Platelet-Activating Factor Acetylhydrolase (PAF-AH) Activity in Patients with Type 1 Diabetes Mellitus

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Summary

Objective: **To evaluate platelet-activating factor acetylhydrolase (PAF-AH) activity and its relationship with clinical and demographic variables, metabolic control, apolipoprotein A and B levels and the susceptibility of low-density lipoprotein (LDL) to in vitro oxidation in patients with type 1 diabetes mellitus (DM 1).**

Methods: **Forty two patients with DM 1 (27 females) and 48 control subjects (16 females) matched for gender, age and body mass index (BMI) were evaluated. The following tests were performed: fast plasma glucose (FG) and postprandial plasma glucose (PPG), lipid profile, uric acid (UA), glycosylated hemoglobin (HbA1c), and low-density lipoprotein (LDL) oxidation rate using colorimetric assay. The PAF-AH activity was analyzed using colorimetric assay (Cayman Chemical).**

Results: **The analysis of PAF-AH activity showed a higher enzyme activity in patients with DM 1 than in control subjects (0.0150 $\pm$ 0.0051 vs. 0.0116 $\pm$ 0.0041; p < 0.001). In patients with DM 1, a direct correlation between PAF-AH activity and age and LDL, and an inverse correlation between PAF-AH and HbA1c and high-density lipoprotein (HDL) were found.**

Conclusion: **In the sample studied, PAF-AH showed a higher activity in patients with DM 1, a factor that may be related to the higher risk of developing cardiovascular diseases observed in these patients. Further studies are necessary to evaluate the real participation of this enzyme in the risk of development of atherosclerotic diseases in patients with DM 1.**

Key words: **PAF-acetylhydrolase, type 1 diabetes, atherosclerotic diseases.**

Introduction

Cardiovascular disease is the major cause of morbidity and mortality in type 1 diabetes mellitus (DM 1), accounting for 44% of all deaths in patients with DM 1[1,2]. Several methods have been used to evaluate the presence of atherosclerotic disease in patients with DM 1, showing that coronary heart disease may already be present in young patients[3-5].

The oxidation process is considered an important component in the initial phase and progression of the atherosclerotic disease. The PAH-AH enzyme activity may be protective in this process, since the association of this enzyme with LDL and HDL prevents the in vitro formation of minimally oxidized LDL molecules and its internalization by macrophages[6-8].

Recently, atherosclerosis has been considered a chronic inflammatory disease. The inflammatory process contributes significantly to the initiation, progression and rupture of atherosclerotic lipid-rich plaques[9,10]. The cells responsible for the tissue damage associated with inflammation are recruited and activated by a series of mediators, and the platelet activating factor (PAF) is a phospholipid involved in this process. Phospholipids with a pro-inflammatory activity may be generated through oxidative fragmentation of the phosphatidylcholine fatty acid, which are known as PAF-like phospholipids. The biological activity of PAF and PAF-like phospholipids is attenuated by phospholipase A2 enzymes (PLA2)[11].

Studies have shown that PLA2 is involved in the inflammatory process and in atherogenesis, and that both the secretory type II PLA2 and the lipoprotein-associated PLA2 (PAF-acetylhydrolase/ PAF-AH) may be related to the risk of coronary heart disease[12,13]. Theoretically, this enzyme may promote atherogenesis if the products it releases from LDL phospholipids have a deleterious effect on arterial walls[14], or it may be protective if, when hydrolyzing PAF, it reduces the inflammatory and thrombotic tendency of the blood[15]. Plasma PAF-AH is mainly associated with LDL (preferentially subfraction 5); however, a small amount (< 20% of the enzyme activity) is associated with HDL (preferentially subfraction 1)[16,17].

The objective of the present study is to evaluate PAF-AH

activity and its relationship with clinical and demographic variables, metabolic control, apolipoproteins A and B levels, and the susceptibility of low-density lipoprotein (LDL) to in vitro oxidation in patients with DM 1, to observe the interference of variables classically related to a higher risk of cardiovascular disease in patients with diabetes and this new factor studied.

Methods

Patients - Fifty patients with DM 1 followed regularly in the outpatient clinic of diabetes of the Hospital Universitário Pedro Ernesto (HUPE) – UERJ, and 48 control subjects matched for gender, age, and body mass index (BMI) were evaluated after giving their written consent previously approved by the ethics committee of HUPE. Clinical and demographic characteristics of the two groups at the moment of sample collection for tests are shown in Table 1.

