nificant physiologic consequences. The finding of a varied pattern in the adult suggests that the precise type of monomer units synthesized in the adult could vary from that synthesized during growth. If a different type of splicing pattern were to occur in human tissues, particularly in pathological situations such as atherosclerosis, this change may contribute to the disease process.

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Lipoprotein *a* Inhibits Streptokinase-Mediated Activation of Human Plasminogen†

Jay M. Edelberg, Mario Gonzalez-Gronow, and Salvatore V. Pizzo*

Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: Lipoprotein *a* [Lp(*a*)] inhibits human plasminogen (Pg) conversion to plasmin (Pm) by streptokinase- (SK-) mediated activation. Kinetic and binding studies indicate that Lp(*a*) inhibits Pg activation by competitive and uncompetitive inhibition. Lp(*a*) competes with Pg for SK and forms a stable complex. Lp(*a*) does not, however, inhibit Pg activation by the proteolytic SK–Pm complex. The SK–Pg and SK–Pg(act) intermediate complexes are possible targets of the Lp(*a*) uncompetitive inhibition. The competitive inhibition constant (K_{ic}) is 45 nM or 14 mg/dL, and the uncompetitive inhibition constant (K_{iu}) is 140 nM or 42 mg/dL, corresponding to physiologic and pathophysiologic Lp(*a*) concentrations, respectively.

Lipoprotein *a* [Lp(*a*)] is a low-density lipoprotein first identified by Berg (1963). Plasma levels of Lp(*a*) strongly correlate with atherosclerotic lesions [see Brown and Goldstein (1987) and Scanu (1988) for brief reviews]. Elevated levels of Lp(*a*) (greater than 30 mg/dL) are associated with a risk of atherosclerotic disease two to five times that of control subjects (Albers et al., 1977; Frick et al., 1978; Rhoads et al., 1986; Dahlen et al., 1986). The elevated Lp(*a*) levels are linked not only to coronary artery disease but also to stenosis of carotid and cerebral arteries (Murai et al., 1986; Zenker et al., 1986) and saphenous vein bypass grafts (Hoff et al., 1988).

Structural studies of Lp(*a*) demonstrate that like low-density lipoproteins it contains an apoprotein B (apo B) subunit, but also contains an apoprotein *a* [apo(*a*)] subunit linked by a disulfide bond to the apo B subunit [see, for review, Scanu (1988)]. The apo B subunit is a M_r ~510 000 polypeptide (Law et al., 1986; Knott et al., 1986), and the apo(*a*) subunit

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* To whom correspondence should be addressed.

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is also a large apoprotein with isoforms larger, smaller, and equal in size to the apo B subunit (Gaubatz et al., 1983; Armstrong et al., 1985; Fless et al., 1986; Eaton et al., 1987; Karadi et al., 1988). The apo(*a*) subunit has extensive homology to the fibrinolytic protein zymogen plasminogen (Pg) (McLean et al., 1987; Eaton et al., 1987). The apo(*a*) subunit contains a region 94% homologous to the Pg proteinase domain. The apo(*a*) subunit also contains the lysine binding domains, termed kringles, found in Pg and other serine proteinases [for review, see Patthy (1985) and Furie and Furie (1988)]. While Pg has a single copy of kringles 1–5, the apo(*a*) subunit has 37 copies of kringle 4 and 1 copy of kringle 5, with 75–85% and 95% homology, respectively, to their Pg counterparts. The apo(*a*) subunit lacks a critical Pg cleavage site, Arg_{560}-Val_{561}, necessary for activation (Robbins et al., 1967). The apo(*a*) subunit has a Ser in the place of the Arg found in Pg, and it cannot be converted to a proteinase by typical Pg activators (Eaton et al., 1987).

Studies by Karadi et al. (1988) showed that Lp(*a*) decreases streptokinase- (SK-) mediated lysis of fibrin clots, but the reaction mechanism was not studied. We have sought to quantify the inhibition by Lp(*a*) of SK-mediated fibrinolysis. In the present study kinetic experiments by initial rate and time course have been employed to determine the Lp(*a*) inhibition constants for SK-mediated activation of fibrinolysis and the sites of inhibition for the Pg activation.

Materials and Methods

Reagents. Bovine serum albumin and heparin–agarose were purchased from Sigma, $Na^{125}I$ was from New England Nuclear, and lactoperoxidase–Sepharose was from Bio-Rad. The SK–Pm and Pm substrate, H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide dihydrochloride (S-2251), was purchased from Helena. Sephacryl S-200 was obtained from Pharmacia. All other reagents were of the best grade commercially available.

