Regulation of fibrinolysis by non-esterified fatty acids

Abd Al-Roof HIGAZI,* Rifat AZIZA, Abd Al-Ruhman SAMARA and Michael MAYER
Department of Clinical Biochemistry, Hadassah Medical Center and Hebrew University-Hadassah Medical School, P.O.B. 12000, Jerusalem IL-91120, Israel

The ability of oleic acid to modulate fibrinolysis was measured by following the urokinase-mediated and plasminogen-dependent cleavage of ^{125}I-labelled fibrin clots. Oleic acid levels within the physiological range exerted a concentration-dependent inhibition of urokinase-mediated fibrinolytic activity. SDS/PAGE revealed that oleic acid enhances urokinase activity but simultaneously increases the autolytic cleavage of the newly formed low-molecular-mass subunit of plasmin. Oleic acid-induced cleavage of this subunit containing the catalytic site of plasmin was suppressed by the plasmin substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) and was prevented by α_2-antiplasmin. A concentration-dependent inhibition of the activity of purified plasmin on ^{125}I-labelled fibrin clot was also observed; 93% and 50% inhibition was noted with 150 μM and 32 μM oleic acid respectively. Oleic acid at 200 μM also effectively displaced plasmin prebound to a polylysine–Sepharose column. Examination of the fatty acid specificity showed that a minimal chain length of 16 carbon atoms and the presence of at least one double bond, preferably in a *cis* configuration, were required for inhibition of the fibrinolytic activity of plasmin. Oleic acid at a concentration that produced only a minimal inhibition of plasmin activity induced a marked inhibition by palmitic acid, while palmitic acid alone is ineffective. The findings suggest that oleic acid stimulates plasminogen activation and modulates the fibrinolytic and autolytic activities of plasmin.

INTRODUCTION

The fibrinolytic cascade is initiated by plasminogen activators in a process that involves cleavage of the Arg^{560}–Val^{561} peptide bond in plasminogen to form the non-specific serine proteinase, plasmin [1–3]. Plasmin digests fibrin clots to soluble degradation products. In addition to the fibrinolytic function of plasmin, the localized production of this endoproteinase plays a major role in a variety of biological processes including tissue remodelling, cell migration and invasiveness [4–6]. The two major enzymes of the fibrinolytic cascade, tissue-type plasminogen activator (tPA) and plasmin, as well as the proenzyme plasminogen, contain specific lysine-binding sites (LBS) [7–9]. In plasmin(ogen) these LBS mediate specific interactions with the fibrin clot [7,10]. The LBS are also involved in regulation of plasmin [11–14] and tPA [15] activities, as binding of ligands to these sites modulates the catalytic activity of the enzymes.

We have recently shown that plasminogen activation by urokinase and the catalytic activity of plasmin on its chromogenic substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) are enhanced by oleic acid [13]. This effect appeared to be associated with binding of oleic acid to one or more of the LBS in plasmin. In the present study we examine the effect of oleic acid and other non-esterified fatty acids on plasminogen activation and on the activity of plasmin on its physiological substrate fibrin. We find that oleic acid and some of its analogues suppress the urokinase-mediated fibrinolytic activity by a mechanism involving enhanced autodigestion of the plasmin that is formed from plasminogen.

MATERIALS AND METHODS

Materials

N-Terminal glutamic acid plasminogen (Glu-plasminogen) was prepared from human plasma by affinity chromatography on lysine–Sepharose [16]. Plasmin, produced by activation of human Glu-plasminogen in the presence of ϵ-aminohexanoic acid, and substrate S-2251, were obtained from Kabi Diagnostics, Stockholm, Sweden. The plasmin stock solution contained 0.5 casein units (CU) of plasmin/ml in solution comprised of 50% (v/v) glycerol, 2 mM HCl and 5 g/l poly(ethylene glycol) 6000. High-molecular-mass urokinase (3000 units/ampoule) was obtained from Calbiochem Corp., La Jolla, CA, U.S.A. Thrombin from human plasma (10 NIH units/vial), α_2-antiplasmin and various non-esterified fatty acids were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Na^{125}I for labelling of fibrinogen was purchased from New England Nuclear, Boston, MA, U.S.A., and the iodide remaining in a free form after labelling of the fibrinogen was removed by passage through a Bio-Gel B-6 column (Enzymobead Radioiodination Reagent; Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Preparation of ^{125}I-fibrinogen and ^{125}I-plasminogen

