Lipoprotein(a)-Associated PAF-Acetylhydrolase Activity in Patients with Coronary Artery Disease

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Key words:
Lipoprotein(a), apolipoprotein(a) isoforms, PAF-acetylhydrolase, coronary artery disease.

Introduction: It has previously been shown that lipoprotein (a) [Lp(a)] is enriched in PAF-acetylhydrolase (PAF-AH) activity. Thus, a functional characteristic of Lp(a) is its capability to hydrolyze and inactivate PAF and oxidized phospholipids that exert potent pro-inflammatory and pro-atherogenic activities. In the present study we examined the Lp(a)-associated PAF-AH activity in comparison to LDL-associated enzyme activity in patients with a history of a coronary artery disease (CAD) as well as in normal subjects (Controls).

Material-Methods: One hundred and thirty-one CAD patients and 33 age- and gender- matched normal subjects (Controls) were enrolled. Lp(a) was measured by ELISA, and apo(a) isoform size was determined by 1.5% SDS-agarose gel electrophoresis and expressed as number of Kringle 4 (K4) repeats. Lp(a) was isolated from plasma by affinity chromatography onto a Lysine-Sepharose 4B column, while LDL by ultracentrifugation. PAF-AH activity was measured by the trichloroacetic acid (TCA) precipitation procedure.

Results: The median plasma Lp(a) levels were significantly elevated in CAD patients compared to Controls [14.9 (0.8-84.1) mg/dl vs 8.1 (0.8-55.0) mg/dl, $p<0.05$]. The percentage of low molecular weight apo(a) isoforms (<26 K4) in CAD was higher compared to Controls (52% vs 30%, $p<0.03$). The specific activity of the Lp(a)-associated PAF-AH in CAD was significantly lower than in Controls (4.8 ± 1.5 nmol/mg lipoprotein mass/min vs 14.8 ± 3.9, respectively, $p<0.00001$). There was no difference in the specific activity of the LDL-associated PAF-AH between the two groups.

Conclusions: The Lp(a)-associated PAF-AH activity in CAD patients is lower compared to Controls, a phenomenon which is not observed in the enzyme activity associated with LDL. Thus the low PAF-AH activity on Lp(a), is a feature characteristic of Lp(a) in CAD patients and confers this lipoprotein with a diminished anti-inflammatory potency. The phenomenon, may constitute an additional, to the high plasma Lp(a) levels, contributing factor in the pathogenesis of CAD.

Manuscript received:
April 26, 2002;
Accepted:
October 21, 2002.

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Lipoprotein(a) [Lp(a)] resembles low density lipoprotein (LDL) in both lipid composition and the presence of apolipoprotein B-100 (apoB-100). Lp(a) is distinguishable from LDL by the presence of an additional protein moiety designated apolipoprotein(a) [apo(a)], which is covalently linked to apoB-100 by a single disulfide bond[1]. Apo(a) is composed of repeated motifs called kringles. Apo(a) contains multiple copies of kringle 4 domain (K4) and one copy of kringle 5 (K5)[2]. The varying number of plasminogen-like K4 domain is the major determinant of apo(a) size polymorphism and gives rise to the large number of apo(a) isoforms detected in human plasma[3]. There is a structural homology between apo(a) and human plasminogen and *in vitro* studies have shown that Lp(a) interferes the fibrinolytic pathway[4]. Furthermore, Lp(a) may play a role in atherogenesis as it is indicated by its presence in atheromatous plaques[5]. High Lp(a) plasma levels (>30 mg/dl) have been recognized as independent risk factor for CAD in Caucasian populations[6,7]. However, prospective clinical studies

have shown conflicting results[8,9], thus the exact pathophysiological link between Lp(a) and atherosclerosis remains unknown.

Human plasma platelet activating factor acetylhydrolase (PAF-AH; EC 3.1.1.47), is a Ca^{2+}-independent phospholipase A_2, associated with lipoproteins, especially with low density lipoprotein (LDL) and high density lipoprotein (HDL). With the exception of PAF, this enzyme hydrolyses phospholipids containing oxidatively fragmented residues at the sn-2 position. Such phospholipids are formed during the oxidative modification of LDL and are thought to play key roles in the inflammatory reactions and particular in vascular inflammation and atherosclerosis[10,11]. It has previously been shown that Lp(a) is enriched in PAF-AH activity[12]. Thus, a functional characteristic of Lp(a), which has not been adequately studied yet, is its capability to hydrolyze and inactivate PAF and oxidized phospholipids molecules that exert potent pro-inflammatory and pro-atherogenic activities.