Proteins. Lp(*a*) was isolated from ten subjects with normal levels of Lp(*a*) by using a two-step chromatography procedure. The pooled frozen plasma was filtered for insoluble impurities and applied to a heparin–agarose column, 3 × 45 cm, at 4 °C. The protein was eluted with a NaCl gradient from 200 to 500 mM in 50 mM Tris-HCl, pH 7.4. The peak, between 300 and 350 mM NaCl, was concentrated by lyophilization and subjected to gel filtration over a Sephacryl S-200 column, 3 × 120 cm, with 50 mM ammonium bicarbonate. The protein in the void volume was collected and lyophilized, and the Lp(*a*) identity was confirmed by Ouchterlony immunodiffusion (Campbell et al., 1970) and Western blot techniques (Towbin et al., 1979) with a polyclonal IgG raised against human Pg as described (Gonzalez-Gronow & Robbins, 1984), which has immunoreactivity with the Lp(*a*) kringles 4 and 5 and protease-like domain. Pg was purified by affinity chromatography as previously described by Deutsch and Mertz (1970) and modified by Brockway and Castellino (1972). SK was isolated as described by Einarsson et al. (1979).

Protein Concentration. The concentration of isolated Lp(*a*) was determined by the modified Lowry method (Peterson, 1979) and by using a protein M_r of ~510 000 for apo B subunit (Law et al., 1986; Knott et al., 1986) and M_r ~500 000 for the apo(*a*) subunit estimated from the size of the immunoreactive band on SDS–PAGE. Concentrations of SK and Pg were determined by the following extinction coefficients and molecular weights: SK, $A^{1\%}_{1cm}$ = 7.5 and M_r ~50 200 (Einarsson et al., 1979); Pg, $A^{1\%}_{1cm}$ = 16.8 and M_r ~92 000 (Sjoholm et al., 1973).

SDS–PAGE. Protein molecular weight and purity were examined by SDS–PAGE on 5–15% gradient slab gels. The proteins were visualized by silver staining (Morrissey, 1981). Molecular weights were estimated relative to α_2-macroglobulin half-molecules of M_r ~360 000 (Hall & Roberts, 1978).

Protein Radioiodination. Proteins were radioiodinated by the solid-state lactoperoxidase method as described by David and Reisfeld (1974). Radioactivity was determined by a LKB-Wallac Clinigamma 1272, Gaithersberg, MD.

Determination of Lipoprotein a Kinetic Inhibition Constants. Steady-state kinetics as determined by initial rate measurement were performed as previously described (Rajagapolan et al., 1985). SK, 10 nM, in quadruplicate sets, was incubated for 15 min at 25 °C, with increasing concentrations of Lp(*a*) from 0 to 500 nM in a total of 50 μL of 50 mM Tris-HCl, pH 7.4. Pg and substrate, S-2251, were then added to the samples. S-2251 and Pg at 20, 40, 100, and 200 nM were added in the same buffer to 1 mL to each set of SK and Lp(*a*) incubations of 0.3 mM. The incubations were continued for 7 min at 37 °C and the reactions terminated with 50 μL of 50% acetic acid. Measurements of the A_{405nm} were employed to determine the velocity of the reactions.

Numerical analysis of the kinetics was carried out as previously described by Dixon (1953) and modified by Knight (1986) by using the equation

$$1/V = (K_m + [S])/V_m[S] + \{(K_m/K_{ic}) + ([S]/K_{iu})\}[I]/V_m[S]$$

with K_{ic} and K_{iu} standing for the inhibition constants for competitive and uncompetitive inhibition, respectively.

Streptokinase–Lipoprotein a Complex Formation Determination. Radioiodinated SK alone and SK with Lp(*a*) were incubated at 130 and 10 nM, respectively, for 30 min at 25 °C and 10 min at 4 °C. The incubates were then subjected to gel filtration on a Sephacryl S-200 column (1 × 20 cm). The radioactivity of the elution fractions was counted. The counts per minute versus fraction number was plotted for both SK and SK–Lp(*a*) incubations.

