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Lipoprotein(a) enhances plasma clot lysis in vitro

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Human plasma lipoprotein(a) (Lp(a)) is a macromolecular complex that contains a protein homologous to plasminogen, the precursor of plasmin. We confirmed recent reports that Lp(a) is not activated by streptokinase or tissue plasminogen activator (t-PA) to yield plasmin-like activity. In testing the hypothesis that Lp(a) can competitively inhibit plasma clot lysis mediated by plasmin, the present study shows that Lp(a) significantly enhanced plasma clot lysis mediated by streptokinase or t-PA The enhancement, however, was not observed in a plasmin-mediated clot lysis using a purified fibrinogen system. The addition of α-$_2$-antiplasmin (α_2-plasmin inhibitor) to this system inhibited fibrinolysis; however, no inhibition was observed in the presence of Lp(a). One potential explanation for the Lp(a)-enhanced plasma clot lysis is that Lp(a) neutralizes the activity of α_2-antiplasmin present in plasma, thereby restoring the activity of plasmin to lyse the clot.

Lipoprotein(a); Plasminogen; Fibrinolysis; α_2-Plasmin inhibitor; α_2-Antiplasmin

1. INTRODUCTION

Lipoprotein(a) (Lp(a)) was first identified by Berg in 1963 [1]. The particle contains one copy of apolipoprotein(a) which is disulfide-linked to apolipoprotein B. The molecular mass of Lp(a) is approximately 3.8×10^6 [2]. Plasma Lp(a) levels are positively correlated with atherosclerosis (see [3] for review) and is an independent risk factor for myocardial infarction [4–8]. The amino acid sequence of apolipoprotein(a) has a high degree of homology with plasminogen. Lp(a) contains 37 copies of kringle 4-like, 1 copy of kringle 5-like, and 1 copy of catalytic-like domain of plasminogen [9,10]. Plasminogen is a zymogen that has to be converted into plasmin by plasminogen activators prior to fibrinolysis (see [11] for review). However, Lp(a) cannot be activated by tissue plasminogen activator (t-PA), urokinase, or streptokinase to obtain plasmin-like activity [9,10]. Since a proteolytically active form of Lp(a) is not produced, it has been speculated that Lp(a) may act as a competitive inhibitor for plasminogen activation [9,10]. Whether or not Lp(a) can protect fibrin from plasmin degradation has not been established. Harpel et al. [12] reported that Lp(a) binds to fibrin and fibrinogen. They suggested that the binding might inhibit plasmin-mediated thrombus (fibrin) degradation. This hypothesis, however, has never been tested. In the present study we show that Lp(a) did not retard plasma clot lysis; rather, Lp(a) enhanced lysis in vitro. The possible mechanism by which Lp(a) enhances plasma clot lysis is discussed.

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2. MATERIALS AND METHODS

2.1. *Preparation of Lp(a)*

Human Lp(a) was a generous gift from Dr Thomas Parker (Rogosin Institute, New York, NY). The plasma sample was obtained from a donor with an elevated Lp(a). Lp(a) was isolated according to the procedure described in [12]. Purified Lp(a) showed a single band, distinct from LDL, by non-denaturing polyacrylamide gradient gel (2–16%) electrophoresis.

2.2. *Preparation of a plasma clot*

Plasma collected in a final EDTA concentration of 0.1% was filtered and aliquoted into 1 ml microcentrifuge tubes and stored at −80°C. For the preparation of clots, 100 μl of plasma (1:5) in a phosphate-buffered saline (PBS) containing 0.01 M phosphate, 0.12 M NaCl and 0.1% bovine serum albumin (BSA), pH 7.4, were added to microtiter wells. To these wells 50 μl of bovine thrombin (Sigma) containing 0.25 NIH units were added according to the method of [13]. The final volume in each well was 150 μl. Clot formation occurred after 45 min at 24°C. Clot turbidity was recorded at 380 nm with an automated EIA plate reader (EL309, Bio-tek instruments, VA).

