Stimulation of plasmin activity by oleic acid

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The amidolytic activity of plasmin with the chromogenic substrate H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide (S-2251) is stimulated by oleic acid in a dose-dependent and saturable fashion. The activity of plasmin on S-2251 in the presence of oleic acid followed a sigmoidal kinetic pattern, with an almost 4-fold stimulation of activity at 60 μ M-oleic acid. Half-maximal stimulation occurred at an oleic acid level of 19.5 μ M. The amino acid analogue 6-aminohexanoic acid (AHA), which is known to bind to lysine-binding sites in plasmin, suppressed the stimulatory effect of oleic acid in a concentration-dependent manner; at 0.3 mM-AHA, about 70 % of the oleic acid-dependent enhancement of plasmin activity was abolished. The $1/v$ versus $1/[S]$ plot for plasmin changed in the presence of oleic acid from a linear to a non-linear curve, suggesting positive co-operativity. 14 C-labelled oleic acid bound to plasmin, and the bound ligand was displaced by an excess of unlabelled oleic acid. Oleic acid also produced a marked (40-fold) stimulation of the plasminogen-dependent cleavage of S-2251 by urokinase. A half-maximal effect on plasminogen activation was obtained at 40 μ M-oleic acid. The present findings suggest that the ability of oleic acid to stimulate plasmin activity and to enhance the conversion of plasminogen to plasmin depends on the interaction of oleic acid with specific lysine-binding sites in plasmin.

INTRODUCTION

Plasmin is a non-specific serine proteinase that is generated from its inactive precursor plasminogen by specific plasminogen activators. Plasmin has a pivotal function in thrombolysis and fibrinolysis by virtue of its ability to effectively digest the insoluble fibrin clots into soluble fragments [1]. In addition, the localized production of plasmin by plasminogen activators plays a major role in a variety of biological processes involving extracellular proteolysis, tissue remodelling, invasion and cell migration [2–4]. Plasmin also converts Glu-plasmin(ogen) to Lys-plasmin(ogen), converts pro-urokinase to urokinase (uPA) and degrades fibrinogen and other plasma proteins as well as miscellaneous matrix proteins [3–5].

Tissue-type plasminogen activator, plasminogen and plasmin contain structures, called lysine-binding sites (LBS), which interact specifically with lysine, 6-aminohexanoic acid (AHA) and various lysine analogues [6,7]. Ligands such as fibrin [8,9], polylysine [10], denatured proteins [11] and fragments of fibrin [12] bind to LBS in tissue-type plasminogen activator and/or in Glu-plasminogen. Binding of the ligands to plasminogen gives rise to non-covalent modulation(s) that stimulate its conversion to plasmin [13,14]. Two distinct types of LBS, differing in their affinity toward lysine and AHA, were observed in plasminogen [15,16]. In the plasmin molecule the LBS are located in the A-chain, while the catalytic centre of the enzyme resides in the B-chain [17,18]. The LBS in plasmin(ogen) mediate the binding of plasmin(ogen) to fibrin(ogen), plasmin to its specific inhibitor α_2 -antiplasmin, and plasmin to fibrin(ogen) [19–21]. Therefore the LBS appear to play a crucial role in the regulation of fibrinolytic activity [16,22].

Initially, plasmin activity was only found to be subject to inhibition by a limited number of specific or unspecific inhibitors [1,22–24]. However, more recent studies suggest that plasmin is a regulatory enzyme, and that it can be inhibited in a reversible fashion by ligands that interact with the enzyme at LBS distant from the catalytic site [25–27].

In the course of our studies on ligands that regulate the fibrinolytic cascade, we have examined the effect of the abundant endogenous fatty acid oleic acid. We report here that oleic acid enhances the amidolytic activity of plasmin and stimulates the conversion of Glu-plasminogen into plasmin.

MATERIALS AND METHODS

Materials

Glu-plasminogen was prepared from outdated human plasma by the method of Deutsch & Mertz [28]. Agarose–L-lysine for preparation of plasminogen was from Bio Makor, Rehovot, Israel. The chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide) and plasmin were purchased from Kabi Diagnostica, Stockholm, Sweden. uPA reference standard [B grade, 3157 units/ampoule (2210 Ploug units)] was obtained from Calbiochem, San Diego, CA, U.S.A. The uPA used in the electrophoretic assay of plasminogen activation was human urine high-molecular-mass uPA (3000 units/ampoule) from Calbiochem, La Jolla, CA, U.S.A. Oleic acid was from Sigma Chemical Co., St., Louis, MO, U.S.A., and 14 C-labelled oleic acid (50 mCi/mmol) was from Amersham International, Amersham, Bucks., U.K. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden.