Exclusion criteria for diabetic patients were: smoking, alcohol consumption, systemic infection and/or use of drugs that could affect the results of determination of the susceptibility of LDL to oxidation, such as ACE inhibitors, ferrous sulphate, and vitamin and mineral supplements containing vitamin C and E, Zn, Se, Fe and Cu, and history of previous cardiovascular events, diabetic retinopathy and nephropathy, and clinical diabetic neuropathy. In the control group, these criteria also included the presence of direct family members with diabetes mellitus.

In accordance with the exclusion criteria, eight patients with type 1 diabetes mellitus were excluded from the study because they had incipient diabetic nephropathy and/or diabetic retinopathy.

The participants had their blood drawn after a 12-hour fast and 2 hours after a 400-kcal breakfast standardized in relation to carbohydrate, protein and fat content. The following tests were performed: fast plasma glucose (FG), postprandial plasma glucose (PPG) (glucose oxidase); total cholesterol (TC), HDL-cholesterol, triglycerides (TG) and uric acid (UA) using colorimetric assay measured on a Cobas-Mira analyzer (Roche); glycosylated hemoglobin (HbA1c – HPLC measured on a Merck Hitachi 9100 analyzer – RV 2.6 to 6.2%, and intra and interassay variation coefficient lower than 1%). LDL was

calculated using the Friedewald formula[18]. The TC/HDL and LDL/HDL ratios were calculated.

All patients had three samples of overnight urine collected in a period of three months, with a minimum two-week interval between each. They were instructed to discard urine at 8p.m. and to collect all the urine until 6 a.m. the next day to determine the urinary albumin excretion rate (UAE). The urine volume was aliquoted and stored in glass flasks at 70º C for further analysis. Urinary albumin concentration was determined using radioimmunoassay (Diagnostic, California, United States, 0.3-μg/mL sensitivity) with intra and interassay variation coefficient of 8.7% and 8.3%, respectively. With base on UAE, only the patients with normal urinary albumin (UAE < 20 μg/min in two out of three urine samples)[19] were included. The patients with DM 1 underwent dilated fundoscopy which was performed by the same ophthalmologist.

For the analysis of the susceptibility of LDL to *in vitro* oxidation, 20 ml of blood were collected in EDTA vacuum tubes which were centrifuged at 4ºC for 20 minutes at 800 x g. The plasma was separated and immediately processed for LDL isolation, in accordance with the following phases: density adjustment at 1.3 g/ml by adding potassium bromide (4.5g of KBr for each 9 ml of plasma) with further preparation of ultracentrifugation tubes with 20 ml of 0.9% saline solution and the 9 ml of plasma previously prepared. These tubes were centrifuged at 4ºC for three hours at 150.00 x g. After centrifugation, the lipoprotein band with density between 1.019 and 1.063 g/ml, compatible with that of LDL, was collected[20,21]. Protein concentration of this material was measured using the Biuret method, and adjusted for 0.2 mg/ml. Copper sulphate ($CuSO_4$) 20 mM was added to the material obtained in the proportion of 1 μl for each 1 ml of LDL and this solution was placed in a water bath at 37ºC for 24 hours.

Oxidized LDL was indirectly assessed using the calculation of its oxidation rate [23,24], in whose formula absorbance at 3 different UV light wavelengths is used, namely: 205 nm, 232 nm and 280 nm. The oxidation rate was calculated using the formula: Abs 205 – Abs 280 / Abs 232 – Abs 280, where Abs 205 = reading of double bonds of polyunsaturated fatty acids of the LDL phospholipids; Abs 232 = reading of conjugated dienes; and Abs 280 = reading of LDL protein fraction.

Variables	DM 1	Control	P
N	42	48	
Gender (F/M)	16/26	27/21	-
Age (years)	24.3 ± 9.6	24.9 ± 7.8	-
Age at diagnosis (years)	14.6 ± 8.0	-	
Duration of disease (years)	9.7 ± 7.3	-	
Body mass index (kg/m²)	21.8 (15.8 – 29.8)	22.2 (18.5 – 36.2)	-
SBP (mmHg)	110.0 (90.0 – 130.0)	110.0 (90.0 – 148.0)	0.393
DBP (mmHg)	70.0 (50.0 – 90.0)	70.0 (60.0 – 95.0)	0.513

SBP = systolic blood pressure; DBP = diastolic blood pressure

Table 1 - Characteristics of control and DM 1 groups at the moment of sample collection for tests

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The readings were taken in a Shimadzu spectrophotometer at the wavelengths mentioned above at the timepoints pre-addition, 1h, 3h, 6h and 24h post-addition of $CuSO_4$ to assess LDL susceptibility to the oxidative process.

