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The effect of different apo(a) isoforms on plasminogen activation in black South African subjects

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Summary

***Objective*: The present study evaluates the effect of different apolipoprotein(a) [apo(a)] isoforms on plasminogen activation.**
***Design*: A cross-sectional study.**
***Setting*: A rural village (Dikgale district) in the Northern Province of South Africa.**
***Subjects*: A total of 90 apparently healthy subjects (64 females and 36 males) aged 43 to 67 years participated in the study.**
***Results*: The mean lipoprotein(a) [Lp(a)] level in the subjects was 38.14 ± 22.34 mg/dl. No association was found between Lp(a) levels and apo(a) isoforms. When the ratio of Lp(a):plasminogen was less than 1.3, a competitive inhibition was observed, but when the ratio exceeded 1.3, an uncompetitive inhibition was observed with all isoforms.**
***Conclusion*: The results of the present study suggest that the inhibition of plasminogen activation by Lp(a) is not dependent on apo(a) size.**

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Lp(a) lipoprotein was first described by Berg in 1963[1] and is now considered as an independent risk factor for cardiovascular disease.[2–5] Structurally, Lp(a) is similar to low-density lipoprotein (LDL) as it contains the lipid core and apolipoprotein B-100 (apo B-100). The only difference between Lp(a) and LDL was believed to be the presence of apolipoprotein(a) in Lp(a), however, apo E-free and apo E-containing Lp(a) have been described.[6,7]

Partial amino acid sequencing of apo(a) and cDNA apo(a) gene sequencing have demonstrated homology between apo(a) and plasminogen.[8,9] The homology between the two was shown to be approximately 87%.[8] Both have the protease domain at their N-terminals and their C-terminals are arranged in loops called kringles. Plasminogen contains kringles I–V whereas Lp(a) contains one copy of kringle V and several copies of kringle IV. Comparison of the protease domains of the two proteins showed that they are approximately 95% homologous.[8] The major difference between the two domains is that at the cleavage site on the protease domain of apo(a), Arg_{560} is substituted by serine. As a result of this, apo(a) cannot be cleaved by plasminogen activators and consequently does not have protease activity.[8] Introduction of the Arg-Val cleavage site on the apo(a) molecule by recombinant DNA technology did not produce

plasmin activity or cleavage by different plasminogen activators.[10]

Due to its homology to plasminogen, recent studies have focused on the effect of Lp(a) on plasminogen activation[10,11] It has been shown that Lp(a) inhibits euglobin clot lysis via both streptokinase and the tissue plasminogen activator (tPA).[13,14] This inhibition was further shown to be concentration dependent[11,12] and the mechanism was suggested to be due to either inhibition of plasminogen activation or reduction of plasmin activity.[14] Competitive inhibition was found at physiological levels of Lp(a)[15,17] while an uncompetitive inhibition was found only at high concentrations.[14] Lack of inhibition has however been reported by other investigators.[17–19] These differences could have been due to variable assay conditions, including temperature, pH, buffer, and the concentration of the reactants. [20]

In a another study it was found that different apo(a) isoforms exhibited different affinities to fibrin.[21–22] This also raised the possibility that individual apo(a) isoforms may exhibit different types of inhibition on plasminogen activation. Our aim in the present study was therefore to determine the effect of different apo(a) isoforms on plasminogen activation.

Materials and methods

The subjects in the study came from a non-urban community of the Dikgale district in the Northern Province of South Africa. This district is situated 15 km north-east of the University of the North and 40 km from the Province's capital, Polokwane (Pietersburg).

The study was approved by the research and ethics committees of the University of the North and informed consent was obtained from the village authorities and individual subjects in the study.

Blood was collected by venesection in plain vacutainer tubes from 90 apparently healthy adult non-urban subjects (54 females and 36 males) with an age range of 43 to 67 years. Serum was separated and stored at –70°C until analysis.

