# Antifibrinolytic Effect of Recombinant Apolipoprotein(a) *in Vitro* Is Primarily Due to Attenuation of tPA-Mediated Glu-Plasminogen Activation†

Waheed Sangrar, Laszlo Bajzar, Michael E. Nesheim, and Marlys L. Koschinsky*

*Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6*

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ABSTRACT: The effect of a 17-kringle form of recombinant apo(a) [r-apo(a)] on *in vitro* fibrin clot lysis was studied. In these assays, fibrin clots were formed in the wells of microtiter plates, and lysis of the clots was monitored by measurement of the turbidity at 405 nm. The results indicate that r-apo(a) produces a dose-dependent antifibrinolytic effect in clots formed using either purified components or barium-adsorbed plasma. This effect was found to be independent of clot structure, since lysis of clots formed using both high and low concentrations of thrombin was prolonged by r-apo(a) to the same extent. The two components of the antifibrinolytic effect of r-apo(a) were determined to be (i) attenuation of tPA-mediated plasminogen activation (the major component) and (ii) inhibition of plasmin degradation of fibrin, although r-apo(a) did not directly attenuate plasmin activity, as measured by S-2251 hydrolysis. r-Apo(a) interfered most substantially with tPA-mediated activation of Glu-plasminogen and less substantially with tPA-mediated Lys-plasminogen activation and urokinase-mediated activation of plasminogen. In summary, we have demonstrated that apo(a) is able to attenuate fibrin clot lysis *in vitro*, primarily as a consequence of the interference by apo(a) with tPA-mediated Glu-plasminogen activation. These studies illuminate possible mechanisms by which Lp(a) may contribute to the development of vascular disease *in vivo*.

Lipoprotein(a) [Lp(a)][1] has been identified in a number of studies as an independent risk factor for the development of coronary heart disease [reviewed in Scanu (1992) and Rader and Brewer (1992)]. Plasma Lp(a) concentrations vary over 1000-fold in the human population, ranging from less than 1 to greater than 100 mg/dL (Albers *et al.*, 1977). Lp(a) levels are under strict genetic control, and are generally not affected by diet, exercise, or drug intervention (Rader & Brewer, 1992). Roughly 25% of the human population possesses Lp(a) levels above an apparent coronary risk threshold of 20 mg/dL, which more than doubles their risk of developing coronary heart disease (Rhoads *et al.*, 1985; Dahlen *et al.*, 1986; Durrington *et al.*, 1988). On the other hand, more recent prospective studies have failed to demonstrate an association between Lp(a) levels and risk for coronary heart disease (Simons *et al.*, 1993; Ridker *et al.*, 1993; Marburger *et al.*, 1994). The mechanism(s) by which Lp(a) exerts its pathogenic effects remain(s) largely unclear at present (Scanu, 1992; Rader & Brewer, 1992), which may underlie the conflicting epidemiological data.

Lp(a) closely resembles low-density lipoprotein (LDL) with respect to both lipid composition and the presence of apolipoprotein B-100 (apoB-100). Lp(a) is distinguishable from LDL by the presence of apolipoprotein(a) [apo(a)] which is covalently linked to apoB-100 by a single disulfide bridge (Sommer *et al.*, 1991; Koschinsky *et al.*, 1993). Human apo(a) consists of multiple tandem repeats of a sequence resembling plasminogen kringle IV, followed by sequences exhibiting approximately 90% identity to the kringle V and protease regions of plasminogen (McLean *et al.*, 1987). Because of the structural homology between apo(a) and plasminogen, it has been postulated that Lp(a) may inhibit the normal fibrinolytic function of plasminogen, thus generating a hypercoagulable state *in vivo*.

The results of studies undertaken to define the effect of Lp(a) on the fibrinolytic system have been highly variable and often conflicting. Early studies demonstrated that apo(a) binds to lysine residues present in fibrin, thereby inhibiting the binding of both plasminogen (Harpel *et al.*, 1989; Rouy *et al.*, 1992) and tissue plasminogen activator (tPA) (Loscalzo *et al.*, 1990) to fibrin. In addition, evidence for the inhibition of tPA-mediated plasminogen activation by apo(a) has been presented by several investigators (Leerink *et al.*, 1992; Rouy *et al.*, 1991; Anglès-Cano *et al.*, 1994); the nature of this inhibition is unclear as both competitive (Edelberg *et al.*, 1990) and uncompetitive (Loscalzo *et al.*, 1990; Leerink *et al.*, 1991) mechanisms of inhibition have been reported. However, recent studies have suggested that apo(a) promotes rather than attenuates tPA-mediated plasminogen activation (Liu *et al.*, 1993). The same investigators have also reported that apo(a) promotes rather than inhibits the binding of plasminogen to fibrin and that in the presence of high concentrations of both D-dimer (a fibrin degradation product) and tPA apo(a) enhances plasminogen activation (Liu *et al.*, 1993). Studies examining the effect of Lp(a) on the lysis of fibrin clots *in vitro* have also produced controversial results; these studies have reported both antifibrinolytic (Loscalzo *et al.*, 1990) as well as profibrinolytic effects of Lp(a) (Mao & Tucci, 1990). Although several studies measuring fibrinolytic parameters in plasma provided evidence for an antifibrinolytic effect of

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* To whom correspondence should be addressed. Phone: (613) 545-6586. Fax: (613) 545-2497.

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); r-apo(a), recombinant apo(a); tPA, tissue plasminogen activator; r-tPA, recombinant tPA; lmw UK, low molecular weight urokinase; apoB-100, apolipoprotein B-100; LDL, low-density lipoprotein.