The aim of the present study was to determine the PAF-AH activity on Lp(a) in comparison to the LDL-associated enzyme activity in patients with coronary artery disease (CAD) as well as in subjects without evidence of coronary artery disease (Controls).

Material and Methods

Study population

Participants in this study were recruited from CAD patients who visited the outpatient Cardiology clinic and subjects who visited the outpatient Lipid Research clinic. Subjects with chronic renal failure, history of alcohol abuse or liver cirrhosis, thyroid function abnormalities, history of acute coronary syndromes or coronary angioplasty or coronary artery bypass graft within the previous 3 months were excluded[13-16]. Nobody was on treatment with any hypolipidaemic agent known to affect Lp(a) concentrations (eg, nicotinic acid or N-acetylcysteine)[17].

The diagnosis of CAD was based on typical description of symptoms, electrocardiographic findings, biochemical markers, functional tests for myocardial ischemia and angiographic results. CAD patients (n=131) were included if their hospital records indicated any of the following criteria: (1) definite or suspected myocardial infarction based on the World Health Organization criteria[18] of typical chest pain, typical enzyme patterns and diagnostic ECG changes, (2) history of unstable angina (Braunwald criteria)[19], (3) chronic stable angina with functional tests positive for myocardial ischemia and significant angiographic stenoses (>70% lumen diameter stenosis).

Control subjects (n=33) selected from individuals without cardiovascular disease (by history, physical examination and electrocardiogram) (Rose questioner[20]) who visited the outpatient lipid clinic for a routine clinicolaboratory investigation.

The study protocol was approved by our Institutional Ethics Committee and each patient gave written informed consent.

Measurement of Lp(a) concentrations and apo(a) phenotype

Lp(a) was measured by an sandwich enzyme immunoassay method (Macra Lp(a), Terumo Medical Corp., Elkton, MD, USA)[21]. Apo(a) isoform size was determined by agarose gel electrophoresis in 1.5% sodium dodecyl sulfate (SDS) agarose gels followed by immunoblotting, according to the method of Kamboh et al[22] as modified by Doucet et al[23]. Isoform size was expressed as number of K4 repeats by using a standard with five different apo(a) isoforms (14, 19, 23, 27 and 35 K4) obtained from Immuno-France (S.A.R.L Rungis, France).

Isolation of Lp(a)

Nine CAD patients and 6 Controls with Lp(a) levels ≥ 20mg/dl and apo(a) size ≤ 21 K4 or ≥ 27 K4 were evaluated [plasma Lp(a) levels ≥20mg/dl is sufficient to isolate Lp(a) and determine its PAF-AH activity]. Briefly, the density of plasma was adjusted to 1.107 g/mL by addition of solid KBr and centrifuged for 10h at 40,000 rpm 14°C in a Beckman L7-65 ultracentrifuge using a type NVT 65 rotor. The Lp(a) containing lipoprotein fraction recovered on the top of the tube was dialysed against 20 mmol/L PBS buffer, pH 7.4, for 12h and loaded onto a Lysine-Sepharose 4B column (Pharmacia Biotech). Unbound lipoproteins (VLDL, LDL, and a small proportion of HDL) were eluted with the above PBS buffer and collected as one fraction (fraction I). The column was then washed with 3 bed volumes of PBS buffer, and Lp(a) was then eluted with a buffer of 200 mmol/L ε-aminocaproic acid (ε-ACA) containing 20 mmol/L PBS and 0.15 mol/L NaCl, pH 7.4.