Chromogenic assays of plasminogen activation

In the chromogenic assays, the activation of plasminogen to plasmin was determined by monitoring the appearance of the amidolytic activity of plasmin. The ability of plasmin to cleave the chromogenic substrate S-2251 was monitored by measuring the production of the chromophore *p*-nitroaniline in an end-point reaction. For assays of plasminogen activation, 0.14 unit of uPA was incubated in 0.9 ml of 0.2 M-Tris/HCl buffer, pH 8.0, containing 1.23 μ M-Glu-plasminogen and 0.56 mg of poly(ethylene glycol) 6000 (5 g/l)/ml. The substrate S-2251 was added in the concentrations indicated in the text and Figure legends. After incubation of 37 °C for 30 min, 100 μ l of 50 % (w/w) acetic

Abbreviations used: AHA, 6-aminohexanoic acid; LBS, lysine-binding sites; uPA, urokinase; S-2251, H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide; c.u., casein units.

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acid was added and the mixture was centrifuged at 12000 *g* for 15 min to remove occasional turbidity. Absorbance was measured at 405 nm in a Gilford 300 spectrophotometer. Controls were run in the absence of Glu-plasminogen to confirm that uPA does not cleave S-2251 directly in either the presence or the absence of oleic acid. A control without uPA was also tested to confirm the stability of the substrate as well as the absence of plasmin contamination in Glu-plasminogen.

Chromogenic assay of plasmin activity

The activity of plasmin was determined by a continuous kinetic method with substrate S-2251. Release of *p*-nitroaniline from S-2251 was measured by monitoring the rate of absorbance change at 405 nm in a Kontron Uvicon 930 spectrophotometer. The reaction mixture contained 2.5×10^{-3} c.u. (casein units) of plasmin/ml in 0.2 M-Tris/HCl buffer, pH 8.0, and S-2251 at the concentrations indicated for the different experiments. Usually the reaction was monitored for up to 10 min, and rates were calculated for the linear portion of the activity curves.

Binding of ^{14}C -labelled oleic acid to plasmin

A 10 μl sample of ^{14}C -labelled oleic acid (50 mCi/mmol; 250 μCi in 2.5 ml of toluene) was evaporated and redissolved in 50 μl of absolute ethanol. A 20 μl portion of this solution (8 nmol) was added to 2.5 mg of plasmin in 0.5 ml of phosphate-buffered saline (12.6 mM- KH_2PO_4 , 64 mM- Na_2HPO_4 , 86 mM- NaCl , adjusted with HCl to pH 7.4), in the absence or presence of 125 nmol of unlabelled oleic acid. After 10 min of incubation at room temperature, the mixtures were applied on to a 10 ml Sephadex G-52 column and eluted with phosphate-buffered saline, pH 7.4. Fractions of 1.0 ml were collected for counting of radioactivity and absorbance reading at 280 nm.

Oleic acid solution

A stock solution of 24 mM-oleic acid in ethanol was prepared. Dilutions were done in 0.2 M-Tris/HCl buffer, pH 8.0. Controls with the vehicle alone indicated that the diluted ethanol had no effect on the enzymes.

RESULTS

The effect of increasing levels of oleic acid on plasmin activity was assayed by employing the chromogenic peptide substrate S-2251. Fig. 1 shows that at a S-2251 concentration equivalent to the K_m value, i.e. 0.29 mM, oleic acid produced a marked, dose-dependent and saturable stimulation of plasmin activity. The stimulation exhibited a sigmoidal kinetic pattern, with a maximal enhancement of 375% at 60 μM -oleic acid. Half-maximal stimulation was obtained at an oleic acid concentration of 19.5 μM .

To determine whether the stimulatory effect of oleic acid is a result of a specific interaction of the ligand with the enzyme, we examined the ability of plasmin to bind oleic acid. Incubation of plasmin with [^{14}C]oleic acid resulted in comigration of the label with the protein on a Sephadex G-25 gel-filtration column, indicating binding of oleic acid to plasmin (results not shown). Addition of an excess of unlabelled oleic acid decreased the binding by about 90%, signifying that the binding of the ligand is specific.

Fig. 2 is a $1/v$ versus $1/[S]$ reciprocal plot of plasmin activity with S-2251. In the absence of oleic acid the enzyme exhibits typical Michaelis-Menten kinetics. The addition of 19.5 μM -oleic acid (a concentration that produces 50% stimulation; see Fig. 1)

Fig. 1. Effect of oleic acid on plasmin activity, measured by cleavage of chromogenic substrates

Increasing concentrations of oleic acid were present during the chromogenic assays of plasmin activity with 0.29 mM S-2251, i.e. $[S] = K_m$. Purified plasmin (1.25×10^{-3} c.u.) was incubated with the substrate for 30 min. Under the conditions of the assay, 2.61 μmol of S-2251/min was cleaved in the absence of oleic acid (100%). The assay was performed in absence of AHA (○), in presence of 65 μM -AHA (△) or in presence of 650 μM -AHA (□). Values are means $\pm$ S.D. for five repeats.