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Lp(a) *in vivo* (Glueck *et al.*, 1993; Szczeklik *et al.*, 1992), an additional study utilizing similar methodologies failed to detect such an effect (Aznar *et al.*, 1992).

The inconsistencies described above may, in part, be due to variations in experimental conditions or to the use of plasma-derived Lp(a). Lp(a) has been shown to bind to several different plasma and extracellular matrix components such as fibrinogen and fibrin, tPA, platelets, and fibronectin (Harpel *et al.*, 1989; Rouy *et al.*, 1992; Simon *et al.*, 1991; van der Hoek *et al.*, 1994; Ezratty *et al.*, 1993); contamination of Lp(a) preparations with these components may influence its effects in fibrinolysis assays. Furthermore, Lp(a) exists in plasma in a number of differently-sized isoforms (Fless *et al.*, 1984; Gaubatz *et al.*, 1987; Utermann *et al.*, 1987, 1988a,b) which arise due to variations in the number of kringle IV type 2 domains present in apo(a) (van der Hoek *et al.*, 1993; Lackner *et al.*, 1993). Additionally, a donor-dependent, variable fraction of Lp(a) has been shown to be unable to bind lysine or fibrin (Leerink *et al.*, 1992), an effect which appears to be independent of isoform size or plasma levels of the lipoprotein. Taken together, these properties have complicated the isolation and direct comparison of homogeneous, highly purified Lp(a) preparations of the same isoform type, and may underlie the discrepancies in the results that have been obtained to date concerning the role of Lp(a) in fibrinolysis.

In the present study, we have analyzed the effect of a recombinant form of apo(a) [r-apo(a)] (Koschinsky *et al.*, 1991) on fibrinolysis *in vitro* using both purified components as well as a plasma-based *in vitro* clot lysis system. Our results clearly demonstrate an antifibrinolytic effect of r-apo(a) in both the purified and plasma-based systems. Most significantly, we show that the major component of this antifibrinolytic effect resides in the ability of r-apo(a) to attenuate tPA-mediated Glu-plasminogen activation.

## EXPERIMENTAL PROCEDURES

*Materials.* The plasmin-specific chromogenic substrate S-2251 (D-Val-Leu-Lys-*p*-nitroanilide) was obtained from Kabi. Dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared according to Nesheim *et al.* (1979). Barium-adsorbed plasma was prepared by dropwise addition of 1.0 M barium chloride to thawed fresh-frozen human plasma (8 mL/100 mL of plasma) at 4 °C. The barium precipitate was removed by centrifugation, and the supernatant was aliquoted and frozen at −70 °C. Two-chain human low molecular weight urokinase (lmw UK) was obtained from American Diagnostics, and human thrombin was prepared as described previously (Lundblad *et al.*, 1976; Nesheim, 1983). Human recombinant tissue plasminogen activator (r-tPA) was obtained from Genentech Inc.; lyophilized r-tPA was resuspended in 20 mM sodium citrate (pH 5.0) containing 300 mM NaCl, 0.01% Tween-80 and stored at −70 °C. The lysine analog ϵ-aminocaproic acid (ϵ-ACA) was obtained from Sigma.

*Purification of Human Fibrinogen, Plasminogen, and Recombinant Apo(a).* Fibrinogen (99% clottable) was prepared from fresh-frozen, citrated plasma according to the method of Straughn and Wagner (1966). Briefly, fibrinogen was precipitated by dropwise addition of β-alanine. The precipitate was dissolved in 5 mM citrate (pH 8.2), 150 mM NaCl and dialyzed against this buffer prior to passage over lysine−Sepharose CL-4B (Pharmacia) in order to remove trace amounts of plasminogen. The flow-through was dialyzed against 50 mM Tris-HCl (pH 8) and applied to a DEAE-cellulose column; fibrinogen was eluted with 150 mM NaCl, dialyzed extensively against 20 mM HEPES (pH 7.4) containing 150 mM NaCl and 0.01% Tween-80, and stored at −20 °C.

Glu-plasminogen was purified from fresh-frozen plasma by adsorption to lysine−Sepharose CL-4B, followed by elution with ϵ-ACA (Castellino & Powell, 1981). The dialyzed sample was precipitated with 70% ammonium sulfate, and the precipitate was dissolved in a minimal volume of 50% glycerol.

Lys-plasminogen was derived from incubation of Glu-plasminogen (10 μM) with plasmin (0.16 μM) in 23 mL of 50 mM Tris-HCl (pH 8), 50 mM ϵ-ACA as previously described (Violand *et al.*, 1978). After 2 h, the reaction was stopped with the addition of VFK-CMK (Val/Phe/Lys chloromethyl ketone derivative) (0.16 μM). Analysis by acid−urea gel electrophoresis (Panyim & Chalkley, 1969) indicated that the preparation contained no Glu-plasminogen. Lys-plasminogen was then dialyzed against 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 0.01% Tween-80 and stored at −20 °C. Lys-plasmin was produced by activation of plasminogen with 100 units or urokinase (Wiman & Collen, 1977) in 2 mL of 0.1 M sodium phosphate (pH 8) containing 25% glycerol; reaction products were diluted 5-fold with 0.1 M sodium phosphate (pH 8) and applied to a lysine−Sepharose CL-4B column. Bound material was eluted with 20 mM ϵ-ACA and precipitated with 80% saturated ammonium sulfate. Precipitated plasmin was dissolved in 0.1 M sodium phosphate/25% glycerol and stored at −20 °C. The purified fibrinogen and plasminogen were judged to be free of contaminating Lp(a) on the basis of SDS−PAGE and silver staining.

