Lipoprotein(a): A Kinetic Study of Its Influence on Fibrin-Dependent Plasminogen Activation by Prourokinase or Tissue Plasminogen Activator†

Jian-ning Liu,‡ Peter C. Harpel,§ Ralph Pannell,‡ and Victor Gurewich*,‡

Vascular Research Laboratory, Institute for the Prevention of Cardiovascular Disease, New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02215, and Division of Hematology, Mount Sinai Medical Center, New York, New York 10029

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ABSTRACT: Lipoprotein(a) [Lp(a)] has been postulated to inhibit fibrinolysis due to its structural homology to plasminogen. Indeed, it has been reported that Lp(a) competitively inhibits the promotion by fibrin of tissue plasminogen activator (t-PA)-catalyzed plasminogen activation. However, it has also been reported that this inhibition is uncompetitive. No studies have been published, to our knowledge, of the effect of Lp(a) on prourokinase (pro-UK)-catalyzed plasminogen activation. Plasminogen activation by pro-UK or a plasmin-resistant mutant pro-UK was previously shown to be promoted by fibrin fragment E_2, whereas that by t-PA is promoted by fragment D. Therefore, the influence of Lp(a) on the kinetics of these two reactions was examined. When Lp(a) was added (90–600 nM), no change in the rate of plasmin generation by Ala^{158}-pro-UK was observed. Consistent with this, immobilized Lp(a) also failed to bind to fragment E_2, whereas it did bind to D dimer. When t-PA-catalyzed plasminogen activation in the presence of D dimer was measured, uncompetitive inhibition by Lp(a) was found, but only at low concentrations of D dimer (<0.5 μM) or t-PA (0.05 nM). At higher concentrations of D dimer and t-PA, instead of inhibition, Lp(a) induced a 2.4-fold promotion of plasminogen activation. Similarly, Lp(a) enhanced (up to 2.5-fold) plasminogen binding to immobilized fibrin in both buffer and plasma milieus at the physiological concentration of plasminogen (2.0 μM). In conclusion, Lp(a) had no effect on plasminogen activation by pro-UK and induced only limited inhibition of activation by t-PA. Since this inhibition was of the uncompetitive type, it could not be attributed to competitive inhibition of plasminogen binding to fibrin by Lp(a). These findings put into question the hypothesis that Lp(a) inhibits physiological fibrinolysis.

Lipoprotein(a) [Lp(a)][1] is a plasma, low-density lipoprotein (LDL) made up of lipid and apoprotein B-100 (apoB). Its distinguishing feature is an additional glycoprotein subunit, apoprotein(a) [apo(a)], which is linked by a disulfide bridge to apoB (Fless *et al.*, 1986; Scanu, 1988). This apo(a) component is highly homologous to plasminogen by virtue of its multiple repeats of plasminogen kringle 4 (McLean *et al.*, 1987; Eaton *et al.*, 1987), a fibrin- or lysin-binding domain. Therefore, like plasminogen, Lp(a) binds both to lysine–Sepharose and to immobilized fibrin (Harpel *et al.*, 1989), a property which, for plasminogen, mediates fibrinolysis. However, although Lp(a) contains one copy of the catalytic-like domain of plasminogen, it cannot be converted to an active proteinase by plasminogen activators (Eaton *et al.*, 1987).

A number of studies have documented that the Lp(a) concentration in plasma is strongly and independently correlated with atherosclerotic cardiovascular disease (Albers, *et al.*, 1977; Seed *et al.*, 1990; Dahlen *et al.*, 1986; Rhoads *et al.*, 1986; Muria *et al.*, 1986). Since an inverse correlation between cardiovascular diesease and fibrinolytic activity has also been frequently reported (Chakrabati *et al.*, 1986; Hamsten *et al.*, 1985; Juhan-Vague & Collen, 1988; Prins & Hirsh, 1991), it has been postulated that the atherogenic effect of Lp(a) is related to the impairment of fibrinolysis due to competitive inhibition of plasminogen binding to fibrin. Indeed, it has been reported that Lp(a) inhibits the heparin-mediated promotion of plasminogen by UK (Edelberg *et al.*, 1991) or strepokinase (Edelberg *et al.*, 1989) and the fibrin-mediated promotion of plasminogen activation by tissue plasminogen activator (t-PA) (Edelberg *et al.*, 1990; Loscalzo *et al.*, 1990; Edelberg & Pizzo, 1990; Leerink *et al.*, 1990). Inhibition has been claimed to be either of the competitive type (Edelberg *et al.*, 1990; Edelberg & Pizzo, 1990) or of the uncompetitive type (Loscalzo *et al.*, 1990; Leerink *et al.*, 1991). The latter, however, is inconsistent with competitive inhibition of fibrin binding, which was also reported (Loscalzo *et al.*, 1990). Furthermore, most of these studies were conducted with nonphysiological concentrations of salt or plasminogen (Edelberg *et al.*, 1989, 1990, 1991; Edelberg & Pizzo, 1990; Leerink *et al.*, 1991). Other investigators failed to find evidence of inhibition of fibrinolysis by Lp(a) either *in vitro* (Kluft *et al.*, 1989; Eaton *et al.*, 1990; Lu *et al.*, 1990; Halvorsen *et al*, 1992) or *in vivo* (Oshima *et al.*, 1991; Garcia Frade *et al.*, 1991; Smith & Crosby, 1991; Donders *et al.*, 1992, 1993). In some studies Lp(a) was even shown to promote fibrinolysis (Mao & Tucci, 1990).

