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ORIGINAL ARTICLE

# Chemical Modification Induced by Glycation Increased Lysine Binding Site Activity of Human Serum Lipoprotein (a)

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**Lipoprotein (a) is a major and independent risk factor for cardiovascular disease. The pathogenicity of Lp(a) as a risk factor may depend upon its Lysine binding site(LBS) activity. It is suggested that non enzymatic glycation of Lp(a) resulting from high plasma glucose level found in diabetic patients may be one of the factors contributing to the severity of this disease. The purpose of this research was to study the effect of glycation on Lysine binding site activity of lipoprotein(a).**

**Materials & Methods: Lp(a) was glycated by incubation of 100 ml serum in vitro with 0.25 to 350 mmol of glucose for 10 days at 37oC. Glycated Lp(a) was separated by using m–aminobronate affinity column chromatography and Lysine binding site properties of the glycated Lp(a) were compared with native Lp(a) by using lysine sepharose affinity chromatography.**

**Results: Glucose uptake by Lp(a) was linear as a function of concentration and time up to 7 days for all given concentrations. Glycation increased the negative charge of Lp(a) as monitored by electrophoresis and increased the affinity of Lp(a) for Lysine sepharose affinity column chromatography.**

**Conclusion: Chemical modification induced by glycation of lp(a) affected its lysine binding site activity and increased LP(a) lysine positive subspecies. Therefore it is suggested that nonenzymatic glycation of Lp(a) may contribute to premature atherogenesis of patients with diabetes mellitus by increasing its LBS activity. and diverting lipoprotein catabolism from non-athero genic to atherogenic pathways.**

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## Introduction

Lipoprotein (a) was identified as a novel antigen in plasma by Kare Berg in 1963.[1] Lp(a) is similar to LDL in both lipid composition and the presence of apoprotein B-100.

Lp(a) is distinguishable from LDL by the presence of an additional protein moiety designated apo(a). In Lp(a), the highly polymorphic multikringle structure and glycosylated apo (a) is covalently linked by a single disulfide bridge to apo B-100.[2] Apo(a) bears a remarkable structureal similarity with plasminogen.[3] Plasminogen contains distinct kringle domains (K1-K5), followed by a serine protease domain.[4] Apo(a) contains no se-

quences corresponding to the plasminogen kringle 1-3 but does have multiple copies of a sequence that closely resemble plasminogen kringle 4, followed by sequences that bear a high degree of similarity to the plasminogen kringle 5 and the serine protease domain. Unlike plasminogen, apo(a) is catalytically inactive, its protease domain cannot be cleaved by plasminogen activators.[4] Experimental studies have implicated high levels of plasma lipoprotein (a) with increased risk for atherosclerotic cardiovascular disease. Since atherosclerosis is a major complication associated with diabetes, much effort has been spent on elucidating the possible role of Lp(a) in this disease. It is suggested that non enzymatic glycation of Lp(a) resulting from high plasma glucose levels found in diabetes is one of the factors contributing to the severity of this disease.[5] Hyperglycemia may cause tissue damage by several mechanisms, one of which is nonenzymatic glycation of intra and extracellular proteins. Previous studies have shown that Lp(a) in plasma according to its ability in binding to lysine has different subspecies,[6] and it is suggested that glycation of lipoproteins alters both structure and function of lipoproteins. Therefore in the present study the aim was to study the effect of nonenzymatic glycation on Lp(a) lysine binding site activity.

## Materials and Methods

**Sample preparation:** Blood samples were collected from healthy volunteers (four male and four female, age 25-45 years) with normal blood glucose levels and lipid profile. Serums were obtained after centrifugation at 3000 g for 10 min; 800 ml pooled serum was filtered through a Whatman 4 filter paper and stored at -20oC. Within one month 100 ml of that was used as a native Lp(a) and the remaining used for incubation with different concentration of glucose separately.

**Lp(a) measurement:** Lp(a) was measured by the immuno turbidometric test( Pars Azmon). End point determination of the concentration of Lp(a) was done through photometric measurement of antigen–antibody reaction between antibodies to lp(a) and Lp(a) present in the serum. Precision studied showed CVs of 3% for intra-assay and 2.5% for inter assay which were in agreement with the manufacturer claim.

**In vitro glycation of Lp(a):** Glycation of Lp(a) was performed by incubating 100 ml serum containing 100 mg Lp(a) with glucose (0.25, 50, 100 and 200 mM/L) and 1mmol/L EDTA at 37ºC for 10 days. Sodium azide (0.02%) was added to prevent bacterial growth. Throughout EDTA was present at a concentrations >0.5 mM to minimize Lp(a) oxidation. Throughout days 1-10 days, every day incubated Lp(a) was removed and dialyzed against one liter of PBS at 4oC in the dark, and glycation was determined by measuring amadori products.[7] Nonenzymatic glycation occurs through the covalent binding of aldehyde or ketone groups of reducing sugars to free amino groups of proteins, forming a labile schiffs base. The initial schiffs base undergoes rearrangements to a much more stable ketoamine, called “Amadori's product”.

