Antifibrinolytic Activities of α-N-Acetyl-L-Lysine Methyl Ester, ε-Aminocaproic Acid, and Tranexamic Acid

Importance of Kringle Interactions and Active Site Inhibition

Patrick K. Anonick, Jayanand Vasudevan, and Steven L. Gonias

α-N-acetyl-L-lysine methyl ester (NALME) is a lysine analogue that reportedly binds to low-affinity lysine binding sites in plasmin(ogen) and miniplasmin(ogen). In the studies presented here, we show that NALME has antifibrinolytic activity; however, unlike the therapeutic agents ε-amino-n-caproic acid (eACA) and tranexamic acid (TEA), the activity of NALME is based on inhibition of the plasmin active site. NALME (0.1-10 mM) significantly inhibited the amidase activity of plasmin, miniplasmin, and streptokinase-plasmin complex without affecting α-thrombin or tissue plasminogen activator. eACA and TEA (0.1-10 mM) did not affect the amidase activity of plasmin or miniplasmin. A kinetic analysis showed that NALME is a competitive inhibitor of D-Val-L-Leu-L-Lys-p-nitroanilide HCl (S-2251) hydrolysis by plasmin; NALME binding to plasmin completely prevented S-2251 binding. The Kᵢ for the plasmin–NALME interaction was 0.4 mM. eACA and TEA inhibited fibrin monomer digestion by plasmin and miniplasmin without binding to the active site of either enzyme. This result suggests that eACA and TEA function as antifibrinolitics by disrupting the noncovalent association of fibrin monomer with a domain common to both plasmin and miniplasmin (probably kringle 5). NALME inhibited fibrin monomer digestion principally by decreasing amidase activity. NALME was the only lysine analogue that prevented fragment X formation; TEA and eACA primarily inhibited the formation of fragments Y and D. When plasmin was incubated simultaneously with α₂-antiplasmin and α₂-macroglobulin, eACA increased the fraction of plasmin reacting with α₂-macroglobulin; NALME had no effect on the plasmin distribution. eACA, TEA, and NALME increased the euglobulin clot lysis time of normal plasma. NALME did not prolong the prothrombin time or activated partial thromboplastin time. These studies demonstrate that the antifibrinolytic activity of NALME is based on inhibition of the plasmin active site, whereas eACA and TEA are active due to kringle domain interactions. (Arteriosclerosis and Thrombosis 1992;12:708-716)

KEY WORDS • plasmin • fibrin • α₂-antiplasmin • α₂-macroglobulin • lysine • ε-aminoacaproic acid • tranexamic acid • kringle domains

In the circulation, fibrinolysis and fibrinogenolysis are mediated primarily by plasmin, the active serine proteinase counterpart of the zymogen plasminogen.1-2 The intact structure of plasminogen includes 791 amino acids with an N-terminal glutamic acid2-3 and either one or two oligosaccharide chains.4-8 Plasminogen activation results from the cleavage of the Arg89-Val106 peptide bond.9 Low concentrations of plasmin cleave [Glu] plasminogen to form [Lys78]-plasminogen, a second more readily activatable form of the zymogen.10 Plasminogen activation and the subsequent function of plasmin are modulated by noncovalent binding interactions with a variety of macromolecules, including fibrin,11-19 extracellular matrix proteins,20 and cellular receptors.21-23 These noncovalent interactions are mediated by a series of five homologous triple-disulfide-bonded plasmin(ogen) domains termed kringles.2-3 Each of the kringles apparently expresses a single binding site for lysine analogues such as ε-amino-n-caproic acid (eACA) and trans-(4-aminomethyl)-cyclohexanecarboxylic acid (tranexamic acid, or TEA) (Figure 1).24-34 Kringle 1 (K1), near the N-terminus of plasmin(ogen), has the highest affinity for eACA (9.0 μM) and TEA (1.1 μM).24,35; K4 contains a binding site with intermediate affinity, which is probably expressed only in [Lys78]-plasmin(ogen).27 The other kringles contain binding sites with lower affinities (0.1-10 mM).24,27,28 A sixth binding site for lysine analogues may be the plasmin active site.29,36