Lp(a) levels and apo(a) phenotyping

Serum Lp(a) levels were determined by enzyme-linked immunosorbent assay kits (Biopool, Sweden). The assay was independent of apo(a) size. According to the manufacturer, the cross reactivity of the antibodies with LDL or plasminogen is negligible and the detection limit of the assay is from 0 mg/dl to 60 mg/dl. Samples with Lp(a) levels above 60 mg/dl were diluted and Lp(a) estimation was redone.

Apo(a) isoforms were determined by a modified method as previously described.[23] Serum samples were diluted 1:2 and apo(a) standards were diluted 1:1. These were subjected to agarose gel electrophoresis at 25W and 4°C for 7 hours. The protein bands on the agarose gel were electro-transferred onto a nitrocellulose membrane by a semi-dry system at 240 mA for 30 minutes. The membrane was then blocked with Tris:boric acid:saline buffer (TBS) containing 0.2% gelatin for 90 minutes. The Lp(a) bands were visualised using the Lp(a) phenotyping kit (Immuno-GMBH, Austria).

Lp(a) isolation

Using the WGA method as previously described,[24] serum samples from subjects homozygous for a single apo(a) isoform were used for the isolation of Lp(a). Contamination by other serum proteins was excluded with the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis of samples was carried out on 7.5% polyacrylamide gels at 4°C and 150 V for 5 hours. The gel was fixed in a mixture of 5:1:4 methanol:acetic acid:water (volume for volume) for 1 hour and stained in 5 g Coomasie brilliant blue R-250, dissolved in a mixture of 500 ml methanol, 100 ml acetic acid and 400 ml distilled water. The gel was then destained in a 0.5:0.7:8.8 methanol:acetic acid:distilled water solution. The bands obtained were confirmed by immunoblotting to be those of Lp(a). A standard techinque was used to exclude contaminating fibrinolytic factors such as tPA, plasminogen, fibrinogen and plasminogen activator inhibitor type 1 (PAI-1). Tissue plasminogen activator was determined by the Biopool ELISA kit method (Sweden). The detection limit for this method is 0 ng/ml to 30 ng/ml. PAI-1 was determined by the Biopool immunoactivity assay (Sweden). The detection limit for this method is 2.0 IU/ml to 50 IU/ml. Fibrinogen was determined by the ACL 2000 coagulation autoanalyser.

Inhibition of plasminogen activation assay

Plasmin substrate (Tos-Gly-Pro-Lys-Nitranaline), tissue plasminogen activator, and glu-plasminogen were purchased from Sigma (USA) and dissolved appropriately with the assay buffer (50 mM Tris-HCL, 0.05% gelatin, 0.1% Tween 80 at pH 7.35). Appropriate concentrations (K_m) for tPA, glu-plasminogen and plasmin substrate were determined according to standard methodology and were found to be 0.2666 µg/µl, 0.234 µM and 0.7874 mM, respectively. The assays were performed at a temperature of 20–25°C, pH of 7.35, and the change in absorbance was monitored at 405 nm.

The inhibition of plasminogen activation by different apo(a) isoforms was then carried out according to the modified method of Edelberg *et al*.[15] The concentration of tPA was kept constant at 3.8 µM and plasminogen concentrations varied from 0.112 µM to 0.896 µM. Lp(a) concentrations, except Lp(a) containing S1, varied between 0.01 µM and 0.07 µM. The concentration of Lp(a) containing isoform S1 varied from 0.03 µM to 0.12 µM. The above concentrations corresponded to a ratio of Lp(a):plasminogen that varied from 0.13 to 0.2. To determine the effect of higher Lp(a):plasminogen ratios on inhibition, ratios between 0.13 and 12.89 were used.

Statistical analysis

The SPSS software package was used for all statistical analyses. The means and standard deviations of Lp(a) and

inhibition parameters were determined. Comparisons of inhibition parameters were performed by the Kruskal-Wallis test.