A 17-kringle form of human recombinant apolipoprotein(a) [r-apo(a)] was purified from roller bottle cultures of the stably-expressing cell line 293/apo(a).24 (Koschinsky *et al.*, 1991). Supernatants (2 L) were harvested and concentrated 10-fold by ultrafiltration; concentrated medium was treated with PMSF (1 mM), precipitated with 50% ammonium sulfate, and dissolved in 10 mL of 20 mM HEPES (pH 7.4) containing 150 mM NaCl (HBS) and chromatographed over a 200 mL gel filtration column (BioGel 1.5M; BioRad). r-Apo(a)-containing fractions were pooled and passed over a lysine−Sepharose CL-4B column. The column was washed with 20 mM HEPES (pH 7.4) containing 0.5 M NaCl, and bound r-apo(a) was eluted with 20 mM HEPES (pH 7.4) containing 1.0 M NaCl and 200 mM ϵ-ACA; eluted r-apo(a) was dialyzed at 4 °C against HBS, and stored at −70 °C. The purified r-apo(a) was free of contaminating tPA, since no lysis of fibrin clots formed in the presence of r-apo(a) was observed in the absence of added tPA (data not shown).

The molecular weights and extinction coefficients utilized in calculating concentrations were as follows: thrombin [37 000, $\epsilon_{1\%}(280) = 18.3$]; r-tPA [68 000, $\epsilon_{1\%}(280) = 15.5$]; Glu-plasminogen [92 000, $\epsilon_{1\%}(280) = 16.1$]; Lys-plasminogen [84 000, $\epsilon_{1\%}(280) = 17$]; fibrinogen [340 000, $\epsilon_{1\%}(280) = 16$]; and r-apo(a) [250 000, $\epsilon_{1\%}(280) = 1.94$].

*In Vitro Fibrinolysis Experiments.* Clots were formed in final volumes of 0.1 or 0.2 mL, depending on the experiment. The clots were prepared by adding individual microaliquots

of $CaCl_2$ (5−10 mM), r-tPA (0−0.15 nM), or lmw UK (200 units), and thrombin (3, 6, or 60 nM) to the bottom of microtiter wells. A solution of Glu- or Lys-plasminogen (0.6 $\mu$M) and fibrinogen (2.9 $\mu$M) in the absence or presence of r-apo(a) (0−1.2 $\mu$M) was then added to the wells. Where indicated, barium-adsorbed, dialyzed (0.02 M HEPES, pH 7.4, 0.15 M NaCl) plasma was substituted for Glu-plasminogen and fibrinogen. The final concentration of the barium-adsorbed plasma within the clot was one-third its initial concentration. Lysis of the resulting fibrin clots was monitored by measuring the absorbance (405 nm, 37 °C) at 2.0 or 2.5 min intervals in a Titertek Twin reader (Bajzar *et al.*, 1990; Bajzar & Nesheim, 1993). In experiments addressing the effect of r-apo(a) on Lys-plasmin-mediated fibrinolysis, individual microaliquots of $CaCl_2$ (5 mM) and thrombin (6 nM) were added to microtiter wells. Subsequently, fibrinogen (2.9 $\mu$M) and plasmin (12 nM) in the absence or presence of r-apo(a) (0.41 and 0.74 $\mu$M) were added to give a final clot volume of 200 $\mu$L.

*Determination of the Effect of r-Apo(a) on r-tPA-Dependent Plasminogen Activation.* These assays were performed by forming a series of identical 100 $\mu$L fibrin clots containing 22.1 pM r-tPA, 3 nM thrombin, 10 mM $CaCl_2$, 0.6 $\mu$M Glu-plasminogen, and 2.9 $\mu$M fibrinogen. A second series of fibrin clots was formed in the presence of 0.41 $\mu$M r-apo(a). Measurement of turbidity (see above) was initiated upon clot formation (zero time point). At 10 min intervals, 100 $\mu$L of 0.2 M sodium acetate, pH 4.5, was added sequentially to one well from each series to arrest clot lysis and solubilize the remaining fibrin clot. Immediately following clot dissolution, 100 $\mu$L of a solution containing 60 $\mu$M DAPA, 0.75 mM S-2251, 15 mM EDTA, and 15 mM $\epsilon$-ACA [in Tris-HCl (pH 8), 0.01% Tween-80] was added, and measurement of the absorbance at 405 nm was resumed. The concentration of Lys-plasmin was measured by the rate of appearance of the color (as assessed by $A_{405}$) produced by hydrolysis of S-2251. $A_{405}$ values corresponding to the chromogenic signal were multiplied by 3 in order to correct for dilution and subsequently converted to molar concentrations by use of a standard curve. Lysis profiles, representative of both series of fibrin costs, were obtained from the last clot of each series.

In order to assess the direct effect of r-apo(a) on S-2251 hydrolysis by plasmin in solution, r-apo(a) (0.41 $\mu$M) was incubated with a solution of 0.25 mM S-2251, 10 mM $CaCl_2$, and 10 nM plasmin in a final volume of 100 $\mu$L. The rate of S-2251 hydrolysis was monitored at 405 nm (37 °C) at 0.5 min time intervals for 12 min.