Streptokinase–Plasmin Complex and Plasmin Activity When Incubated with Lipoprotein a. SK–Pm complexes were formed as previously described by Bajaj and Castellino (1977). SK and Pg were incubated at a 1:1 molar ratio at 2 μM in 100 μL of 50 mM Tris-HCl, pH 7.4, and incubated at 25 °C for 10 min. Substrate solutions with increasing concentrations of Lp(*a*) at 0, 2.5, 12.5, 25, and 50 nM, and substrate S-2251, 0.3 mM, in 50 mM Tris-HCl, pH 7.4, were added to 3 μL of the SK–Pm complexes in 1-mL final volume. The reaction was incubated at 37 °C for 10 min and terminated by the addition of 50 μL of 50% acetic acid. The velocity was measured by recording the A_{405nm}. A plot of the velocity versus Lp(*a*) concentration determined the Lp(*a*) inhibition of the SK–Pm complex activity. Pg was activated by SK as previously described (Gonias et al., 1982). Pg, 200 nM, and SK, 10 nM, were incubated together in 100 μL of 50 mM Tris-HCl, pH 7.4, at 25 °C for 50 min. Ten-microliter aliquots were then added to 1 mL of 0.3 mM S-2251 plasmin substrate with increasing concentrations of Lp(*a*), 0, 2.5, 5, 25, and 50 nM, in 50 mM Tris-HCl, pH 7.4. The samples were incubated for 7 min at 37 °C, the reactions were stopped with 50 μL of 50% acetic acid, and then the velocity was measured by recording the A_{405nm}. A plot of the velocity versus Lp(*a*) concentration determined the Lp(*a*) inhibition of the Pm activity.

Lipoprotein a Inhibition of Plasminogen Activation as a Function of Time. Pm activity as a function of time was examined as previously described by Rajagopalan et al. (1985). SK and Lp(*a*) were incubated at 8.8 and 0–6 nM, respectively, for 30 min at 25 °C in 50 μL of 50 mM Tris-HCl, pH 7.4. Pg was then added to a final concentration of 2.2 μM in

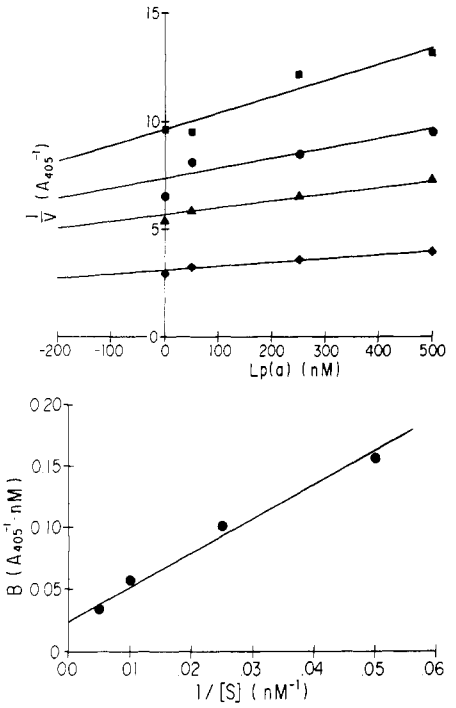

FIGURE 1: Kinetic analysis of the effect of Lp(*a*) on fibrinolysis. Upper panel: Dixon plot of the plasmin activity reciprocal versus Lp(*a*) concentration during SK preincubation. SK, 10 nM, and Lp(*a*), 0–500 nM, were preincubated in 50 μL of 50 mM Tris-HCl, pH 7.4. Pg, at 20 (■), 40 (●), 100 (▲), and 200 nM (◆), and 0.3 mM S-2251 substrate in 50 mM Tris-HCl, pH 7.4, were added to a total of 1 mL for incubation at 37 °C for 7 min. Lower panel: Plot of Dixon slopes versus Pg concentration reciprocal. The slope is a function of the competitive inhibition, and the *y*-axis intercept is a function of the uncompetitive inhibition.

100-μL total volume. The samples were incubated at 25 °C. At specific times, 20 μL of the incubations was added to 1 mL of substrate solution, 0.3 mM S-2251 in 50 mM Tris-HCl, pH 7.4, and incubated for 10 min at 37 °C. The reactions were then stopped with 50 μL of 50% acetic acid. The Pm activity was determined by A_{405nm}.