Fig. 2. Lineweaver-Burk reciprocal plot for plasmin activity with S-2251

Plasmin activity with substrate S-2251 was assayed in the absence (○) and presence (●) of 19.5 μM -oleic acid.

converted the curve from a linear to a concave pattern. A concave curve is typical of positive co-operativity. The experiment depicted in Fig. 2 thus indicates that oleic acid has a dual effect on plasmin: it stimulates the activity of plasmin throughout the tested range of substrate concentrations, and it induces a positive co-operativity pattern of plasmin activity.

The present observation that plasmin is subject to positive co-operativity conforms with our earlier finding that polymerized ampicillin induces positive co-operativity in plasmin [25]. In the case of the ampicillin polymer, the change that the ligand induces in plasmin could be abolished by the amino acid analogue AHA, indicating the involvement of LBS in this effect. To test whether the presently observed effect of oleic acid on plasmin similarly involves interaction with LBS, we determined the effect of AHA on the oleic acid-induced stimulation. Fig. 3 shows that increasing

Fig. 3. Effects of glutamine and AHA on oleic acid-stimulated plasmin activity

Glutamine at the indicated concentrations was added to plasmin at a S-2251 concentration of 0.29 mM ($[S] = K_m$; $\blacktriangledown$) and in the presence of 19.5 μM -oleic acid. AHA at the indicated concentrations was added to plasmin in presence of 19.5 μM -oleic acid at several substrate concentrations: $[S] = K_m$ ($\square$), $[S] = 2K_m$ ($\triangle$) and $[S] = K_m/2$ ($\bullet$). The chromogenic activity was assayed as outlined in the legend to Fig. 1.

levels of AHA decreased, in a concentration-dependent manner, the stimulation of plasmin by oleic acid. At $[S] = K_m$ and with 19.5 μM -oleic acid, an amount that induces a $\sim 50\%$ stimulation of the chromogenic activity of plasmin, 65 μM -AHA was needed to decrease the oleic acid-induced stimulation by 50%. AHA at 0.3 mM decreased the stimulatory effect of oleic acid by 70%. A further increase in the concentration of AHA above 0.3 mM failed to produce a larger suppression of the effect of oleic acid, and a plateau was consequently obtained. Fig. 3 also indicates that glutamine had no effect on the response of plasmin to oleic acid. This observation rules out the possibility that AHA elicits a non-specific interaction with oleic acid through carboxyl-amino-group interactions.

Since the experiment shown in Fig. 3 indicated that AHA was capable of decreasing the oleic acid-induced enhancement of plasmin, it was of interest to determine the kinetics of the effect of oleic acid in the presence of AHA. The results of such an experiment are shown in Fig. 1. AHA at 65 μM , a concentration that decreased the response of plasmin to oleic acid by 50%, markedly suppressed the oleic acid-induced stimulation throughout the tested range of oleic acid concentrations. This effect is manifested by a rightwards shift of the curve. A 10-fold higher level of AHA (650 μM) abolished the positive co-operativity induced by oleic acid (Fig. 1), whereas the dose-dependent and saturable stimulation of plasmin by increasing levels of oleic acid was maintained.

The co-operativity shown in Fig. 2 suggested that the substrate, at certain concentrations, affects plasmin at more than one site. If this is the case, the response of plasmin to modulating ligands might be different at different substrate concentrations. This possibility can be tested by comparing the effect of AHA on plasmin in the presence of stimulatory levels of oleic acid and at various substrate concentrations (Fig. 3). The results obtained at $[S] = K_m$ were discussed above. In addition, Fig. 3 shows that at $[S] = 2 \times K_m$, AHA had no effect on the stimulation produced by oleic acid. In contrast, when $[S] = K_m/2$, AHA produced the same effect as observed at $[S] = K_m$. At the three substrate

Fig. 4. Effect of oleic acid on the activation of plasminogen by uPA

The activation of plasminogen by uPA was assayed by measuring the plasminogen-dependent cleavage of the chromogenic substrate S-2251. Because of the extensive stimulation of uPA activity by oleic acid, different concentrations of the enzyme were employed: at 0, 8 and 24 μM -oleic acid, 0.14 unit; at higher concentrations of oleic acid, 0.06 unit. The enzyme was incubated with plasminogen and S-2251 for 30 min as detailed in the Materials and methods section. 100% activity of uPA at 0.14 unit corresponded to 0.1 absorbance unit at 405 nm. Values are means $\pm$ S.D. for five repeats.

concentrations tested, the sigmoidal pattern of the response of plasmin to oleic acid was maintained in spite of the presence of 65 μM -AHA.