2.3. *Clot lysis*

Fifty μl of streptokinase (2.5 mg/ml; Sigma) or recombinant t-PA (5 IU/ml; Genentech) were added to 20 μl of Lp(a) at concentrations from 0 to 4 mg/ml. The reaction mixture was then carefully layered on top of the microtiter well containing the plasma clot. Clearance of clot turbidity was recorded at 380 nm for 50–60 min.

2.4. *Plasmin assay*

Plasmin activity was determined using the chromogenic substrate S-2251 (H-D-Val-Leu-Lys-*p*-nitroanilide·2HCl (KabiVitrum, Stockholm, Sweden)). The activity is based on the rate of pNA formation from the hydrolysis of S-2251. Plasmin (10 U/ml) purchased from Sigma was appropriately diluted (in PBS containing 0.1% BSA) so that the rate of hydrolysis of S-2251 was linear during the first 30 min. For a typical assay, 50 μl of S-2251 (1.75 mM) was added to 50 μl of plasmin (diluted to 1:320) preincubated with or without Lp(a). The increase in absorbance was monitored at 405 nm and data recorded over a 35 min period. To activate plasminogen (Sigma),

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50 μl of plasminogen (0.5 U/ml in 0.1% BSA) were preincubated with 50 μl of streptokinase (2 mg/ml, Sigma) for 30 min at 24°C. Fifty μl of the reaction mixture were then added to 50 μl of S-2251 (1.75 mM) and the hydrolysis of S-2251 was monitored over a 15 min period

2.5. *Determination of the effect of Lp(a) on α_2-antiplasmin activity on fibrinolysis induced by plasmin*

Fibrinogen (Sigma) was dissolved in PBS containing 0.1% BSA and centrifuged at 3000 rpm to remove any insoluble materials. For the preparation of the clot, 30 μl of 1 mg/ml Coomassie blue R-250 (Bio-Rad) were added to 200 μl of 5 mg/ml fibrinogen in polypropylene test tubes (12 × 75 mm). After 5 min incubation at 24°C, 100 μl of bovine thrombin (Sigma) containing 0.25 NIH units were added to initiate the fibrin clot. Clots were formed after 30 min incubation at 24°C.

Fibrinolysis was initiated by plasmin addition and the rate of fibrinolysis was determined by the chromogenic substances released from the clots. Assaying for clearance of turbidity could not be used due to the lack of turbidity of the fibrin clot. The fibrinolysis was allowed to proceed for 60 min at 24°C, at which time about 50% of maximal lysis was achieved. At this time 100 μl of the supernatant fraction were removed from each clot and placed into the wells of a microtiter plate. Absorbance was recorded at 540 nm. To study the effect of Lp(a) on the activity of α_2-antiplasmin, 100 μl of Lp(a) (8 mg/ml) was preincubated with 100 μl α_2-antiplasmin (American Diagnostica, NY) (90 μg/ml) for 30 min at 24°C before addition of 100 μl plasmin (3.5 U/ml). Final volume of the reaction mixture was adjusted to 1.16 ml with 0.1% BSA in PBS.

3. RESULTS

3.1. *Effect of Lp(a) on plasmin activity*

Effect of Lp(a) on the plasmin activity was studied. The enzyme activity was based on the hydrolysis of a chromogenic substrate S-2251. Fig. 1 shows that Lp(a) at a final concentration of 0.33 mg/ml did not affect the activity of plasmin ranging from 0.016 to 0.5 U/ml. The activity remained the same when Lp(a) concentration was increased to 4 mg/ml (data not shown). In the absence of plasmin, no detectable hydrolysis of S-2251 was seen in the reaction mixture with and without Lp(a). Thus, the data indicate that even though Lp(a) is homologous with the catalytic domain of plasmin, it neither enhanced nor competitively inhibited plasmin activity.

Since Lp(a) is structurally similar to plasminogen, we tested whether or not Lp(a) could be activated by a plasminogen activator, streptokinase. As shown in Fig. 2, plasminogen (0.5 mg/ml) was maximally activated at streptokinase concentrations between 0.25 and 1.25 mg/ml. Streptokinase at a concentration of 1.25 mg/ml failed to activate Lp(a) (2 mg/ml). Similar results were seen when Lp(a) concentrations between 0.1 and 4 mg/ml were tested (data not shown). The finding suggests that Lp(a) is not readily activated by streptokinase.