Isolation of LDL

LDL (d=1.019-1.063 g/mL) was isolated from the above Lp(a)-deficient fraction I by sequential ultracentrifugation at KBr densities of 1.019 g/mL and 1.063 g/mL, in a Beckman L7-65 ultracentrifuge at 40,000 rpm 14 °C for 10h. LDL and Lp(a) were extensively dialyzed against Hepes buffer, pH 7.4, containing 4.2 mmol/L Hepes, 137 mmol/L NaCl, 2.6 mmol/L KCl and 0.01% EDTA, for 12h. The purity of isolated lipoproteins was evaluated by agarose gel electrophoresis [Hydradel Lipo and Lp(a) kit, Sebia] and gradient SDS-PAGE in 5-19% SDS-gradient polyacrylamide gels.

PAF-AH assay

PAF-AH activity was measured by the trichoroacetic acid (TCA) precipitation procedure[24] using 1-O-hexadecyl-2-[^{3}H-acetyl]-sn-glycero-3-phosphocholine [^{3}H]-PAF (10 Ci/mmol; DuPont-New England Nuclear, Boston, MA, USA) as a substrate and 5 μg protein of Lp(a) or LDL as the source of the enzyme. The reaction was performed for 30 minutes at 37°C by adding 10 μl of various [^{3}H]-PAF solutions in BSA saline varying from 2.5 to 100 μM final concentration. PAF-AH activity was expressed as nmol PAF degraded per min, per mg of protein or per mg of lipoprotein. The apparent Km and Vmax values of the enzyme were calculated using the Hanes-Dixon representation of the data.

Analytical methods

Protein content of Lp(a), LDL was determined by the BCA procedure (Pierce). The plasma levels of triglycerides and total cholesterol were measured by standard enzymatic methods (bioMerieux). HDL cholesterol was also determined enzymatically, in the supernatant after precipitation of the apoB containing lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using the Friedwald formula[25].

Statistical analysis

Continuous variables were expressed as mean (±SD) and categorical variables as number (percentage). Serum Lp(a) levels were expressed as median values (range)[7,26]. Groups were compared with Students *t* test for continuous variables and x^2 for categorical variables. For variables with non normal distribution [Lp(a) and triglyceride levels] a non-parametric analysis (Mann-Whitney test) was used. Correlation analysis was performed either with Kendal test or with Spearman rank test. The level of statistical significance was defined as $p< 0.05$.

Table 1. Clinical characteristics and lipid profile of the study population.

	Controls n=33	CAD n=131
Gender, male, n (%)	24 (73)	99 (76)
Age, years	61.5 ± 9.2	61.2 ± 8.8
Body mass index (Kg/m^2)	26.8 ± 3.8	27.1 ± 3.5
Family history of CAD, n (%)	5 (15)	22 (17)
Diabetes, n (%)	5 (15)	30 (23)
Hypertension, n (%)	11 (33)	52 (40)
Smokers, n (%)	13 (40)	41 (31)
Total Chol, mg/dl	258.3 ± 53.2	251.3 ± 47.8
Triglycerides, mg/dl	194.1 ± 104.6	188.4 ±106.2
LDL-Chol, mg/dl	169.5 ± 29.2	168.7 ± 45.2
HDL-Chol, mg/dl	41.7 ± 5.6	40.2 ± 8.5
Lipid lowering therapy, n (%)	6 (18)*	54 (41)

Data are expressed as number (n) or as mean ± S.D.
*p<0.02 vs Controls
CAD: Coronary artery disease

Results

Lp(a) plasma levels and apo(a) isoforms

One hundred and thirty one CAD patients and 33 control subjects were studied. There were no differences between the 2 groups in respect to risk factors for CAD. However, more CAD patients were on hypolipidaemic therapy (Table 1). The median plasma Lp(a) levels were significantly elevated in CAD patients compared to controls (Table 2). There

Table 2. Lp(a) concentrations and apo(a) isoforms in the study population.

