Original Article

Relationship of non-LDL-bound apo(a), urinary apo(a) fragments and plasma Lp(a) in patients with impaired renal function

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Abstract

Background. Plasma lipoprotein (a) [Lp(a)] has been shown to be a risk factor for atherosclerosis in numerous studies. However, the catabolism of this lipoprotein is not very clear. We and others have shown that Lp(a) is excreted into urine in the form of fragments. Lp(a) has also been shown to exist in a low-density non-lipoprotein (LDL)-bound form. Since Lp(a) is increased in all forms of kidney disease with reduced excretory kidney function and decreased excretion of apo(a) fragments could be partially responsible for this increase, we investigated the relationship of non-LDL-bound apo(a), urinary apo(a) fragments and plasma Lp(a) in patients with impaired renal function.

Methods. Plasma Lp(a), non-LDL-bound apo(a) and urinary apo(a) fragments were measured in 55 kidney disease patients (28 males and 27 females) and matched controls.

Results. Plasma Lp(a) and non-LDL-bound apo(a) were increased in patients, whereas urinary apo(a) was decreased, especially in patients with a creatinine clearance < 70 ml/min. There was a significant correlation between plasma Lp(a) and non-LDL-bound apo(a) in patients and controls.

Conclusion. We conclude that decreased urinary apo(a) excretion could be one possible mechanism of increased plasma Lp(a) and non-LDL-bound apo(a) in patients with decreased kidney function.

Keywords: lipoprotein (a); non-LDL-bound apo(a); renal function; urinary apo(a)

Introduction

Lipoprotein (a) [Lp(a)] has been established as an independent risk factor for atherosclerotic disease, due to its strong correlation with coronary artery disease, stroke and peripheral atherosclerosis [1]. However, up to now, little is known about the physiological function and catabolism of Lp(a).

There are several indications that the kidney plays an important role in Lp(a) metabolism. Various publications have appeared with the unanimous finding that patients with uraemia and nephrotic syndrome have a 3- to 5-fold increase in their plasma Lp(a). Patients with nephrotic syndrome, for example, exhibit excessively high plasma Lp(a) concentrations, which can be reduced by antiproteinuric therapies [2]. Patients with end-stage renal disease (ESRD) treated with haemodialysis are also found to have elevated Lp(a) levels, and these are even higher in patients treated with continuous ambulatory peritoneal dialysis. Interestingly, Lp(a) is present in the form of different pools. Most studies have measured low-density lipoprotein (LDL)-bound Lp(a), which consists of apolipoprotein B-100 and apo(a) which are covalently bound by a disulfide bridge, and appears in plasma in up to 30 high or low molecular weight isoforms.

In 1987, Gries et al. [3] reported the existence of non-LDL-bound apo(a) in human serum. We and others have published findings that apo(a) immunoreactivity is found in urine in the form of apo(a) fragments [4,5]. We previously reported a highly significant correlation between these urinary apo(a) fragments and plasma Lp(a) levels in healthy volunteers and patients with coronary artery disease [6]. Herrmann et al. [7] also reported increased concentrations of non-LDL-bound apo(a) in patients with coronary artery disease. The same authors also
found a strong elevation of non-LDL-bound apo(a) in patients with ESRD and in patients with nephrotic syndrome [8].

Recently, Kronenberg et al. [9] showed an arteriogenous difference in Lp(a) of ~10% and concluded that substantial amounts of this atherogenic lipoprotein are removed by the kidney. The same authors could also show that plasma concentrations of non-LDL-bound apo(a) are increased in patients with ESRD [10]. However, the relationship between non-LDL-bound apo(a), plasma Lp(a) and urinary apo(a) fragments still remains unclear.

Therefore, in the present study, we determined the relationship between plasma Lp(a), non-LDL-bound apo(a) and urinary apo(a) fragments in kidney disease patients and compared them with a matched control group.