## RESULTS AND DISCUSSION

*The Effects of a 17-Kringle Recombinant Form of Apo(a) on Fibrinolysis.* The *in vitro* clot lysis assay used in these studies has been previously characterized (Jones & Meunier, 1990; Bajzar *et al.*, 1990; Bajzar & Nesheim, 1993) and provides a highly sensitive measure of the rate of fibrinolysis. In order to provide a measure of the effect of r-apo(a) in a plasma-based system, microaliquots of thrombin, $CaCl_2$, and r-tPA are added to the bottom of microtiter wells, and barium-adsorbed, dialyzed plasma (lacking the vitamin K-dependent clotting factors) is added to give a final clot volume of 0.2 mL. Thrombin-induced clot formation occurs within the first 2 min and is marked by an initial rapid

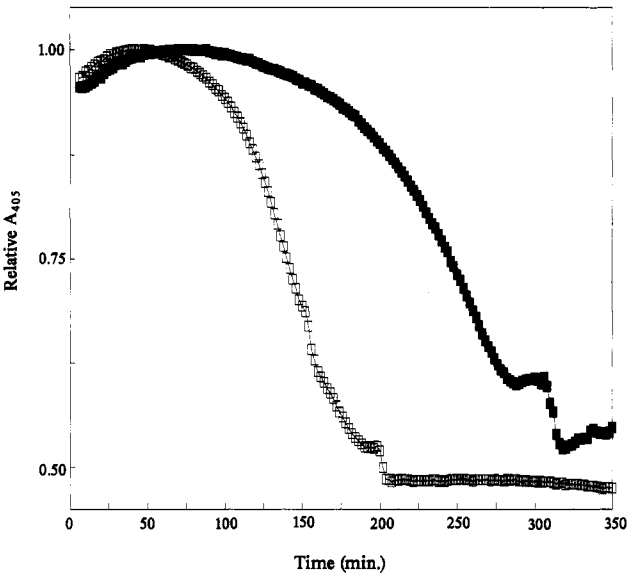


FIGURE 1: Effect of r-apo(a) on fibrinolysis *in vitro*. Clots were formed in microtiter wells from barium-adsorbed plasma (diluted 1/3), 6 nM thrombin, 5 mM $CaCl_2$, and 0.15 nM r-tPA in the absence (□) or presence (■) or 0.14 $\mu$M r-apo(a). Clot lysis was monitored by measurement of turbidity at 405 nm.

increase in turbidity, as measured by $A_{405}$. Subsequent clot lysis is indicated by a rapid return of the turbidity signal to base-line levels. The parameter $t_m$ (transition midpoint) is taken as the standard measure of lysis time and is defined as the time point on the lysis curve that is halfway between the minimum and maximum excursions; the percentage increase in $t_m$ in the presence of r-apo(a) is defined as $\Delta t_m$. In order to assess the effect of r-apo(a) on clot lysis *in vitro*, clots containing thrombin (6 nM), $CaCl_2$ (5 mM), and r-tPA (0.15 nM) were formed from barium-adsorbed, dialyzed plasma in the presence and absence of r-apo(a). In the absence of r-apo(a), the $t_m$ was approximately 142 min (Figure 1). Inclusion of 0.14 $\mu$M r-apo(a) resulted in an increase in $t_m$ to approximately 245 min ($\Delta t_m$ = 73%; Figure 1), indicating that r-apo(a) has a significant inhibitory effect on clot lysis *in vitro*.

In order to assess the antifibrinolytic effect of r-apo(a) in an *in vitro* clot lysis assay consisting of purified components, solutions of Glu-plasminogen and fibrinogen were substituted for barium-adsorbed plasma. When r-apo(a) was tested in this system, a dose-dependent antifibrinolytic effect was observed (Figure 2); inclusion of 0.07 $\mu$M, 0.14 $\mu$M, or 0.27 $\mu$M r-apo(a) resulted in $\Delta t_m$ values of approximately 16%, 29%, and 64%, respectively.

A significantly enhanced antifibrinolytic effect was observed in the plasma-based assay using 0.14 $\mu$M r-apo(a) compared to that produced by 0.27 $\mu$M r-apo(a) in the clot lysis assay utilizing purified components. This observation suggests that other plasma components present in barium-adsorbed plasma may be interacting with r-apo(a) such that the antifibrinolytic effect is enhanced. This may be expected, as a number of physiological components derived from both blood and the extracellular matrix environment have been shown to interact with apo(a). These components include fibronectin and laminin (van der Hoek *et al.*, 1994), $\alpha_2$-antiplasmin (Edelberg & Pizzo, 1992), glycosaminoglycans (Kostner & Bihari-Varga, 1990), platelets (Ezratty *et al.*, 1993), and vitronectin (Kluft *et al.*, 1989). In particular, Lp(a) has been shown to promote plasmin inhibition by $\alpha_2$-

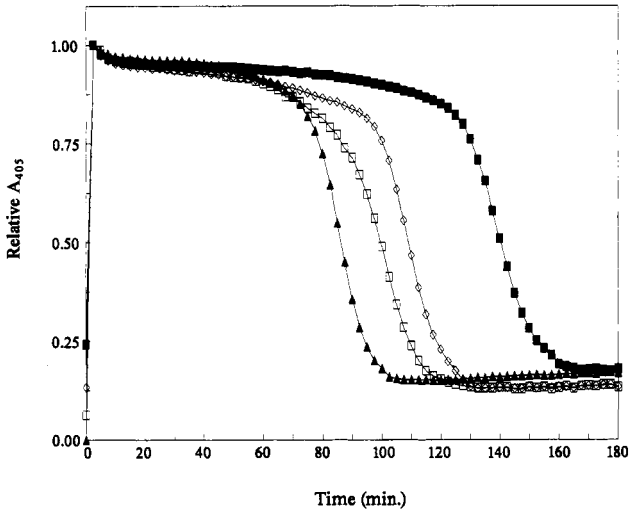


FIGURE 2: Effect of r-apo(a) on fibrinolysis *in vitro* using clots formed from purified components. Fibrin clots were formed in microtiter wells and their lysis monitored by measurement of turbidity at 405 nm. Clots contained 0.6 $\mu$M Glu-plasminogen, 2.9 $\mu$M fibrinogen, 6 nM thrombin, 5 mM $CaCl_2$, and 13.2 pM r-tPA, in the absence (▲) or presence of increasing concentrations of r-apo(a): 0.07 $\mu$M (□); 0.14 $\mu$M (◇); 0.27 $\mu$M (■).

