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 ± 0.0051 vs. 0.0116 ± 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. 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.

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.

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. 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).

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. Theoretically, this enzyme may promote atherogenesis if the products it releases from LDL phospholipids have a deleterious effect on arterial walls, or it may be protective if, when hydrolyzing PAF, it reduces the inflammatory and thrombotic tendency of the blood. 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).

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 diabetology 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 using 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. 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 8 p.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 70°C for further analysis. Urinary albumin concentration was determined using radioimmunoassay (Diagnostica, 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) 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 4°C 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 4°C 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. Protein concentration of this material was measured using the Biuret method, and adjusted for 0.2 mg/ml. Copper sulphate (CuSO4) 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 37°C for 24 hours.

Oxidized LDL was indirectly assessed using the calculation of its oxidation rate, with 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.

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

| 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) | 0.393 |
| SBP (mmHg)                       | 110.0 (90.0 – 130.0) | 110.0 (90.0 – 148.0) | 0.513 |
| DBP (mmHg)                       | 70.0 (50.0 – 90.0) | 70.0 (60.0 – 95.0) | -    |

SpBP = systolic blood pressure; DBP = diastolic blood pressure.
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 CuSO₄ 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 protocol⁴ 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 infarction⁵.

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:

\[
\text{PAF-AH activity} = \Delta A_{405/min/10 \text{ mM} \times 1} \times (0.225 \text{ ml/mol/0.01 ml} \times \text{ sample dilution, where} \Delta A_{405/min} = [\Delta A_{405/\text{timepoint 2} - 2} - \Delta A_{405/\text{timepoint 1} - 1}] / [\text{timepoint 2 (min)} - \text{timepoint 1 (min)}].
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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 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 CuSO₄ 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,

**Table 2 - Metabolic control variables**

| 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 = postprandial plasma glucose, CR = control rate, TC = total cholesterol, HDL = high-density lipoprotein, LDL = low-density lipoprotein, TG = triglycerides, UA = uric acid, UAE = urinary albumin excretion
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 explanatory variables showed HDL and LDL as independent explanatory variables of changes in PAF-AH activity (step 1: $r = 0.231$, $r^2 = 0.053$, beta = -0.231, $p = 0.039$; step 2: $r = 0.316$, $r^2 = 0.100$, 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. Some studies demonstrate that patients with DM 1 have low levels of PAF-AH, unlike patients with DM 2. 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.

In regard to patients with DM 1, Cavallo-Perin et al 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 and Nathan et al 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 or may exert pro-inflammatory and pro-atherogenic effects.

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. 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.

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Potential Conflict of Interest

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

References

1. Laing SP, Swerdlow AJ, Slater SD, Botha JL, Burden AC, Waugh NR, et al. The British Diabetic Association Cohort Study II: cause-specific mortality in patients with insulin-treated diabetes mellitus. Diabet Med. 1999; 16: 466-71.

2. Morrish NJ, Wang S-L, Stevens LK, Fuller JH, Keen H. The WHO Multinational Study Group: Mortality and causes of death in the WHO multinational study of vascular disease in diabetes. Diabetologia. 2001; 44(Suppl 2): 514-521.

3. McGill HC, McMahan CA, Zieske AW, Tracy RE, Malcom GT, Herderick EE, et al. Association of coronary heart disease risk factors with microscopic qualities of coronary atherosclerosis in youth. Circulation. 2000; 102: 374-9.

4. Tuzcu EM, Kapadia SR, Hobbs RE, McCarthy PM, et al. High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults. Circulation. 2001; 103: 2705-10.

5. Järvisalo MJ, Patto-Laurila A, Jarti L, Lehtimäki T, Ronnemaa T, et al. Carotid artery intima-media thickness in children with type 1 diabetes. Diabetes. 2002; 51: 493-8.

6. Stafforini DM, Zimmerman GA, McIntyre TM, Prescott SM. The platelet-activating factor acetylhydrolase from human plasma prevents oxidative modification of low-density lipoprotein. Trans Assoc Amer Phys. 1992; 105: 44-63.

7. Watson AD, Navab M, Hana SY, Sevanian A, Prescott SM, Stafforini DM, et al. Effect of platelet-activating factor acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. J Clin Invest. 1995; 95: 774-82.

8. Stafforini DM, Rollins EN, Prescott SM, McIntyre TM. The platelet activating factor acetylhydrolase from human erythrocytes. Purification and properties. J Biol Chem. 1993; 268: 3857-65.

9. Ross R. Atherosclerosis: an inflammatory disease. N Engl J Med. 1999; 340: 115-26.

10. Libby P. Changing concepts of atherogenesis. J Intern Med. 2000; 247: 349-58.

11. Tijssen LW, Stafforini DM. Platelet-activating factor acetylhydrolases in health and disease. Biochim Biophys Acta. 2000; 1488: 102-23.

12. Witzum JL, Berliner JH. Oxidized phospholipids and isoprostanes in atherosclerosis. Curr Opin Lipidol. 1998; 9: 441-8.