Fig. 4 shows that oleic acid produced a dramatic stimulation of the activation of Glu-plasminogen by uPA, with a maximal enhancement of more than 30-fold over the activity in the absence of oleic acid. Half-maximal stimulation was obtained at 38 μM -ligand. In this experiment the activation of Glu-plasminogen by uPA was assayed by a dual-stage method in which the plasmin production was monitored by measuring its activity with S-2251. Under similar conditions the activity of plasmin alone (Fig. 1) was only stimulated by 375%, indicating that most of the dramatic stimulation presented in Fig. 4 involves an effect of oleic acid on plasminogen activation rather than on plasmin activity. Control experiments confirmed that, regardless of the presence or absence of oleic acid, uPA does not cleave S-2251 in the absence of plasminogen.

The direct stimulatory effect of oleic acid on the activation of plasminogen was also demonstrated by an electrophoretic assay of uPA-mediated plasminogen activation. Electrophoresis of heat-denatured Glu-plasminogen on 10% polyacrylamide gels containing 1% SDS exhibited one major band of protein (molecular mass ~ 90 kDa). The activation of plasminogen by uPA was documented by disappearance of the plasminogen band and the emergence of a new band corresponding to the heavy chain of plasmin (molecular mass ~ 55 kDa). Inclusion of increasing concentrations of oleic acid in the activation stage enhanced this conversion of Glu-plasminogen to plasmin in a concentration-dependent manner (results not shown).

DISCUSSION

This study demonstrates that oleic acid binds to plasmin and stimulates plasmin activity. Oleic acid is known to bind avidly to many proteins including albumin and several intracellular fatty-acid-binding proteins [29,30]. It also interacts with additional

proteins such as the tri-iodothyronine receptor [31,32] and fibronectin [33] through specific binding sites. The high-affinity binding of oleic acid to fibronectin was found to induce changes in the protein conformation and to increase the susceptibility of fibronectin to proteolysis [33]. The observed binding of oleic acid to plasmin involves specific binding sites, since bound [14 C]oleic acid could be displaced by an excess of unlabelled oleic acid. This contention is also supported by the direct effect of oleic acid on plasmin activity, which is dose-dependent and saturable. Furthermore, the effects of oleic acid on activity can be suppressed by AHA, a ligand known to bind to specific sites on plasmin [34], but not by glutamine, a ligand that fails to bind to these sites but possesses similar carboxyl and amino groups. These findings make it unlikely that the observed effect of oleic acid on plasmin activity is due to a non-specific detergent effect or micelle formation. Furthermore, octanoic acid, at a concentration that is expected to form micelles and to exert a detergent effect (600 μ M), failed to affect plasmin activity (results not shown).

The sigmoidal curve observed as a consequence of increasing oleic acid concentrations (Fig. 1) indicates co-operativity. The co-operative effect suggests binding of oleic acid to more than one binding site and mutual interactions among these sites. Co-operativity between binding sites was previously noted in plasminogen, the parent molecule of plasmin which contains similar binding sites (Fig. 7 in [33], [35]); Takada *et al.* also suggested mutual interactions between LBS in plasminogen [36]. A second phenomenon of positive co-operativity induced by oleic acid relates to the interaction of plasmin with the substrate S-2251 (Fig. 2). This phenomenon is similar to that induced by another ligand that binds to LBS, namely the polymer of ampicillin [25]. Thus two types of positive co-operativity are induced by oleic acid: one in which oleic acid facilitates its subsequent interaction with the enzyme (Fig. 1), and a second type by which oleic acid enhances the interaction of additional substrate molecules with the enzymes (Fig. 2). Both types of co-operativity may be explained by the same model in which the binding of oleic acid to plasmin induces a conformational change in one or more of the LBS. This change either facilitates the subsequent interaction of a second molecule of oleic acid or enables additional substrate molecules to bind to plasmin and to modulate its activity.

The ability of S-2251 to modulate plasmin in the presence of oleic acid is further documented by the response to AHA. As shown in the experiment described in Fig. 3, at low S-2251 levels AHA suppressed the oleic acid-induced stimulation of plasmin activity, whereas at high S-2251 levels plasmin failed to respond to AHA.

Oleic acid also enhances the conversion of Glu-plasminogen to plasmin. It is possible to ascribe at least a part of this effect to the oleic acid-stimulated plasmin activity. Plasmin is known to convert Glu-plasminogen into Lys-plasminogen, and the resulting Lys-plasminogen is known to be a better substrate for activation [13,14]. An alternative explanation could be related to the presence of LBS in plasminogen. An interaction of oleic acid with these LBS could increase the susceptibility of plasminogen to activation by uPA.

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