3.2. *Effect of Lp(a) on plasma clot lysis*

Under physiological conditions, the specificity of plasmin is determined primarily by its lysine-binding sites (substrate-binding) rather than by its protease-

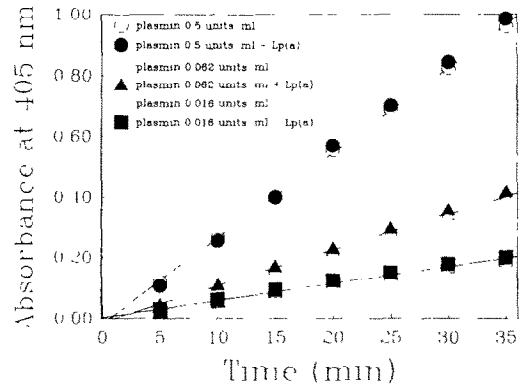

Fig. 1. Effect of Lp(a) on the catalytic activity of plasmin. Plasmin activity was determined using S-2251 (D-Va-Leu-Lys-pNA) as substrate. The reaction mixture contained 50 μl of plasmin (0.016–0.5 U/ml), 50 μl of substrate, and 50 μl of Lp(a) (1 mg/ml) or buffer. The hydrolysis of S-2251 was monitored at 405 nm. Each point represents the mean of duplicate determinations.

catalytic site [14,15]. The lack of inhibition of Lp(a) on the hydrolysis of substrate S-2251 (as shown above) indicates that Lp(a) did not compete with the catalytic domain of plasmin. The lysine-binding domain responsible for the binding of the fibrin substrate might interfere with Lp(a) due to the sequence homology [9,10]. For this reason the effect of Lp(a) on clot lysis was studied. Plasma clots were first allowed to form in microtiter plates; streptokinase in the presence of various amounts of Lp(a) (0.125–4 mg/mg) was then added to mediate clot lysis. Clearance of clot turbidity was recorded at 380 nm. Fig. 3A shows that the streptokinase-mediated clot lysis was enhanced by Lp(a) rather than inhibited. This enhancement was dependent on the amount of Lp(a). Regression analyses of the rate of clot lysis showed a significant difference

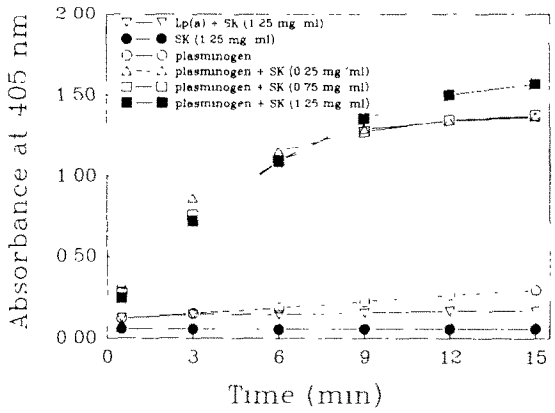

Fig. 2. Effect of streptokinase (SK) on the activation of plasminogen and Lp(a). Plasminogen (0.5 mg/ml) or Lp(a) (2 mg/ml) was preincubated with streptokinase (0.25–1.25 mg/ml) in 100 μl for 20 min at 24°C prior to the assay. 50 μl of substrate S-2251 were then added to the assay well. The hydrolysis of S-2251 was monitored at 405 nm. Each point represents the mean of duplicate determinations.