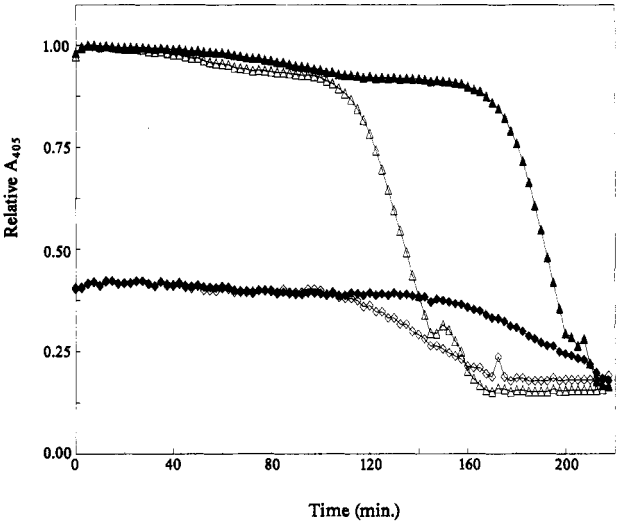


FIGURE 3: Effect of fibrin clot structure on the antifibrinolytic properties of r-apo(a). The profiles correspond to lysis of 0.2 mL clots containing 0.6 $\mu$M Glu-plasminogen, 2.9 $\mu$M fibrinogen, 5 mM $CaCl_2$, 3 or 60 nM thrombin, and 3.3 pM r-tPA in the presence or absence of r-apo(a): 60 nM thrombin/0 $\mu$M r-apo(a) (◇); 60 nM thrombin/0.48 $\mu$M r-apo(a) (♦); 3 nM thrombin/0 $\mu$M r-apo(a) (△); 3 nM thrombin/0.48 $\mu$M r-apo(a) (▲).

antiplasmin (Edelberg & Pizzo, 1992). This may provide an explanation for the enhanced antifibrinolytic effect which we observed using the plasma-based system. However, the nature of the contribution of the other plasma components to the antifibrinolytic effect of apo(a) remains unknown. Irrespective of the difference in the magnitude of the effect observed, the ability of r-apo(a) to inhibit clot lysis *in vitro* in systems based on both plasma and purified components strongly suggests that Lp(a), through its apo(a) component, acts as an antifibrinolytic agent *in vivo*.

*The Effect of Clot Structure on the Antifibrinolytic Effect of r-Apo(a).* The effect of r-apo(a) at two different thrombin concentrations was determined. Lysis of 0.2 mL clots containing 3 or 60 nM thrombin was monitored at 405 nm as a function of time. At the lower thrombin concentration, lateral association of fibrin monomers is favored while at the higher thrombin concentration a greater number of free fibrin ends are generated, resulting in a longitudinal association of fibrin monomers and a subsequent clot structure of lower density (Carr *et al.*, 1977). The difference in clot density is reflected in the initial excursions produced by the unlysed clots (Figure 3). When 0.48 $\mu$M r-apo(a) was included, an identical $\Delta t_m$ value (35%) was observed at either thrombin concentration (Figure 3). These observations suggest that the antifibrinolytic effect produced by r-apo(a) occurs independently of thrombin and its effect on fibrin clot organization.

*The Effect of r-Apo(a) on Lys-Plasmin-Mediated Fibrinolysis.* In an attempt to ascertain the point(s) of inhibition by r-apo(a) within the fibrinolytic cascade, the effect of r-apo(a) on Lys-plasmin-mediated fibrinolysis was studied. Lysis of 0.2 mL clots (in which 12 nM plasmin was substituted for Glu-plasminogen and r-tPA in order to bypass the plasminogen activation step) in the presence of various concentrations (0.41 and 0.74 $\mu$M) of r-apo(a) was monitored at 405 nm as a function of time. As in the experiments described above, a dose-dependent inhibition of fibrinolysis was observed in the presence of r-apo(a) ($\Delta t_m$ values of 19% and 25%, respectively; Figure 4), although the magnitude of the antifibrinolytic effect was lower than that observed

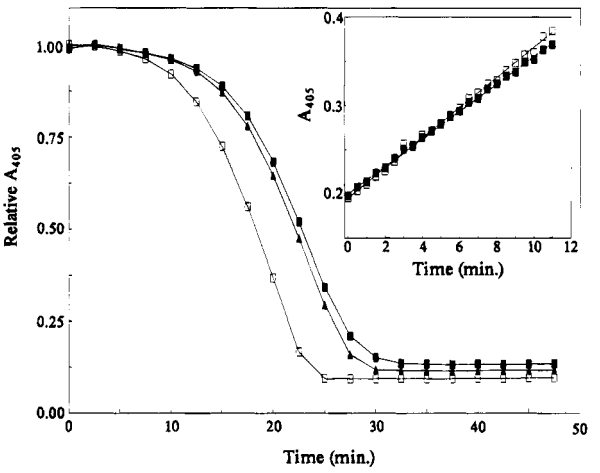


FIGURE 4: Antifibrinolytic effect of r-apo(a). The profiles represent lysis of 0.2 mL clots containing 2.9 $\mu$M fibrinogen, 12 nM plasmin (plasmin was substituted for Glu-plasminogen and r-tPA in order to bypass the plasminogen activation step), 6 nM thrombin, 5 mM $CaCl_2$, and various concentrations of r-apo(a): 0 $\mu$M r-apo(a) (□); 0.41 $\mu$M r-apo(a) (▲); 0.74 $\mu$M r-apo(a) (■). INSET: The effect of r-apo(a) on S-2251 hydrolysis by plasmin in solution. A solution of 10 nM plasmin, 0.25 mM S-2251, and 10 mM $CaCl_2$ in HBS, pH 7.4, 0.01% Tween-80 was incubated in the absence (□) or presence (■) of 0.41 $\mu$M r-apo(a). The total volume of the reactions was 0.1 mL. The absorbance at 405 nm was measured at 0.5 min time intervals.

