The plasma levels of lipoprotein-associated phospholipase A₂ are increased in patients with β-thalassemia

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Abstract

Lipoprotein-associated phospholipase A2 (Lp-PLA₂) is an independent cardiovascular risk factor. We investigated the plasma levels of Lp-PLA₂ activity and mass as a function of plasma lipid levels, LDL subclass profile and oxidative stress in patients with β-thalassemia. Thirty-five patients with β-thalassemia major (β-TM) and 25 patients with β-thalassemia intermedia (β-TI) participated in the study. Lp-PLA₂ activity and mass were measured in total plasma, in apo B-depleted plasma (HDL-Lp-PLA₂) and in LDL subclasses. Lp-PLA₂ activity produced and secreted from peripheral blood monocytes in culture was also determined. Patients with β-thalassemia are characterized by a predominance of small-dense LDL particles, increased oxidative stress as well as very high plasma levels of Lp-PLA₂ mass and activity, despite the low LDL-cholesterol levels. A significant positive correlation between plasma Lp-PLA₂ activity or mass with 8-isoprostane and ferritin levels as well as with intima-media thickness (IMT) values was observed. An increase in secreted and cell-associated Lp-PLA₂ activity from monocytes in culture was observed in both patient groups. The HDL-Lp-PLA₂ activity and mass as well as the ratio of HDL-Lp-PLA₂ / plasma Lp-PLA₂ were significantly higher in both patient groups compared with controls. In conclusion, patients with β-thalassemia exhibit high plasma Lp-PLA₂ levels, attributed to increased enzyme secretion from monocytes/macrophages and to the predominance of sDLDL particles in plasma. Plasma Lp-PLA₂ is correlated with carotid IMT suggesting that this enzyme may be implicated in premature carotid atherosclerosis observed in β-thalassemia.
**Supplementary Key words:** Lipoprotein-associated phospholipase A\(_2\), β-thalassemia, LDL, HDL, oxidative stress, atherosclerosis

**Abbreviations:** Lipoprotein-associated phospholipase A\(_2\); Lp-PLA\(_2\), β-thalassemia major; β-TM, β-thalassemia intermedia; β-TI, intima-media thickness; IMT, Platelet-activating factor; PAF, low-density lipoprotein; LDL, high-density lipoprotein; HDL, tumor necrosis factor-α; TNF-α, interleukin-6; IL-6, thiobarbituric acid-reactive substances; TBARS, very low-density lipoprotein; VLDL, intermediate-density lipoprotein; IDL, small dense LDL; sdLDL, lipoprotein(a); Lp(a), platelet microparticles; PMPs, paraoxonase 1; PON1, 8-isoprostanes; 8-epiPGF2a, apolipoprotein B; apoB
**Introduction**

Platelet-activating factor (PAF) is a proinflammatory phospholipid that may be implicated in atherogenesis. PAF is degraded by PAF-acetylhydrolase, an enzyme that exhibits a Ca$^{2+}$-independent phospholipase A$_2$ activity (1). PAF-acetylhydrolase also degrades oxidized phospholipids, which are formed during oxidation of low-density lipoprotein (LDL) and may play key roles in vascular inflammation and atherosclerosis (2). Four intracellular (Ia, Ib, II and erythrocyte form) and one secreted (plasma form) isoenzymes have been described. Among the intracellular forms, the erythrocyte PAF-acetylhydrolase is a distinct 25-kDa protein that is found primarily in the cytosol (3). The plasma form of PAF-acetylhydrolase is complexed to lipoproteins (1), thus it is also referred to as lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) (4). Lp-PLA$_2$ is primarily associated with LDL, whereas a small proportion of circulating enzyme is also associated with high-density lipoprotein (HDL) (1). Lp-PLA$_2$ is produced by hematopoietic cells primarily from monocytes/macrophages (5, 6) and it is located with and highly expressed by macrophages within the necrotic core and the fibrotic cap of advanced rupture-prone plaques (7, 8). The Lp-PLA$_2$ associated with LDL is the major determinant of plasma enzyme levels and most of studies over the last years suggest that this enzyme plays a proinflammatory role in the artery wall thus promoting vascular inflammation and atherosclerosis (9, 10). The proinflammatory and proatherogenic role of plasma Lp-PLA$_2$ is primarily attributed to the fact that during the hydrolysis of oxidized phospholipids, this enzyme generates lysophosphatidylcholine and oxidized free fatty acids both of which exhibit proatherogenic activities (4). In this regard, a substantial
body of peer-reviewed studies in Caucasian population has supported Lp-PLA$_2$ as a new independent cardiovascular risk factor (11).

