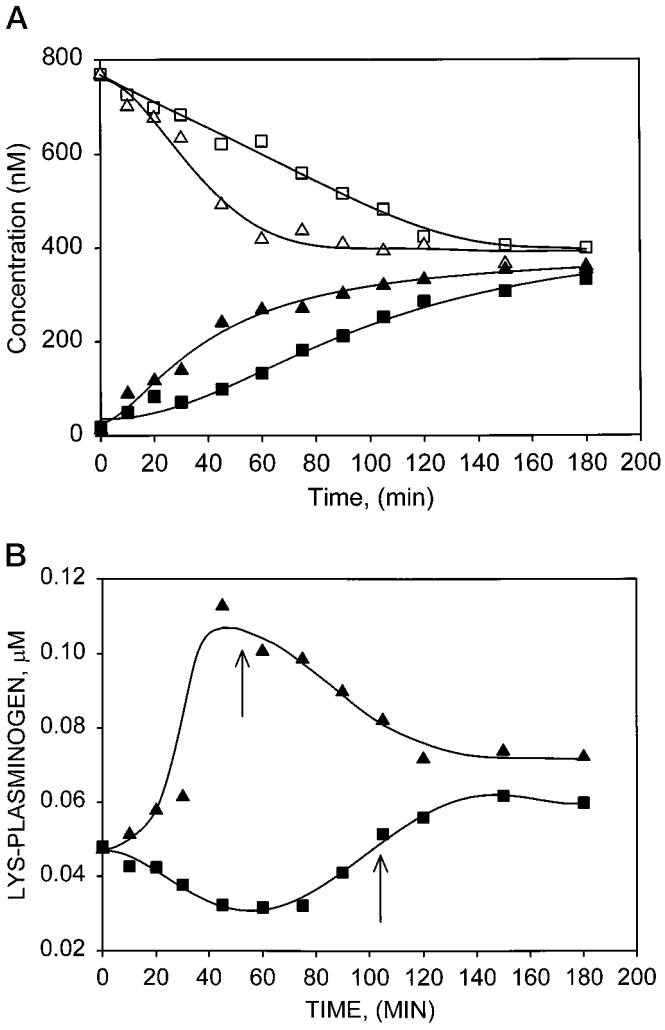

FIG. 4. **Effect of TAFIa on the activation of plasminogen and the conversion of Glu-plasminogen to Lys-plasminogen during fibrinolysis.** Shown in *panel A* are the time courses of the concentrations of Glu-plasminogen (△, □) and plasmin-antiplasmin complexes (▲, ■) when fibrinolysis was initiated by t-PA in a clot. The *triangles* represent results obtained without TAFIa and the *squares,* results with it. The concentrations of Lys-plasminogen are shown in *panel B.* Lysis times were 53 min in the absence of TAFIa and 104 min in the presence of TAFIa (*arrows*).

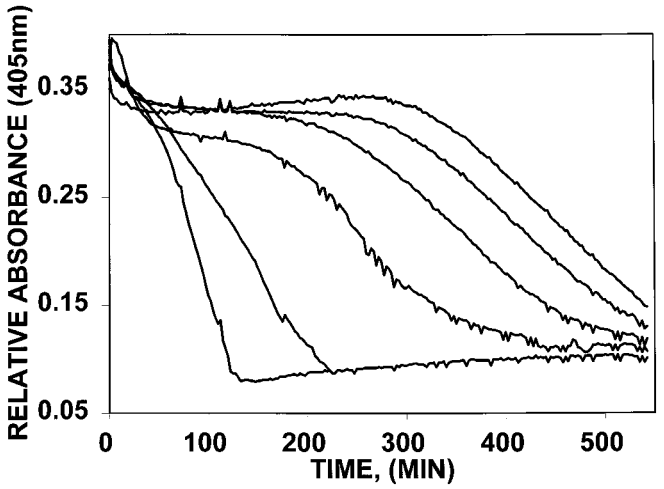

FIG. 5. **Inhibition of plasmin-induced clot lysis by TAFIa.** TAFIa solutions (7 μl) were pipetted into the wells of a microtiter plate containing small, separated aliquots of plasmin (2 nM, final), thrombin (6 nM, final), and $CaCl_2$ (10 mM, final). Clots were produced by adding 100 μl of a fibrinogen solution (3 μM, final) to the wells. Turbidity at 405 nm was then recorded at 37 °C over 10 h. The final TAFIa concentrations for the profiles from *left* to *right* were 0, 30, 60, 80, 100, and 120 nM.