A resting 12-lead electrocardiogram (ECG) was performed and classified by two doctors according to the Minnesota protocol[25] in a double-blind manner. Based on this coding, the patients were defined in the following categories: without coronary heart disease, possible or definite coronary heart disease, or possible or definite myocardial infaction[26].

PAF-AH activity was analyzed using the colorimetric method with a commercial kit according to instructions provided by the manufacturer (Cayman Chemical). Ten microliters of serum samples frozen in a freezer at - 70° C distributed in a 90-well plate were used. In each well, 5 μl of buffer solution, 10 μl of developing substance and 200 μl of substrate (2-thio PAF) were added. The kit material is enough to analyze 60 samples, including the negative and positive controls. Absorbance readings at 405 nm were further taken at timepoints 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 minutes. PAF-AH activity is expressed in μmol/min/ml and was calculated using the following formula:

PAF-AH activity = ΔA405/min/10 mM^{-1} * (0.225 ml/0.01 ml) * sample dilution, where ΔA405/min = [ΔA405 (timepoint 2) - ΔA405 (timepoint 1)] / [timepoint 2 (min) – timepoint 1 (min)].

Statistical analysis - The results of all variables with a normal distribution analyzed were expressed as mean ± standard deviation, and those with a non-Gaussian distribution were expressed as median (minimum – maximum). The non-paired Student's t test was used for the comparison of the means of variables with a normal distribution between the two groups analyzed, and the Mann-Whitney test was used for those with a non-Gaussian distribution. The Pearson and Spearman correlation tests, according to variable distribution, were used to test the correlations between PAF-AH activity and metabolic control variables and susceptibility of LDL to in vitro oxidation.

Results

The glycemic control of the patients studied was inadequate and no statistically significant difference of total cholesterol, and LDL and HDL levels was observed between the two groups assessed (Tab. 2). Uric acid and triglyceride levels were lower in diabetics than in control subjects [3.65 ± 0.98 mg/dl vs. 4.69 ± 1.34 mg/dl; p < 0.001 and 69.00 (26.00-201.00) vs. 87.50 (20.00-278.00); p = 0.027, respectively].

Patients with DM 1 had a mean baseline LDL oxidation rate similar to that of control subjects. However, 3 hours after $CuSO_4$ addition this rate was lower in the diabetic group (6.98 ± 1.36 vs. 7.91 ± 1.48; p = 0.007) (Fig. 1; Tab. 3).

According to the Minnesota Coding, no patient had possible or definite coronary heart disease, or possible or definite myocardial infarction.

PAF-AH activity analysis showed a higher enzyme activity in patients with DM 1 than in control subjects (0.0150 ± 0.0051 vs. 0.0116 ± 0.0041; p < 0.001 μmol/min/ml) (Fig. 2).

When the correlation between PAF-AH activity and the other variables was analyzed in patients with type 1 diabetes,
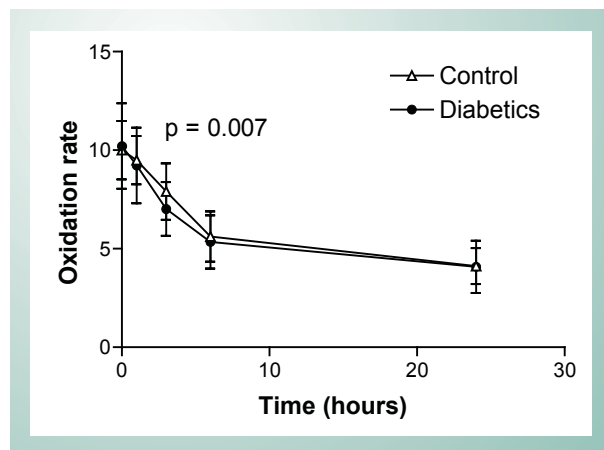

Fig. 1 - *Oxidation rate before and 1h, 3h, 6h and 24 hours after copper sulphate addition in patients with type 1 diabetes mellitus and control subjects*

Variables	DM 1	Control	P
FG (mg/dl)	147.5 (33.0-574.0)	78.0 (57.0-98.0)	-
PPG (mg/dl)	199.0 (91.0-620.0)	95.0 (62.0-147.0)	-
HbA1c (%)	8.9 (4.9 – 15.5)	5.1 (3.1 – 6.0)	-
TC (mg/dl)	169.4 ± 37.7	182.9 ± 41.6	0.113
HDL (mg/dl)	44.5 (21.0 – 67.0)	42.5 (25.0 – 73.0)	0.521
LDL (mg/dl)	104.3 (68.0 – 164.0)	116.2(68.0 – 209.0)	0.204
TG (mg/dl)	69.0 (26.0 – 201.0)	87.5 (20.0 – 278.0)	0.027
AU (mg/dl)	3.6 ± 0.9	4.6 ± 1.3	<0.001
EUA (μg/min)	9.6 (2.2 – 19.2)	3.9 (0.3 – 11.7)	<0.001