In contrast to the Lp-PLA$_2$ associated with LDL, several lines of evidence suggest that HDL-associated Lp-PLA$_2$ (HDL-Lp-PLA$_2$) although present at low levels, may contribute to the antiatherogenic effects of this lipoprotein. However, the clinical value of HDL-Lp-PLA$_2$ as an inhibitor of the atherosclerotic process needs further investigation (1).

Beta-thalassemias result from either reduced synthesis (i.e., thalassemia intermedia, [β-TI]) or complete absence (i.e., thalassemia major, [β-TM]) of structurally normal β-globin subunits of hemoglobin. Patients with β-TI require sporadic, if any, blood transfusions during life, while patients with the major phenotype are on a regular transfusion program since their first months after birth (12). Thalassemic patients are at risk for iron overload due to increased gastrointestinal iron absorption in β-TI and frequent blood transfusions in β-TM. Furthermore, a high incidence of strokes and thromboembolic episodes despite rarity of coronary artery disease has been found and accounted for by interplay of profound endothelial activation, immunmodulation and a characteristic, rather antiatherogenic, plasma lipid profile (13-15). In accordance to the above observations, we have previously demonstrated a premature carotid artery disease as well as a globally disturbed vasorelaxation in β-thalassemic patients (16).

According to previously published results, the plasma Lp-PLA$_2$ activity in β-thalassemia/Hemoglobin E patients is significantly increased, a phenomenon that could be attributed to the increased oxidative stress observed in these patients (17). Therefore, the aim of the present study was to investigate the plasma levels of Lp-PLA$_2$ activity and
mass as a function of plasma lipid levels, LDL subclass profile and oxidative stress and to further elucidate the mechanisms that may underline the alterations of the above enzyme observed in patients with β-thalassemia.

Materials and methods

Study design

Thirty-five patients with β-TM, who were on regular blood transfusion regimen since their first years of life participated in the study. All patients were receiving desferrioxamine approximately 40-50 mg/kg subcutaneously overnight and ascorbic acid orally. Compliance with chelation therapy was considered optimal if patients were >90% adherent to the instructions given by the hematologists. Twenty-five patients with β–TI, who were either not transfused or received only sporadically blood transfusions (i.e. less than 4 times each year) were also included in the study. All β-TI patients were instructed to receive chelation therapy with desferrioxamine if serum ferritin levels exceeded 2000 ng/ml. Serum ferritin levels in both patient groups were determined three to five times each year. All patients participated in the study were free from diabetes mellitus and cardiac disease and demonstrated an echocardiographically normal bi-ventricular systolic function. For comparison, 30 healthy normolipidemic volunteers, who were matched with the patient group for age, sex and body surface area and smoking habits, also participated in the study (control group). Post hoc carotid artery ultrasonography was obtained in all patients with β-TM; however, this was not possible for the rest of the participating study subjects due to logistic constraints. As we previously described (16) the left and right common carotid arteries were examined in multiple directions. We used the beginning of
the dilatation of the carotid bulb as a reference point for measurement of the intima-media thickness (IMT). IMT was defined as the distance between the boundaries of lumen-intima and media-adventitia interfaces at the far common carotid artery wall. The average IMT of the six measurements in each patient was obtained from the frozen magnified images of the screen display. Plaque was defined as a focal structure encroaching into the arterial lumen at least 0.5mm or 50% of the surrounding IMT. The Ethics Committee of the University Hospital of Patras approved the research protocol and written informed consent was obtained from all subjects.