Fibrinogen was radioiodinated with lactoperoxidase as suggested by the manufacturer of the Enzymobead Radioiodination Reagent. The labelled product was diluted with unlabelled fibrinogen to yield a final concentration of 3 mg of protein/ml (compatible with the physiological concentration range for circulating fibrinogen) and a specific radioactivity of 8.3×10^4 c.p.m./mg. Plasminogen was iodinated by the same method, and diluted with non-labelled plasminogen to obtain a stock solution containing 10 mg/ml (1×10^5 c.p.m./mg).

Preparation of ^{125}I-fibrin clots

Portions (200 μl) of ^{125}I-labelled fibrinogen, diluted with unlabelled fibrinogen as detailed above, were introduced into wells of tissue culture plates with an inner diameter of 16 mm (Costar, Cambridge, MA, U.S.A.). To initiate clot formation, 25 μl of a thrombin solution containing 2 NIH units of thrombin/ml of PBS (12.6 mM KH_2PO_4, 64 mM Na_2HPO_4, 86 mM NaCl, adjusted to pH 7.4 with HCl) was added. The

Abbreviations used: tPA, tissue-type plasminogen activator; LBS, lysine-binding sites; CU, casein units.
* To whom correspondence should be addressed.

plates were immediately shaken manually to ensure a complete and uniform covering of the bottoms of the wells by the forming clots. After the initial shaking, the wells were incubated at room temperature for 10 min without shaking, resulting in formation of visible opaque fibrin clots which adhered to the bottom of the wells. The clots were washed three times with 400 μl of PBS, the wash solution being left in the wells for 10 min during each wash to ensure efficient removal of the remaining fibrinogen. The prolonged washes also ensured aging of the clot for a fixed time period of 40 min. The washing removed up to 25% of the counts initially applied to the wells.

Digestion of ^{125}I-fibrin clots by plasmin

Fibrinolysis of the ^{125}I-fibrin clots attached to the wells of tissue culture plates was initiated by the addition of 400 μl of PBS containing 0.025 CU of plasmin, 50% (v/v) glycerol, 2 mM HCl and 5 g/l poly(ethylene glycol) 6000. The plates were rotated at 37 °C for 2 h and samples of 50 μl were removed every 30 min for counting of the solubilized label in a Packard 5160 auto-gamma scintillation spectrometer.

Activation of plasminogen by urokinase

Activation of plasminogen by urokinase was initiated by mixing plasminogen (250 μg), 21 units of high-molecular-mass urokinase and various concentrations of oleic acid in a total volume of 400 μl of PBS. The mixture was added directly to the wells containing the preformed ^{125}I-fibrin clots, and the assay of fibrinolysis was carried out as described above for the assay of plasmin.

Electrophoresis under denaturing conditions

Plasminogen (60 μl; 10 mg/ml) and high-molecular-mass urokinase (40 μl; 400 units/ml) were incubated for 30 min at 37 °C in a final volume of 250 μl of PBS, pH 7.4. Oleic acid, dissolved in PBS, was added as indicated. Incubation was terminated by addition of Coomassie Blue containing 10% (w/v) SDS. After boiling for 2 min, 25 μl portions were applied to 12% (w/v) SDS/10% (w/v) polyacrylamide gels by the method of Laemmli [17]. When electrophoresis of systems containing ^{125}I-labelled plasminogen was performed, the volume applied was adjusted to contain a fixed radioactivity corresponding to 1×10^5 c.p.m.