gel electrophoresis the activation of plasminogen and the conversion of Glu-plasminogen to Lys-plasminogen during fibrinolysis in the absence and presence of TAFIa. Fig. 4*A* shows the time course of Glu-plasminogen consumption and concomitant formation of plasmin-antiplasmin complexes for clots formed in the presence and absence of TAFIa. The lysis times were 53 min in the absence of TAFIa and 104 min in its presence (40 nM), indicating an approximate 2-fold prolongation. In both instances the amount of Glu-plasminogen consumed equals the sum of the amounts of plasmin-antiplasmin complexes and Lys-plasminogen accumulated. In the absence of TAFIa, plasminogen consumption shows its characteristic acceleration as the lysis time is approached. This is also reflected in the formation of the plasmin-antiplasmin complexes. This characteristic acceleration, however, does not occur in the presence of TAFIa. These effects are very similar to those indicated in Fig. 2. In addition, the conversion of Glu-plasminogen to Lys-plasminogen occurs in the absence of TAFIa but not in its presence (Fig. 4*B*). The autoradiogram also indicated both the Glu and Lys forms of the plasmin-α_2-antiplasmin complex. In the presence of TAFIa only the Glu- form was evident, whereas in the absence of TAFI both forms were evident. We conclude that TAFIa not only prevents the feedback enhancement of plasminogen activation but also interferes with the fibrin-dependent conversion of Glu-plasminogen to Lys-plasminogen and attenuates the contribution of this reaction to the accelerated phase of plasminogen activation. These data are consistent with the conclusions of others that partially degraded fibrin, but not intact fibrin, is the cofactor for the conversion of Glu-plasminogen to Lys-plasminogen (17, 18).

Effect of TAFIa on Plasmin-induced Fibrinolysis and Plasmin Activity—To determine whether TAFIa inhibits fibrinolysis by modes other than inhibition of plasminogen activation and the conversion of Glu-plasminogen to Lys-plasminogen, the lysis of fibrin directly catalyzed by plasmin was studied in the presence and absence of TAFIa. Fig. 5 shows the time courses of plasmin-induced lysis of clots. In the absence of TAFIa, the clot lysed at about 60 min with 2 nM plasmin. In the presence of TAFIa, the lysis times were prolonged in a TAFIa concentration-dependent manner. The concentrations of TAFIa required to prolong lysis initiated by plasmin are considerably greater than those needed to prolong lysis initiated by t-PA plus plasminogen (the half-maximal effect is observed at 1.0 nM TAFIa (2)). Nonetheless, because the concentration of TAFI in human plasma is about 75 nM (9) and the TAFI is exquisitely sensitive to activation by thrombin-thrombomodulin (2), sufficient TAFIa possibly could be generated *in vivo* to attenuate plasmin-catalyzed fibrin degradation directly. The observation that TAFIa markedly inhibits lysis of fibrin by plasmin suggests that TAFIa can inhibit plasmin activity directly. Therefore, plasmin activity during fibrinolysis was measured (Fig. 6). In the absence of TAFIa, plasmin activity in the clot was stable. In the presence of TAFIa, however, plasmin activity was progressively lost. Thus, the inhibition of plasmin activity in the presence of TAFIa is consistent with prolongation of lysis initiated by plasmin. Although the data are not shown here, very similar results were obtained when fibrinogen was deleted from the experiment, thus indicating that fibrin or fibrin degradation products are not in some way responsible for the apparent inhibition. Binding of TAFIa to plasmin does not explain the inhibition because the kinetics of inhibition are too slow to be consistent with simple binding (7). To determine whether TAFIa can catalyze removal of the carboxyl-terminal