**Separation of Lp(a) subspecies:** In order to determine whether glycation affects Lp(a) LBS activity, serum containing higher concentration of glycated Lp(a) was used to separate Lp(a) subspecies using lysine sepharose affinity chromatography.[6]

**Chromatographic procedure:** Dry lysine sepharose, 15 gm (Pharmacia) was treated with distilled water, packed in a 40 x 2.6 cm ID chromatographic column and extensively washed with 500 ml of 50 mM (NH4)HCO3, pH 7.95. Lysine is bound to the matrix with no spacer arm by its α aminogroup, leaving ε-amino group to interact with kringle containg proteins. 100 ml of pooled serum was applied in the column. The column was eluted with 500 ml of 50 mM (NH4)HCO3, pH 7.95 and the eluate following the pooled

serum injection was collected. In this step Lp(a) Lys-which did not bind to the lysine was removed. The eluate was collected in 5 ml fractions, read at 280 nm and tested for Lp(a). Lysine was positive throughout all the elutions.

The release of Lp(a) bound to the ε-amino group of lysine sepharose was obtained employing three sequential elution steps:1) 100 ml continuous linear gradient from 0 to 0.2 M sodium phosphate buffer, pH 7.4. 2) 50 ml 0.5 M sodium phosphate buffer pH 4.4. 3) 50 ml 0.5 M sodium phosphate buffer, 0.2 M ε-amino caproic acid (ε-ACA) pH 4.4.ε-ACA acts as a strong competitor for the elution of high affinity proteins for lysine. All pH adjustments were performed by mixing 0.5 M NaH2PO4 and 0.5M Na2HPO4 in the appropriate amounts. Following each elution step, the gel was washed with 50 ml of 50 mM(NH4)HCO3 pH 7.95. The whole procedure was performed at 8°C.

**Protein assay:** Total proteins were measured according to the method described by Lowry et al.[8]

**Electrophoretic methods:** The electrophoretic mobility of Lp(a) native and glycated was compared by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 2% to 16% polyacrylamide gel followed by coomassie brilliant blue staining.[9]

**Statistical analysis:** Data are expressed as mean of all three separate experiments. Statistical analysis was performed by paired student's t-test to determine difference in subspecies of Lp(a) glycated and native and also the effect of glucose concentration on Lp(a) glycation.

## Results

Glucose incorporation into Lp(a) was investigated as a function of time and concentration. These studies established that glucose uptake by Lp(a) was linear as a function of concentration and time. However, according to Fig. 1, glucose uptake peaks at day 7 for all given concentrations and glucose uptake is maximal at a glucose concentration well below that 400 mM which is the maximum concentration used. After 7 days there is a reduction in glycation of Lp(a) for all given concentrations ($p<0.004$). Sequential elution profile of lp(a) subspecies separation by Lysine sepharose affinity chromatography is demonstrated in Fig. 2. Lp(a) Lysine negative which is unable to bind to lysine was eluted immediately after eluting the column with 50mM(NH4)HCO3, pH7.95 following the injection of pooled serum. The amount of this fraction was 50 mg/L. A significant amount of protein was eluted at this step. Lp(a) Lys positive fractions (Lp(a) Lys+1) was identified at the second elution. The Lp(a) Lp(a) Lysine1+ fractions represent 10.6 mg/L. The Lp(a) Lp(a) Lysine 2+ were eluted when the column was eluted with 0.5 M sodium phosphate buffer pH 4.4 and represent 16.8 mg/L. Third elution profile accounted for 10.6 mg/L. Subspecies separation of glycated lp(a) by lysine sepharose affinity chromatography is shown in Fig. 2. The amount and relative values of Lp(a) subspecies in native and glycated serum are shown in Table 1. Lp(a) lysine positive subspecies separated from glycated Lp(a) are higher than those from native Lp(a). In glycated Lp(a), Lysine positive subspecies increased, which was statistically significant $p<0.001$). Electrophoretic mobility of glycated and native Lp(a) shown in Fig.3, showed a slight shift in mobility of Lp(a) incubated with 100 mM of glucose whereas Lp(a) glycated in 200 mM showed more mobility towards the anode, indicating that glycation increased the negative charge of Lp(a) was incubated with 0-400 mM glucose in phosphate buffer and 1 mmol/L EDTA, pH 7.4 at 37°C.

