Urinary Excretion of Apolipoprotein(a) Fragments in Type 1 Diabetes Mellitus Patients

M. Clodi, R. Oberbauer, G. Bodlaj, J. Hofmann, G. Maurer, and K. Kostner

High levels of plasma lipoprotein(a) [Lp(a)] represent an independent risk factor for cardiovascular morbidity; however, Lp(a) has not yet been identified as a risk factor for type 1 diabetic patients. Results from the limited number of available studies on plasma Lp(a) levels in relation to renal function in type 1 diabetes mellitus are inconclusive. We hypothesized that only type 1 diabetes mellitus patients with impaired renal function show increased plasma Lp(a) levels, due to decreased urinary apolipoprotein(a) [apo(a)] excretion. We therefore measured urinary apo(a) levels in 52 type 1 diabetes mellitus patients and 52 matched controls, and related the urinary apo(a) concentration to the plasma Lp(a) level, kidney function, and metabolic control. Our findings indicate that patients with incipient diabetic nephropathy as evidenced by microalbuminuria (20 to 200 μg/min) exhibit significantly higher plasma Lp(a) levels (median, 15.6 mg/dL) in comparison to normoalbuminuric patients (median, 10.3 mg/dL) and healthy controls (median, 12.0 mg/dL). Urinary apo(a) normalized to creatinine excretion was significantly elevated in both normoalbuminuric (median, 22.3 μg/dL) and microalbuminuric type 1 diabetic patients (median, 29.1 μg/dL) compared with healthy subjects (median, 16.0 μg/dL) and correlated significantly with Lp(a) plasma levels in both patient and control groups ($P < .003$). No correlation existed between the Lp(a) plasma level or urinary apo(a) concentration and metabolic control in type 1 diabetes mellitus patients. From these studies, we conclude that urinary apo(a) excretion is significantly increased in type 1 diabetic patients and correlates with plasma Lp(a) levels, and only type 1 diabetic patients with microalbuminuria have higher plasma levels of Lp(a) compared with patients with normoalbuminuria and healthy controls.

HIGH PLASMA LEVELS OF lipoprotein(a) [Lp(a)] represent an independent risk factor for cardiovascular morbidity.[1-6] Due to its high structural homology to plasminogen,[7] Lp(a) has the ability to inhibit fibrinolysis via inhibition of plasminogen activation.[8] Furthermore, a strong Lp(a) affinity for proteoglycans, the major constituents of atherosclerotic plaques, may be the reason that Lp(a) is present in abundance in atherosclerotic plaques.[9]

Although Lp(a) has become a widely studied lipoprotein in lipid research, both the catabolism and renal handling of Lp(a) in type 1 diabetes mellitus remain relatively unclear. Cardiac events are one of the leading causes of death in type 1 diabetic patients; however, Lp(a) has not been identified as a risk factor for these patients.[10-14] Various studies on Lp(a) levels in type 1 diabetes mellitus have yielded controversial results. Jenkins et al,[15] Haffner,[16] and Rudberg and Persson[17]found increased plasma Lp(a) levels in type 1 diabetic patients with microalbuminuria, whereas Westerhuis and Venekamp[18] and Purnell et al[19] did not find elevated Lp(a) levels in such patients. Furthermore, Csaszar et al[20] found no significant difference in apolipoprotein(a) [apo(a)] isoform frequency and plasma Lp(a) levels between type 1 diabetes mellitus patients and controls.

Apo(a) immunoreactivity is found in urine.[21-25] However, it is not the intact Lp(a) that appears in urine, but rather fragments of the apo(a) antigen. Independent of the apo(a) isoform, more than 10 distinct apo(a) bands were consistently found in urine with a molecular mass between 50 and 160 kd.[22] These fragments were glycosylated and were not complexed to apoB.[22] In addition, a significant correlation between urinary apo(a) concentrations normalized to creatinine excretion and plasma Lp(a) levels in healthy subjects has also been described by our group.[22] In the present study, urinary apo(a) excretion and its relationship to elevated plasma Lp(a) was determined in 52 type 1 diabetes mellitus patients both with and without incipient diabetic nephropathy (albumin excretion rate [AER], 20 to 200 μg/min) compared with 52 nondiabetic healthy controls.