The role of the various kringle domains and the interrelation between these structures in promoting plasmin activity remain incompletely understood. Considerable attention has focused on the fibrin–plasmin(ogen) interaction.12-19 Christensen and colleagues50,37,38 suggested that there are two classes of functional fibrin
binding sites in plasminogen. The lysine binding site in K1 provides a high-affinity interaction; this interaction requires C-terminal lysine residues that are present in fibrin only after partial digestion. By contrast, a low-affinity binding site in K5 (termed the aminohexyl or AH site) may mediate the initial association of plasminogen with fibrin. The role of K5 in fibrin binding is supported by studies demonstrating fibrin's association with miniplasminogen (an elastase digestion fragment of plasminogen that includes the active site and the K5 domain). Urano et al proposed that the antifibrinolytic activity of eACA is based on disruption of plasminogen-fibrin interactions and is not due to inhibition of the tissue plasminogen activator (t-PA)-fibrin interaction. The same investigators also showed that eACA comparably inhibits fibrin clot lysis by plasmin or miniplasmin.

A second potentially important role for the lysine binding sites involves the conformation of plasminogen. In the presence of physiological concentrations of chloride ion, [Glu']-plasminogen exists in a "closed," poorly activated conformation while the conformation of [Lys']-plasminogen is "open" and more readily activated. Concentrations of eACA near 2.0 mM convert the conformation of [Glu']-plasminogen into the open form. Based on the affinity of the eACA-K5 interaction, Christensen and Mølgaard suggested that the binding of eACA to K5 may be critical in promoting plasminogen conformational change.

In studies with AH-Sepharose, Christensen identified α-N-acetyl-L-lysine methyl ester (NALME) (Figure 1) as a high-affinity ligand ($K_D$ 30 μM) for the AH site in miniplasminogen and [Lys']-plasminogen. By comparison, the $K_D$ for the binding of eACA in the same system was 300 μM. Because both eACA and TEA are used as intravascular antifibrinolytic agents in patients, the affinity of NALME for the AH site (K5 domain) suggested a possible therapeutic role for this lysine analogue. Thewes et al studied purified K5 by $^1$H nuclear magnetic resonance spectroscopy. These investigators demonstrated that the $K_D$ for the binding of NALME to purified K5 is 1.7 mM, significantly higher than the constant determined with eACA (94 μM).

The antifibrinolytic activity of lysine analogues may involve multiple mechanisms, including 1) inhibition of plasminogen binding to sites of rapid activation, 2) dissociation of active plasmin from substrate, and 3) direct inhibition of the plasmin active site. In this investigation, the antifibrinolytic activity of NALME was studied in comparison with eACA and TEA. Plasmin amidase activity, fibrinolytic activity, and reaction with α-antiplasmin (αAP) and α2-macroglobulin (αM) were studied in the presence of different concentrations of each agent. Our results demonstrate that the antifibrinolytic activity of NALME is unique because it is based on inhibition of the plasmin active site, unlike eACA and TEA, which function primarily by interacting with the plasminogen kringle domains. NALME was the only lysine analogue that inhibited the initial steps of fibrin digestion, which may be important in priming the clot for enhanced plasminogen activation. Therefore, NALME could potentially be used to regulate fibrinolysis in a manner that complements the activity of eACA or TEA.

Materials

- $p$-Nitrophenyl-$p'$-guanidinobenzoate hydrochloride (PNPGB), N-$p$-benzoyl-D-$l$-arginine-$p$-nitroanilide hydrochloride (BAPNA), eACA, TEA, and NALME were purchased from Sigma Chemical Co., St. Louis, Mo.
- D-Val-$l$-Leu-$l$-Lys-$p$-nitroanilide hydrochloride (S-2251) and D-Ile-$l$-Pro-$l$-Arg-$p$-nitroanilide hydrochloride (S-2288) were from Kabi Vitrum, Stockholm, Sweden.
- Na$I^2$I was from Amersham, Amersham Corp., Arlington Heights, Ill., and Iodobeads were from Pierce Chemical Co., Rockford, Ill.