**Biochemical measurements**

Antecubital venous blood samples were obtained after 12 hours of fasting. Serum total cholesterol, HDL–cholesterol, triglyceride glucose and ferritin levels as well serum aminotransferase levels, were determined on the Olympus AU560 Clinical Chemistry analyser (Hamburg, Germany) as previously described. Serum LDL–cholesterol levels were calculated using the Friedewald formula (16, 18). Serum Lipoprotein [a] (Lp[a]) levels were measured by an enzyme immunoassay method (Macra Lp[a], Terumo Medical Corporation Diagnostic Division, Elkton, MD) (18). The levels of high-sensitivity tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured in duplicate by the quantitative sandwich enzyme-linked immunoassay technique (ELISA, R&D Systems, Minneapolis, MN, USA) (16). Creatinine levels in urine were measured by the Jaffe’ method (19). Hematologic parameters were also determined in all subjects participating in the present study (16). Products of lipid peroxidation in lipoprotein subclasses were detected through the measurement of thiobarbituric acid-reactive
substances (TBARS) by using a spectrophotometric assay, essentially as previously described (20).

**LDL subclass analysis**

LDL subclass analysis was performed electrophoretically by use of high-resolution 3% polyacrylamide gel tubes and the Lipoprint LDL System (QuantiMetrix, Redondo Beach, CA) as we have previously described (21). After electrophoresis, very low-density lipoprotein (VLDL) remained in the origin [retention factor (Rf) = 0.0], whereas HDL migrated at the front (Rf = 1.0). In between, several bands can be detected: MID bands C, B, and A, which correspond mainly to intermediate-density lipoprotein (IDL), as well as up to 7 LDL bands. The LDL-1 and LDL-2 bands correspond to large, buoyant LDL particles, whereas bands LDL-3 to LDL-7 correspond to small dense LDL (sdLDL) particles. We determined the cholesterol mass of each apoB-lipoprotein subfraction, the mean LDL particle size (in Å), and the proportion (%) of the cholesterol mass of sdLDL particles over the total LDL-cholesterol mass (21).

**Subfractionation of plasma apoB-containing lipoproteins**

ApoB-containing lipoproteins were fractionated by isopycnic density gradient ultracentrifugation as we described previously (22). Total plasma was subjected to ultracentrifugation, and 30 fractions of 0.4 ml each were collected and analyzed for their protein content. Equal volumes of gradient fractions 1 to 12 corresponding to apoB-containing lipoproteins, were pooled to form the following subfractions: VLDL - IDL (d = 1.019 g/ml), LDL-1 (d = 1.019–1.023 g/ml), LDL-2 (d = 1.023–1.029 g/ml), LDL-3 (d
= 1.029–1.039 g/ml), LDL-4 (d = 1.039–1.050 g/mL), and LDL-5 (d = 1.050–1.063 g/ml) (22). All subfractions were dialyzed extensively at 4°C in 10 mmol/L phosphate-buffered saline, pH 7.4 (PBS) containing 2 mmol/L EDTA, filter-sterilized, and maintained at 4°C under nitrogen until analysis. Under these storage conditions, no oxidation was observed in each LDL subfraction, as it was revealed by agarose gel electrophoresis and the determination of the TBARS values (0.9 ± 0.4 malondialdehyde equivalents per mg of protein for all lipoprotein preparations).