Non-esterified fatty acid solutions

Stock solutions (24 mM in ethanol) were diluted in PBS. Controls with the vehicle alone indicated that the diluted ethanol had no effect on the enzymes.

All experiments were run in triplicate and repeated at least three times; the results are means ± S.D.

RESULTS

Fibrinolytic activity of different components of the plasminogen-activation cascade was determined by following their ability to release labelled soluble fibrin-degradation products from ^{125}I-labelled fibrin clots. Figure 1 depicts the effect of oleic acid on fibrinolytic activity resulting from urokinase-mediated activation of plasminogen. Under the conditions of the assay, 717 ± 42 μg/ml ^{125}I-fibrin was cleaved to soluble products over 120 min in the absence of oleic acid. The time-dependent solubilization of ^{125}I-labelled fibrin clot as a result of urokinase-mediated plasminogen activation was markedly inhibited by oleic acid in a dose-dependent manner.

In an attempt to clarify the mechanism of this effect, ^{125}I-labelled plasminogen was incubated with urokinase and unlabelled fibrin clot in the presence or absence of oleic acid. After 30 min of incubation, samples were withdrawn and subjected to SDS/PAGE. Figure 2 shows that oleic acid enhanced the consumption of 90 kDa plasminogen and simultaneously decreased the level of the 25 kDa low-molecular-mass chain of plasmin which contains the catalytic subunit of the enzyme [18]. The decrease in plasmin catalytic subunit in the presence of oleic acid explains the reduction in fibrinolysis (Figure 1).

To determine whether the decreased amount of the low-molecular-mass chain of plasmin in the presence of oleic acid is a consequence of decreased generation or increased cleavage of this fragment, conversion of unlabelled plasminogen into plasmin was followed by SDS/PAGE (Figure 3). In this experiment, oleic

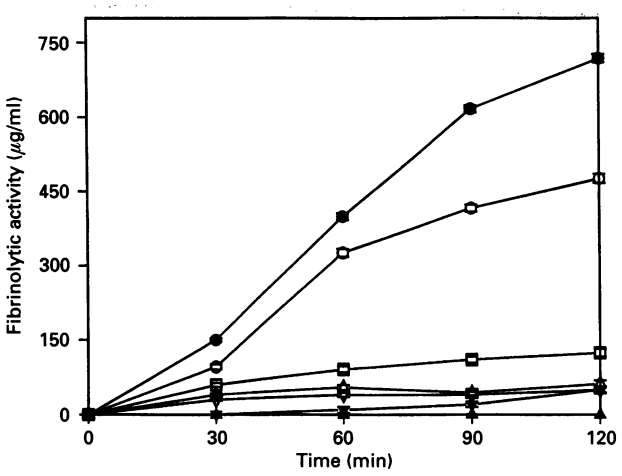

Figure 1 Effect of oleic acid on urokinase activity

Increasing levels of oleic acid were incubated with 21 units of urokinase in the presence of 0.25 mg of plasminogen and ^{125}I-labelled fibrin clot. Cleavage of the clot was determined as a function of time. ●, No oleic acid; ○, 15 μM; □, 30 μM; △, 60 μM; ▽, 120 μM; ▲, no urokinase.

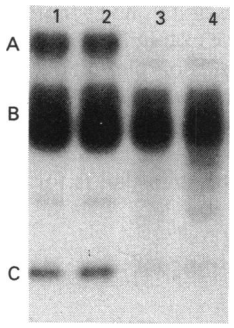

Figure 2 Effect of oleic acid on plasminogen activation by urokinase

^{125}I-labelled plasminogen was activated by urokinase in the presence of fibrin clots and increasing levels of oleic acid for 30 min, and applied to 12% SDS/10% polyacrylamide gels. Lanes 1 and 2, controls in the absence of oleic acid; lane 3, 75 μM oleic acid; lane 4, 100 μM oleic acid. A, Plasminogen, 90 kDa; B, high-molecular-mass chain of plasmin, 60 kDa; C, low-molecular-mass chain of plasmin, 25 kDa.