in Figure 2. The effect of r-apo(a) on S-2251 hydrolysis by Lys-plasmin was also tested (see inset, Figure 4). A solution containing 10 nM plasmin, 0.25 mM S-2251, and 10 mM $CaCl_2$ in the presence or absence of 0.41 $\mu$M r-apo(a) was prepared, and S-2251 hydrolysis by plasmin was measured at 405 nm as a function of time. Using this assay, it was determined that the rate of S-2251 hydrolysis by plasmin was not altered to any significant extent in the presence of r-apo(a). These observations suggest that the inhibitory effect of r-apo(a) on Lys-plasmin-mediated fibrinolysis is not the result of direct inhibition of the enzymatic activity of Lys-plasmin. The ability of plasmin to access its substrate fibrin may be hindered by the binding of apo(a) to the fibrin

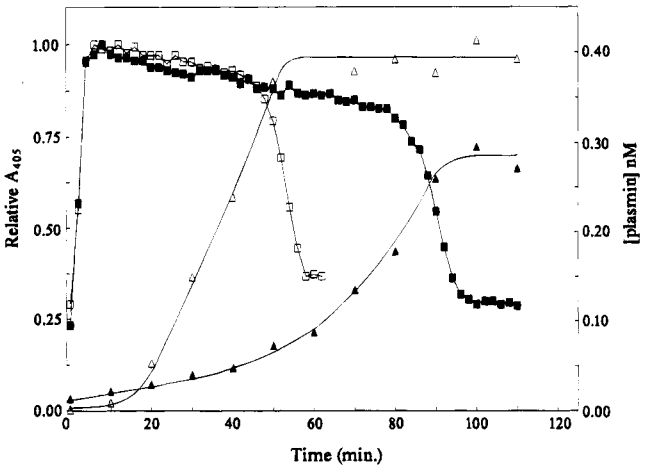


FIGURE 5: Effect of r-apo(a) on Glu-plasminogen activation. A set of 12 identical 0.1 mL clots was formed containing 0.6 $\mu$M Glu-plasminogen, 2.9 $\mu$M fibrinogen, 3 nM thrombin, 10 mM $CaCl_2$, and 22.1 pM r-tPA. A second set of clots was prepared which were identical to the first set except that 0.41 $\mu$M r-apo(a) was added. Lysis of both sets of clots was monitored by $A_{405}$ every 2.0 min. Every 10 min, one clot from each set was dissolved by adding 0.1 mL of 0.2 M acetic acid, followed by addition of a solution containing 0.75 mM S-2251, 60 $\mu$M DAPA, 15 mM EDTA, and 15 mM $\epsilon$-ACA (in Tris-HCl, pH 8, 0.01% Tween-80). Monitoring of the absorbance at 405 nm was resumed to measure the rate of S-2251 hydrolysis by plasmin. Plasmin concentration was determined using a standard curve relating plasmin concentration to the rate of S-2251 hydrolysis. Lysis profiles were obtained from the last clot to be dissolved in each set. The figure illustrates the changes in plasmin concentration [no r-apo(a) (△); 0.41 $\mu$M r-apo(a) (▲)] during the time course of the lysis profiles [no r-apo(a) (□); 0.41 $\mu$M r-apo(a) (■)].

surface, since both plasmin and apo(a) bind to exposed lysine residues in fibrin (Harpel *et al.*, 1989; Rouy *et al.*, 1992).

*The Effect of r-Apo(a) on r-tPA-Dependent Glu-Plasminogen Activation.* The effect of r-apo(a) on r-tPA-dependent Glu-plasminogen activation was investigated by measuring plasmin activity concurrently with fibrin clot lysis (see Figure 5). Two sets of identical fibrin clots were prepared in the wells of microtiter plates in the presence or absence of 0.41 $\mu$M r-apo(a), and turbidity was measured by monitoring $A_{405}$ at 2.0 min intervals. At 10 min intervals, 0.2 M sodium acetate was added sequentially to each well to arrest clot lysis and dissolve the remaining clot. To measure the rate of plasmin generation, the chromogenic substrate S-2251 was added to the dissolved clots, and color development was measured by $A_{405}$; the concentration of plasmin at each time point was determined using a standard curve relating S-2251 hydrolysis ($A_{405}$) to plasmin concentration. A clot lysis profile was obtained from the last clot quenched in each series. The results shown in Figure 5 indicate that in the presence of r-apo(a), the rate of plasmin formation during the time course of the experiment is substantially reduced compared to that in the absence of r-apo(a). These results suggest that in addition to interference with fibrin degradation by plasmin, r-apo(a) also inhibits fibrinolysis by attenuating Glu-plasminogen activation; the latter observation is in agreement with previous studies utilizing purified Lp(a) (Loscalzo *et al.*, 1990; Leerink *et al.*, 1991, 1992; Rouy *et al.*, 1991; Anglès-Cano *et al.*, 1994; Edelberg *et al.*, 1990). The $\Delta t_m$ produced by 0.41 $\mu$M r-apo(a) was approximately 68% (Figure 5). When the Glu-plasminogen reaction was bypassed (i.e., when fibrin clots were formed in the presence