Proteins

[Glu']-plasminogen was prepared from human plasma by the method of Deutsch and Mertz. Miniplasminogen was prepared by digesting human plasminogen with porcine pancreatic elastase and was purified by chromatography on lysine-Sepharose. 3 Miniplasminogen and plasminogen were activated with low-molecular-weight urokinase (Calbiochem Corp., La Jolla, Calif.) as previously described. The concentration of active proteinase was determined by the rate of S-2251 substrate hydrolysis at 22°C using a Michaelis constant ($K_m$) of 0.18 mM and a $k_{cat}$ of 11.8 second$^{-1}$ for plasmin and a $k_{cat}$ of 0.13 mM and a $k_{cat}$ of 10.8 second$^{-1}$ for miniplasmin.

αM and αAP were purified from human plasma by the methods of Imber and Pizzo and Wiman, respectively. Bowes melanoma one-chain t-PA was purchased from American Diagnostica (Greenwich, Conn.), and bovine thrombin was purchased from Sigma. Human α-thrombin was a gift from Dr. John Fenton II (Albany, N.Y.). Streptokinase was purified from Kabikinase by the method of Castellino et al. Streptokinase–plasin complex was prepared by incubating plasminogen with a twofold molar excess of streptokinase for 20 minutes at 37°C as previously described. Human fibrinogen, grade I, was obtained from Kabi Vitrum; this fibrinogen was used to prepare plasminogen-free fibrin monomer (Fn-M) as previously described.

Radioiodination

Plasminogen was radioiodinated with Iodobeads as described by the manufacturer (Pierce). Desalting was performed on Sephadex G-25 (Pharmacia Fine Chemi-
Amidase Activity

Plasmin (18 nM) and different concentrations of eACA, TEA, or NALME (0–10 mM) were incubated in the sample cuvette of a Hewlett-Packard 8450 diode-array spectrophotometer for 5 minutes at 22°C. The incubation buffer was 20 mM sodium phosphate, 150 mM NaCl, and 0.05% Tween-80, pH 7.4 (PBS-T). S-2251 (0.09–0.72 mM) was added, and the absorbance at 406 nm was measured every 2 seconds for 100 seconds. The velocity of substrate hydrolysis was determined by transforming absorbance measurements with the first-derivative function (dA406nm/dt). Equivalent studies were performed using miniplasmin and streptokinase-plasmin complex. All experiments were performed at least in triplicate.

The hydrolysis of S-2288 by human α-thrombin (2.8 nM) and t-PA (30 nM) was studied in the presence of NALME (0–10 mM) as described above. The substrate concentration was 0.2 mM for α-thrombin and 0.8 mM for t-PA.

Fibrinolysis by Plasmin and Miniplasmin

Plasmin or miniplasmin (18 nM) was incubated with eACA, TEA, or NALME (0–10 mM) in PBS-T for 5 minutes. Fn-M was then added, and incubation was continued for 30 minutes at 22°C. Reactions were stopped with the addition of PNPG (0.4 mM). Samples were denatured under nonreducing conditions and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 8% slabs using the HEPES-imidazole pH 7.3 buffer system described by McLellan58 as modified by Gionias and Figler.59 Fragments X, Y, and D were identified by comparison with molecular-weight standards (Bio-Rad, Richmond, Calif.). The molecular weights of fragments Y and D were 140,000 and 90,000, respectively, in good agreement with the published values.57,58

Reaction of Plasmin With α2-Antiplasmin

The reaction of plasmin with α2AP was studied in the presence of S-2251 and different concentrations of eACA, TEA, or NALME (0–10 mM). Plasmin (18 nM) was incubated with the lysine analogues in PBS-T for 5 minutes at 22°C. S-2251 (0.54 mM) was then added, and the absorbance at 406 nm was determined at 2-second intervals. At 100 seconds, the solution was made 18 nM in α2AP, and monitoring was continued for an additional 300 seconds. The concentration of active plasmin at each time interval (Pm) was determined by the velocity of substrate hydrolysis (dA406nm/dt). Apparent second-order plasmin inhibition constants (k-2app) were determined from the following relation that corrects for the effect of S-2251:

\[
Pm_0 = \frac{k_{\text{app}} \cdot t \cdot Pm_0}{1 + \frac{[S]}{K_M} + 1}
\]

where Pm0 is the initial concentration of active plasmin (before addition of α2AP), [S] is the concentration of S-2251, and K_M is the Michaelis constant for the hydrolysis of S-2251 by plasmin in the absence of lysine analogues (0.18 mM). Each study was performed at least in triplicate.