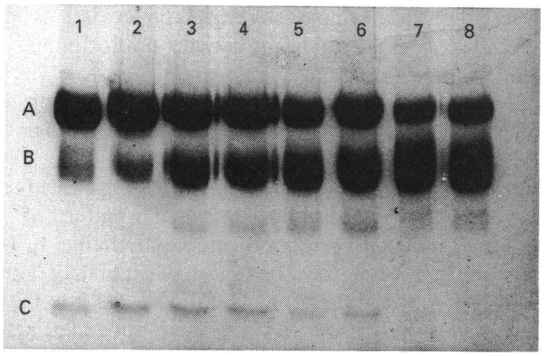

Figure 3 Effect of oleic acid on cleavage of the low-molecular-mass chain of plasmin

Tubes containing plasminogen were incubated with urokinase for 60 min to form plasmin, and samples subjected to SDS/PAGE (lanes 1 and 2). Similarly prepared mixtures were incubated for an additional 30 min in the presence of 25 μM oleic acid (lanes 3 and 4), 50 μM oleic acid (lanes 5 and 6) and 100 μM oleic acid (lanes 7 and 8), and then subjected to SDS/PAGE. A, Plasminogen, 90 kDa; B, high-molecular-mass chain of plasmin, 60 kDa; C, low-molecular-mass chain of plasmin, 25 kDa.

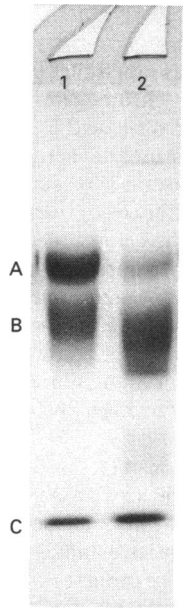

Figure 4 Protective effect of α_2-antiplasmin on the low-molecular-mass chain of plasmin

Plasminogen activation by urokinase was performed for 60 min with no addition (lane 1) or in the presence of 100 μM oleic acid and 0.5 mg/ml α_2-antiplasmin (lane 2). A, Plasminogen, 90 kDa; B, high-molecular-mass chain of plasmin, 60 kDa; C, low-molecular-mass chain of plasmin, 25 kDa.

acid was added only after a certain amount of plasmin had been formed from plasminogen. The results in Figure 3 show that oleic acid promotes the disappearance of the preformed catalytic subunit of plasmin in a concentration-dependent manner.

The enhanced cleavage of the catalytic subunit of plasmin in the presence of oleic acid might reflect increased autolytic digestion of the enzyme. This possibility was confirmed by the experiment shown in Figure 4, in which α_2-antiplasmin was added to prevent plasmin activity. In the presence of the inhibitor