In the present study, the influence of Lp(a) on the kinetics of fibrin fragment E_2-stimulated plasminogen activation by prourokinase (pro-UK) was examined since, to our knowledge, the effect of Lp(a) on this fibrinolytic pathway has not been

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* Address correspondence to: Dr. Victor Gurewich, Vascular Research Laboratory, Burlington, Bldg., Rm 554G, New England Deaconess Hospital, 185 Pilgrim Rd., Boston, MA 02215.

‡ Harvard Medical School.

§ Mount Sinai Medical Center.

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apoprotein(a); apoB, apoprotein B-100; t-PA, tissue plasminogen activator; pro-UK, prourokinase; SK, streptokinase; LDL, low-density lipoprotein; A-pro-UK, plasmin-resistant mutant Ala^{158}-prourokinase; EACA, ε-aminocaproic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PVC, polyvinyl chloride; KIU, kallikrein inactivator units.

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previously reported. Fibrin fragment E_2 was chosen since it was recently shown to selectively promote catalysis by pro-UK or by a plasmin-resistant mutant pro-UK (Ala^{158}-pro-UK) (Liu & Gurewich, 1991, 1992). In addition, the effect of Lp(a) on t-PA-catalyzed plasminogen activation in the presence of fibrin fragment D dimer, the most effective stimulator of this reaction (Hasan *et al.*, 1992), was studied in order to help reconcile some of the contradictory findings reported in the literature.

METHODS AND MATERIALS

A plasmin-resistant mutant *rec*-prourokinase (pro-UK), constructed by site-directed mutagenesis of Lys^{158} to Ala^{158} (A-pro-UK), was a gift from Collaborative Research Incorporated (Bedford, MA). Its concentration was determined from absorbance using the extinction coefficient, $E^{1\%}_{280nm}$ = 13.6 (White & Barlow, 1970). This mutant was previously shown to be a valid surrogate of pro-UK with respect to its amidolytic and plasminogen-activating activity and its promotion by fibrin fragment E_2 (Liu & Gurewich, 1992). Single-chain t-PA was obtained from Genenetech (S. San Francisco, CA). Glu-plasminogen was purified from DFP-treated bank plasma essentially by the method of Castellino and Powell (1981).

Preparation of Lp(a) and LDL. Lp(a) and LDL were purified from a donor with an elevated Lp(a) concentration by ultracentrifugation and gel filtration according to the procedure previously described (Harpel *et al.*, 1989).

Preparation of Fibrin Fragment E_2 and D Dimer. Fragments E_2 and D dimer were prepared by a modification of the method of Varadi and Patthy (1983) as follows: A plasmin digest of fibrin was prepared by treatment of a solution of human fibrinogen (10 mg/mL) in 0.05 M Tris·HCl, 0.15 M NaCl, and 0.01 M $CaCl_2$ (pH 7.4). The solution was equilibrated to 37 °C, and Glu-plasminogen (25 μg/mL) and streptokinase (SK) (Hoechst Pharmaceuticals, Somerville, NJ) (7.5 IU/mL) were added immediately before the addition of highly purified thrombin (8 NIH units/mL). The reaction mixture was incubated (37 °C) for 3 h. Digestion was arrested by the addition of aprotinin (Trasylol, 100 KIU/mL, Miles Inc., West Haven, CT), EACA (0.02 M), and EDTA (0.02 M). The digest was then applied onto a Sephacryl S-200 (Pharmacia, Piscataway, NJ) column (2.5 × 160 cm) equilibrated with 0.3 M NH_4HCO_3 and eluted with the same buffer. Fragments E were obtained from pool B, whereas pool A contained D dimer–E. The latter pool was lyophilized and dissolved in 0.05 M sodium citrate and 3 M urea (pH 5.5) and incubated (37 °C) for 3 h as described by Varadi and Patthy (1983). Fragment E_2 and D dimer were separated from D dimer–E by gel filtration on a Sephacryl S-300 column (2.5 × 160 cm) equilibrated with 0.05 M sodium citrate and 3 M urea. The purified E_2 and D dimer preparations were desalted and lyophilized. To eliminate any traces of residual plasmin, the preparation was treated with diisopropyl phosphorofluoridate (5 mM) for 1 h (37 °C) and then dialyzed exhaustively against 0.05 M sodium phosphate and 0.15 M NaCl (pH 7.8).

The concentrations of fragment E_2 and D dimer were determined from absorbance using the extinction coefficients of fragment E_2 ($E^{1\%}_{280nm}$ = 10.0) and fragment D ($E^{1\%}_{280nm}$ = 20.0). Protein was additionally measured using a Bio-Rad protein assay kit. The final preparation was examined by gradient (5–15%) SDS–PAGE and shown to be pure (Liu & Gurewich, 1991).