Reaction of Plasmin With α2-Macroglobulin

125I-plasmin (3.33 nM) was incubated with the lysine analogues (0–10 mM) in PBS-T at 22°C for 5 minutes. α2M (33.3 nM) was then added, and the incubation was continued for 15 minutes. PNPG (0.4 mM) was added to stop the reactions. Samples were denatured under nonreducing conditions and subjected to SDS-PAGE on 5% slabs. For the reaction of plasmin with α2M, >95% of the complex that forms is covalent.54,59 α2M-plasmin complex was clearly resolved from unreacted 125I-plasmin. Gels were autoradiographed, and each lane was sliced into 6-mm sections for counting in an LKB model 1275 Minigamma gamma counter (efficiency >80%). This procedure quantifies the free 125I-plasmin and the 125I-plasmin bound to α2M.

Reaction of Plasmin With Mixtures of α2-Antiplasmin and α2-Macroglobulin

125I-plasmin was incubated with eACA or NALME (0–10 mM) in PBS-T for 5 minutes. A premixed solution of α2AP and α2M was then added. The final concentrations of plasmin, α2AP, and α2M were 3.33 nM, 33.3 nM, and 1.96 μM, respectively. These concentrations were selected so that the plasmin would distribute nearly equally between the two inhibitors in the absence of lysine analogues. After incubation for 15 minutes at 22°C, reactions were terminated with addition of 0.4 mM PNPG and analyzed by SDS-PAGE, autoradiography, and gel slicing.

Prothrombin Time and Activated Partial Thromboplastin Time Determinations

Normal pooled plasma (3.8% wt/vol sodium citrate) was incubated with eACA, TEA, or NALME (0.2–10 mM) for 5 minutes at 4°C. Prothrombin time (PT) and activated partial thromboplastin time (APTT) values were determined by standard methods using a Coagamate X-2 automated analyzer. The PT reagent was Simplastin Excel (prepared from rabbit brain by Organon Teknika, Rockville, Md.). The APTT reagent consisted of micronized silica and rabbit brain phospholipid (Organon Teknika). Each value was determined in duplicate.

Euglobulin Clot Lysis Times

The euglobulin clot lysis time (ECLT) provides an index of plasminogen activation by intrinsic t-PA in the absence of plasmin inhibitors and in the presence of a fibrin clot.60 The effects of eACA, TEA, and NALME on the ECLT were determined by the method of Triplett and Harms.61 Briefly, plasma from normal human donors was anticoagulated with 3.8% sodium citrate, and aliquots (0.5 ml) were placed into several
Table 1. Effect of α-N-Acetyl-L-Lysine Methyl Ester on Amidase Activity of Proteinases

<table>
<thead>
<tr>
<th>NALME concentration (mM)</th>
<th>Proteinase</th>
<th></th>
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<tr>
<td></td>
<td>Plasmin</td>
<td>Miniplasmin</td>
<td>Thrombin</td>
<td>t-PA</td>
<td>SkPm</td>
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<td>100.0±1.0</td>
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<td>113.±4.2</td>
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<td>65.5±3.8</td>
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<td>100.5±9.5</td>
<td>56.0±2.2</td>
</tr>
<tr>
<td>10.0</td>
<td>14.3±1.8</td>
<td>15.4±1.2</td>
<td>94.3±2.0</td>
<td>98.4±0.1</td>
<td>10.0±1.3</td>
</tr>
</tbody>
</table>

NALME, α-N-acetyl-L-lysine methyl ester; t-PA, tissue plasminogen activator; SkPm, streptokinase-plasmin complex. Values are in percent activity±SEM. Activity is defined as the relative substrate hydrolysis velocity of the proteinase in the presence and absence of NALME.