SUBJECTS AND METHODS

Patients and Control Subjects

We recruited 52 consecutive type 1 diabetes mellitus patients from the outpatient clinic of the Division of Endocrinology and Metabolism, University of Vienna, between January and May 1996. The selection criteria were as follows: onset of diabetes before age 30 years, insulin treatment within the first 3 months of diagnosis, basal C-peptide concentration less than 1.0 ng/mL, and AER less than 200 μg/min. The mean duration of diabetes was 14.2 ± 8.9 years (mean ± SD) and hemoglobin A_{1c} (HbA_{1c}) was 7.7% ± 0.9% (mean ± SD). To assess the influence of incipient diabetic nephropathy on plasma Lp(a) and urinary apo(a) levels, the patients were assigned to two groups according to AER (group I, AER < 20 μg/min; group II, AER > 20 < 200 μg/min). Urine AERs were determined in urine samples collected for 24 hours except in four patients, where a 12-hour urine sample was collected due to compliance problems. The results for these four patients were then multiplied by two to compare them with the others. Urinary tract infections were excluded by urine microscopy and culture.

The control group consisted of 52 nondiabetic healthy subjects matched for age, sex, hormonal status, and socioeconomic status during a university health survey program. None of the control subjects were on medication, and the only treatment in the diabetic patients was insulin as functional insulin therapy. Therefore, none of the subjects were affected by conditions or medications known to influence apo(a) levels (alcohol intake <20 g/d, ACE inhibitors, hormonal replacement therapy, steroids, nicotinic acid, and tranexamic acid). All investigations were performed with the consent of each patient and according to the Declaration of Helsinki. Demographic data for the type 1 diabetic patients and healthy controls are provided in Table 1.

From the Divisions of Endocrinology and Metabolism and Nephrology and Dialysis, Third Department of Medicine, and Division of Cardiology, Second Department of Medicine, University Hospital of Vienna, Vienna, Austria.

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Address reprint requests to K. Kostner, MD, AKH Wien, Division of Cardiology, Waehringerguertel 18-20, A-1090 Vienna, Austria.

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Table 1. Demographic Data for the Type 1 Diabetic Patients and Controls

Variable	Patients	Controls	Significance
No. of subjects	52	52	NS
Sex ratio (male/female)	27/25	29/23	NS
Age (yr)‡	38.5 ± 12.5	39.5 ± 14.0	NS
BMI (Kg/m²)‡	23.8 ± 5.1	24.1 ± 5.5	NS
CAD (n)	5	—	
PVD (n)	4	—	—
Smokers (n)	12	10	NS
Duration of diabetes (yr)‡	14.2 ± 8.9	—	
Alcohol consumption (g/d)	<20	<20	NS
Creatinine clearance (mL/min)‡	86 ± 22	—	
Albuminuria (μg/min)	10†	Negative*	
HbA_{1c} (%)‡	7.7 ± 0.9	4.3 ± 1.2	$P < .005$

Abbreviations: NS, not significant; PVD, peripheral vascular disease; CAD, coronary artery disease.

*Determined by dipstick.

†Due to nonparametric distribution of albuminuria, the median is given.

‡Mean ± SD.

Blood and Urine Samples

Following an overnight fast, venous blood was drawn from the forearm, allowed to clot for 30 minutes at room temperature, and then centrifuged for serum collection. Samples were stored at −20°C until analysis. In previous experiments, we ascertained that freezing once did not affect the assays performed. A 24-hour (in four patients, 12-hour) urine sample was collected and also frozen at −20°C. Serum and urine were assayed together in one determination.

Measurement of Lp(a) and Apo(a)

A sandwich Dissociation Enhanced Lantanoid Fluorescence Immuno Assay (DELFIA; LKB-Pharmacia, Vienna, Austria) was used as previously described[22] to determine blood Lp(a) concentrations. Urinary excretion of apo(a) was analyzed from the 24-hour, ie, 12-hour, urine collection. The measurement was also performed by DELFIA using a Europium (Eu)-labeled polyclonal (POAB) anti-apo(a) antibody from rabbit (a-a DELFIA). Apo(a):apoB complexes were depicted by Eu-labeled anti-apoB antibody from rabbit as the detection antibody (a-B DELFIA), as described previously.[22] Highly purified recombinant apo(a), and low-density lipoprotein (LDL) samples were used as standards, and the recovery was 98% ± 3%. The assay was linear between 1 and 100 ng apo(a) per well, exhibiting a coefficient of variation less than 4%. Polyacrylamide gel electrophoresis and Western blotting were performed as described previously[22] to characterize the size of urinary apo(a) fragments in type 1 diabetic patients and controls. Total cholesterol, high-density lipoprotein cholesterol, triglycerides, and albumin were determined with commercially available kits from Boehringer (Mannheim, Germany). LDL cholesterol was calculated using the Friedewald equation and the modified Friedewald formula, which takes into consideration the contribution of cholesterol in Lp(a) to LDL cholesterol in patients with high Lp(a) levels. In patients with plasma triglyceride levels higher than 5 mmol/L, direct measurement of LDL cholesterol after precipitation of HDL and VLDL was performed with commercially available kits from Boehringer. The creatinine level was measured by the Jaffee method using commercial assay kits from Boehringer. All chemicals were reagent-grade (Merck, Darmstadt, Germany) unless stated otherwise.