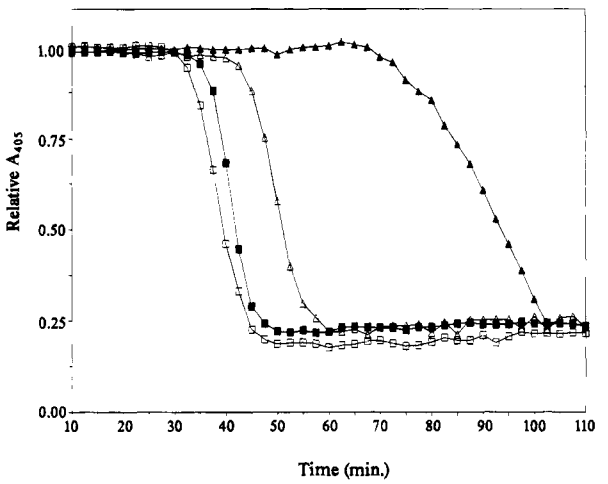


FIGURE 6: Comparison of the effect of r-apo(a) on lysis of clots formed in the presence of either Glu- or Lys-plasminogen. Lysis profiles were obtained for 0.1 mL clots containing 0.6 $\mu$M Glu- or Lys-plasminogen, 2.9 $\mu$M fibrinogen, 6 nM thrombin, 17.6 pM r-tPA, and 10 mM $CaCl_2$ formed in the presence or absence of 0.44 $\mu$M r-apo(a). Lys-plasminogen/0 $\mu$M r-apo(a) (□); Lys-plasminogen/0.44 $\mu$M r-apo(a) (■); Glu-plasminogen/0 $\mu$M r-apo(a) (△); Glu-plasminogen/0.44 $\mu$M r-apo(a) (▲).

of Lys-plasmin), the same r-apo(a) concentration resulted in a $\Delta t_m$ of only 19% (Figure 4). Thus, there appears to be two components of the antifibrinolytic effect of r-apo(a): a larger component which is attributable to the inhibition of plasminogen activation and a smaller component which is attributable to the inhibition of Lys-plasmin degradation of fibrin.

*Comparison of the Effect of r-Apo(a) on Lysis of Clots Formed in the Presence of either Glu- or Lys-Plasminogen.* Lys-plasminogen is derived from native Glu-plasminogen by plasmin-catalyzed cleavage between $Lys^{76}$ and $Lys^{77}$ (Castellino, 1984) and is superior to Glu-plasminogen in fibrin binding and as a substrate for tPA (Hoylaerts *et al.*, 1982; Fredenburgh & Nesheim, 1992). Lysis of fibrin clots containing either Lys- or Glu-plasminogen (0.6 $\mu$M) in the absence or presence of r-apo(a) (0.44 $\mu$M) was monitored by $A_{405}$ as a function of time. As shown in Figure 6, the antifibrinolytic effect of r-apo(a) observed using clots formed in the presence of Lys-plasminogen was much smaller ($\Delta t_m$ = 8%) than that observed for clots formed in the presence of Glu-plasminogen ($\Delta t_m$ = 82%). The inhibitory effect of r-apo(a) on the lysis of clots formed in the presence of Lys-plasminogen (Figure 6) is comparable to that observed when plasminogen activation was bypassed in the presence of a similar concentration of r-apo(a) (see Figure 4). This suggests that r-apo(a) has little or no effect on Lys-plasminogen activation by r-tPA and that the minor antifibrinolytic effect observed in Figure 6 is likely attributable to the direct inhibitory effect of r-apo(a) on the degradation of fibrin by Lys-plasmin.

Studies by Fredenburgh and Nesheim (1992) have shown that *in vitro*, Glu-plasminogen consumption is equally distributed between direct activation to plasmin and conversion to Lys-plasminogen. These authors also demonstrated that Lys-plasminogen is superior to the native Glu-plasminogen as a substrate for activation by tPA. As a result, the formation of Lys-plasminogen within a clot constitutes an important positive feedback mechanism which enhances the progress of fibrinolysis. The ability of r-apo(a) to inhibit

Glu-plasminogen activation suggests that less Lys-plasminogen would be generated due to reduction of Glu-plasmin levels; Glu-plasmin is probably responsible for the initial conversion of Glu-plasminogen to Lys-plasminogen. Thus, apo(a), by inhibiting Glu-plasmin formation, may delay the occurrence of positive feedback which serves to enhance fibrinolysis. On the other hand, the inability of r-apo(a) to significantly affect Lys-plasminogen activation may serve to limit the degree to which apo(a) inhibits fibrinolysis.

Previous analyses of the antifibrinolytic mechanism of action of apo(a) have shown that Lp(a) competitively inhibits the binding of Glu-plasminogen to fibrin (Harpel *et al.*, 1989; Rouy *et al.*, 1992; Loscalzo *et al.*, 1990). These results have led to the suggestion that apo(a) inhibits Glu-plasminogen activation by displacing Glu-plasminogen from its binding sites on fibrin. However, this manner of inhibition likely does not apply in our system since Glu-plasminogen exhibits little or no binding to intact fibrin under our conditions (Nesheim *et al.*, 1990). Studies based on kinetic analysis of the reactions of fibrinolysis (Hoylaerts *et al.*, 1982) have proposed a model in which plasminogen activation requires the initial formation of a binary complex consisting of tPA and fibrin; this complex in turn favors the formation of a ternary complex with plasminogen which facilitates proteolytic cleavage of plasminogen by tPA and subsequent liberation of plasmin. As such, the mechanism of inhibition of fibrinolysis by Lp(a) may involve displacement or exclusion of plasminogen from the tPA/fibrin complex, thereby resulting in an overall decrease in plasmin generation. The $K_m$ for the formation of the ternary complex with Glu-plasminogen is 0.16 $\mu$M, while the $K_m$ is approximately 8-fold lower for Lys-plasminogen (Hoylaerts *et al.*, 1982). This implies that Lys-plasminogen is a better substrate for activation by tPA and may thus explain the inability of r-apo(a) to significantly inhibit Lys-plasminogen activation.