*Proteinase was incubated with NALME for 5 minutes in the reaction cuvette before addition of substrate as described in the text. Absorbance at 406 nm was measured every 2 seconds for 100 seconds. The velocity of substrate hydrolysis was determined from the graph of dA/dt.

Results

Plasmin Amidase Activity

The amidase activity of plasmin was studied at 22°C in the presence of TEA and eACA (0.1–10 mM) using a single concentration of S-2251 (0.54 mM). Neither agent affected the rate of substrate hydrolysis by more than 7% in the specified concentration range (data not shown), consistent with the results of previous investigations.2940–62–63 Equivalent results were obtained with miniplasmin and streptokinase-plasmin complex.

NALME markedly inhibited the hydrolysis of S-2251 by plasmin, miniplasmin, and streptokinase-plasmin complex (Table 1). The hydrolysis of S-2251 by human α-thrombin and t-PA was unaffected by NALME, eACA, or TEA (0.1–10 mM).

Figure 2A shows a reciprocal plot of the kinetics of S-2251 hydrolysis by plasmin in the presence of NALME. The apparent KM was increased without a significant change in apparent kcat. A replot of the slopes from panel A versus NALME concentration was linear (correlation coefficient, 0.99) (Figure 2B), indicating that NALME is a simple competitive inhibitor of S-2251 hydrolysis by plasmin. The KI for the binding of NALME to plasmin, based on the replot, was 0.4 mM.

Fibrinolysis by Plasmin in the Presence of Lysine Analogues

The lysis of Fn-M by preactivated plasmin was studied after incubating the plasmin with eACA, TEA, or NALME (Figure 3). eACA and TEA at concentrations of 1.0 mM almost completely inhibited the formation of fibrin fragments Y and D. Marked inhibition was observed with 0.1 mM TEA, and a slight effect (mostly in the digestion of fragment Y) was seen with 0.1 mM eACA. Importantly, the concentrations of eACA and TEA that inhibited Fn-M digestion had no effect on plasmin amidase activity. The formation of fragment X was not significantly inhibited by eACA or TEA even at the highest concentration studied (10.0 mM).

Fn-M digestion by plasmin was not affected by 0.1 mM NALME (Figure 3). Inhibition of Fn-M digestion was observed with 1.0 mM NALME; however, the extent of inhibition was less than that demonstrated with 1.0 mM eACA or TEA. NALME at 10 mM almost completely inhibited the formation of fragments Y and D. Fragment X formation was also decreased; this activity was unique to NALME compared with eACA and TEA.

Fibrinolysis by Miniplasmin in the Presence of Lysine Analogues

Fn-M digestion by preactivated miniplasmin was studied after incubating the miniplasmin with eACA,
FIGURE 3. Fibrin monomer (Fn-M) digestion by plasmin in the presence of lysine analogues. Lane 1 shows the undigested Fn-M preparation. Lane 2 shows digestion products after incubation with plasmin (18 nM) at 22°C for 30 minutes. Lanes 3–5 show digestion in the presence of 0.1, 1.0, and 10.0 mM eACA, respectively. Lanes 6–8 show digestion in the presence of 0.1, 1.0, and 10.0 mM TEA, respectively. Lanes 9–11 show digestion in the presence of 0.1, 1.0, and 10.0 mM NALME, respectively. Frag. fragment; eACA, ε-amino-n-caproic acid; TEA, trans-(4-aminomethyl)-cyclohexanecarboxylic acid; NALME, α-N-acetyl-L-lysine methyl ester. Coomassie blue-stained gel.

TEA, or NALME (Figure 4). As demonstrated previously,63,64 the Fn-M fragments generated by miniplasmin were equivalent to those observed with plasmin. The extent of Fn-M digestion by miniplasmin after 30 minutes was somewhat decreased compared with the equivalent concentration of active plasmin, confirming the work of Ney and Pizzo.64 eACA and TEA inhibited Fn-M digestion by miniplasmin, and the concentration dependency of this activity was almost identical to that observed with plasmin. Miniplasmin lacks four of the five kringle domains found in plasmin.3 Because the miniplasmin active site was unaffected by eACA and TEA (as determined by amidase activity), interaction of these lysine analogues with the K5 domain was probably responsible for the inhibition of Fn-M digestion. eACA and TEA (even at 10 mM) had little effect on the formation of fragment X by miniplasmin.