Results

Isolation of Lipoprotein a. Lp(*a*) was purified to homogeneity by a two-step chromatography procedure, taking advantage of the apo(*a*) subunit affinity for heparin–Sepharose (Fless et al., 1984) as described under Materials and Methods. Affinity chromatography on heparin–agarose, followed by gel filtration with Sephacryl S-200, was employed. These two steps resulted in isolation of a homogeneous Lp(*a*) fraction with apo(*a*) and apo B subunits with similar sizes, M_r ~500 000, with a similar total molecular weight to previously isolated Lp(*a*) from normal donors (Gaubatz, 1983). The identity of the Lp(*a*) and the apo(*a*) bands was confirmed by Ouchterlony immunodiffusion and Western blotting with polyclonal IgG raised against human Pg, taking advantage of the immunochemical relation between Lp(*a*) and Pg (Karadi et al., 1988).

Lipoprotein a Inhibits Streptokinase-Mediated Plasminogen Activation. The kinetic experiments were performed to demonstrate that increasing concentrations of Lp(*a*) preincubated with SK decreased Pg activation as determined by Pm substrate hydrolysis. A Dixon plot of the reciprocal velocity versus Lp(*a*) concentration of fixed Pg concentrations illustrates this inhibition (see Figure 1, upper panel). Lp(*a*) inhibition constants were derived from a plot of the Dixon slopes versus the reciprocal Pg concentrations (Figure 1, lower panel). Lp(*a*) has both competitive and uncompetitive inhibition constants of 45 nM and 140 nM, respectively.

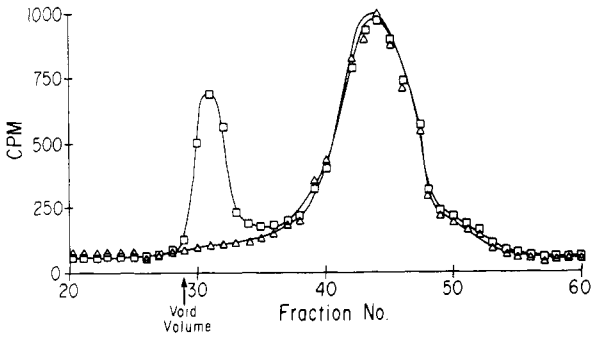

FIGURE 2: Sephacryl S-200 chromatography elution profile of radiolabeled SK. Radiolabeled SK incubated alone (△) or with Lp(*a*) (□) was eluted on a Sephacryl S-200 column (1 × 20 cm).

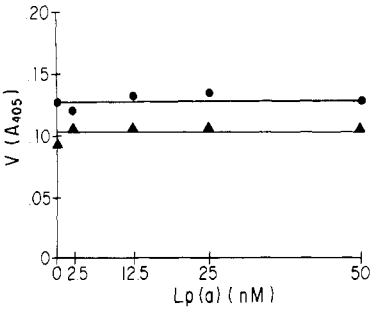

FIGURE 3: Pm and SK–Pm complex activity versus Lp(*a*) concentration. Lp(*a*), 0–50 nM, substrate (S-2251), 0.3 mM, and preformed Pm (▲) and SK–Pm complex (●), 6 nM, were incubated in 1 mL of 50 mM Tris-HCl, pH 7.4.

Determination of Competitive Inhibition Sites. Sites of competitive inhibition, the SK binding site for Pg and the site of SK–Pm complex proteolysis of Pg and hydrolysis of S-2251, were tested. Competitive inhibition at the site of SK binding of Pg was demonstrated by SK binding to Lp(*a*). Radioiodinated SK was incubated with Lp(*a*) and then subjected to gel filtration. Figure 2 shows the labeled SK shifts into the void volume, were Lp(*a*) elutes. Competitive inhibition at the sites of SK–Pm complex and Pm proteolysis of Pg was excluded by the following studies. Pm and SK–Pm complexes were preformed and incubated with the substrate S-2251 with increasing Lp(*a*) concentrations. Figure 3 demonstrates that there is no inhibition of either the Pm or the SK–Pm complex activity with increasing Lp(*a*) concentrations.

Lipoprotein a Inhibition of the Plasminogen Activation Is Stable as a Function of Time. SK was preincubated with increasing Lp(*a*) concentrations. A fixed concentration of Pg was added to these incubations and the incubation continued. At various times, Pm formation was measured by the addition of the substrate S-2251. A plot of the Pm activity versus time for increasing concentrations of Lp(*a*) is shown in Figure 4. The plot demonstrates stable inhibition of Pg activation by Lp(*a*) over time. There is no evidence that the Lp(*a*) inhibition is altered by the 40-min, 37 °C incubation or by the increasing concentrations of Pm formed.