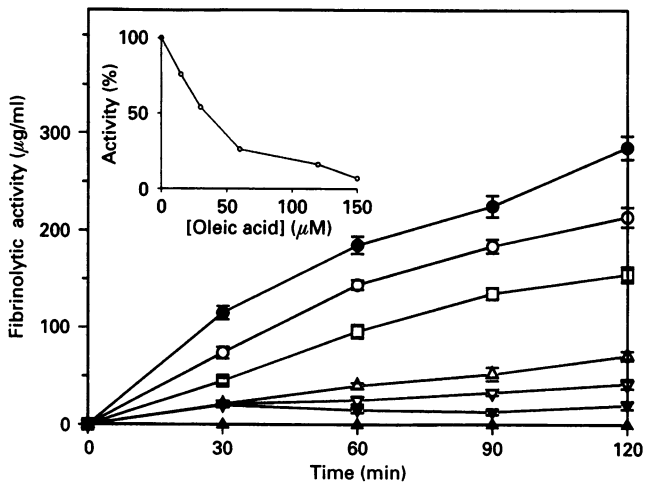

Figure 5 Effect of oleic acid on the fibrinolytic activity of plasmin

Increasing levels of oleic acid were incubated with 60 mCU of plasmin and ^{125}I-labelled fibrin clots. Cleavage of the clot was determined as a function of time. ●, no oleic acid; ○, 15 μM; □, 30 μM; △, 60 μM; ▽, 120 μM; ▼, 150 μM; ▲, no plasmin. Inset: dependence of the inhibitory effect on the concentration of oleic acid. End-point plasmin activity was determined after 120 min of incubation.

and oleic acid, high levels of the catalytic subunit of plasmin are detected. It is therefore suggested that α_2-antiplasmin prevents oleic acid-mediated stimulation of autolytic digestion of newly formed plasmin.

To test whether the ability of oleic acid to decrease the amount of the catalytic subunit of plasmin parallels a reduction in plasmin activity, we measured the effect of oleic acid on the fibrinolytic activity of purified plasmin. Figure 5 shows the direct effect of increasing levels of oleic acid on the fibrinolytic activity of plasmin on ^{125}I-fibrin clots as a function of time. Under these conditions, 60 mCU of plasmin cleaved 285 ± 27 μg/ml fibrin during 120 min of incubation. Oleic acid produced a marked concentration-dependent inhibition of this activity, with half-maximal inhibition at 32 μM and 93% inhibition at 150 μM oleic acid.

We have shown previously that plasminogen activation and plasmin activity, measured with S-2251 as the plasmin substrate, are enhanced by oleic acid [13]. The apparent contradiction between these earlier findings and the present observation that oleic acid inhibits fibrinolysis may reflect the presence of plasmin fragments that retain amidolytic but not fibrinolytic activity. Alternatively, this difference may be due to S-2251-mediated protection of plasmin against autocleavage. These possibilities were examined in the experiment shown in Figure 6. The presence of S-2251 with oleic acid is seen to increase the amount of the low-molecular-mass chain of plasmin, with no apparent increase in plasminogen consumption over that observed with oleic acid alone. A computerized scanning densitometry profile of the low-molecular-mass plasmin band in the presence of oleic acid indicates that its intensity in the absence of S-2251 (Figure 6, lanes 5 and 6) is only 40% of its intensity in presence of the chromogenic substrate (Figure 6, lanes 7 and 8). Thus S-2251 protects plasmin against autolytic cleavage.

The ability of oleic acid to inhibit fibrin cleavage and to stimulate plasmin autodigestion may be related to its interaction with LBS in plasmin, sites known to mediate plasmin–fibrin interaction [9]. This possibility is supported by the ability of

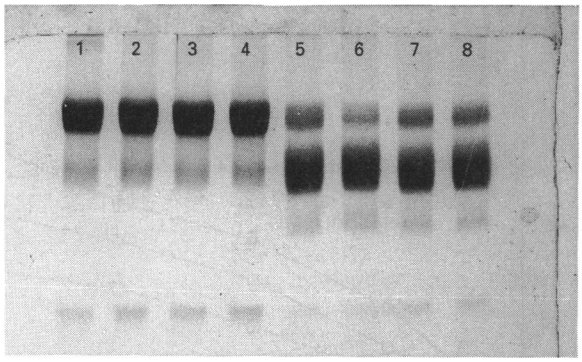

Figure 6 Protective effect of S-2251 on the low-molecular-mass chain of plasmin

SDS/PAGE analysis of plasminogen activation by urokinase was performed after incubation for 60 min with no addition (lanes 1 and 2), in the presence of S-2251 at [S] = K_m = 0.29 mM (lanes 3 and 4), in the presence of 100 μM oleic acid (lanes 5 and 6) and in the presence of both 100 μM oleic acid and 0.29 mM S-2251 (lanes 7 and 8).