FIGURE 4. Fibrin monomer (Fn-M) digestion by miniplasmin in the presence of lysine analogues. Lane 1 shows the undigested Fn-M preparation. Lane 2 shows digestion products after incubation with miniplasmin (18 nM) at 22°C for 30 minutes. Lanes 3–5 show digestion in the presence of 0.1, 1.0, and 10.0 mM eACA, respectively. Lanes 6–8 show digestion in the presence of 0.1, 1.0, and 10.0 mM TEA, respectively. Lanes 9–11 show digestion in the presence of 0.1, 1.0, and 10.0 mM NALME, respectively. Frag. fragment; eACA, ε-amino-n-caproic acid; TEA, trans-(4-aminomethyl)-cyclohexanecarboxylic acid; NALME, α-N-acetyl-L-lysine methyl ester. Coomassie blue-stained gel.

NALME inhibited Fn-M digestion by miniplasmin when present at 1.0 mM or greater. NALME (10 mM) also inhibited fragment X formation. These results were comparable to those obtained with plasmin.

Reaction of Plasmin With α2-Antiplasmin in the Presence of Lysine Analogues

α2AP is the primary antiplasmin in human plasma.65,66 The second-order rate constant (kapp) for the inhibition of plasmin by α2AP is ~2.0×10^7 M⁻¹·sec⁻¹; this rapid reaction depends on noncovalent association of α2AP with the plasmin Kl-3 domain.62 Table 2 shows kapp values determined for the inhibition of plasmin by α2AP in the presence of lysine analogues. eACA or TEA (0.1 or 1.0 mM) significantly decreased the kapp; the 30-fold decrease caused by 10 mM eACA is consistent with the results of previous studies.62-67 As much as a 40-fold decrease in kapp was observed with TEA. These results probably reflect binding of eACA or TEA to the Kl-3 domain of plasmin. By contrast, 0.1 mM NALME did not significantly affect the reaction of plasmin with α2AP. With 1.0 mM NALME, the kapp was decreased by <25%. This minor change in reaction rate was close to the 40% decrease that would be predicted for 1.0 mM NALME functioning strictly as a competitive inhibitor (0.54 mM S-2251). Experiments with the highest concentration of NALME (10 mM) were precluded by the decrease in S-2251 hydrolysis rate (Table 1).

<table>
<thead>
<tr>
<th>Lysine analogue</th>
<th>eACA</th>
<th>TEA</th>
<th>NALME</th>
</tr>
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<tr>
<td>Concentration (mM)</td>
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<td>10.0</td>
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<td>2.9±0.6×10⁵</td>
<td>ND</td>
</tr>
</tbody>
</table>

kapp values (in M⁻¹·sec⁻¹) were determined as described in text and represent mean±SEM of at least three experiments. eACA, ε-amino-n-caproic acid; TEA, trans-(4-aminomethyl)-cyclohexanecarboxylic acid; NALME, α-N-acetyl-L-lysine methyl ester; ND, not determined.

TABLE 2. Plasmin Inhibition by α2-Antiplasmin in the Presence of Lysine Analogues

αM is the second major antiplasmin; the rate constant for the reaction of plasmin with αM is 5×10^5 M⁻¹·sec⁻¹.19,68-70 Unlike α2AP, the kringle domains probably do not play an important role in the plasmin/αM reaction; eACA does not significantly affect the rate of reaction.69 Although plasmin binding to αM is almost always covalent,54-59 nucleophilic amines can inhibit covalent bond formation without affecting irreversible αM–proteinase complex formation. To address this possibility, 125I-plasmin (3.3 nM) was preincubated with eACA (0.01–10.0 mM) and then reacted with αM (33 nM) for 1 hour. The extent of covalent bond formation between 125I-plasmin and αM was not changed, as determined by SDS-PAGE (data not shown). This preliminary study demonstrated that the SDS-PAGE method could be used to study the reaction.
Inhibition of Plasmin by Mixtures of α2-Macroglobulin and α2-Antiplasmin

125I-plasmin (3.33 nM) was incubated with either εACA or NALME (0–10 mM) for 5 minutes. Premixed solutions of α2AP (33.3 nM) and α2M (1.96 μM) were added, and reactions were conducted for 15 minutes.