Results

Lp(a) levels and apo(a) phenotype frequencies

The mean and standard deviation of Lp(a) levels was found to be 38.14 ± 22.34 mg/dl. The apo(a) phenotype frequencies and corresponding Lp(a) levels are shown in Table I. Of the homozygous phenotypes, S4 was the most common at 26.7%, and of the heterozygous phenotypes, S3S4 was the most common at 17.8%, followed by S4>S4 at 13.3%. None of the rare phenotypes (BB and FF) was found. Overall, the larger apo(a) isoforms were predominant and Lp(a) concentrations did not vary with different apo(a) isoforms, as shown in Table I.

TABLE I. APO(a) PHENOTYPES AND CORRESPONDING LP(a) LEVELS

Apo(a) phenotype	*Frequency* n *(%)*	*Lp(a) concentration (mg/dl) (mean ± SD)*
S1	3 (3.3%)	38.60 ± 23.66
S2	4 (4.4)	36.88 ± 12.21
S3	5 (5.6)	27.78 ± 12.77
S4	24 (26.7)	41.07 ± 22.89
>S4	6 (6.7)	36.00 ± 18.50 B
S4	1 (1.1)	47.00*
S1S2	1 (1.1)	9.70*
S1S3	3 (3.3)	23.50 ± 21.96
S1S4	3 (3.3)	36.97 ± 24.62
S2S3	1 (1.1)	23.00*
S2S4	5 (5.6)	43.80 ± 28.13
S2>S4	4 (4.4)	44.52 ± 27.34
S3S4	16 (17.8)	41.46 ± 29.19
S3>S4	2 (2.2)	28.90 ± 18.53
S4>S4	12 (13.3)	31.81 ± 22.12

*SD not available as only one phenotype was found in each case.

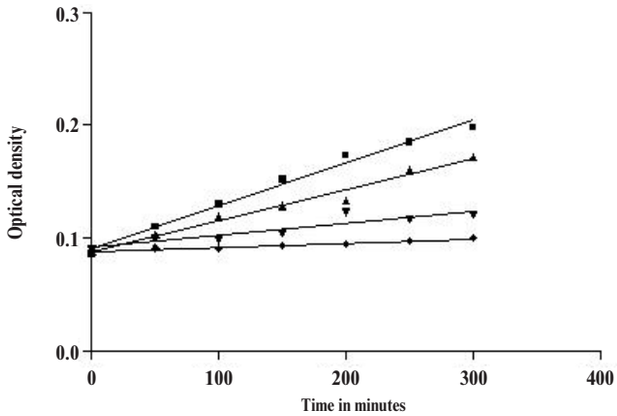

Fig. 1. Effect of Lp(a) (isoform S1) concentration on plasminogen activation. (◆) Lp(a) = 0.000 μM, s = 0.03 x 10^{-2}; (▼) Lp(a) = 0.02889 μM, s = 0.027 x 10^{-2}; (▲) Lp(a) = 0.05778 μM, s = 0.011 x 10^{-2}; (■) Lp(a) = 0.11556 μM, s = 0.004 x 10^{-2}; (s = slope).

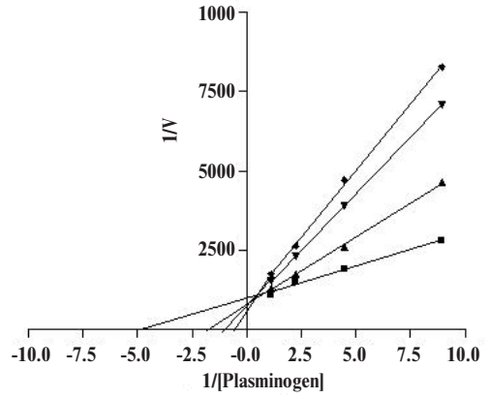

Fig. 2. Lineweaver-Burk plot of inhibition of plasminogen activation by Lp(a) with isoform S1. (■) Lp(a) = 0.000 μM, (▲) Lp(a) = 0.02889 μM, (▼) Lp(a) = 0.05778 μM, (◆) Lp(a) = 0.11556 μM.