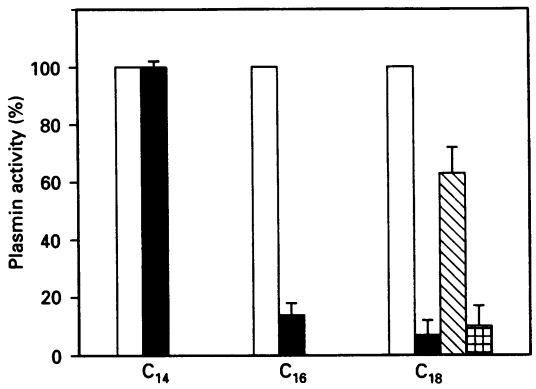

Figure 7 Effect of different non-esterified fatty acids on plasmin activity

The number of carbon atoms in the molecule is indicated. □, Saturated fatty acid; ■, *cis*-Δ^9-unsaturated fatty acid; ▨, *trans*-Δ^9-unsaturated fatty acid; ▦, *cis*-Δ^9- and *cis*-Δ^{12}-unsaturated fatty acid. All fatty acids were employed at 150 μM, and cleavage of ^{125}I-labelled fibrin clot was determined after 60 min of incubation. An activity of 100% corresponds to 185 ± 20 μg/ml fibrin cleaved.

200 μM oleic acid to displace plasmin prebound to a polylysine–Sepharose column (results not shown).

To examine the structural requirements for inhibition of plasmin activity by non-esterified fatty acids, several fatty acids were examined. Figure 7 shows that saturated fatty acids with increasing chain lengths from C_{14} to C_{18}, all with an even number of carbon atoms, failed to inhibit the activity of plasmin on fibrin. The importance of double bonds for the inhibitory capacity of the fatty acids is indicated by the observation that C_{16} and C_{18} with a double bond in the *cis* configuration are inhibitory. As the C_{18} *trans*-Δ^9-unsaturated fatty acid was much less potent than its *cis* analogue, a *cis* configuration seems to increase the inhibitory effect of the unsaturated long-chain fatty acid. The response of plasmin to unsaturated fatty acids that are readily available in the circulation was also examined. Linoleic acid (*cis*, *cis*-Δ^9,Δ^{12}-octadecadienoic acid) and arachidonic acid (all-*cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid) inhibit the fibrinolytic activity of plasmin. Additional experiments corroborate these observations by showing that saturated non-esterified fatty acids with 8 or 12 carbons failed to inhibit plasmin activity on fibrin clots. These results imply that a minimal chain length of at least 16 carbons and a double bond are essential for plasmin inhibition by non-esterified fatty acids, and that a *cis* configuration is more effective than a *trans* configuration.

In a previous study on the effect of oleic acid on the chromogenic activity of plasmin, we noted a pattern of positive co-operativity, suggesting a conformational change in plasmin [13]. An initial conformational change in plasmin may potentially facilitate the interaction of plasmin with additional ligand molecules. This hypothesis is based on an experiment (not shown) in which plasmin was incubated with 25 μM oleic acid, a concentration that produces low (21%) inhibition of plasmin activity. The addition of increasing concentrations (30–125 μM) of palmitic acid produces a concentration-dependent potentiation of the inhibitory effect of oleic acid on fibrinolysis, whereas palmitic acid alone at a high concentration of 150 μM fails to inhibit plasmin. Thus oleic acid displays a permissive effect on palmitic acid-mediated inhibition. Such an effect implies that oleic acid, possibly through a conformational change in plasmin, induces susceptibility to inhibition by a ligand that is not inhibitory when presented alone.