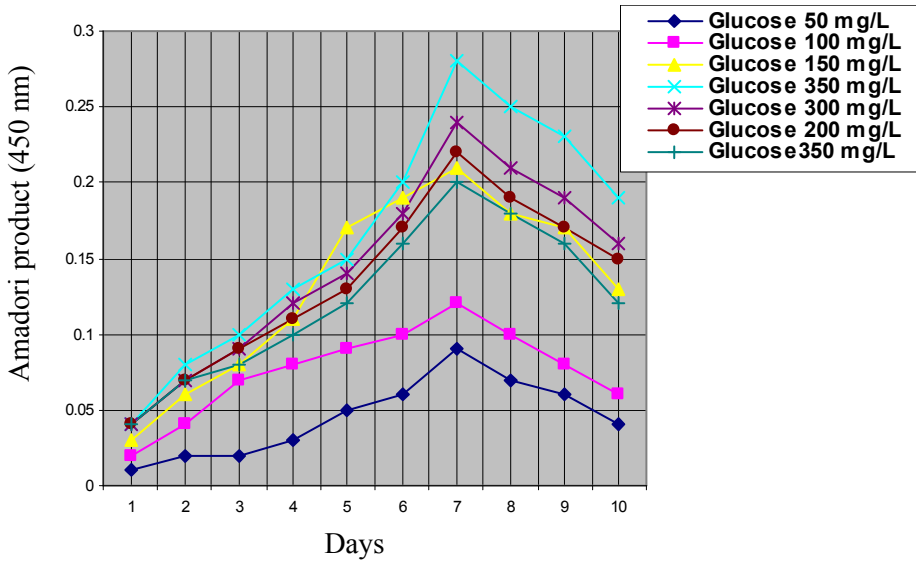


**Fig.1. Line graphs showing the effect of incubation time and glucose concentration on glycation of Lp(a).**

**Table 1. Relative yield of Lp(a) fractions from native and glycated serum following each sequential elution after lysine sepharose affinity chromatography. Data are presented as mean of three measurements. There was significant different between Lp(a) Native and glycated suhspecies ( p= 0.04)**

| | Native serumLp(a) | | Glycated Lp(a) | |
|---|---|---|---|---|
| ***Lp(a) subspecies*** | ***Lp(a) mg/L*** | ***Relative value*** | ***Lp(a) mg/L*** | ***Relative value*** |
| Lp(a) | 100 | 100 | 100 | 100 |
| Lp(a) $Lys^{-}$ | 50 | 50 | 12 | 12 |
| Lp(a) $Lys^{+}$ | 10.6 | 10.6 | 22 | 22 |
| Lp(a) $Lys^{++}$ | 16.8 | 16.8 | 30 | 30 |
| Lp(a) $Lys^{+++}$ | 10.6 | 10.6 | 36 | 36 |