Identification of Fibrin Fragments Bound to Lp(a). ELISA plates (Dynatech Immulon) were coated with 100 μL per well of Lp(a) (57 μg/mL), LDL (20 μg/mL), or BSA (20 mg/mL) in 0.05 M Tris·HCl and 0.15 M NaCl (pH 7.4) at 37 °C for 2 h and then washed with 200 μL per well of 0.05 M Tris·HCl, 0.15 M NaCl, 0.01% NaN_3, 0.02% Tween 80, and 2% BSA (pH 7.4) at 4 °C overnight in order to occupy extra protein-binding sites on plates. Next, 100 μL of fibrin fragment E_2 (10 μM), D dimer (1 μM) ± Lp(a) (0.45 μM), or a mixture of these was added per well and incubated at 37 °C for 1 h after the coating treatment described above. This concentration of D dimer is >30-fold higher than the K_D for Lp(a) binding to D dimer (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990). The coating treatment was followed by a five-time wash with 200 μL per well of 0.05 M Tris·HCl, 0.15 M NaCl, 0.01% NaN_3, 0.02% Tween 80, and 2.0% BSA (pH 7.4). Then, 100 μL per well of rabbit antibodies specific for fragment D or fragment E (1:1000 dilution) in 0.05 M Tris·HCl, 0.15 M NaCl, 0.01% NaN_3, 0.01% Tween 80, and 0.25% BSA (pH 7.4) was incubated at 37 °C for 1 h, followed by a five-time wash again with 200 μL per well of 0.05 M Tris·HCl, 0.15 M NaCl, 0.01% NaN_3, 0.02% Tween 80, and 2% BSA (pH 7.4). After that, 100 μL per well of goat anti-rabbit IgG antibody labeled with alkaline phosphatase (Sigma, St. Louis, MO) (1:1000 dilution) in 0.05 M Tris·HCl, 0.15 M NaCl, 0.01% NaN_3, 0.01% Tween 80, and 0.25% BSA (pH 7.4) was added and incubated at 37 °C for another hour, followed again by the same washing process. Finally, 150 μL per well of *p*-nitrophenyl phosphate (1.4 mg/mL) in 1.0 M diethanolamine with 0.01% $MgCl_2$ was added and incubated at room temperature. The OD increase was measured over time at 410 nm against a reference wavelength of 490 nm (410/490 nm) on the microtiter plate reader.

Covalent conjugation of Lp(a) and LDL (or BSA as control) to the plates was also used in these studies. Briefly, a polyvinyl chloride (PVC) microtiter plate was treated with 100 μL per well of 2.5% glutaraldehyde in 0.1 M sodium bicarbonate (pH 9.5) at room temperature for two hours. Then the plates were coated with Lp(a), LDL or BSA and recoated with excess BSA to occupy extra protein binding sites as described above.

Effect of Lp(a) on pro-UK-Induced Plasminogen Activation in the Presence and Absence of Fragment E_2. Since pro-UK-induced plasminogen activation has been shown to be substantially and selectively promoted by fibrin fragment E_2 (Liu & Gurewich, 1991, 1992), the effect of Lp(a) on plasminogen activation by Ala^{158}-pro-UK in the presence and absence of fragment E_2 was determined by measuring the OD increase with time in the reaction mixture at 410 nm against a reference wavelength of 490 nm (410/490 nm) on the microtiter plate reader. The reaction mixture contained 1.5 mM S-2251, Glu-plasminogen (0.1 and 2.0 μM) with or without 5.0 μM fragment E_2, and 2.0 nM of Ala^{158}-pro-UK in the presence and absence of a range of concentrations of Lp(a) (0.09–0.6 μM) or LDL (0.18–6.9 μM) in 0.05 M sodium phosphate, 0.15 M NaCl, 0.2% BSA, and 0.01% Tween 80 (pH 7.8) at room temperature.

Effect of Lp(a) on t-PA-Induced Plasminogen Activation in the Presence and Absence of Fibrin D Dimer. Plasminogen activation by t-PA was shown to be selectively promoted by the fragment D domain of fibrin (Liu & Gurewich, 1991). Therefore, the effect of Lp(a) on plasminogen activation by t-PA in the presence and absence of D dimer was determined by measuring the OD increase with time in the reaction mixture at 410 nm against a reference wavelength of 490 nm (410/490 nm) on the microtiter plate reader. The reaction mixture contained 1.5 mM S2251, Glu-plasminogen (0–2.0 μM) ± D dimer (0.1–1.0 μM), and t-PA (0.05 and 50.0 nM) in the

presence and absence of varied concentrations of Lp(a) (0.13–0.45 μM) or LDL (0.54 μM) in 0.05 M sodium phosphate, 0.15 M NaCl, 0.2% BSA, and 0.01% Tween 80 (pH 7.8) at room temperature. The coupled reactions on the activation of plasminogen to plasmin and the release of *p*-nitroaniline (pNA) from S-2251 can be described by the following two reactions:

$$\text{plasminogen} \xrightarrow{\text{A-pro-UK/t-PA}} \text{plasmin}$$

$$\text{S-2251} \xrightarrow{\text{plasmin}} \text{peptide} + \text{pNa}\ (\Delta A_{410\,nm})$$

The reaction rate was then determined directly by curve fitting using the integrated rate equation (eq 1) adapted from Liu and Gurewich (1992). The time–absorbance curves of these reactions were plotted from the assay described above.

$$A(t)_i = V_i t^2 + B \tag{1}$$

where V_i is the reaction rate and B the background (t_0 absorbance). Curve fitting was performed on early time points, before depletion of substrates became significant, using the Enzfitter program (Elsevier-Biosoft, New York).