	Controls n=33	CAD n=131
Lp(a) concentrations		
Median value (range)	8.1 (0.8-55.0)	14.9 (0.8-84.1)*
≥30 mg/dl, n(%)	3 (9)	28 (21)**
Apo(a) isoforms		
LMW, (%)	10/33 (30)	68/131 (52)***
HMW, (%)	19/33 (58)	50/131 (38)***
Null phenotype, (%)	4/33 (12)	13/131 (10)

LMW: low molecular weight, HMW: high molecular weight.
*p<0.05, **p<0.05 and ***p<0.03 vs Controls.
CAD: Coronary artery disease

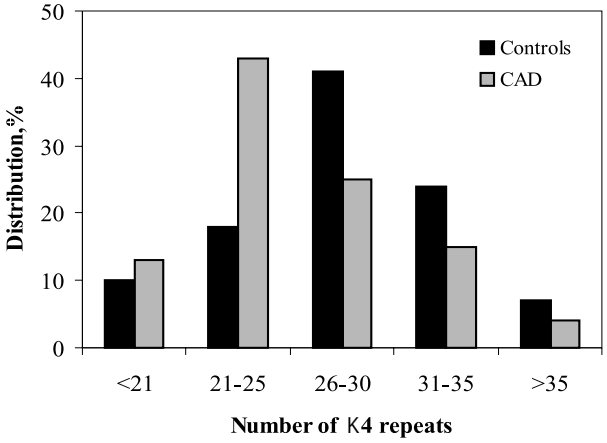

Figure 1. Distribution (%) of apo(a) isoform size among the study population.

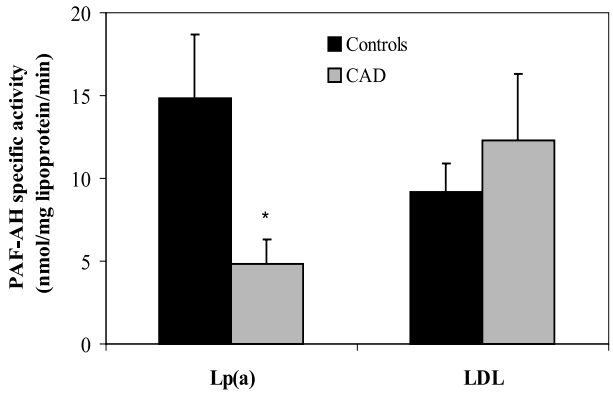

Figure 2. Specific activity of PAF-AH on Lp(a) and LDL in patients with coronary artery disease and controls.
*p<0.00001

was no correlation between Lp(a) plasma levels and the other lipid parameters in both groups. The median apo(a) isoform size was 26 K4 repeats. The percentage of low molecular weight apo(a) isoforms (<26 K4) in CAD patients was higher compared to Controls (Table 2, Figure 1). No differences were observed in the null phenotype frequencies (Table 2). An inverse correlation (Rho = – 0.261; p = 0.01) was observed between plasma Lp(a) levels and the size of the dominating apo(a) isoform in the study population.

PAF-AH activity on Lp(a) and LDL

The specific activity of Lp(a)-associated PAF-AH in CAD patients was significantly lower than in Controls (4.8±1.5 nmol/mg lipoprotein/min vs 14.8±3.9, respectively, p<0.00001, Figure 2). Similarly, the enzyme kinetic constants, Km and Vmax, in CAD patients were significantly lower compared to Controls (p<0.001 for Km and p<0.00001 for Vmax, Table 3). The specific activity of the Lp(a)-associated PAF-AH was similar in CAD patients with 1 vessel disease and those having more than 1 vessel disease (4.7±1.3 nmol/mg lipoprotein/min vs 4.9±1.6, p=NS). No difference was also observed in the enzyme specific activity between smokers and nonsmokers in CAD patients (4.8±1.5 nmol/mg lipoprotein/min vs 4.9±1.6, p=NS) and Controls (14.6±4.1 nmol/mg lipoprotein/min vs 14.9±3.7, respectively, p=NS).

There was no difference in the specific activity of the LDL-associated PAF-AH between the two groups (12.3±4.0 nmol/mg lipoprotein mass/min vs 9.2±1.7, respectively, Figure 2), as well as in the Km, Vmax values (Table 3). There were no differences in the enzyme kinetic constants between Lp(a) and LDL in the Control population. Although, the Km and Vmax values of Lp(a)-associated PAF-AH activity was significant lower than the LDL-associated enzyme activity in CAD patients (p<0.0001 for both Km and Vmax values, Table 3).

Table 3. Kinetic constants of PAF-AH on Lp(a) and LDL of the study population.