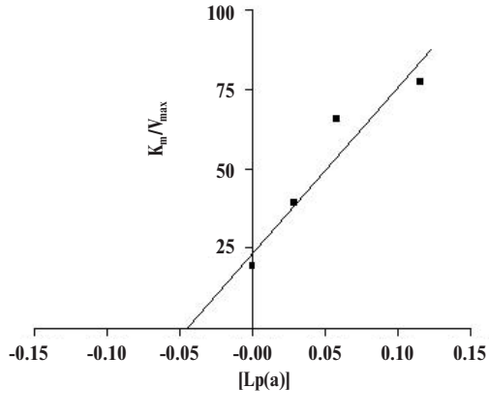

Fig. 3. Determination of K_i for lipoprotein(a) with isoform S1.

Effect of different apo(a) isoforms on plasminogen activation

There was no contamination by plasma proteins or fibrinolytic factors. Lpa(a) inhibited plasminogen activation and the inhibition was concentration dependent (Fig. 1). The inhibition was also found to be competitive as shown by the Lineweaver-Burk plot (Fig. 2) when the ratio of Lp(a):plasminogen concentration was below 1.3. The inhibition however changed from competitive to uncompetitive with the use of high concentrations of Lp(a) relative to plasminogen concentrations [Lp(a):plasminogen ratio exceeding 1.3] (Fig. 3). All apo(a) isoforms exhibited competitive inhibition and there was an insignificant difference in catalytic efficiency of tPA in the presence of different apo(a) isoforms (Table II).

Discussion

Lp(a) levels and apo(a) phenotypes

The mean Lp(a) level of 38.14 mg/dl found in our black subjects was comparable to that found in previous studies on black subjects.[25-27] It was, however, much higher than the concentration quoted for Caucasians (0–30 mg/dl).[30] The distribution of Lp(a) levels was found to be Gaussian and similar to that found in Asians, African-Americans and the

TABLE II. INHIBITION PARAMETERS OF LP(a) ISOFORMS

Lp(a) isoforms	*Type of inhibition*	K_i *(μM)*	K_{cat} $(V_{max}/[tPA])$ *[μl/min]*	K_{cat}/Ki *[μl.min^{-1}/μM]*
S1	Competitive	0.04952	0.3111×10^4	0.062803×10^6
S2	Competitive	0.02433	0.3774×10^4	0.155117×10^6
S3	Competitive	0.01597	0.2837×10^4	0.177645×10^6
S4	Competitive	0.02549	0.3107×10^4	0.121890 x 10^6
>S4	Competitive	0.02423	0.2917×10^4	0.120387 x 10^6

Congolese.[24,25,29] The present study also shows that the frequency of apo(a) phenotypes in our black subjects is similar to those found in other black population groups, with larger isoforms being predominant in all populations.[26,30]

An inverse relationship between Lp(a) levels and apo(a) phenotypes has previously been reported in both Caucasians[28] and Nigerians.[33] No such association was found in the present study. Whilst low Lp(a) levels were associated with larger apo(a) isoforms among Caucasians,[28] in this study, we found high Lp(a) levels associated with larger apo(a) isoforms. This agrees with data reported by Trommsdorf *et al*.[30] and Kraft *et al*.[27] and further suggests that Lp(a) levels may not be apo(a) size dependent in black subjects.

Inhibition of plasminogen activation by Lp(a)

When isolating blood constituents for biochemical testing, structural damage of that particular constituent during the isolation should be minimised as this may alter its biochemical function *in vitro*. Among the objectives of the present study was to obtain a purified Lp(a) at its physiological structure. Ultracentrifugation and chromatography has been used previously in preparation of Lp(a) lipoproteins.[15,19,28] Recently, Seman *et al*.[24,31] developed a wheat germ agglutinin-based Lp(a) isolation method which is rapid, thus minimising exposure of Lp(a) to chemical agents and non-physiological conditions that may affect its structure. In the present study the wheat germ agglutinin method was used and the Lp(a) isolates showed no contamination by other carbohydrate-rich proteins (results not shown) or fibrinolytic factors.