Kinetic Analysis. Uncompetitive inhibition was observed by Lp(a) in t-PA-induced plasminogen activation in the presence of certain concentrations of D dimer and Lp(a). The uncompetitive inhibition constant (K_{iu}) was obtained from modified Lineweaver–Burk plots as follows:

$$\frac{1}{v} = \frac{K_M}{V_{max}}\frac{1}{[S]} + \left(1 + \frac{[I]}{K_{iu}}\right)\frac{1}{V_{max}} \tag{2}$$

when $1/v = 0$, then

$$\frac{1}{K_{M,app}} = \left(1 + \frac{[I]}{K_{iu}}\right)\frac{1}{K_M} = -\frac{1}{[S]} \tag{3}$$

$$\frac{1}{K_{M,app}} = \left(1 + \frac{[I]}{K_{iu}}\right)\frac{1}{K_M} \tag{4}$$

when $1/K_{M,app} = 0$, then

$$1 + \frac{[I]}{K_{iu}} = 0 \quad \text{and} \quad K_{iu} = -\frac{1}{[I]} \tag{5}$$

Each experiment was performed in quadruplicate. At the concentrations used, none of the activators had any effect on S-2251. At the end of measurement, less than 5% of the Glu-plasminogen had been consumed, and Lys-plasminogen generation was undetectable by acid/urea gel electrophoresis.

Fibrin Binding of Plasminogen in the Presence of Lp(a). (*a*) *Preparation of Plasminogen-Charged, Solid-Phase Fibrin Microtiter Plates.* Solid-phase, fibrin-bound plasminogen plates were prepared according to the method of Angles-Cano (1986). Briefly, a polyvinyl chloride (PVC) microtiter plate was treated with 2.5% glutaraldehyde in 0.1 M sodium bicarbonate (pH 9.5) at room temperature for 2 h. The 100 μL of 0.3 μM purified fibrinogen in 1 mM calcium chloride and 0.1 M phosphate buffer (pH 7.4) was added to each well, incubated (4 °C for 18 h), and then washed three times with 0.05 M sodium phosphate (pH 7.4), 0.15 M NaCl, 0.2% BSA, and 0.01% Tween 80 (assay buffer). The fibrinogen–PVC plate was treated with 1 NIH units/mL thrombin at 37 °C for 20 min in assay buffer containing 1 mM calcium chloride (100 μL per well) and then washed with 200 μL of 0.5 M NaCl, 8 mM calcium chloride, and 0.05% Tween 70 and then three times with 200 μL of 5 mM phosphate buffer and 0.05% Tween 80. Plasmin treatment (100 μL of 0.5 μM at 37 °C for 15 min) was used to degrade the bound fibrin in order to increase the plasminogen-binding sites. The plate was washed with 200 μL of 0.5 M EACA and then three times with assay buffer.

(*b*) *Plasminogen Binding.* Plasminogen was bound to the plasmin-degraded fibrin immobilized in microtiter plate wells by incubation for 2 h at 37 °C with 100 μL per well of plasminogen (0.2 mg/mL) in assay buffer or of bank plasma with Lp(a) added at various concentrations up to 200 μg/mL. The wells were washed three times with the same buffer. Plasminogen was activated by a 20-min incubation with 100 μL of streptokinase (2500 IU/mL) in 0.2 M Tris (pH 7.4), 0.2 M NaCl, and 0.4% BSA. Plasmin substrate S-2251 (100 μL, 1.5 mM) was added, and the development of color at 37 °C was monitored for 2 h.

Table I: Identification of Fibrin Fragment Which Binds to Immobilized Lipoprotein(a)

	column					
	1	2	3	4	5	6
fibrin fragment	D_2	E	D_2 + Lp(a)[c]	E + D_2	E + D_2	(BSA)
antibody	anti-D	anti-E	anti-D	anti-D	anti-E	anti-D/E
matrix[d]						
Lp(a), covalent[a]	0.112	0.010	0.041	0.109	0.029	0.014
Lp(a), physical[b]	0.129	0.000	0.045	0.108	0.028	0.007
LDL, covalent	0.033	0.009		0.031	0.005	0.010
LDL, physical	0.005	0.000		0.000	0.004	0.004

[a] Attached to glutaraldehyde-treated PVC microtiter plate wells. [b] Attached by passive protein binding. [c] The concentration of Lp(a) was 0.45 μM. [d] Numbers represent ΔA/min for ELISA detection of bound antibody with alkaline phosphatase conjugated anti-rabbit IgG.

RESULTS

Binding of Fragments D and E to Immobilized Lp(a) and LDL (Table I). The ELISA results showed significant binding of fragment D but not E to Lp(a). Neither fragment bound to LDL. The results obtained when Lp(a) or LDL was bound physically or covalently to the plate were similar, except that nonspecific binding to the fragments was slightly higher with the latter. The results of control experiments with the opposite antibody (column 2) showed that there was negligible nonspecific binding to the Lp(a). The slight apparent binding indicated by anti-E against a mixture of D dimer and E (column 5) was probably related to some fragment E_2 bound to D. The Lp(a) in solution inhibited binding of fragment D to immobilized Lp(a) (column 3).

Effect of Lp(a) and LDL on pro-UK-Induced Plasminogen Activation (Table II). Little activation of plasminogen at 0.1 or 2.0 μM by A-pro-UK alone was seen. However, in the presence of fragment E_2 (5 μM), 50- and 34-fold promotion of plasmin generation was obtained at 0.1 and 2.0 μM plasminogen, respectively, consistent with previous observations (Liu & Gurewich, 1992). Neither Lp(a) (0.09–0.6 μM) nor LDL (0.18–6.9 μM) affected the rate of plasmin generation under any of these conditions (±E_2). This finding was consistent with the binding studies above showing no binding between fragment E_2 and Lp(a). When the fragment E_2 concentration was decreased, the promoting effect was substantially diminished, but Lp(a) again had no effect on the reaction rate.