Statistical Analysis

The Macintosh version of the SPSS statistical program (SPSS, Chicago, IL) was used for analysis of the data. Serum lipids were calculated and analyzed by one-way ANOVA. Student's t test was applied to assess differences in continuous variables among groups. Differences in serum Lp(a) and urine apo(a) values among groups were calculated by the Wilcoxon test or by ANOVA after logarithmic transformation. The Spearmann rank correlation test was performed for the description of correlations for serum Lp(a) and urine apo(a) values. Data are presented as the mean ± SD, and a P value less than .05 was considered statistically significant.

RESULTS

Plasma lipoprotein values for patients and controls are shown in Table 2. No significant difference was found in total cholesterol, triglycerides, and LDL or HDL cholesterol between patients and controls. Urinary apo(a) immunoreactivity consisted of apo(a) fragments of molecular weight 50 to 160 kd that were mainly apo(a) kringle IV type 1 and type 2 domains. In patients with type 1 diabetes mellitus, immunoblotting determined that apo(a) fragments were present with features identical to those described for the normal population.

Urinary apo(a) concentrations normalized to 100 mg creatinine and plasma Lp(a) levels in both type 1 diabetic patients and healthy controls are depicted in Table 3. The median and 25th and 75th percentiles for plasma Lp(a) concentrations were comparable in patients and controls; however, patients with microalbuminuria had significantly higher plasma Lp(a) levels compared with healthy controls and patients without incipient diabetic nephropathy (Table 3). The median values for both parameters are presented in Fig 1. Measurement of urinary apo(a) excretion showed that the entire group of type 1 diabetic patients had a higher urinary apo(a) concentration compared with controls. The median and 25th and 75th percentiles for urinary apo(a) were all higher in patients versus controls (19, 26, and 40 v 3, 16, and 34 μg/dL, respectively). Furthermore, this difference in urinary apo(a) excretion between patients and controls was statistically significant ($P < .01$; $Z = -11.2$, Wilcoxon signed-rank test). Interestingly, this difference was even more pronounced in patients with microalbuminuria (median, 29 μg/dL) compared with patients without microalbuminuria (median, 22 μg/dL; $P < .001$, $Z = -5.3$, Wilcoxon signed-rank test).

To further determine the origin of the higher urinary apo(a) levels in type 1 diabetes mellitus patients, we calculated the

Table 2. Lipid and Lipoprotein Concentrations in Type 1 Diabetic Patients and Matched Controls

Group	Triglycerides (mmol/L)	Cholesterol (mmol/L)	LDL Cholesterol (mmol/L)	HDL Cholesterol (mmol/L)
Patients (n = 52)	1.2 ± 0.9	5.3 ± 0.9	3.2 ± 0.8	1.5 ± 0.4
Controls (n = 52)	1.2 ± 0.6	4.9 ± 1.2	3.2 ± 0.8	1.2 ± 0.4

NOTE. Differences were nonsignificant by ANOVA (triglycerides were tested by Wilcoxon signed-rank test).

Table 3. Plasma Lp(a) and Urinary Apo(a) in Type 1 Diabetic Patients and Controls

Group	Plasma Lp(a) (mg/dL)			Urinary Apo(a) (μg/dL)*		
	25%	50%	75%	25%	50%	75%
Patients (n = 52)	4.2	11.4	27.8	18.7	25.7	40.4
Controls (n = 52)	5.1	12.0	24.0	3.4	16.0	33.5
Z†					−11.2	
P†		NS			.01	
Patients with AER <20 μg/min (n = 31)	3.4	10.3	29.4	18.0	22.3	37.1
Patients with AER >20 μg/min (n = 21)	11.2	15.6	31.0	21.2	29.1	69.0
Z†		−5.7			−5.3	
P‡		.001			.001	

NOTE. The 25th, 50th, and 75th percentiles (%) are indicated.

*Normalized to 100 mg/dL creatinine.

†Wilcoxon signed-rank test, patients with type 1 diabetes mellitus *v* controls.