It has been reported that there are plasminogen binding sites on the endothelial cell surfaces.[32] Binding of plasminogen to these sites allows the formation of plasmin at the cell surface, thus minimising proteolysis by plasma α_2-antiplasmin. Inhibition of pro-urokinase-mediated plasminogen activation by Lp(a) is more pronounced in the presence of fibrin fragments.[19] In the present study Lp(a) was found to inhibit plasminogen activation in the absence of fibrin or fibrin fragments. This difference may be the result of using different assay conditions such as isolation procedure, assay buffer, pH and tPA concentration.

The type of inhibition was competitive (Fig. 1) whenever the Lp(a):plasminogen ratio was between 0.13 and 0.2 (Table II) and uncompetitive (Fig. 4) whenever the Lp(a):plasminogen ratio exceeded 0.2. The catalytic efficiency of an enzyme (K_{cat}), which is given by V_{max}/[enzyme],

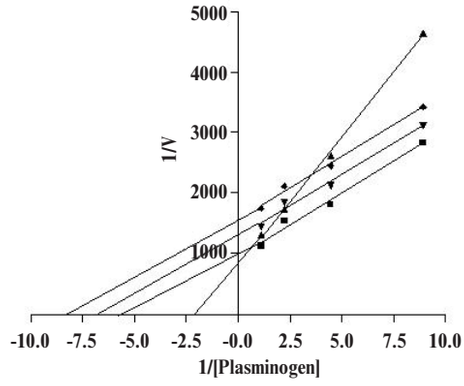

Fig. 4. Effect of high Lp(a) concentrations on the inhibition of plasminogen activation. (■) Lp(a) = 0.000 μM; (▲) Lp(a) = 0.02889 μM; (▼) Lp(a) = 0.2889 μM; (◆) Lp(a) = 2.8890 μM.

can be used to confirm that two different inhibitors are competitive inhibitors.[34] That is, if a competitive inhibition is observed, K_{cat} should be the same since a competitive inhibitor does not affect V_{max} when the same enzyme concentration (tPA in the present study) is used.

The inhibition constants for each apo(a) isoform were also determined (Table II). The inhibition constant reveals the degree of inhibition.[34] That is, a high inhibition constant reflects a higher degree of inhibition, and vice versa. Our results show that except for S1, the inhibition constants between different apo(a) isoforms were comparable. The higher inhibition constant for apo(a) S1 might be the result of using higher concentrations of this isoform compared to other isoforms. This may suggest that all apo(a) isoforms, when kept at same concentrations, inhibit plasminogen activation to the same degree.

The inhibition parameters, as shown in Table II, also indicate that there is no statistical difference in the catalytic efficiency of tPA (K_{cat}) in the presence of different apo(a) isoforms ($\rho = 0.149$). This suggests that even though Lp(a) inhibits plasminogen activation in a concentration-dependent manner, use of different Lp(a) particles with different apo(a) isoforms do not have different effects on the catalytic efficiency of tPA.

It should, however, be noted that in the present study the whole Lp(a) molecule was used in the inhibition assay. It would therefore be interesting to determine the effect of purified apo(a) isoforms on the catalytic efficiency of tPA.

In conclusion, this study confirms that plasminogen activation is inhibited by Lp(a) in black subjects, as is the case in Caucasians. The inhibition does not appear to depend on the size of the apo(a) isoform but depends on the Lp(a) concentration relative to the plasminogen concentration. The results of the present study cannot be compared with previous studies, as different Lp(a) isolation and assay conditions were used. The serum Lp(a) concentrations were, however, found to be higher in our black subjects as compared to the Caucasians, yet the incidence of coronary heart diseases is known to be lower in the black population.[35-37] Whilst Lp(a) is now considered to be an independent risk factor for cardiovascular disease, its role in the pathogenesis of this disorder in the black population requires further investigation.

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