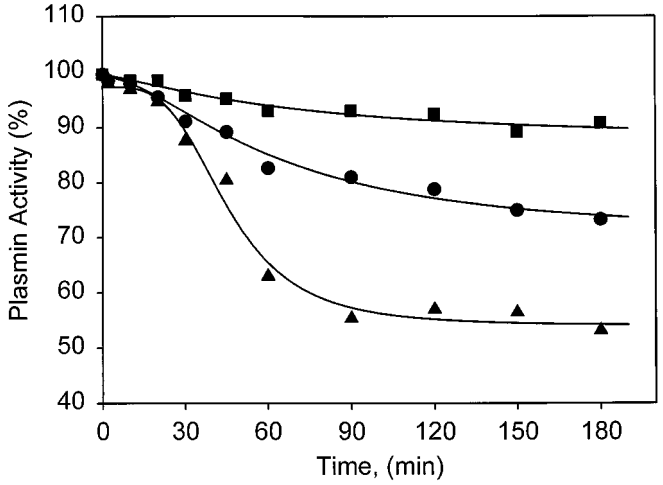

FIG. 6. **Inhibition of plasmin activity by TAFIa during fibrinolysis.** Three series of 12 identical clots containing 4 nM plasmin were formed at 37 °C, one in the absence (■) of TAFIa and another two in the presence of 25 nM (●) and 50 nM (▲) TAFIa, respectively. At various times clots were solubilized and quenched by adding acetic acid (0.1 M, final). Equal volumes of a solution of S-2251 (500 μM) in 437 mM HEPES, 10 mM EDTA, 2 mM ϵ-amino caproic acid, and 40 μM DAPA, pH 8.0, were added to the wells, and the time courses of increase of absorbance at 405 nm were then monitored. The initial rates of S-2251 hydrolysis, relative to that at zero time, were determined and are plotted *versus* time.

arginine of the heavy chain, active-site blocked plasmin (4.0 μM) was incubated with TAFIa (100 nM) for 30 min. Over this interval arginine (3.6 μM) was released. Thus, TAFIa is able to remove the single carboxyl-terminal arginine that appears when plasminogen is converted to plasmin. This, however, is not sufficient to account for loss of plasmin activity because porcine pancreatic carboxypeptidase B also catalyzes release of arginine from active-site-blocked plasmin but does not inactivate native plasmin (data not shown). Perhaps TAFIa has other exo peptidase activities not revealed by our current studies.

DISCUSSIONS

Numerous previous studies have shown that a carboxypeptidase with specificity for COOH-terminal arginine and lysine residues can attenuate several reactions and interactions associated with fibrinolysis. For example, de Vries *et al*. (20) showed that immobilized, plasmin-treated fibrin, after exposure to porcine carboxypeptidase B, exhibits reduced capacity for the binding of t-PA. Miles *et al*. (21) showed that treatment of U937 cells with the same carboxypeptidase reduces their capacity to bind plasminogen, presumably by removing the COOH-terminal lysine residue of the enolase moiety that appears to serve as a plasminogen receptor. Carboxypeptidase B-catalyzed removal of the COOH-terminal lysine residue of the heavy chain of urokinase decreases its catalytic efficiency about 2-fold in the activation of plasminogen (22). Fleury and Angles-Cano (23) demonstrated increased plasminogen binding capacity of immobilized fibrin that had been pretreated with plasmin, and the excess capacity could be eliminated with pancreatic carboxypeptidase B (23). In addition, carboxypeptidase B treatment of partially degraded fibrin reduces urokinase-mediated plasminogen activation (24) and reduces the quantity of plasminogen associated with fibrin during the lytic process (25).

The studies cited above clearly indicate the importance of carboxyl-terminal lysine and possibly arginine residues of partially degraded fibrin or cellular receptors in modulating binding of fibrinolytic components and fibrinolysis. These studies, however, did not allow the inference to be drawn that physiologically significant regulation could be associated with removal of these residues because the results were obtained with the pancreatic carboxypeptidase B. Eaton *et al*. (6), however, showed that plasma contains a precursor of carboxypeptidase B-like enzyme. Because it binds plasminogen they suggested that it might influence fibrinolysis when activated. Further work reported by Redlitz *et al*. (10) showed that this protein reduces the rate of whole blood clot lysis induced by t-PA. Other work led to the isolation of the protein (1, 4, 26) and showed that it was activated by the thrombin-thrombomodulin complex to the expected carboxypeptidase B-like enzyme (2). The activated enzyme was shown to suppress fibrinolysis potently in a system of defined components as well as in plasma (1, 2, 26). Thus, because the precursor is found in plasma and is activated by a physiologic activator, physiologic modulation of fibrinolysis by this carboxypeptidase B-like enzyme is plausible.

The present studies show that when fibrin is exposed to plasmin, stoichiometric levels of COOH-terminal arginine and lysine residues are generated which can be removed by TAFIa. To determine whether the removal of these residues influences plasminogen activation, the kinetics of cleavage of Glu^1-Plg(S741C)-fluorescein were measured in the presence and absence of TAFIa under conditions where limited plasmin degradation was allowed by including a trace of plasma plasminogen. The results showed that the rate constant for cleavage increases 2.5-fold during the reaction when plasma plasminogen is included, an effect that is eliminated completely with TAFIa present at 5.0 nM. A half-maximal effect is achieved at a TAFIa concentration of 1.0 nM, which is the same concentration needed to prolong fibrinolysis half-maximally (2). We conclude therefore that one of the means by which TAFIa suppresses fibrinolysis is by down-regulating the cofactor activity of partially degraded fibrin.