Preparation of erythrocyte lysates

Erythrocyte lysates were prepared as we have described previously (19). In brief, venous blood samples were collected using acid citrate as anticoagulant and centrifuged at 1500xg for 20 min. The supernatant plasma along with the buffy coat was removed by aspiration. The remaining erythrocyte pellet was washed three times with a 4.2 mM Hepes buffer solution pH 7.4. A small portion of this suspension was used for erythrocyte count. The remaining suspension was centrifuged as above and the sedimented erythrocytes were lysed by mixing with 5 volumes of a 7 mM sodium phosphate buffer solution, pH 7.4 for 20 min at -4°C (19). The erythrocyte lysate was stored at -80°C until analysis.

Measurement of Lp-PLA₂ activity and mass

Lp-PLA₂ activity in total plasma, in apo B-depleted plasma, after the sedimentation of all apo B-containing lipoproteins with dextran sulfate-magnesium chloride (HDL-Lp-PLA₂ activity) and in LDL subclasses, was determined by the trichloroacetic acid
precipitation procedure using [³H]-PAF (100 μM final concentration) as a substrate (18, 22). Fifty microlitres of total plasma diluted 1:50, v/v with HEPES buffer, pH 7.4, or the apo B-depleted plasma (diluted 1:3, v/v with HEPES) or 5 μg protein of each LDL subclass, were used as a source of the enzyme. The reaction was performed for 10 min at 37°C and Lp-PLA₂ activity was expressed as nmol PAF degraded per min per ml of plasma or mg of total protein. Lp-PLA₂ activity was also determined in the supernatants and cell lysates of monocytes in culture as we previously described (23). In some experiments Lp-PLA₂ activity was determined in 90 microliters of erythrocyte lysates. The reaction was performed for 10 min at 37°C and enzyme activity was expressed as nmol PAF degraded per min per 10⁹ cells (19).

Lp-PLA₂ mass was determined by a dual monoclonal antibody immunoassay standardized to recombinant Lp-PLA₂ (PLAC test, diaDexus, Inc.), following the manufacturer instructions (24). Lp-PLA₂ mass was measured in plasma and in apo B-depleted plasma, prepared as above (HDL-Lp-PLA₂ mass), as well as in LDL subclasses as we have previously described (21). We used 10 μl of undiluted plasma or apoB-depleted plasma or 10–20 μg of total protein from each LDL subfraction as the source of the enzyme. Lp-PLA₂ specific activity was expressed as a ratio of the enzyme activity to the enzyme mass (nmol/ng/min) (21). Finally we determine the Lp-PLA₂ mass in 10-50 microlitres of erythrocyte or monocyte lysates or monocyte supernatants.

**Determination of Lp-PLA₂ activity associated with platelet microparticles in plasma**

Plasma-containing microparticles was prepared as we previously described (25). Blood samples from patient groups and controls were drawn into tubes containing acid-
citrate dextrose as an anticoagulant (Vacutainer, Becton Dickinson) and centrifuged at 150×g for 10 min to obtain platelet-rich plasma. Platelet-rich plasma was then mixed with 0.1% EDTA/saline, at a ratio of 3:2 (v/v) and then centrifuged at 1,500×g for 20 min to obtain the plasma-containing microparticles. Flow cytometry was then performed, to verify the presence of platelet microparticles by using annexin-V-PE, which recognizes the population of all microparticles that express anionic phospholipids and anti-CD41a-FITC, which is specific for platelet microparticles (PMPs) (25). The Lp-PLA<sub>2</sub> activity associated with PMPs in plasma was then measured by a captured ELISA method previously described by our group using ELISA plates were coated with the monoclonal antibody anti-CD61 (it specifically recognizes the β<sub>3</sub> integrin of the platelet integrin α<sub>IIb</sub>β<sub>3</sub> located on PMPs) or the isotype-matched mouse monoclonal IgG (25).