Effect of Lp(a) on t-PA-Induced Plasminogen Activation (Figure 1A–E). (*a*) *Effect of Lp(a) at Lower Concentrations of t-PA (0.05 nM).* In the presence of 0.2 μM D dimer, uncompetitive inhibition by Lp(a) (0.15–0.45 μM) of t-PA-induced plasminogen (0.05–0.4 μM) activation was observed.

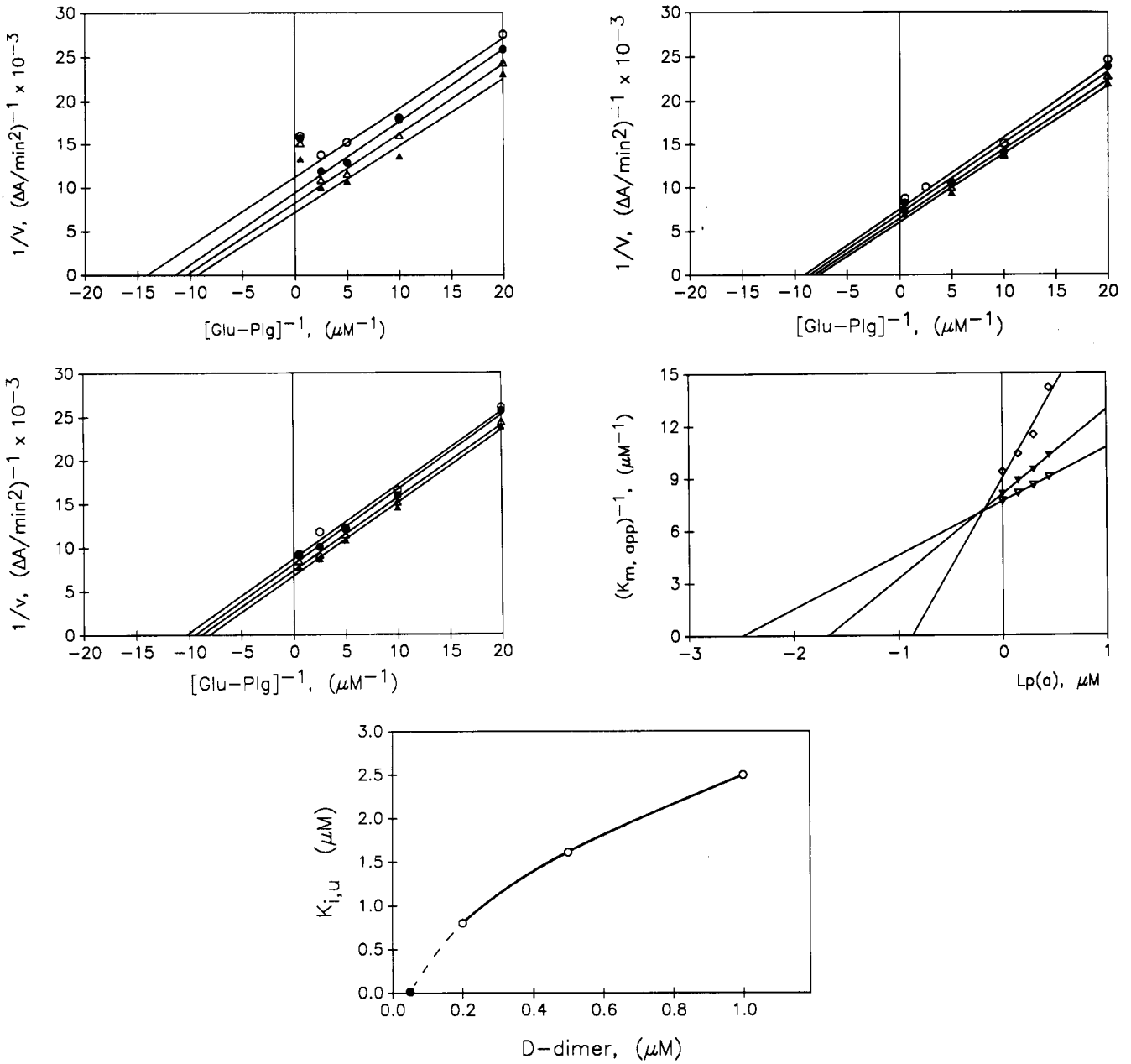

FIGURE 1: Lineweaver–Burk plot analysis of the effect of Lp(a) on plasminogen activation by t-PA (0.05 nM). The reaction mixtures contained Lp(a), t-PA, plasminogen, and D dimer: (A, top left) 0.2 μM; (B, middle left) 0.5 μM; (C, top right) 1.0 μM. Lp(a) concentrations were (○) 0.45 μM, (●) 0.30 μM, (△) 0.15 μM, and (▲) 0. (D, middle right) Determination of apparent inhibition constants of Lp(a) at various D dimer concentrations (0.2 μM (◇), 0.5 μM (▼), and 1.0 μM (▽)) by replotting of data from A–C. (E, bottom) Influence of D dimer concentration on the apparent inhibition constant of Lp(a).

The Lp(a) induced a reduction in the V_{max} from 0.140 to 0.089 × 10^3 $\Delta A/min^2 \cdot nM$ associated with a reduction in apparent K_M from 0.107 to 0.071 μM. At physiological concentrations of plasminogen (2.0 μM), the reaction did not fit Michaelis–Menten kinetics (Figure 1A), probably due to substrate inhibition (Liu & Gurewich, 1993). These data were, therefore, not included in the calculation of uncompetitive inhibition.