We also showed that TAFIa, at relatively elevated concentrations, interferes directly with plasmin-catalyzed fibrin degradation. The interplay between plasmin and TAFIa is complex. TAFIa can inhibit plasmin and vice versa. In addition, TAFIa activity decays spontaneously, especially at 37 °C (27). When TAFIa and plasmin are together, the long term result is that plasmin activity declines to either none or a finite, stable amount, whereas TAFIa decays completely. The decline of plasmin activity to a stable, finite level that depends on the initial concentration of TAFIa is evident in Fig. 6.

A previous study from this laboratory indicated that when plasminogen, α_2-antiplasmin, and t-PA are present within fibrin, approximately 50% of the plasmin formed during the lytic process is produced through the Lys-plasminogen intermediate (19). Because Lys-plasminogen is a much better substrate than Glu-plasminogen, the conversion of Glu-plasminogen to Lys-plasminogen potentially creates positive feedback in plasminogen activation and thereby promotes fibrinolysis. The formation of Lys-plasminogen is promoted by fibrin (18, 28) and consequently might be susceptible to modulation by TAFIa. The present work indicates that the accumulation of Lys-plasminogen during plasminogen activation and subsequent fibrinolysis is eliminated by TAFIa, and the time to achieve lysis is increased. These observations suggest that one of the means by which TAFIa suppresses fibrinolysis is by down-regulating the conversion of Glu-plasminogen to Lys-plasminogen. They also suggest that the reaction is dependent on, and promoted by, COOH-terminal lysine and/or arginine residues in fibrin. Notably, the data of Bajzar *et al*. (1) indicate that TAFIa has little influence on the activation of Lys-plasminogen. Thus, the conversion of Glu-plasminogen to Lys-plasminogen provides a means through which the fibrinolytic system can attenuate the inhibition elicited by TAFIa.

Hortin *et al*. (29) demonstrated that the COOH-terminal lysine residue of α_2-antiplasmin is removed in the presence of porcine pancreatic carboxypeptidase B with a consequent substantial loss of functional activity. Whether TAFIa modulates the properties of α_2-antiplasmin was not investigated in this work. TAFIa activity expressed *in vivo*, however, could account for the observation that plasma has two forms of α_2-antiplasmin, one of which both binds to plasminogen and has a COOH-terminal lysine and the other of which has neither of these properties (30, 31).

A recent study by Sakharov *et al*. (32) showed that the time to lyse clotted fibrin could be prolonged up to 4-fold by adding thrombomodulin, and this effect correlated with the activation of procarboxypeptidase B (TAFI). The effect was sustained over a wide range of t-PA concentrations, including those that would be expected during thrombolytic therapy. In addition, they showed that fluorescently labeled plasminogen accumulates on fibrin in the absence of added thrombomodulin but not in its presence. This latter effect could be eliminated with an inhibitor of TAFIa. From these observations, the conclusion was reached that TAFIa attenuates fibrinolysis by eliminating, by virtue of its carboxypeptidase B activity, plasminogen binding sites in partially degraded fibrin. The work by Sakharov and the present work are thus highly complementary. Sakharov *et al*. demonstrated TAFIa-dependent diminishing of plasminogen binding to fibrin but did not study the impact of this on plasminogen activation. In the present work, the binding of plasminogen to fibrin was not studied, but loss of the up-regulation of fibrin cofactor activity was demonstrated. The two studies suggest that loss of plasminogen binding and attenuation of cofactor activity are related, and both are caused by the TAFIa-catalyzed removal of carboxyl-terminal lysine (and possibly arginine) residues.

In summary, the current work shows that TAFIa suppresses fibrinolysis by down-regulating plasminogen activation and the conversion of the Glu-plasminogen to Lys-plasminogen, effects most likely caused by the removal of COOH-terminal lysine and/or arginine residues from partially degraded fibrin. These effects occur at very low relative concentrations of TAFIa. In addition, at higher concentrations, TAFIa also inhibits plasmin directly. Whether TAFIa also influences α_2-antiplasmin or other interactions associated with fibrinolysis is not known, but further work relating to modulation by TAFIa of other fluid phase or cellular reactions or interactions will contribute new insights into the role or roles of this carboxypeptidase B-like molecule in regulating fibrinolysis and coupling the coagulation and fibrinolytic cascades. A recent report by Redlitz *et al*. (33) is particularly intriguing. They showed, in a canine model of coronary thrombosis and thrombolysis, that a carboxypeptidase B-like activity, which is induced in serum and can be suppressed by a carboxypeptidase inhibitor from potatoes, correlates with both the time needed for reperfusion *in vivo* and for clots to lyse *in vitro*. The latter observations suggest that carboxypeptidase B-like activity modulates fibrinolysis not only *in vitro* but also *in vivo*.