**Isolation and culture of peripheral blood monocytes**

Peripheral blood from patients with β-thalassemia as well as from controls was drawn into EDTA-containing tubes. Blood was centrifuged and monocytes were isolated from the buffy coats as we previously described (23). Cells from each donor were cultured and grown in duplicate in 24-well plastic tissue culture dishes (1 x 10<sup>6</sup> cells/well) with RPMI medium, containing L-glutamine, 40 µg/mL gentamycin and 1% Nutridoma (Boehringer Mannheim GmbH, Germany) (23). After 48h of culture, the cell supernatants were recovered and the cell layers were washed twice with PBS and then detached and lysed by the addition of 0.2 ml of a lysis solution containing 1% EDTA and 0.1% Triton X-100. Both supernatants and cell lysates were centrifuged (500 x g for 10 min at 4°C), stored at 4°C and analyzed for Lp-PLA<sub>2</sub> activity and lactate dehydrogenase.
activity (kit from Boehringer Mannheim GmbH, Germany) within 24h from the collection (23, 26). Cell lysates were further analyzed for their protein content, determined with the Lowry method (26). Viability under all culture conditions was determined by trypan blue dye exclusion and the absence of lactate dehydrogenase release; viability was > 95%.

**Measurement of PON1 activities**

PON1 activities in serum were measured using paraoxon as a substrate (paraoxonase activity), as well as using phenyl acetate as a substrate (arylesterase activity). Both PON1 activities were determined in the presence of 2 mM Ca^{2+} in 100 mM Tris-HCl buffer (pH 8.0) for paraoxon and in 20 mM Tris-HCl buffer (pH 8.0) for phenyl acetate (26).

**Determination of 8-isoprostanes**

For estimation of oxidative stress in all subjects participated in the present study, the plasma and urine levels of 8-isoprostanes (8-epiPGF2a), were determined. Measurement of 8-epiPGF2a levels in plasma samples (expressed as pg/ml) was carried out by means of a competitive enzyme immunoassay (commercial 8-isoprostane EIA kit, Cayman Chemicals, Ann Arbor, MI, USA), following the manufacturer instructions as we previously described (27). The 8-epiPGF2a levels in urine were also determined by the same technique and were expressed as ng/mg creatinine.

**Statistical analysis**
Data are presented as the mean (SD) except for nongaussian-distributed variables, which are presented as the median (range). Statistical analyses were performed using ANOVA followed by least significant difference (LSD) test for comparisons between individual groups. The nonparametric Mann-Whitney U test and the Kruskal-Wallis test were applied to discriminate differences in nongaussian-distributed variables. Correlations between Lp-PLA<sub>2</sub> and other parameters determined in the present study were estimated using linear regression analysis and Spearman’s rank correlation coefficients (for nongaussian-distributed variables), while Yates’s corrected chi-square test was used for differences in proportions. A P value of <0.05 was considered to be significant.

Results

Characteristics of the study population

The clinical and biochemical characteristics of the study population are shown in Table 1. Both thalassemic patients groups exhibited elevated serum ferritin levels compared with controls. Liver biopsy was performed in 12 patients. Histologically, hepatitis and cirrhosis were found in four and one of these patients, respectively. Moreover, severe hepatic siderosis could be shown in 2 out of the 12 patients. Nineteen (55%) of the patients were on optimal desferrioxamine therapy, whereas history of splenectomy, endocrinopathy and active or healed hepatitis was evident in a significant minority of them. Both patient groups demonstrated significantly lower serum total-, LDL- and HDL-cholesterol levels and increased concentrations of both serum triglycerides and liver transaminases compared with controls, whereas no difference in
the above parameters was observed between β-TI and β-TM. No difference in the serum Lp(a) and glucose levels was also observed among the studied groups. The atherogenic ratio LDL-cholesterol to HDL-cholesterol was significantly lower in both patient groups compared with controls (Table 1). The proinflammatory cytokines IL-6 and TNF-α were significantly higher in both patient groups compared with controls, the β-TM patients exhibiting higher levels of both parameters compared with β-TI (Table 1). Finally on ultrasonography, the mean IMT values in β-TM patients were 0.50±0.14 mm, in accordance to our recently reported values on these patients (16).