When the concentration of D dimer was increased to 0.5 (Figure 1B) and 1.0 μM (Figure 1C), the sensitivity to inhibition by Lp(a) (0.05–2.0 μM) was diminished but the mode of inhibition remained uncompetitive. The K_{iu} was determined from modified Lineweaver–Burk plots according to eqs 2–5 using the data shown in Figure 1A–C. The K_{iu} values were 0.8, 1.6, and 2.5 μM at D dimer concentrations of 0.2, 0.5, and 1.0 mM, respectively (Figure 1D).

The relationship between the D dimer concentration and the K_{iu} for Lp(a) was plotted. Included in the figure is a point (closed circle) taken from the literature (Loscalzo *et al.*, 1990). As shown (Figure 1E), inhibition diminished substantially with increasing concentrations of D dimer. Above 1.0 μM, slight stimulation of Lp(a) occurred.

(b) Effect of Lp(a) at a Higher Concentration (50 nM) of t-PA (Figure 2A–D). When the concentration of t-PA was increased in order to reduce the effect of substrate inhibition, uncompetitive inhibition by Lp(a) (0.13–0.45 μM) was again seen, but this was limited to low concentrations of D dimer (0.1 μM) (Figure 2A) and was associated with a K_{iu} of 1.41 μM (Figure 2D). At higher concentrations of D dimer (0.5 and 1.0 μM), Lp(a) (0.45 μM) induced an ~2.4-fold promotion (Figure 2B,C).

Effect of LDL on t-PA-Induced Plasminogen Activation in the Presence of D Dimer. No inhibition by LDL was obtained under the conditions described. In some experiments, slight stimulation of plasmin generation was, in fact, seen (data not shown).

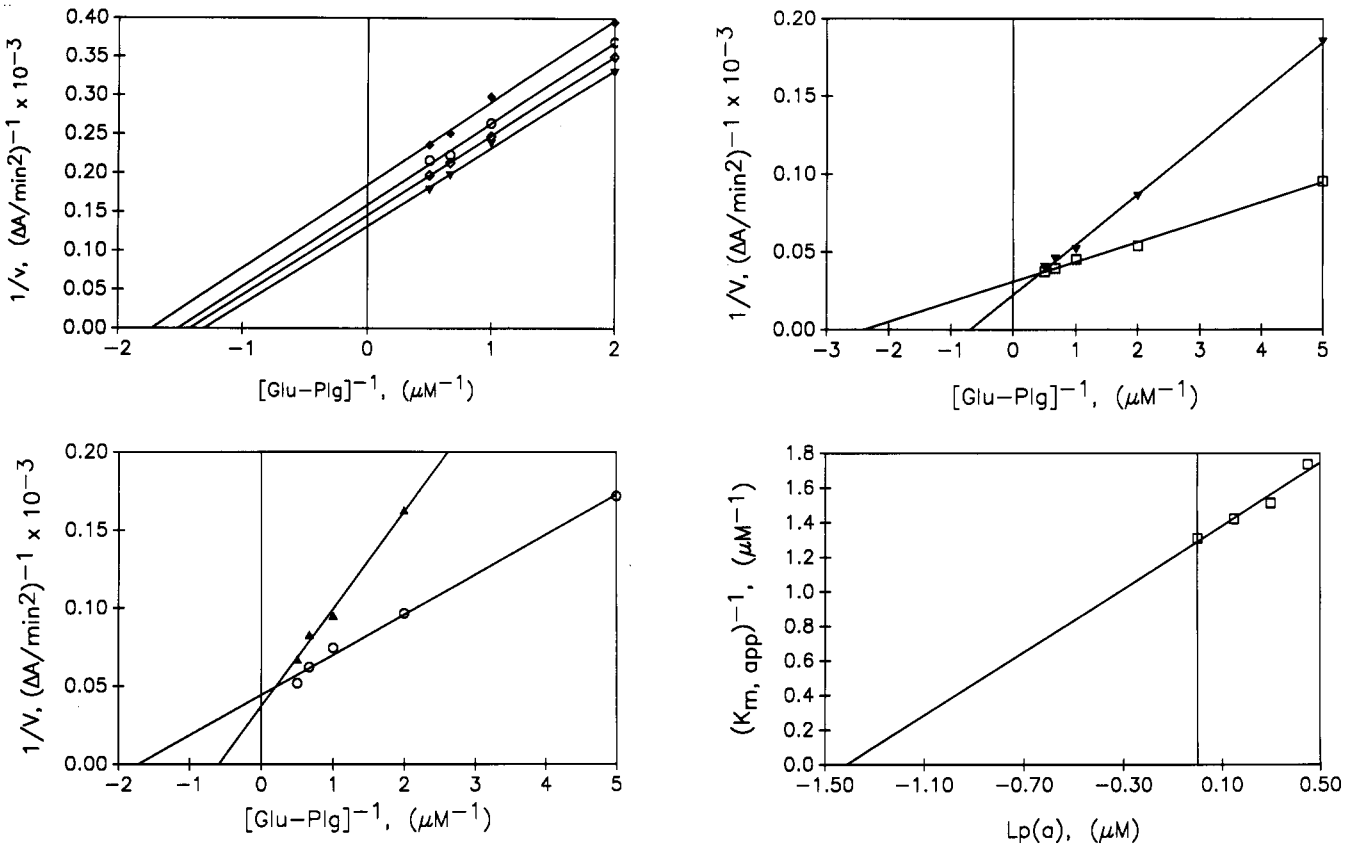

FIGURE 2: Lineweaver–Burk plot analysis of the effect of Lp(a) on plasminogen activation at a higher concentration of t-PA (50 nM). The reaction mixtures contained Lp(a), t-PA, and plasminogen and D dimer: (A, top left) 0.1 μM, where Lp(a) was 0.45 μM (◆), 0.30 μM (○), 0.13 μM (◇), or not added (▼); (B, bottom left) 0.5 μM, where Lp(a) was 0.45 μM (○) or not added (▲); (C, top right) 1.0 μM, where Lp(l) was 0.45 μM (□) or not added (▼). (D, bottom right) Determination of the apparent inhibition constant of Lp(a) by replotting data from A.