‡Patients with *v* without microalbuminuria.

apparent fractional excretion rate (FE) of apo(a) according to the equation, urinary apo(a) × plasma creatinine/plasma apo(a) × urinary creatinine. The FE of apo(a) in type 1 diabetic patients with microalbuminuria was 0.13% ± 0.20%, as compared with 0.08% ± 0.11% in patients without microalbuminuria. This was not statistically significant. In addition, no significant correlation was found between plasma Lp(a), urinary apo(a), or FE of apo(a) and metabolic control in our type 1 diabetic patient group. Urinary apo(a) and plasma Lp(a) were significantly correlated in patients ($P < .003$, $\rho = .32$, $Z = 3.2$, Spearman rank correlation), as reported previously for healthy subjects.[22-25]

DISCUSSION

The main goal of this study was to determine the relationship between urinary apo(a) excretion, albuminuria, metabolic control, and plasma Lp(a) levels in patients with type 1 diabetes mellitus. We found that the entire group of patients had a higher urinary apo(a) concentration compared with the controls. A significant correlation was found between urinary apo(a) excretion, plasma Lp(a), and microalbuminuria, but no correlation was apparent between apo(a) and metabolic control, plasma lipoproteins, or excretory renal function, which was normal or even elevated in terms of hyperfiltration in early type 1 diabetes mellitus.[26] However, apo(a) excretion was even more elevated in microalbuminuric patients. Although the exact mechanism of apo(a) excretion by the kidney remains to be determined, it is concluded from this study and earlier studies that apo(a) fragments may be actively secreted into the urinary space by the tubulus.[23,24]

From our study, it appears that "basal" apo(a) secretion in type 1 diabetic patients is higher than in controls. The fact that no correlation between HbA_{1c} and plasma Lp(a) or urinary apo(a) could be found may be due to large population fluctuations of these parameters. Furthermore, although our patient population was very well selected, the patients had fairly well-controlled diabetes and the group was relatively small. The data may therefore be limited to determining only whether metabolic control alters Lp(a) levels and/or apo(a) urine concentrations. To study such correlations, single individuals must be studied over prolonged periods.

We further addressed the question of whether plasma Lp(a) levels are elevated in patients with type 1 diabetes in comparison to age-matched controls and, if so, to what extent the plasma levels depend on the level of renal impairment due to incipient diabetic nephropathy or metabolic control. Previous studies that also addressed this question remain fairly inconclusive.[10] In the present study, plasma Lp(a) levels were not different between patients and controls. However, when the patient group was subdivided into patients with or without microalbuminuria, a significant elevation of Lp(a) was found in patients with incipient diabetic nephropathy; however, there was no significant correlation between the level of glycemic

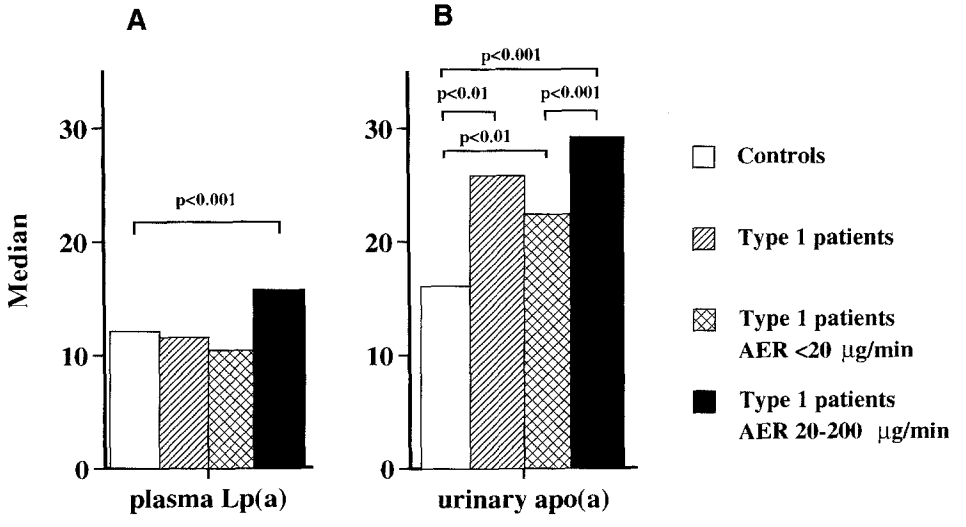

Fig 1. (A) Median plasma Lp(a) concentration (mg/dL) in controls, the entire type 1 diabetic patient group, and the 2 subgroups (type 1 patients with microalbuminuria <20 μg/min and type 1 patients with microalbuminuria 20-200 μg/min). (B) Median urinary apo(a) excretion (μg/dL) normalized to 100 mg/dL creatinine in the same groups.

control and the urinary apo(a) level in the subgroups. Again, this may be due to the limitations already mentioned.

In conclusion, we have shown that urinary apo(a) excretion is significantly increased in type 1 diabetic patients and correlates with the plasma Lp(a) level, and only type 1 diabetes mellitus patients with microalbuminuria have higher plasma levels of Lp(a) compared with patients with normoalbuminuria and healthy controls.

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