DISCUSSION

In our earlier work it was noted that plasminogen activation and plasmin activity on a chromogenic tripeptide substrate are markedly stimulated by oleic acid [13]. In contrast, the present results show that the fibrinolytic activity obtained as a result of urokinase-mediated plasminogen activation or by the use of purified plasmin is inhibited by oleic acid and several other unsaturated non-esterified fatty acids. A similar substrate-dependent differential response of plasmin to a modulator has already been described for ϵ-aminohexanoic acid; certain levels of this ligand stimulate plasmin activity on low-molecular-mass synthetic substrates [19,20] but inhibit plasmin activity on fibrin clots [21–23]. Similarly, differential effects of ϵ-aminohexanoic acid on plasminogen activation by urokinase were previously noted [7,24,25].

The mechanism responsible for the apparent contradiction between the effect of oleic acid on plasmin activity on fibrin and on small synthetic substrates can be explained by the present results. As shown in Figures 2 and 3, plasminogen consumption is increased in the presence of oleic acid as a result of stimulation of plasminogen activation by urokinase, as reflected by the increased level of the high-molecular-mass chain of plasmin. This stimulation is further indicated by emergence of increased amounts of the low-molecular-mass subunits of plasmin in the presence of oleic acid and α_2-antiplasmin (Figure 4). Figures 2 and 3 show that oleic acid simultaneously enhances cleavage of the newly formed low-molecular-mass chain of plasmin. This cleavage seems to be due to autodigestion, as it is prevented by α_2-antiplasmin (Figure 4).

The finding that plasmin is displaced from polylysine–Sepharose by oleic acid suggests that the LBS-mediated interaction of plasmin with fibrin is impaired in the presence of oleic acid, leading to a decreased local concentration of the enzyme on the fibrin clot surface. As a consequence, the oleic acid-stimulated plasmin does not bind to fibrin and is therefore not subjected to the protective effect of the substrate [26]. Thus the unbound plasmin is susceptible to autodigestion which reduces the amount of active plasmin (Figures 2 and 3), thereby causing a reduction in fibrinolysis (Figures 1 and 5). In contrast, interaction with LBS is not required for plasmin activity on low-

molecular-mass substrates, and therefore these substrates protect the enzyme against autodigestion in the presence of oleic acid (Figure 6). The effect results in a stimulation of plasmin chromogenic activity by oleic acid [13].

The results shown in Figures 2–4 suggest that the high-molecular-mass subunit of plasmin is much less susceptible to cleavage. Indeed, plasmin is known to cleave its catalytic subunit more efficiently than its high-molecular-mass subunit [26]. In spite of the lower susceptibility of the high-molecular-mass chain, it also yields a cleavage product in the presence of oleic acid. This cleavage may explain why only small amounts of α_2-antiplasmin–plasmin complex are present in the gel shown in Figure 4.

The response of plasmin to the different non-esterified fatty acids (Figure 7) indicates that the interaction with plasmin is fairly specific. A minimal chain length of 16 carbons and at least one double bond, preferably in a *cis* configuration, are required for the inhibitory effect of fatty acids. This observation supports the earlier indications that the effect of oleic acid is specific and does not reflect a detergent effect [13]. As albumin competes with fibrin and with the chromogenic substrates of plasmin, the effect of albumin-bound fatty acids on plasmin activity could not be determined with the assays used here.

Palmitic acid markedly inhibits plasmin activity in the presence of oleic acid at a level that has only minimal inhibitory effect when presented alone. This observation supports the contention that oleic acid binds to more than one LBS in plasmin [13]. It seems likely that an initial interaction of the low level of oleic acid with the enzyme permits subsequent interactions of other molecules with newly exposed binding sites. These newly exposed sites appear to be less specific than the sites that are continually available on the protein. Thus oleic acid, in addition to being a regulator of plasmin, has a permissive effect that enables interaction of plasmin with other potential modulators of plasmin activity.

The present results support the contention that plasmin is a complex regulatory enzyme, containing mutually interacting regulatory sites. Several physiological compounds, e.g. fibrinogen [12], prourokinase [14] and oleic acid [13], regulate plasmin activity by interacting with these sites.

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