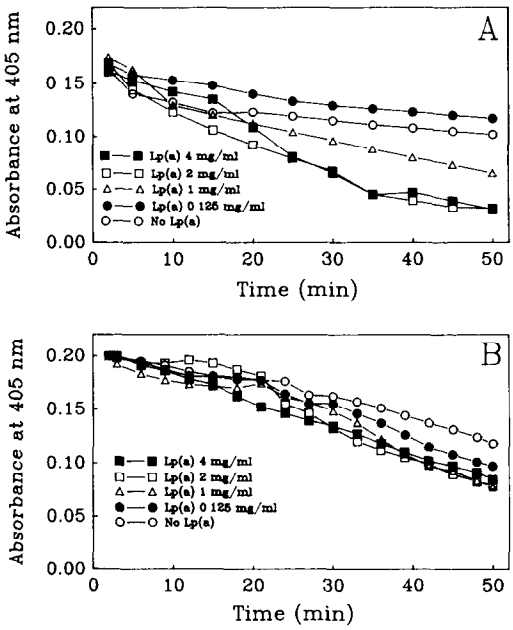

Fig. 3. Effect of Lp(a) on the clot lysis mediated by streptokinase (A) and t-PA (B). In the absence of streptokinase or t-PA, Lp(a) did not lyse the clot. Autolysis of the clot was not evident during the entire duration of the study. The assay contained 20 μl of Lp(a) from concentrations of 0–4 mg/ml. Each point represents the mean of duplicate determinations.

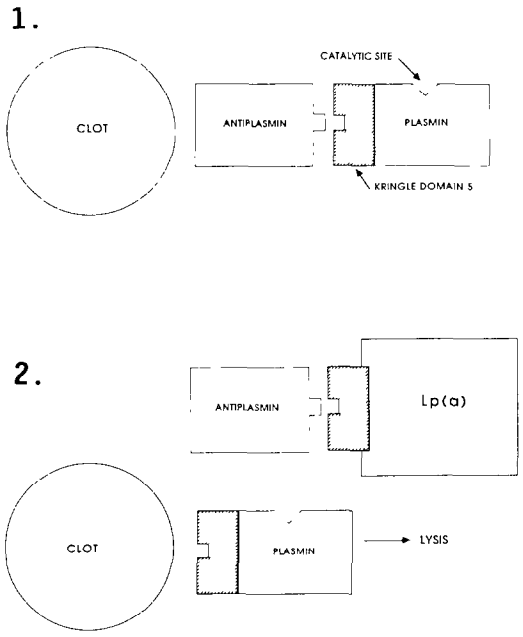

Fig. 4. A simple schematic drawing of a potential mechanism of which Lp(a) enhances the streptokinase or t-PA mediated clot lysis in plasma. Step 1: in the absence of Lp(a), α_2-antiplasmin binds to the kringle 5 domain of plasmin and inhibits clot lysis. Step 2: in the presence of Lp(a), α_2-antiplasmin competitively binds to Lp(a) and allows plasmin interaction with the clot. The size of each molecule drawn does not necessarily correspond to the actual molecular mass.

as compared to the control ($P < 0.001$). Lp(a) alone in the absence of streptokinase did not affect lysis (data not shown). A 20-fold molar excess of human low density lipoproteins (LDL) or high density lipoproteins (HDL) had no effect on clot lysis (data not shown).

In addition, the same enhancement by Lp(a) was observed using tissue plasminogen activator (t-PA) (Fig. 3B). At 4 mg/ml of Lp(a), the enhancement was significant ($P < 0.001$). Thus, the result provides evidence that there is no direct link between Lp(a) and inhibition of thrombolysis in vitro.

Table I

Effect of Lp(a) on α_2-antiplasmin activity of fibrinogen clots

Condition	% Lysis ± SD[a]
Plasmin (control)	50 ± 2
Buffer without plasmin	0 ± 1.5
Lp(a) + buffer	0.1 ± 4.2
Plasmin + Lp(a)	48 ± 2
Plasmin + α_2-antiplasmin	2.5 ± 1.2
Plasmin + α_2-antiplasmin + LDL	2.2 ± 1.5
Plasmin + α_2-antiplasmin + Lp(a)	54 ± 1.5

[a] Each value represents the mean ± SD of 3 determinations

Fibrinolysis was terminated at 60 min to obtain 50% of maximal lysis. Thrombin (100 μl) was added to 230 μl of purified fibrinogen (3.85 mg/ml) containing Coomassie blue R-250 (0.025%) to mediate clot formation. A typical assay was performed using a 100 μl of plasmin (3.5 U/ml) and 100 μl of tested components in a final volume of 1.16 ml. The initial concentration of α_2-antiplasmin, LDL, and Lp(a) was 0.09, 10, and 8 mg/ml, respectively