Because of the extremely high concentrations of protease inhibitors used in these experiments, the plasmin completely reacted within the allotted incubation time (with or without εACA and NALME). In the absence of εACA or NALME, approximately 50% of the plasmin bound to each inhibitor (Table 4). εACA altered the distribution of 125I-plasmin so that more α2M-plasmin was formed, with 10 mM εACA, ~80% of the plasmin bound to α2M. This result is consistent with the known effect of εACA on the noncovalent interaction of α2AP with plasmin.

NALME had no effect on the distribution of 125I-plasmin between α2AP and α2M, supporting the hypothesis that NALME principally affects the plasin active site. In a reversible equilibrium system when NALME is not bound to the plasmin active site, the plasmin reacts with α2AP or α2M with unchanged specificity.

Effect of α-N-Acetyl-L-Lysine Methyl Ester on Coagulation

NALME, εACA, and TEA at concentrations up to 10 mM were incubated with normal pooled plasma. The PT and APTT were not affected by any of the lysine analogues (data not shown).

Effect of Lysine Analogues on the Euglobulin Clot Lysis Time

The ECLT may be affected by inactivation of t-PA, disruption of the fibrin clot–dependent plasminogen activation mechanism, dissociation of plasmin from the clot, or direct inhibition of plasmin. Table 5 compares the effects of εACA, TEA, and NALME on the ECLT of normal plasma. εACA and TEA, even at 0.1 mM, prolonged the ECLT, probably reflecting disruption of the fibrin clot–dependent plasminogen activation mechanism. NALME also prolonged the ECLT but to a lesser extent than εACA or TEA. With 10 mM NALME, the ECLT was 2.2 times the normal value (9.6 hours). This prolongation is probably caused by the interaction of NALME with the plasmin active site; however, we cannot exclude other mechanisms in this experimental system.

Discussion

While it is clear that noncovalent binding of plasminogen to fibrin is critical in fibrinolysis, the relative importance of the various kringle domains in mediating this interaction remains unresolved. One theory proposes that the K5 domain mediates the initial interaction of plasminogen with fibrin. Binding of K5 to fibrin is thermodynamically weak but necessary for initiating the cleavage of fibrin, a process that generates new C-terminal lysine residues. These C-terminal lysine residues then provide new plasminogen binding sites that
interact preferentially with the K1 plasminogen domain.37

Christensen30 identified NALME as a reagent that can displace plasminogen from AH-Sepharose by interacting with a low-affinity lysine binding site termed the AH site. Subsequent studies suggested that the AH site is equivalent to the K5 lysine binding site.27,38 Because the K5 domain of plasminogen may play a critical role in fibrinolysis, we hypothesized that NALME might demonstrate unique antifibrinolytic properties compared with the established therapeutic agents eACA and TEA.

Any lysine analogue may affect fibrinolysis by interacting with the plasminogen kringle domains or the plasmin active site. In the present investigation, we compared the antifibrinolytic activities of NALME, eACA, and TEA using chromogenic substrate hydrolysis experiments, fibrin cleavage studies, proteinase inhibitor experiments, and ECLT assays. The results of our studies and the work of others strongly suggest that each lysine analogue is capable of interacting with multiple sites in the plasminogen (structure). Nevertheless, for each agent, a specific interaction or class of interactions is primarily responsible for the antifibrinolytic activity. For eACA and TEA, our studies confirm that antifibrinolytic activity is due primarily to interaction with the kringle domains.39 As shown here and elsewhere,29 eACA and TEA interact minimally with the plasmin active site when present at concentrations that are sufficient to significantly alter fibrinolytic activity (10 mM or less). By contrast, the activity of NALME is based primarily on inhibition of the plasmin active site. While indeed NALME may interact with K5 when present in sufficient concentration, this interaction probably contributes minimally to the antifibrinolytic activity of this agent.