FG = fast plasma glucose, PPG = posprandial plasma glucose, CR = control rate, TC = total cholesterol, HDL = high-density lipoprotein, LDL = low-density lipoprotein, TG = tryglicerides, UA = uric acid, UAE = urinary albumin excretion

Table 2 - Metabolic control variables

Oxidation rate	DM 1	Control	P
Baseline	10.24 ± 2.16	10.04 ± 1.50	0.669
1 hour	9.23 ± 1.95	9.55 ± 1.24	0.424
3 hours	6.98 ± 1.36	7.91 ± 1.48	0.007
6 hours	5.36 ± 1.30	5.68 ± 1.29	0.303
24 hours	3.96 (2.08 – 7.58)	3.95 (2.83 – 6.94)	0.692

Table 3 - LDL oxidation rate

we observed significant data with: age (r=0.328; p=0.034), HbA1c (r=-0.319; p=0.039), HDL (r=-0.348; p=0.028) and LDL (r=0.324; p=0.041) (Fig. 3). The stepwise multiple regression using PAF-AH activity as a dependent variable and age, gender, BMI, HbA1c, HDL and LDL as independent variables showed HDL and LDL as independent explanatory variables of changes in PAF-AH activity (step 1: r = 0.231, r2
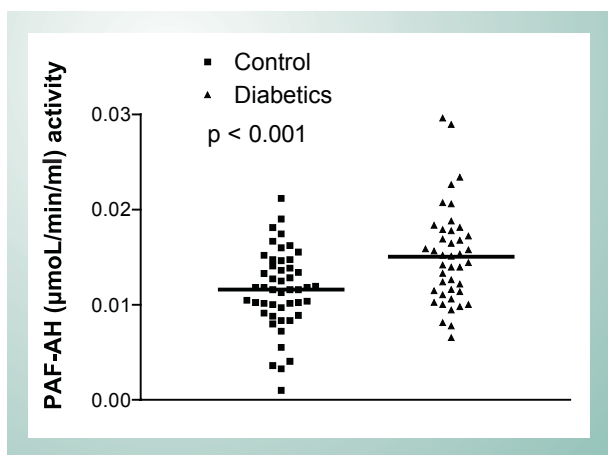

Fig. 2 - *PAF-AH (μmoL/min/mL) activity in patients with type 1 diabetes mellitus and control subjects*

= 0.053, beta = - 0.231, p = 0.039; step 2: r = 0.316, r2 = 0.100, beta = - 0.252, p = 0.017).

Discussion

There is controversy in the literature regarding the importance of the PAF-AH activity and concentration in the atherosclerotic process[27-33]. Some studies demonstrate that patients with DM 1 have low levels of PAF-AH, unlike patients with DM 2[27,29]. The literature also correlates changes in PAF-AH activity with several inflammatory diseases including atherosclerosis, and shows that these changes may reflect a slight increase or decrease in the enzyme activity. However, it remains unclear whether these changes have an impact on the progression, severity and resolution of the diseases associated with them[11].

In regard to patients with DM 1, Cavallo-Perin et al[28] did not find any difference in PAF-AH activity among groups of patients with DM 1 without microalbuminuria, with microalbuminuria, and control subjects. Although they did not find any difference in PAF-AH activity among the groups

studied, Cavallo-Perin et al[28] and Nathan et al[27] demonstrated that PAF levels are increased in patients with DM 1, and that this would be basically associated with a higher production of this particle. The disagreement between data found by these authors and our own may reflect the characteristics of the population studied – our sample had a lower age range, no microvascular complications and lower BMI, as well as the sample size – 42 diabetics versus 7 patients with microalbuminuria and 7 without microalbuminuria assessed by Cavallo-Perin et al. The higher PAF-AH activity in patients with DM 1 may represent an attempt at protection against pathophysiological mechanisms induced by increased levels of PAF found in these patients[34] or may exert pro-inflammatory and pro-atherogenic effects[30,32,33].

The analysis of oxidation rate evidenced a higher LDL oxidation in patients with type 1 diabetes 3 hours after the addition of the oxidative agent than in control subjects. These data are also controversial in the literature[35-38]. The differences may result from the number of patients studied and from differences in their clinical and demographic characteristics. No correlation between PAF-AH activity and oxidation rate was observed, and apparently no relation between the higher susceptibility of LDL to oxidation in patients with diabetes and PAF-AH activity levels in the sample studied was observed either.