**Subjects and methods**

**Patients and controls**

Fifty-five patients (28 males and 27 females) from the outpatient clinic of the Department of Nephrology of the University Hospital of Vienna, with different stages of renal insufficiency, were included in this case-control study. All included patients had serum triglyceride levels < 350 mg/dl, and were of Caucasian ancestry. The aetiology of renal failure was chronic glomerulonephritis (n = 15), interstitial nephritis (n = 8), polycystic disease (n = 5), diabetic nephropathy (n = 17), hypertensive nephropathy (n = 4) and others or unknown (n = 6). For statistical analysis, we divided the patients into subgroups (i) with normal creatinine clearance (CrCl > 70 ml/min); and (ii) with reduced CrCl (< 70 ml/min). Demographic data of patients and controls are listed in Table 1. The control group consisted of 55 healthy individuals (28 males and 27 females), who had serum creatinine determination and a urinary dip stick test for protein performed, and participated in a health survey programme. The controls were matched for age and gender (Table 1). Apart from occasional pain relievers, the controls did not take any medication. Both patients and controls gave informed consent to participate in this study.

**Blood and urine samples**

Following an overnight fasting period, antecubital vein blood samples were collected in EDTA tubes, centrifuged and the plasma was frozen at –20°C. In previous experiments, we ascertained that freezing once does not affect the DELFIA assays performed. Urine was collected over 24h starting early in the morning. Urine was also frozen at –20°C for 1 month and assayed in one run together with the serum samples. Urine was concentrated by pressure dialysis by a factor of ~100 and immediately processed for apo(a) fragment analysis. CrCl was measured directly.

**Immuoquantitation of Lp(a) and apo(a)**

Analyses were carried out by a sandwich DELFIA as recommended by the manufacturer, (LKB-Pharmacia) and described in detail previously [4,6]. Briefly, polyclonal affinity-purified antibody from rabbit produced in our own laboratory [4,6] was passed over an affinity column loaded with plasminogen and was used to coat 96-well Costar plates. The purified antibody was free of any detectable cross-reactivity against plasminogen, plasmin or elastase as tested by immunoblot analysis, but had a slight cross-reactivity with catalase. For control experiments, we used a monoclonal antibody and obtained identical results. Non-specific binding sites were blocked with 250 μl of 0.5% (w/v) bovine serum albumin for 30min. Aliquots (200 μl) of the samples were added to the wells and incubated for 2h at 20°C. After three successive washing steps with 50 mM Tris–HCl pH 7.7, the same polyclonal antibody against apo(a) as above, labelled with Europium (Eu), was added to the wells and incubated further for 2h at 20°C. Excess antibody was removed by two further washing steps with 50 mM Tris–HCl pH 7.7. A 200 μl aliquot of enhancement solution (Pharmacia, Uppsala, Sweden) was added, and fluorescence was determined after 15min in a DELFIA reader. For the determination of total apo(a), Eu-labelled polyclonal (POAB) anti-apo(a) from rabbit was used (= a:a DELFIA). Plasma samples were diluted 3000-fold and urine samples were diluted between 10- and 50-fold. The assay was linear between 1 and 100 ng of apo(a) per well; the within-run coefficient of variation was < 3%.

**Table 1. Demographic data of patients and controls**

<table>
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<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
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<th>Patients CrCl &lt; 70</th>
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<td>NS</td>
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<td>8</td>
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<td>NS</td>
<td>&lt;20 g/day</td>
<td>&lt;20 g/day</td>
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</table>

*Tested by ANOVA; all patients vs controls.

bTested by ANOVA; patients CrCl > 70 ml/min vs patients CrCl < 70 ml/min.

cMean ± SD. 

dTwo type I diabetics (one in group CrCl > 70 and one in group CrCl < 70); all others type II.

NS, not significant.
Measurement of non-LDL-bound apo(a)

The enzyme-linked ligand sorbent assay (ELLSA) for fapo(a) (Immuno GmbH, Heidelberg, Germany) captured fapo(a) by a specific peptide fixed on the microwells which carried the amino acid sequence of a non-covalent apo(a)-binding site on apo B (ligand peptide, 20).