The plasmin amidase activity studies demonstrated that NALME is a simple competitive inhibitor of S-2251 hydrolysis. Accordingly, the plasmin–NALME complex is completely incapable of binding S-2251. These data are most simply explained by direct association of NALME with the plasmin active site. Similar competitive inhibition may be observed if NALME binds to a separate area of plasmin, allosterically altering the structure of the active site so that binding of S-2251 is completely prevented. This second explanation is considered less likely, as NALME binding sites other than the active site are probably located in distinct plasmin domains that have minimal potential for altering active site structure.43 In addition, in our studies TEA and eACA probably saturated the kringles domain lysine binding sites without affecting plasmin amidase activity.

The Kᵢ for the plasmin–NALME interaction, based on the analysis of S-2251 hydrolysis, was 0.4 mM. In the presence of other plasmin substrates, such as S-2251 or fibrin, concentrations of NALME somewhat higher than the Kᵢ should be necessary to occupy 50% of the plasmin active sites. Therefore, the Kᵢ (0.4 mM) is consistent with the concentrations of NALME (1-2 mM) that eliminated 50% of the activity of plasmin in the different experimental systems studied here.

In Fn-M digestion experiments, the lowest concentration of TEA (0.1 mM) significantly but incompletely inhibited the formation of fragments Y and D. eACA at the same concentration had only a slight effect. These results indicate that TEA and eACA are functioning at concentrations significantly above the reported Kᵢ values for the respective K1 interactions. In addition, the efficacies of TEA and eACA in preventing Fn-M digestion were essentially identical with plasmin and miniplasmin. These data strongly suggest that cleavage of Fn-M to form fragments Y and D requires noncovalent binding of the Fn-M to a domain present in both plasmin and miniplasmin. Furthermore, because this particular domain behaves as a low-affinity lysine binding site, it is most likely that K5 mediates the critical plasmin–Fn-M interaction, resulting in soluble fibrin digestion. These studies are consistent with the work of Urano et al.39

The initial cleavage of fibrin to form early digestion products (fragment X) may be extremely important because this digestion results in the formation of new high-affinity plasminogen binding sites and enhances the activation of plasminogen by t-PA.18,47,48 NALME was unique among the lysine analogues in its ability to inhibit fragment X formation. In this capacity, eACA and TEA were relatively ineffective. These data suggest that the initial steps of fibrin digestion may occur independent of noncovalent plasmin–fibrin interactions, unlike the later steps that result in the formation of fragments Y, D, and E.

In a previous investigation,19 we presented evidence for a high-affinity interaction of Fn-M with plasmin but not with miniplasmin. The apparent Kᵢ for the plasmin–Fn-M interaction was 20 nM, as determined by an analysis of S-2251 hydrolysis (the actual Kᵢ may have been somewhat higher if each fibrin monomer provided more than one plasmin binding site). As a result of Fn-M binding, the reaction of plasmin with α₂AP was inhibited; however, as suggested in the previous study,19 the Fn-M preparation was partially degraded by plasmin. This modification was probably necessary for the high-affinity interaction. Figures 3 and 4 show that Fn-M binding sites specific for plasmin and not miniplasmin were not important in determining the rate of soluble fibrin cleavage. Of course, a more synergistic relation may exist between binding sites in the lysis of polymerized fibrin as opposed to soluble fibrin. In the intact polymer, different kringles may function together to permit plasmin movement across the degrading clot. Overall, this study demonstrates that the antifibrinolytic activities of lysine analogues vary in a qualitative (mechanism of activity) as well as a quantitative (concentration dependency) manner. The complementary modes of action of NALME and eACA or TEA suggest that these agents might be efficacious as therapeutics when used in combination.

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Antifibrinolytic activities of alpha-N-acetyl-L-lysine methyl ester, epsilon-aminocaproic acid, and tranexamic acid. Importance of kringle interactions and active site inhibition.

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