An alternative mechanism for the inhibition by r-apo(a) of tPA-mediated Glu-plasminogen activation involves disruption of the formation of the initial tPA/fibrin complex. This could result from a direct interaction of apo(a) with tPA, evidence for which has been provided by Simon and co-workers (Simon *et al.*, 1991). Such an interaction may inhibit the formation of the initial tPA/fibrin binary complex, thereby resulting in a decrease in plasmin generation, as reflected in an increased $K_m$. This model may be consistent with the specific effect of r-apo(a) on Glu-plasminogen activation, since the catalytic efficiency of tPA for Lys-plasminogen activation exceeds that for Glu-plasminogen activation by an order of magnitude, an effect expressed primarily through a decrease in $K_m$ (Hoylaerts *et al.*, 1982). As a result, any decrease in Lys-plasminogen activation attributable to r-apo(a), via an elevation in $K_m$, may not be sufficient to detect a prolongation of lysis time under the experimental conditions (in particular, the Lys-plasminogen concentration) employed here. Reduction in the formation of binary tPA/fibrin complexes may also occur as a result of competition between apo(a) and tPA for fibrin binding as has been demonstrated by Loscalzo *et al.* (1990).

*Comparison of the Effect of r-Apo(a) on r-tPA- or lmw UK-Dependent Fibrinolysis.* The specificity of the antifibrinolytic effect of r-apo(a) for tPA-dependent plasminogen activation was evaluated by employing lmw UK in place of r-tPA. Clots (0.1 mL) containing 200 units of lmw UK or 17.6 pM r-tPA were formed in the absence or presence of 0.44 $\mu$M r-apo(a). Urokinase and r-tPA concentrations were chosen such that the $t_m$ values in the respective lysis assays in the absence of r-apo(a) were similar. Whereas inclusion of r-apo(a) resulted in a $\Delta t_m$ value of 82% for tPA-dependent fibrinolysis, inclusion of r-apo(a) resulted in a $\Delta t_m$ of only 15% for lmw UK-dependent fibrinolysis (Figure 7). The latter value is comparable in magnitude to the $\Delta t_m$ observed for Lys-plasmin-mediated clot lysis (see Figure 4), which suggests that r-apo(a) has little or no effect on lmw UK-dependent Glu-plasminogen activation. The minor change in $t_m$ observed in Figure 7 is likely due solely to r-apo(a) inhibition of fibrin degradation by lmw UK-activated plasmin. This hypothesis is supported by the results of a previous study in which Lp(a) had no effect on the rate of plasmin generation by urokinase, although these studies were performed with the single-chain form of urokinase (prourokinase) (Liu *et al.*, 1993). Since apo(a) interferes exclusively with tPA-mediated plasminogen activation, the major antifibrinolytic effects of Lp(a) *in vivo* may be localized to acute responses to vascular injury involving tPA.

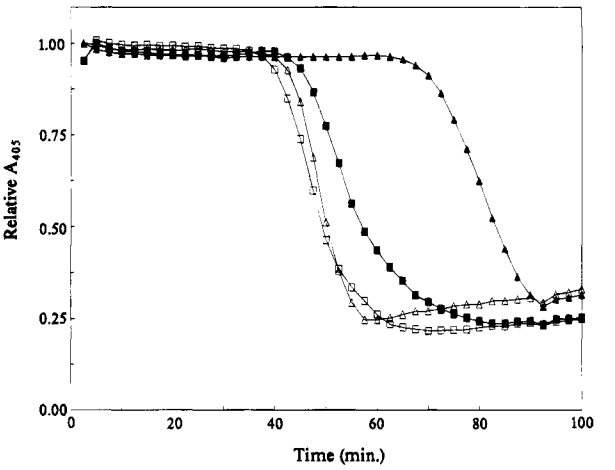


FIGURE 7: Comparison of the effect of r-apo(a) on r-tPA- and lmw UK-dependent fibrinolysis. Lysis profiles were obtained for 0.1 mL clots containing 0.6 $\mu$M Glu-plasminogen, 2.9 $\mu$M fibrinogen, 6 nM thrombin, 10 mM $CaCl_2$, and 200 units of lmw UK or 17.6 pM r-tPA formed in the absence and presence of 0.44 $\mu$M r-apo(a). lmw UK/0 $\mu$M r-apo(a) (□); lmw UK/0.44 $\mu$M r-apo(a) (■); r-tPA/0 $\mu$M r-apo(a) (△); r-tPA/0.44 $\mu$M r-apo(a) (▲).

In summary, we have demonstrated a dose-dependent antifibrinolytic effect of r-apo(a) using an *in vitro* clot lysis assay. The major component of this antifibrinolytic effect is inhibition of tPA-dependent Glu-plasminogen activation. In addition, r-apo(a) attenuates plasmin-mediated fibrin degradation. Additional studies will be required to further elucidate the molecular mechanisms of the antifibrinolytic effect of r-apo(a), which may provide the basis for analysis of the effect of Lp(a) on fibrinolysis *in vivo*.

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