Discussion

Lp(*a*) is implicated in the pathogenesis of atherosclerosis [for reviews, see Brown and Goldstein (1988) and Scanu (1988)], and the formation of the lesions also correlates with decreased fibrinolytic activity (Walker et al., 1977; Gram et al., 1987; Hamsten et al., 1987). The high degree of homology that the apo(*a*) subunit has with Pg (McLean et al., 1987; Eaton et al., 1987) increases speculation about the role of

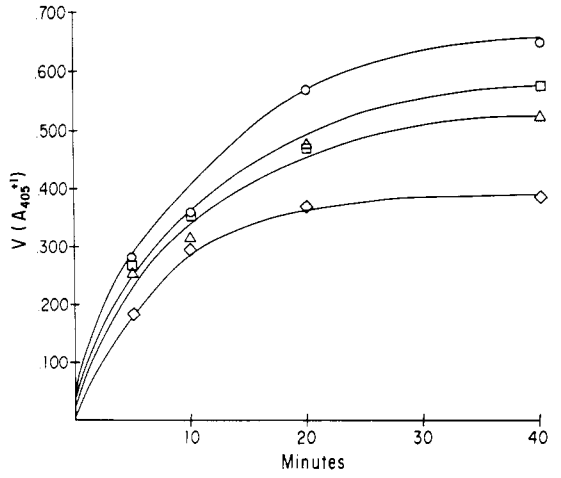

FIGURE 4: Lp(*a*) inhibition of Pg activation as a function of time. SK, 8.8 nM, and Lp(*a*), 0.0 (○), 1.5 (□), 3.0 (△), and 6 nM (◇), were preincubated together in 50 μL of 50 mM Tris-HCl, pH 7.4. Pg was then added for 2.2 μM in 100 μL. At selected times, the Pm activity was measured.

Lp(*a*) in fibrinolytic regulation. Karadi et al. (1988) has demonstrated that Lp(*a*) decreases SK-mediated fibrinolysis activity. We have observed a similar effect (unpublished results), and in the present studies we have attempted to probe the mechanism for the decrease in fibrinolytic activity.

SK-mediated activation of human Pg is a multistep reaction as reviewed by Castellino (1981). In the first step, Pg reversibly binds the SK to form the first SK–Pg complex intermediate, which has no proteolytic activity. The SK–Pg complex is then converted, without cleavage of Pg to Pm, to an intermediate with proteolytic activity. This unstable SK–Pg(act) complex intermediate was first demonstrated by McClintock and Bell (1971) and Reedy and Markus (1972). The activity of the SK–Pg(act) lies in the Pg (Schnick & Castellino, 1973), and the active form of Pg has been isolated from SK–Pg(act) complexes (Summaria et al., 1982). The SK–Pg(act) complex intermediate is quickly converted to the final proteolytic complex SK–Pm. The SK-mediated Pg activation is

$$\text{SK} + \text{Pg} \rightleftarrows \text{SK–Pg} \rightarrow \text{SK–Pg(act)} \rightarrow \text{SK–Pm}$$

in which SK–Pg and SK–Pg(act) are inactive and active intermediates, respectively (Castellino, 1981).

Lp(*a*) is a competitive inhibitor of SK-mediated Pg activation. As a competitive inhibitor it competes with the substrate for binding to the enzyme. Lp(*a*) could competitively inhibit Pg activation at the site of Pg binding to SK, the site of Pg activation by SK–Pm, or the site of Glu–Pg cleavage to Lys–Pg by Pm. Our studies indicate that the only site of Lp(*a*) competitive inhibition with Pg is for the SK, and not the SK–Pm or the Pm. Our data demonstrate that the Lp(*a*) forms a complex with SK which is stable during gel filtration. The studies also indicate that increasing concentrations of Lp(*a*) do not inhibit the proteolytic function of either the SK–Pm complex or the Pm alone.