3.3. *Effect of Lp(a) on the inhibitory activity of α_2-antiplasmin for fibrinolysis*

Clot lysis, using purified fibrinogen as a clot substrate, was conducted. Fibrinolysis induced by the addition of plasmin was followed by the release of Coomassie blue R-250 labeled fibrinogen. Table I shows the plasmin-mediated clot lysis. In the absence of whole plasma, Lp(a) did not enhance fibrinolysis. The data suggest that Lp(a) might interact with a factor(s) that is normally present in plasma to regulate the plasmin activity (see section 4 for more details). In Table I, we show that plasmin inhibitor (α_2-antiplasmin) inhibited the lysis activity of plasmin. In the presence of Lp(a), α_2-antiplasmin was not effective in inhibiting fibrinolysis. Under the same conditions, LDL had no effect on α_2-antiplasmin.

4. DISCUSSION

Using a chromogenic substrate (S-2251), we show that Lp(a) did not affect the catalytic activity of plasmin. This result suggests that although there is structural homology between Lp(a) and the catalytic domain of plasmin, Lp(a) is not a competitive inhibitor for the catalytic-site of plasmin. Presumably, the conformation of the 'proteolytic domain' of Lp(a) is fold-

ed in such a way within the intact lipoprotein particles that it cannot bind the substrate. Whether or not Lp(a) is a zymogen that can be activated by other enzyme systems needs to be determined.

Although Karadi et al. [16] have reported that Lp(a) prolongs the time required for fibrinolysis in an assay where fibrinolytic activity is stimulated with streptokinase, the present study was unable to show the same effect. Under their experimental conditions, the euglobulin fraction from plasma was first precipitated before the addition of thrombin to induce the clot. Initially, the mechanism(s) by which Lp(a) enhanced plasma clot lysis in the present study was not clear. It is not because Lp(a) contains plasmin activity (Fig. 1) nor is it caused by the activation of Lp(a) by streptokinase (Fig. 2). It is known, however, that plasmin in the circulation does not effectively cause fibrinolysis of plasma clots. Plasmin is rapidly bound to circulating proteins, such as α_2-plasmin inhibitor (α_2-antiplasmin) and α_2-macroglobulin which inhibit plasmin activity [17]. It is believed that α_2-antiplasmin specifically binds to the kringle 5 domain of plasmin (lysine-binding sites) and thereby inactivates the plasmin recognition of fibrin clots [15]. Since Lp(a) contains 91% sequence homology with plasmin kringle 5 [10], we speculated that Lp(a) might also interact with α_2-antiplasmin to 'neutralize' its activity. This potential mechanism is diagrammed in Fig. 4. The present study shows that Lp(a) is capable of reversing the inhibitory effects of α_2-antiplasmin and, therefore, supports our speculation. The mode and physiologic role of the interaction between Lp(a) and α_2-antiplasmin are not known and deserve further study.

The lack of direct inhibitory activity of Lp(a) for fibrinolysis may be contributed to its weak binding to fibrin [10]. Although Kluft et al. [18] reported the absence of fibrin binding of Lp(a), a recent study [12] shows that plasmin mediates the binding of Lp(a) to immobilized fibrinogen and fibrin and indicates that Lp(a) possesses a site that interacts with fibrinogen or fibrin. They suggest that such interactions would impair plasminogen binding to fibrin and thereby might inhibit fibrinolysis [12], but the effect of Lp(a) on fibrinolysis in plasma was not studied.

In summary, the present study shows that Lp(a) promotes the lysis of a plasma clot in vitro. As such, it seems unlikely that the postulated atherogenic role of Lp(a) can be explained by its direct inhibition of fibrinolysis. However, on the cellular level, Lp(a) displaces plasminogen from binding to the endothelial surface [19,20]. Therefore, the link between impaired cell surface fibrinolysis on the arterial wall and progressive atherosclerosis as suggested [19,20], cannot be ruled out.

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