Apolipoprotein B-containing lipoprotein subclasses

Both β-TI and β-TM patient groups had higher plasma levels of VLDL-cholesterol and lower levels of IDL-cholesterol and buoyant LDL-cholesterol compared with control group (Table 2). Furthermore, β-TI and β-TM patients had significantly higher levels of sdLDL-cholesterol and a higher proportion of sdLDL, whereas the mean LDL size was lower compared with the subjects of control group. Finally, no difference was observed between the two patient groups in the above parameters (Table 2).

Plasma Lp-PLA₂ activity and mass

Total plasma Lp-PLA₂ activity as well as the enzyme mass were significantly higher in both patient groups compared with controls, the β-TM patients having significantly higher enzyme activity and mass compared with β-TI patients (Table 1). Lp-PLA₂ activity and mass in thalassemic patients were positively correlated with 8-epiPGF2a levels in serum (r=0.245, p<0.03 and r=0.288, p<0.03, respectively) and in urine.
A positive correlation was also observed between Lp-PLA\(_2\) activity and mass with serum ferritin levels (Fig. 1A,B) whereas no correlation was found between Lp-PLA\(_2\) activity or mass and LDL-cholesterol (Fig. 1C,D), total cholesterol, or Lp(a) levels in both patient groups. Finally a significant correlation between plasma Lp-PLA\(_2\) activity and mass with IMT values in \(\beta\)-TM patients was observed (\(r= 0.440, p<0.01\) and \(r= 0.463, p<0.01\), respectively).

Importantly, the enzyme specific activity in total plasma (calculated as the activity to mass ratio) in both patient groups was significantly lower (\(p<0.05\)) compared with controls (in nmol/ng/min, 0.17±0.03 for controls, versus 0.13±0.03 for \(\beta\)-TI and 0.13±0.02 for \(\beta\)-TM patients). Similarly, the HDL-Lp-PLA\(_2\) activity and mass were significantly higher compared with controls, the \(\beta\)-TM patients having significantly higher enzyme activity and mass compared with \(\beta\)-TI patients (Table 1), whereas in both patient groups, the enzyme specific activity was significantly lower (\(p<0.05\)) compared with controls (0.045±0.01 for controls, versus 0.039±0.02 for \(\beta\)-TI and 0.038±0.01 for \(\beta\)-TM patients). Finally, the ratio of HDL-Lp-PLA\(_2\) mass to total plasma Lp-PLA\(_2\) mass was significantly higher (\(p<0.001\)) in both patient groups compared with controls (0.11±0.01 for controls, versus 0.14±0.02 for \(\beta\)-TI and 0.15±0.02 for \(\beta\)-TM patients). Similarly, the ratio of HDL-Lp-PLA\(_2\) activity to total plasma Lp-PLA\(_2\) activity was significantly higher (\(p<0.001\)) in both patient groups compared with controls (0.26±0.03 for controls, versus 0.52±0.04 for \(\beta\)-TI and 0.65±0.04 for \(\beta\)-TM patients).

**Lp-PLA\(_2\) activity and mass in apolipoprotein B-containing lipoprotein subfractions**
The higher Lp-PLA$_2$ activity and mass found in plasma of $\beta$-TM and $\beta$-TI patients despite their lower plasma levels of LDL-cholesterol, led us to determine the above enzyme parameters in apolipoprotein B (apoB)-containing lipoprotein subfractions prepared by an isopycnic density gradient ultracentrifugation method. The Lp-PLA$_2$ activity and mass were preferentially associated with the small dense LDL-5 subfraction in all studied groups (Fig. 2). Importantly, $\beta$-TM and $\beta$-TI patients exhibited higher enzyme activity and mass in all lipoprotein subfractions compared with controls, the $\beta$-TM patients having significantly higher enzyme activity and mass compared with $\beta$-TI patients (Fig. 2).