Lp(*a*) is also an uncompetitive inhibitor of Pg activation by binding to an SK–Pg intermediate. As an uncompetitive inhibitor Lp(*a*) inhibits by binding the enzyme after the substrate has bound, but before the product has been formed. The possible enzyme–substrate intermediates include the SK–Pg intermediate before it is converted to SK–Pm, the SK–Pm–substrate intermediate before the substrate is cleaved, or the Pm–substrate intermediate before the substrate is cleaved. Our studies exclude the latter two possibilities as uncompetitive inhibition sites because increasing concentrations of Lp(*a*) do not inhibit either competitively or uncompetitively the reactions described. The site of Lp(*a*) uncompetitive inhibition therefore appears to be an SK–Pg complex intermediate. McClintock and Bell (1971) and Reedy and Markus (1972) have shown that an SK–Pg complex intermediate has activity before conversion to an SK–Pm complex. The SK–Pg complex intermediates include SK–Pg(act) which could be uncompetitively inhibited by Lp(*a*) in its conversion to SK–Pm by binding to its transient proteolytic site. It is therefore predicted that an SK–Pg(act) complex intermediate is uncompetitively inhibited by Lp(*a*).

The Lp(*a*) inhibition of Pg activation not only has significant inhibition constants but also is stable over time. Since Lp(*a*) inhibition of Pg activation is unaffected during a 40-min incubation, Lp(*a*) remains an inhibitor of plasminogen activation in the presence of increasing Pm concentrations.

Our experiments have examined the effect of Lp(*a*) on SK-mediated Pg activation in vitro. Pg activation is sensitive to anion, type and concentration (Chibber et al., 1986), and fibrinogen (Chibber et al., 1985), which were not studied here. Further experiments are necessary to measure the effect of anions and fibrinogen on the Lp(*a*) inhibition.

Lp(*a*) competitive and uncompetitive inhibition constants reflect physiologic and pathophysiologic Lp(*a*) levels, respectively. Though SK is a nonphysiologic Pg activator, the inhibition suggests that physiologic Pg activators, such as urokinase and tissue-type plasminogen activator (t-PA), may also be inhibited by Lp(*a*). Preliminary experiments in our laboratory indicate that Lp(*a*) inhibits t-PA-mediated fibrin clot lysis, as it inhibited SK-mediated lysis. At physiologic concentrations, the Lp(*a*) may regulate fibrinolytic activity, but at elevated pathophysiologic concentrations, it may inhibit fibrinolysis.

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Further Characterization of the Cellular Plasminogen Binding Site: Evidence That Plasminogen 2 and Lipoprotein *a* Compete for the Same Site†

Mario Gonzalez-Gronow, Jay M. Edelberg, and Salvatore V. Pizzo*
Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
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ABSTRACT: Specific cell surface receptors for plasminogen (Pg) are expressed by a wide variety of cell types and serve to promote fibrinolysis and local Pg proteolysis. Pg types 1 and 2, separated by chromatography on concanavalin A–Sepharose, were utilized to determine their binding to the monocytoid U937 cell line. Both forms bind in a dose-dependent manner. However, Pg 2 binds to the cellular receptor considerably better than Pg 1 and at equilibrium demonstrates approximately 10-fold greater binding. Lipoprotein *a* [Lp(*a*)], which possesses a subunit showing considerable homology to Pg, competes with Pg 2 for the Pg receptor in U937 cells. Moreover, Pg 1 is not able to displace Pg 2 from the receptor. These studies suggest that high levels of Lp(*a*) may alter the profibrinolytic activity at the cell surface and increase the risks of atherosclerosis and thrombosis. This hypothesis is in accord with the 2–5-fold increased risk of atherosclerosis in patients having high levels of Lp(*a*).

Plasminogen (Pg), the plasma zymogen of the fibrinolytic enzyme plasmin, is a single-chain glycoprotein. There are two major isozymes of Pg in human plasma, Pg 1 and 2 (Brockway & Castellino, 1972). Pg 1 has a greater molecular weight (93 000) with two carbohydrate chains linked to Asn-280 and Thr-345, while Pg 2 has a lower molecular weight (89 000) with one carbohydrate chain linked to Thr-345 (Hayes & Castellino, 1979a–c). Both forms seem to have identical amino acid composition (Powell & Castellino, 1983), and the amino acid sequence is also identical (Sottrup-Jensen et al., 1978a,b). However, many physicochemical properties of the two forms are different (Takada et al., 1985). An interesting feature of the Pg molecule is the presence of five tandem repeats, called kringles. These structures, containing about 80 amino acids, are present in the amino-terminal region of the protein (Sottrup-Jensen et al., 1978a). They are homologous to two kringles present in the amino-terminal region of tissue plasminogen activator (Pennica et al., 1983), two kringles present

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* To whom correspondence should be addressed.

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