Immolized fapo(a) was detected utilizing horseradish peroxidase-labelled polyclonal apo(a) [7,8]. Cross-reactions with Lp(a)/apo B-containing particles and plasminogen were excluded. The ligand peptide was also coupled to Sepharose and used in affinity chromatography to separate fapo(a) from whole serum, which was demonstrated by immuno blot analysis. Affinity chromatography-purified fapo(a) was free of apo B and did not cross-react with plasminogen. Levels of free apo(a) determined with the ELLSA method correlated well with those measured by electroimmunodiffusion, an agarose gel electrophoresis which precipitates apo B-containing particles [Lp(a) and LDL] and free apo(a) separately during one electrophoretic run.

Determination of other plasma lipids and albumin

Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were determined with commercially available kits from Roche Diagnostics, Mannheim. LDL cholesterol was calculated using the Friedewald equation. Creatinine was measured by the Jaffee method using commercial assay kits from Boehringer Mannheim. All chemicals were reagent grade obtained from E. Merck, Darmstadt if not stated otherwise.

Statistical analyses

The analysis of our data was performed with the Statistical Package for Social Sciences (SPSS/Mac+). For serum lipids, mean values ± SD were calculated and analysed by a one-way analysis of variance (ANOVA). A Student’s t-test was applied to assess significant differences of continuous variables among groups. Because of the abnormal distribution of Lp(a), non-LDL-bound apo(a) and urine apo(a), non-parametric tests were carried out and medians instead of means are given. Comparison of serum Lp(a), non-LDL-bound apo(a) and urine apo(a) values among groups was performed by the Wilcoxon test or by ANOVA after logarithmic transformation of values. Correlations of serum apo(a) and urine apo(a) values were performed by the Spearman rank correlation test.

Results

Patients had higher total cholesterol, triglyceride, LDL-cholesterol and Lp(a) levels compared with controls, and these differences were statistically highly significant (P < 0.0001, Table 2). There was no statistically significant difference in HDL-cholesterol between patients and controls (Table 2).

When we divided the patients into subgroups (i) with normal CrCl (>70 ml/min) and (ii) with reduced CrCl (<70 ml/min), the cholesterol and LDL cholesterol values tended to be higher in the latter group; however, this was not statistically significant (Table 2).

Plasma Lp(a) and morning urinary apo(a) normalized to 100 mg/dl of creatinine, were measured in 55 patients with kidney disease and compared with those of a control group (Table 3). Median plasma Lp(a) concentrations were found to be significantly higher in patients (21.6 mg/dl) compared with controls (12.0 mg/dl). Median non-LDL-bound apo(a) was 0.72 mg/dl in the patients and 0.53 mg/dl in controls (P < 0.01). There was a highly significant correlation between non-LDL-bound apo(a) and plasma Lp(a) as measured by the Spearman rank correlation test (P < 0.0001) in patients and controls.

Urinary apo(a) normalized to creatinine was lower in patients (7.2 μg/dl), when compared with controls (16.1 μg/dl, Table 3), and this was statistically significant. We also found a significant positive correlation between urinary apo(a) and plasma Lp(a) (P < 0.001) and urinary apo(a) and non-LDL-bound apo(a) (P < 0.001) in patients and controls. When the patient group was divided into subgroups with impaired or normal CrCl, plasma Lp(a) was higher in patients with CrCl < 70 ml/min compared with the group with a CrCl > 70 ml/min (35.5 vs 13.4 mg/dl, P < 0.01, Figure 1). Non-LDL-bound apo(a) was also higher in the group of patients with CrCl < 70 ml/min when compared with those with CrCl > 70 ml/min (1.0 vs 0.4 mg/dl). In addition,
patients with a CrCl < 70 ml/min were found to have significantly lower urinary apo(a) values normalized to creatinine as compared with patients with a CrCl > 70 ml/min (5.3 vs 8.4 mg/dl, Table 3, Figure 1).