13. Packard CJ, O’Reilly DS, Caslake MJ, McMahon AD, Ford I, Cooney J, et al. Lipoprotein-associated phospholipase A2 as an independent predictor of coronary heart disease. N Engl J Med. 2000; 343: 1148-55.

14. Marphee CH, Moores KE, Boyd HF, Dhanak D, Ile RJ, Leach CA, et al. Lipoprotein-associated phospholipase A2, platelet-activating factor acetylhydrolase, generates two bioactive products during the oxidation of low-density lipoprotein: use of a novel inhibitor. Biochem J. 1999; 338: 479-87.

15. Berliner J, Leitinger N, Watson A, Huber J, Fogelman A, Navab M. Oxidized lipids in atherogenesis: formation, destruction and action. Thromb Haemost.
1997; 78: 195-9.

16. Teleples AD, Chapman MJ. Inflammation, bioactive lipids and atherosclerosis: potential roles of a lipoprotein-associate phospholipase A2, platelet activating factor, and high density lipoprotein. Arterioscler Thromb Vasc Biol. 2002; 3: 57-68.

17. Teleples AD, Dentan C, Karabina S-AP, Chapman MJ, Ninnis E. PAF-degrading acetylhydrolase is preferentially associated with dense LDL and VHDL-I in human plasma. Catalytic characteristics and relation to the monocyte-derived enzyme. Arterioscler Thromb Vasc Biol. 1995; 15: 1764-73.

18. Friedwald WT, Levy R, Fredrickson DS. Estimations of serum low density lipoprotein cholesterol without use of preparative ultracentrifuge. J Clin Chem. 1972; 18: 499-502.

19. Mogensen CE, Keane WF, Bennett PH, Jerums G, Parving HH, Passa P, et al. Prevention of diabetic renal disease with special reference to microalbuminuria. Lancet. 1995; 346: 1080-4.

20. Chung BH, Wilkinson T, Geer JC, Segrest JP. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. J Lipid Res. 1980; 21: 284-91.

21. Heery JM, Kozak M, Stafforini DM, Jones DA, Zimmerman GA, McIntyre TM, et al. Oxidatively modified LDL contains phospholipids with platelet-activating factor-like activity and stimulates the growth of smooth muscle cells. J Clin Invest. 1995; 96: 2320-30.

22. Cornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the Biuret reaction. J Biol Chem. 1949; 177: 751-66.

23. Kim BS, LaBella FS. Comparison of analytical methods for monitoring autoxidation profiles of authentic lipids. J Lipid Res. 1987; 28: 1110-7.

24. Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and its antioxidant modulators in atherosclerosis. J Appl Biochem. 1987; 7: 70-7.

25. Nathan N, Denizot F, Claverie C, Laubie B, Benveniste J, et al. Increased activity of platelet-activating factor acetylhydrolase in low-density lipoprotein: use of a novel inhibitor. Biochem J. 1999; 338: 48-53.

26. Satoh K, Yoshida H, Iamazumi TA, Takamatsu S, Mizuno S. Platelet-activating factor acetylhydrolase in plasma lipoproteins of healthy men and women. Clin Chim Acta. 1991; 202: 95-103.

27. Jenkins AJ, Klein RL, Chassereau CN, Hermayer KL, Lopez-Virella ME. LDL from patients with well-controlled IDDM is more susceptible to in vitro oxidation. Diabetes. 1996; 45: 762-7.

28. Castro SH, Castro-Faria-Neto HC, Clemente ELS, Gomes MB. Acorrelation of the susceptibility of LDL from patients with diabetes type 1 to oxidation in vitro and its relation to the control of the disease. Archivos de la Academia Nacional de Medicina. 2001; 131: 7-13.

29. Memon RA, Saeed SA, Jabbar A, Jafri A, Gilani AH, Saleem S, et al. Increased activity of platelet-activating factor acetylhydrolase in platelet activating factor metabolism in insulin dependent diabetes mellitus. Diabetes Metab. 1992; 88(2): 156-161.

30. Karabina SA, Elsal F, Bairaktari E, Tzallas C, Stanopoulos KC, Teleples AD. Increased activity of platelet-activating factor acetylhydrolase in low-density lipoprotein: addition of an inhibitor to oxidation in patients with heterozygous familial hypercholesterolemia. Eur J Clin Invest. 1997; 27: 595-602.

31. Murakami M, Kudo I. Phospholipase A2 J. Biochem. 2002; 131: 285-92.

32. Castro SH, Castro-Faria-Neto HC, Clemente ELS, Gomes MB. Acorrelation of the susceptibility of LDL from patients with diabetes type 1 to oxidation in vitro and its relation to the control of the disease. Archivos de la Academia Nacional de Medicina. 2001; 131: 7-13.

33. Satoh K, Imaizumi T, Yoshida H, Kawamura Y, Takamatsu S, Takamatsu M, et al. Platelet-activating factor acetylhydrolase in plasma lipoproteins of healthy men and women. Clin Chim Acta. 1991; 202: 95-103.