**The PAF-acetylhydrolase of erythrocytes does not contribute to the pool of plasma Lp-PLA$_2$ of patients with $\beta$-thalassemia**

The elevated Lp-PLA$_2$ activity and mass in plasma of patients with $\beta$-thalassaemia prompted us to study the source(s) from which this high amount of enzyme derives. We initially searched whether hemolysis, a phenomenon that occurs in these patients could contribute to the increased Lp-PLA$_2$ in plasma. Indeed erythrocytes contain a PAF-acetylhydrolase type that exhibits similar substrate specificity but it is structurally different to that of Lp-PLA$_2$ (3). The enzyme activity determined in erythrocyte lysates prepared from blood of five subjects from each group was similar among the study groups (in nmol/10$^9$ cells/min, 73±8 for controls, 70±10 for $\beta$-TI patients and 75±11 for $\beta$-TM patients). As expected, the enzyme activity in erythrocyte lysates was sensitive to sulfhydryl reagents (28) since it was significantly reduced by 75±5% in all groups after treatment with 1mM 5,5’-dithiobis (2-nitrobenzoic acid), (DTNB) for 30 min at 37°C.
Furthermore, as it was previously reported (28), the enzyme activity in erythrocyte lysates was susceptible to proteolysis since it was significantly reduced by 90±10% in all groups after treatment with 0.25 mg /ml Trypsin for 1h at 37°C. By contrast, the plasma Lp-PLA₂ activity in all studied groups was resistant to the above treatments. Additionally, we were not able to detect Lp-PLA₂ mass in erythrocyte lysates, indicating that the PLAC test does not detect the PAF-acetylhydrolase type that exists in erythrocytes. Overall, the above results suggest that the increase in Lp-PLA₂ mass and activity in plasma of β-TM and β-TI patients is not due to the existence in plasma of the enzyme type derived from erythrocytes.

**Lp-PLA₂ activity associated with platelet microparticles in plasma of patients with β-thalassemia**

As we have previously shown, another source of the Lp-PLA₂ in plasma could be platelets, which secrete this enzyme associated with platelet microparticles (PMPs) (25). By using an ELISA method previously described by our group, we determined the PMPs associated enzyme in plasma of β-TM and β-TI patients as well as of controls. Representative flow cytometric profile of PMPs in plasma of β-TM patients is illustrated in Fig. 3A. Similar profiles were obtained in plasma of β-TI patients and controls. As it is shown in Fig. 3B, The PMPs-associated enzyme activity was similar among all studied groups suggesting that the increase in plasma Lp-PLA₂ observed in β-TM and β-TI patients is not due to an increase in the enzyme secretion from platelets of these patients.

**Lp-PLA₂ secretion from adherent monocytes in culture**
It has been shown that adherent monocytes produce and secrete Lp-PLA₂ during differentiation into macrophages, and these cells represent the major source of the plasma Lp-PLA₂ activity (5, 6). To investigate whether the increase in plasma- and HDL-Lp-PLA₂ activity of patients with β-thalassemia was due to an increase in enzyme secretion from monocytes in culture, we studied the spontaneous enzyme secretion form monocytes of both groups as well as from controls. As it is shown in Fig. 4A, there was an increase in total (secreted plus cell-associated) Lp-PLA₂ activity in both patient groups compared with controls at 48 hours of culture (p<0.01) and this increase reflected mainly the secreted enzyme activity (Fig. 4B). Importantly the total and secreted enzyme activity in β-TM patients was significantly higher compared to those observed in β-TI patients (p<0.03) (Fig. 4A,B). It should be noted that in preliminary experiments we attempted to determine the enzyme mass in the supernatants and in cell lysates using the PLAC test. However, we were not able to detect any mass in our samples possibly due to the fact that the Lp-PLA₂ mass was lower than the detection limit of this kit which is 1.2 ng/ml (24).