**Discussion**

Numerous publications have appeared on the role of the kidney in apo(a) catabolism, over the last decade; they are best summarized by Kronenberg et al. [11]. From these publications it becomes evident that plasma Lp(a) levels are increased in all forms of kidney diseases with impaired renal function [11]. It also becomes evident that plasma Lp(a) levels are reduced to normal in patients with kidney diseases when kidney function is restored by transplantation or angiotensin-converting enzyme (ACE) inhibitor therapy [11]. What is not so clear is the mechanism of this increase in plasma Lp(a).

One possible mechanism is the general increase of protein and therefore also lipoprotein synthesis [Lp(a) synthesis] by the liver due to urinary protein loss. However, since Lp(a) is also increased in kidney diseases without significant proteinuria (ESRD) and other lipoproteins are not increased to the same extent as Lp(a), there must be additional mechanisms responsible for this increase. We showed recently that decreased urinary excretion of apo(a) fragments could be one additional mechanism [12]. Furthermore, Kronenberg et al. [9] showed that there exists a significant renovascular arteriovenous difference in plasma Lp(a), suggesting removal of Lp(a) from the renal circulation.

Lp(a) seems to consist of three pools: (i) the majority seems to circulate in the form of intact apo B (LDL) containing Lp(a), which consists of up to 30 isoforms; (ii) non-LDL-bound or free apo(a); and (iii) apo(a) fragments which appear in urine and plasma. Not very much is known about this non-LDL-bound apo(a), especially since very few assays exist for its measurement. Most of this non-LDL-bound apo(a) seems to consist of apo(a) fragments, identical to those fragments that can be found in urine [4]. Several histopathology studies have shown that atherosclerotic lesions are selectively enriched in non-LDL-bound apo(a). About 50% of the apo(a)
extracted from atherosclerotic lesions is not associated with lipids [13].

It could also be shown that the ratio of plasma concentration of apo B-100 to apo(a) in venous graft lesions is only 3:1, whereas it is 9:1 in plasma [14]. Furthermore, individuals with little to no Lp(a) measurable in plasma have detectable apo(a) in arterial lesions [15]. Lastly, Hoff et al. [16] have identified a series of apo(a)-immunoreactive fragments in the >1.21 g/ml density fraction of human atherosclerotic lesions that were similar in size to those we have characterized.

From the results of the above-mentioned studies, one could speculate that free apo(a) and apo(a) fragments are more atherogenic than apo B-bound apo(a). This would explain why Lp(a) turns out to be a risk factor for coronary artery disease only in some studies. The kidney could play an important role in the metabolism of non-LDL-bound apo(a) by excreting it in the form of apo(a) fragments. To understand better the relationship of these apo(a) fragments, that can be detected in urine and non-LDL-bound apo(a) in plasma in kidney disease patients, we performed the present study. To our knowledge, this is the first study that looks at the relationship between Lp(a), non-LDL-bound apo(a) and urinary apo(a) fragments in patients with kidney disease.

The main finding of the present study was that there exists a positive correlation between non-LDL-bound apo(a), plasma Lp(a) and urinary apo(a) fragments in kidney disease patients and controls. In addition, levels of not only Lp(a), but also non-LDL-bound apo(a) were significantly higher in kidney disease patients with a CrCl <70 ml/min as compared with controls and kidney disease patients with a normal CrCl. We could also reproduce earlier findings from our group that urinary excretion of apo(a) fragments is significantly decreased in kidney disease patients with a CrCl of <70 ml/min.

The limitations of our study are the relatively small number of kidney disease patients and the marked heterogeneity of our patient population. We conclude that both Lp(a) and non-LDL-bound apo(a) are elevated in patients with kidney disease as compared with healthy controls. In addition, a significant correlation exists between both Lp(a) and non-LDL-bound apo(a) in patients and controls. Since urinary apo(a) fragments are significantly decreased in kidney disease patients, especially in patients with a CrCl of <70 ml/min, a decreased ability of the kidney to metabolize Lp(a) could be responsible for the increase of Lp(a) and non-LDL-bound apo(a).

References

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