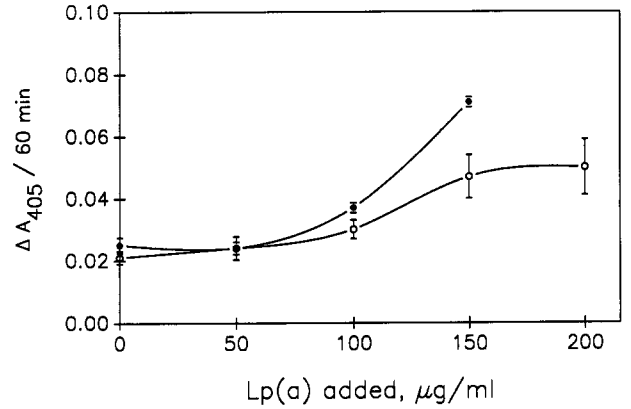

FIGURE 3: Influence of Lp(a) on the binding of plasminogen to plasma-degraded, PVC-immobilized fibrin in a purified system (○) and in a plasma milieu (●).

Effect of LP(a) on the Binding of Plasminogen to Degraded Fibrin (Figure 3). The binding of plasminogen to plasmin-degraded fibrin immobilized in microtiter plate wells was measured. In a buffer milieu, the addition of Lp(a) produced a dose-responsive increase in the amount of plasminogen bound of up to about 2.5-fold increased at 200 μg/mL of Lp(a). A similar promotion of plasminogen binding, instead of inhibition, was observed in a plasma milieu when Lp(a) was added.

DISCUSSION

A remarkable structural homology between Lp(a) and the kringle 4 of the fibrinolytic proenzyme, plasminogen, has been established (McLean *et al.*, 1987; Eaton *et al.*, 1987), giving rise to the hypothesis that Lp(a) may compete with plasminogen for binding sites on fibrin and interfere with its fibrinolytic function. A number of studies designed to investigate this possibility have shown that Lp(a) bound to immobilized fibrin appeared to competitively inhibit plasminogen binding (Harpel *et al.*, 1989; Rouy *et al.*, 1992) and that t-PA-catalyzed plasminogen activation was inhibited (Edelberg *et al.*, 1990; Edelberg & Pizzo, 1990). However, a number of inconsistencies are also found in the current literature. For example, Lp(a)-induced inhibition of plasminogen activation has been reported to be either competitive (Edelberg *et al.*, 1990; Edelberg & Pizzo, 1990) or uncompetitive (Loscalzo *et al.*, 1990; Leerink *et al.*, 1991), the latter being inconsistent with a mechanism related to competitive inhibition of plasminogen binding to fibrin. Moreover, some studies of clot lysis *in vitro* (Mao & Tucci, 1990; Halvorsen *et al.*, 1992; Eaton *et al.*, 1990; Lu *et al.*, 1990) and measures of fibrinolytic activity in subjects (Halvorsen *et al.*, 1992; Oshima *et al.*, 1991; Garcia Frade *et al.*, 1991; Donders *et al.*, 1992, 1993) failed to show any correlation between the results and the Lp(a) concentrations in plasma.

The first objective of the present study was to evaluate the effect of Lp(a) on fibrin-mediated, pro-UK-induced plasminogen activation which has, to our knowledge, not been previously studied. It was recently shown that plasminogen activation by pro-UK or by A-pro-UK, a plasmin-resistant pro-UK mutant, was substantially and selectively promoted by fibrin fragment E_2 (Pannell *et al.*, 1988; Liu & Gurewich, 1991, 1992). The specificity and magnitude of this promotion have provided a mechanism to explain the fibrin dependence of pro-UK-catalyzed plasminogen activation (Liu & Gurewich, 1992). Therefore, the effect of Lp(a) on this reaction is

Table II: Effect of Lp(a) and LDL on the Rate of Plasminogen Activation by Ala^{158}-Prourokinase (2.0 nM), with and without Promotion by Fibrin Fragment E_2 (5.0 μM)

	Glu-plasminogen			
	0.1 μM		2.0 μM	
addition	$+E_2$	$-E_2$	$+E_2$	$-E_2$
none	25.52	0.51	284.80	8.38
Lp(a) (0.09 μM)	28.63	0.53	291.96	8.87
Lp(a) (0.2 μM)	ND	ND	300.83	8.60
Lp(a) (0.6 μM)	ND	ND	281.65	8.31
LDL (0.18 μM)	28.15	0.53	294.82	8.45
LDL (6.9 μM)	ND	ND	296.82	8.59

[a] Numbers represent the initial rate in the coupled reaction described in Methods and Materials; $\Delta A \times 10^6/min^2$.

relevant to the evaluation of its potential antifibrinolytic role.