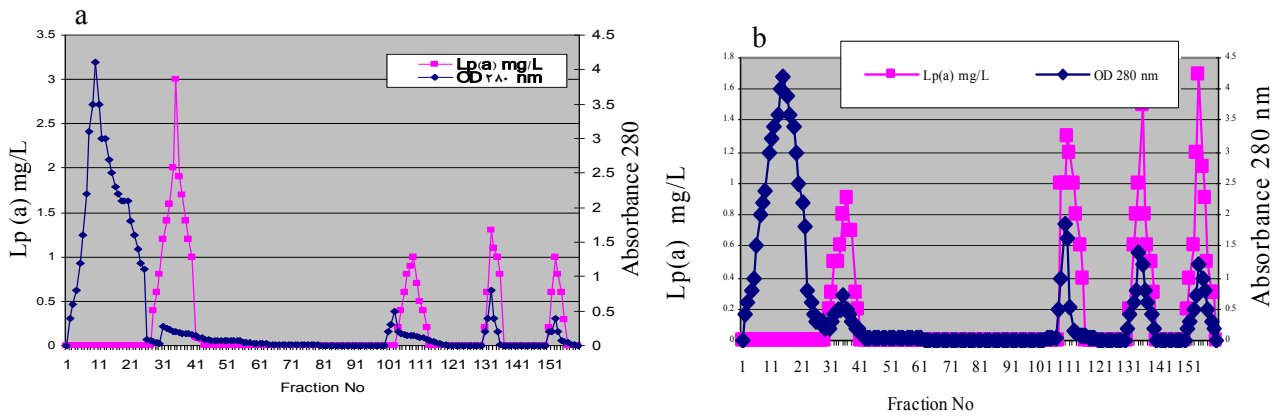


**Fig. 2. Line graphs showing lysine sepharose affinity chromatography of native (a) and glycated (b) Lp(a).**

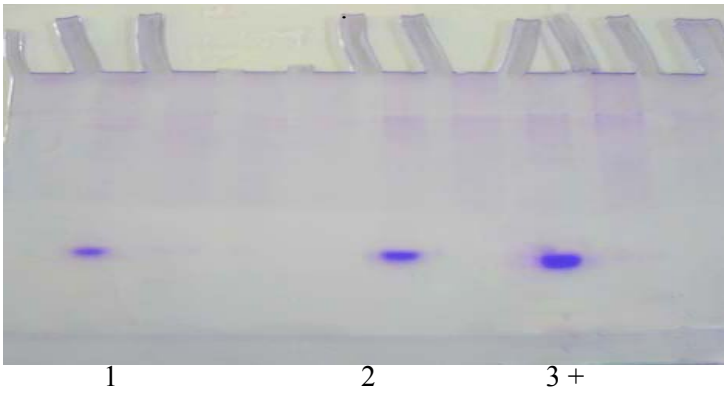


**Fig. 3. SDS –PAGE electrophoresis of native lp(a) (line1) and lp(a) glycated with 100 and 200 mmol of glucose respectively ( line 2 and 3**

## Discussion

The structure of apo(a) is similar to that of zymogen plasminogen; the amino acid sequence of both proteins shows almost 80% homology.[3,10] The two proteins differ mainly in the absence of plasminogen K-1, K-2 and K3 domains and the presence of multiple tandem repeats of plasminogen kringle-4 in apo(a). The role of the kringle domains seem to be essential for the activation of fibrinolysis. The supposed binding between kringle containing proteins and the carboxyl terminal lysine residual of the stabilized fibrin involves a highly hydrophobic pocket in the kringle domain, called the Lysine Binding Site (LBS). The apo(a) LBS properties seem to reside chiefly on kringle IV type 10[11]. The affinity of different kringle for the ε-amino group of lysine can be affected by conformational changes of the lysine binding site. Plasma Lp(a) concentration is variable and its distribution in the general population is highly skewed. Approximately 90% of the

population have serum values below 300 mg/L.

Experimental studies have implicated that high levels of plasma lipoprotein (a) with increased risk for atherosclerotic cardiovascular disease.[12-14] Patients with diabetes mellitus suffer from an increased incidence of complications including atherosclerosis, cardiovascular disease and cataracts; correlation between diabetes and cardiovascular disease (CVD) has been well established.[15-16]

It is believed that part of the atherogenicity of Lp(a) may be explained by its affinity for lesion localized fibrin and much of the binding of Lp(a) is lysine mediated. It has been suggested that glycation of proteins alters both their structure and function and these changes have been linked to diabetic disorders.[17-18] High Lp(a) concentrations as well as an increased rate of nonenzymatic glycation of proteins may be involved in degenerative diabetic complications.[19,20] Lp(a) particles are susceptible to oxidative modification and scavenger receptor uptake, leading to intracellular cholesterol accumulation and foam cell formation which contributes further to atherogenesis. It has been reported that Lp(a) in plasma can be present in heterogeneous forms in respect to its ability to bind lysine sepharose and identified as Lp(a) lys negative and Lp(a) lysine positive.[6] Hoover-Plow and co workers have shown a significant increase in Lp(a) positive fraction with modification of Lp(a) with lipoprotein lipase A2 and this increase was related to the release of fatty acids from Lp(a); they have also shown that CuSO4 oxidation increased LBS activity.21 Doucet et al[22] have studied non-enzymatic glycation of lp(a) in vitro and in vivo, by combination of m-aminophenylboronate affinity chromatography and Enzyme-Linked Immunosorbent Assay (ELIZA) for Lp(a), and they showed that Lp(a) in normal plasma after incubation with glucose can be glycated. In vivo studies have shown that the percentages of glycated Lp(a) in diabetic patients are significantly higher than in the normal population, 2.8% versus 2.0%. Liu and coworkers have shown that lysine binding may facilitate covalent binding which may contribute to the deposition of Lp(a) on endothelial surfaces and its localization with fibrin in atheromas.[23]

Our results are in agreement in part with the findings of Makino et al[24] that show glucose uptake by Lp(a) was linear as a function of time and concentration, we are in agreement that glycation increased the negative charge of Lp(a) as monitored by electrophoresis and ion exchange chromatography; but our results do not agree that glycation does not affect Lp(a) LBS properties; if its negative charge has increased how it is possible that glycation does not affect the attachment to lysine of lysine sepharose. Therfore increase in LBS activity of lp(a) after glycation of Lp(a) and such non enzymatic and chemical modifications may contribute to the variability in LBS function of Lp(a) seen within the population.

In conclusion we can suggest that increased proportion of Lp(a) Lys positive in glycated serum may be associated with endothelial disfunction, independent of other cardiovascular risk factors, and nonenzymatic glycation of Lp(a) may contribute to ` atherogenesis of patients with diabetes mellitus by diverting lipoprotein catabolism from non-atherogenic to atherogenic pathways.

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