	Lp(a)		LDL	
	Km (μmol/l)	Vmax (nmol/mg proteinς/min)	Km (μmol/l)	Vmax (nmol/mg protein/min)
Controls	70.2 ± 28.4	44.6 ± 11.9	79.9 ± 26.5	40.8 ± 8.0
CAD	21.9 ± 11.6*	15.0 ± 4.2**	91.7 ± 34.0Π	54.2 ± 19.4†

CAD: Coronary artery disease
Data are expressed as number (n) or mean ± S.D. Km and Vmax were calculated using the Hanes-Dixon representation of the data.
*p<0.001 and **p<0.00001, vs Controls, Πp<0.0001 for Km value and †p<0.0001 for Vmax value between Lp(a) and LDL in CAD

Discussion

According to the results of our study, patients with CAD had higher concentrations of plasma Lp(a) levels than the Control population. In the early 80's various clinical studies showed that there was an association between elevated Lp(a) levels and the occurrence of a myocardial infarction[27,28]. However the results of large prospective studies[8,9,26,29] opposed this initial hypothesis, leading to questions and doubts about the pathophysiological significance of Lp(a) in CAD.

In our population the prevalence of low molecular weight isoforms of apo(a) in CAD patients compared with normal subjects, was observed. There was an inverse correlation in isoform size, based on the number of K4 repeats, and plasma Lp(a) levels. These findings are in agreement with previously published results[30]. Furthermore, other recent studies by Gazzaruso et al[31] and Paultre et al[32] have shown that the prevalence of low molecular weight isoforms in combination with high plasma Lp(a) levels, increases the risk of CAD. Concerning the biochemical and pathophysiological basis of this phenomenon, Chapman et al[33] have shown that the inhibition of fibrinolysis induced by Lp(a) increases as the size of the apo(a) isoform decreases.

For the first time in the present study we show that the Lp(a)-associated PAF-AH activity in CAD patients is significantly lower than in the Control population. Recently published reports and unpublished data from our laboratory have shown that atorvastatin administration affects only the LDL-associated PAF-AH activity and not Lp(a)-associated enzyme activity[34]. Due to the structural similarity of Lp(a) with LDL, which the major carrier of PAF-AH in plasma, we researched whether the reduction in PAF-AH activity on Lp(a) of CAD patients is also observed on the LDL of these patients. According to our results, there was no observable difference between the LDL-associated PAF-AH activity between the two study populations. Furthermore, the Lp(a) particles of CAD patients transport significantly lower PAF-AH activity compared to their LDL, a phenomenon which is not observed in the Control population. Several studies have shown that PAF-AH plays an important anti-atherogenic role[11], since it degrades PAF and oxidized phospholipids, which are byproducts of the oxidative modification of LDL within the arterial wall. These phospholipids play essential roles in the various biological effects of oxidized LDL (OxLDL) on the cells of the arterial wall (endothelial cells, macrophages, smooth muscle cells)[35]. In addition, these phospholipids play a significant role in the foam cell formation because they are recognized by the macrophage scavenger receptor either as independent molecules or as a complex with apoB-100. Furthermore, they can induce an immune response leading to the formation of autoantibodies that are also important in atherogenesis[36].

Under physiological conditions, Lp(a) is enriched with this anti-atherogenic enzyme. Thus, in contrast to its thrombogenic role (inhibition of fibrinolysis) Lp(a) play an anti-inflammatory and anti-atherogenic role. However, according to our results, the Lp(a) of CAD patients is poor in PAF-AH activity, a phenomenon, which may results in a decrease of the anti-inflammatory and anti-atherogenic properties of Lp(a). The contribution of this phenomenon in the pathogenesis of CAD needs further investigation.

In conclusion, the Lp(a)-associated PAF-AH activity in CAD patients is lower compared to Controls, a phenomenon which is not observed in the enzyme activity associated with LDL. Thus the low PAF-AH activity on Lp(a), is a feature characteristic of Lp(a) in CAD patients and confers this lipoprotein with a diminished anti-inflammatory potency. The phenomenon may, in addition to the high plasma Lp(a) levels, constitute yet another contributing factor in the pathogenesis of CAD.

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