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REFERENCES

1. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) *J. Biol. Chem.* **270,** 14477–14484
2. Bajzar, L., Morser, J., and Nesheim, M. (1996) *J. Biol. Chem.* **271,** 16603–16608
3. Bajzar, L., Nesheim, M. E., and Tracy, P. B. (1996) *Blood* **88,** 2093–2100
4. Hendriks, D., Wang, W., Scharpe, S., Lommaert, M. P., and van Sande, M. (1990) *Biochim. Biophys. Acta* **1034,** 86–92
5. Wang, W., Hendriks, D. F., and Scharpe, S. S. (1994) *J. Biol. Chem.* **269,** 15937–15944
6. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Drayna, D. (1991) *J. Biol. Chem.* **266,** 21833–21838
7. Tan, A. K., and Eaton, D. L. (1995) *Biochemistry* **34,** 5811–5816
8. Campbell, W., and Okada, H. (1989) *Biochem. Biophys. Res. Commun.* **162,** 933–939
9. Bajzar, L., Nesheim, M. E., Morser, J., and Tracy, P. B. (1998) *J. Biol. Chem.* **273,** 2792–2798
10. Redlitz, A., Nicolini, F. A., Malycky, J. L., Topol, E. J., and Plow, E. F. (1996) *Circulation* **93,** 1328–1330
11. Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979) *Biochemistry* **18,** 996–1003
12. Gaede, G., and Grieshaber, M. (1975) *Anal. Biochem.* **66,** 393–399
13. Nakatani, Y., Fujioka, M., and Higashino, K. (1972) *Anal. Biochem.* **49,** 225–231
14. Horrevoets, A. J. G., Pannekoek, H., and Nesheim, M. E. (1997) *J. Biol. Chem.* **272,** 2176–2182
15. Horrevoets, A. J. G., Pannekoek, H., and Nesheim, M. E. (1997) *J. Biol. Chem.* **272,** 2183–2191
16. Panyim, S., and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130,** 337–346
17. Suenson, E., Bjerrum, P., Holm, A., Lind, B., Meldal, M., Selmer, J., and Petersen, L. C. (1990) *J. Biol. Chem.* **265,** 22228–22237
18. Norrman, B., Wallen, P., and Ranby, M. (1985) *Eur. J. Biochem.* **149,** 193–200
19. Fredenburgh, J. C., and Nesheim, M. E. (1992) *J. Biol. Chem.* **267,** 26150–26156
20. de Vries, C., Veerman, H., and Pannekoek, H. (1989) *J. Biol. Chem.* **264,** 12604–12610
21. Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Plow, E. F. (1991) *Biochemistry* **30,** 1682–1691
22. Lenich, C., Pannell, R., and Gurewich, V. (1991) *Thromb. Res.* **64,** 69–80
23. Fleury, V., and Angles-Cano, E. (1991) *Biochemistry* **30,** 7630–7638
24. Pannell, R., Black, J., and Gurewich, V. (1988) *J. Clin. Invest.* **81,** 853–859
25. Sakharov, D. V., and Rijken, D. C. (1995) *Circulation* **92,** 1883–1890
26. Broze, G. J. Jr., and Higuchi, D. A. (1996) *Blood* **88,** 3815–3823
27. Boffa, M. B., Wang, W., Bajzar, L., and Nesheim, M. E. (1998) *J. Biol. Chem.* **273,** 2127–2135
28. Suenson, E., and Thorsen, S. (1988) *Biochemistry* **27,** 2435–2443
29. Hortin, G. L., Gibson, B. L., and Fok, K. F. (1988) *Biochem. Biophys. Res. Commun.* **155,** 591–596
30. Kluft, C., Los, P., Jie, A. F. H., van Hinsbergh, V. W. M., Vellenga, E., Jespersen, J., and Henny, C. P. (1986) *Blood* **67,** 616–622
31. Aoki, N., Sumi, Y., Miura, O., and Hirosawa, S. (1993) *Methods Enzymol.* **223,** 185–197
32. Sakharov, D. V., Plow, E. F., and Rijken, D. C. (1997) *J. Biol. Chem.* **272,** 14477–14482
33. Redlitz, A., Tan, A. K., Eaton, D. L., and Plow, E. F. (1995) *J. Clin. Invest.* **96,** 2534–2538

A Study of the Mechanism of Inhibition of Fibrinolysis by Activated Thrombin-activable Fibrinolysis Inhibitor

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