The present findings indicate that neither Lp(a) nor LDL had any measurable effect on Pro-UK-induced plasminogen activation, and no attenuation of the promotion of this reaction by fragment E_2 was observed (Table II). These findings were supported by the observation that immobilized Lp(a) also failed to bind to fragment E_2 (Table I). In contrast, binding of Lp(a) to fragment D dimer was observed, consistent with the previous findings by Harpel *et al.* (1989) that Lp(a) binds to intact fibrin. These authors additionally showed that Lp(a) binding was considerably promoted by plasmin treatment of the immobilized fibrin. Although the experimental conditions they used differed in that fibrin rather than Lp(a) was immobilized, the present findings suggest that the additional Lp(a) binding sites exposed by plasmin treatment of immobilized fibrin are not functionally comparable to fibrin fragment E_2.

In contrast to pro-UK, some inhibition of t-PA-catalyzed plasminogen activation by Lp(a) in the presence of fibrin fragment D was found. Significantly, the Lp(a)-induced inhibition was of the uncompetitive type, as also previously observed by Localzo *et al.* (1990), but not by Edelberg *et al.* (1990) and Edelberg and Pizzo (1990), who reported competitive inhibition. Uncompetitive inhibition is inconsistent with a mechanism related to competition between Lp(a) and plasminogen for the same binding sites on fibrin. However, uncompetitive inhibition may be explained by plasminogen binding to fibrin-bound Lp(a), which is consistent with the Lp(a)-mediated plasminogen binding observed in the present study (Figure 3).

The observed inhibition by Lp(a) was found to be highly conditional, being limited to low concentrations of both t-PA (≤50 nM) and D dimer (≤0.1 μM), similar to the observations of Leerink *et al.* (1991) in which inhibition was attenuated by increasing the amount of CNBr–fibrinogen fragments. At higher concentrations, a modest stimulation of plasmin generation in the presence of Lp(a) was, in fact, observed (Figure 2B,C). The latter was consistent with the additional observation that Lp(a) slightly enhanced the binding of plasminogen (2 μM) to fibrin (Figure 3). This surprising finding may be explained by the binding of plasminogen to the kringles of Lp(a). Occupancy of the lysine-binding site of one kringle by an internal lysine of another kringle molecule has been previously observed in crystals of t-PA kringle 2 (de Vos *et al.*, 1992).

These results therefore do not support the hypothesis that Lp(a) is a significant inhibitor of plasmin generation on the fibrin surface by either of the two principal plasminogen activators in blood. Although the heterogeneity of Lp(a) may account for certain differences between the present findings and some others, the Lp(a) used in this study was previously shown to bind to fibrin and to compete with plasminogen under certain experimental conditions (Harpel *et al.*, 1989). Moreover, the results obtained are consistent with reports that Lp(a) did not inhibit fibrinolysis (Kluft *et al.*, 1989; Lu *et al.*, 1990; Mao & Tucci, 1990; Halvorsen *et al.*, 1992; Oshima *et al.*, 1991) and with the finding of Eaton *et al.* (1990) that *rec*-apo(a) bound to fibrin without inhibiting plasminogen activation by t-Pa.

Although Lp(a) may compete with the fibrin binding of plasminogen, this property does not appear to mediate the modest inhibition of t-PA-induced plasminogen activation, since this was found to be uncompetitive. The reduction in the V_{max} of t-PA against plasminogen that was seen in the presence of Lp(a) may be related to its binding directly to the substrate, which is consistent with the finding that increasing the substrate concentration, rather than overcoming the inhibition, actually enhanced the inhibition by Lp(a). Rouy *et al.* (1991) and Edelberg *et al.* (1990) also found that Lp(a) induced a modest inhibition of plasminogen activation by t-PA, but in these studies competitive inhibition was reported. The important difference in the mechanism of the inhibition may be related to differences in the experimental conditions. For example, little or no salt was included in their assay buffer, and chloride ion (Bakshy *et al.*, 1986) as well as divalent cations (Stack *et al.*, 1991) has been shown to affect plasminogen activation.

In the present study, Lp(a) was not found to inhibit plasminogen (2 μM) binding to solid-phase fibrin. Instead, a modest promotion was, in fact, seen (Figure 3), consistent with the Lp(a)-induced promotion of plasmin generation observed at the higher concentrations of t-PA and D dimer (Figure 2B,C). These findings are supported by the observation of Mao and Tucci (1990) that Lp(a) enhances clot lysis by t-PA in a plasma milieu. In those studies in which Lp(a) was reported to inhibit plasminogen binding to solid-phase fibrin (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990; Rouy *et al.*, 1991, 1992), low concentrations of plasminogen (<90 nM) or salt (≤0.08 M) were used. In the present study, physiological concentrations of plasminogen (2 μM) and sodium chloride (0.15 M) were used so that the results in buffer and plasma could be compared.

In conclusion, the remarkable homology of Lp(a) with plasminogen has suggested that it may represent a nonactivatable form of plasminogen and thereby may induce competitive inhibition of fibrinolysis. Although this hypothesis remains attractive, the published evidence has been inconclusive. The present study reconciles some of the inconsistencies in the literature but fails to support the hypothesis, suggesting that the atherogenicity of Lp(a) is mediated by another mechanism. However, this study does do not exclude the possibility that Lp(a) may inhibit plasminogen activation on the endothelium (Hajjar *et al.*, 1989; Miles *et al.*, 1989).

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