The evaluation of correlations of PAF-AH activity in patients with type 1 diabetes was positive with age and LDL, and negative with HbA1c and HDL. Several studies demonstrated an increase in the activity of this enzyme with age[39-42]. The cause of this increase is perhaps related to the elevation of LDL levels that accompanies aging[43], since a significant correlation between LDL levels and PAF-AH activity occurs[42,44-47]. Data from the literature corroborate the correlation of PAF-AH activity with HDL and LDL found in our sample[16,17].

Our study had limitations regarding the analysis of other variables associated with atherosclerotic disease, such as acute phase proteins, and the use of other methods to assess this disease in patients with DM 1, such as the measurement of the common carotid artery intima-media thickness[5].

Considering the multiple factors involved in atherosclerotic disease and the controversy on the real function of PAF-AH, the higher activity of this enzyme in patients with DM 1 could reflect both a protective mechanism and a marker of a higher risk for atherosclerotic disease. Further prospective studies are necessary to define the association of PAF-AH with cardiovascular risk in patients with type 1 diabetes.
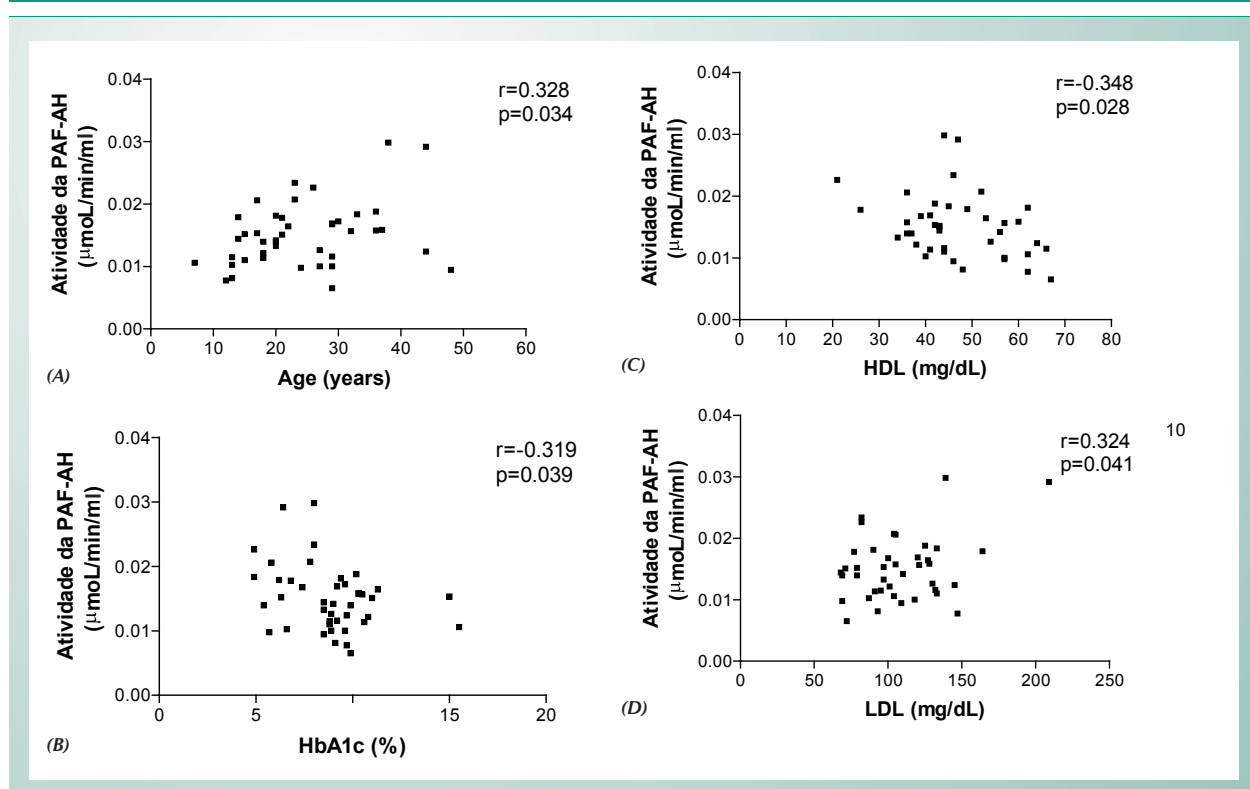
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Fig. 3 - *Correlation of PAF-AH activity with age (A), HbA1c (B), HDL (C) and LDL (D) in patients with type 1 diabetes mellitus.*

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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