**Paraoxonase-1 activities and 8-epiPGF2a levels**

The serum paraoxonase-1 (PON1) activities towards paraoxon as well as towards phenylacetate were significantly lower in both patient groups compared with controls whereas no difference was observed between patient groups (Table 3). Furthermore, both patient groups had significantly higher levels of serum and urine 8-isoprostane (8-epiPGF2a), an established marker of lipid peroxidation, compared with controls, the β-TM patients having significantly higher levels of 8-epiPGF2a compared with β-TI patients (Table 3).
Discussion

The present study shows for the first time that patients with β-thalassemia exhibit very high plasma levels of Lp-PLA2 mass and activity, these parameters being significantly higher in β-TM compared with β-TI patients. Major determinants of Lp-PLA2 levels in plasma are the LDL levels as well as the rate of LDL clearance from the circulation (18, 26). In the present study both patient groups exhibit low LDL-cholesterol levels, thus this is the first report for a population with low LDL-cholesterol levels that exhibits so high plasma levels of Lp-PLA2. Notably, the LDL-cholesterol or the total cholesterol levels in thalassemic patients are not correlated with enzyme mass or activity, in contrast to that expected based on the results from most published to date studies, including those from our group (18, 26). As we have previously shown, the majority of the LDL-associated Lp-PLA2 is bound to atherogenic sdLDL particles in both normolipidemic and dyslipidemic population (18, 22, 26, 29). Our results revealed that both patient groups are characterized by a predominance of sdLDL particles, a finding that is consistent with their elevated fasting triglyceride levels (30). Furthermore, sdLDL particles (LDL-5) carry the majority of LDL-associated Lp-PLA2. Thus the predominance of sdLDL particles enriched in Lp-PLA2 in plasma of β-thalassemic patients could contribute to the high enzyme mass and activity observed in these patients despite the low LDL-cholesterol levels (Fig. 5).

The increased hemolysis occurring in β-thalassemia could also contribute to the elevated plasma enzyme observed in these patients. However this hypothesis is unlikely since neither the method used to determine the enzyme mass in plasma detects the
erythrocyte PAF-acetylhydrolase in the lysates of these cells, despite the relatively high enzyme activity, nor the plasma enzyme in our patients exhibits any sensitivity to the treatments with sulfhydryl reagents or proteases in contrast to the erythrocyte enzyme. A source of Lp-PLA₂ in plasma is platelets that secrete this enzyme associated with PMPs (25). Platelets in β-thalassemia undergo a state of oxidative stress, leading to their activation (31). Thus platelets could contribute to the elevation in plasma Lp-PLA₂ of our patients since they exhibit increased oxidative stress as it is indicated by the elevation of serum and urine 8-epiPGF2a, an established marker of lipid peroxidation, which is correlated with Lp-PLA₂ activity and mass. However the above hypothesis is unlikely since the Lp-PLA₂ activity associated with PMPs in plasma of our patients is not different compared with controls.

In contrast to platelets, monocytes/macrophages, the major cellular source of plasma Lp-PLA₂ may significantly contribute to the elevation in plasma enzyme observed in β-thalassemia since an increased Lp-PLA₂ production and secretion from monocytes in culture is observed in both patient groups (Fig. 5). This phenomenon could be at least partially due to the increased oxidative stress that has been attributed to the high plasma iron turnover observed in patients with β-thalassemia (32, 33). Indeed, the plasma ferritin levels, a measure of body and macrophage iron content and a potent pro-oxidant, are a potential modulator of Lp-PLA₂ activity in plasma (34). Consistent to the above suggestion are the results of the present study showing that the increased plasma ferritin levels in β-thalassemic patients are strongly correlated with Lp-PLA₂ activity and mass.

Patients with β-thalassemia exhibit increased inflammation (32) that may also contribute to the elevation in plasma Lp-PLA₂. In this regard it has been reported that the
only proinflammatory mediator that stimulates the expression and secretion of Lp-PLA₂ by monocyte/macrophages is PAF (35). As we (36) and others (37) have shown, PAF and structurally related oxidized phospholipids are formed during LDL oxidation; these molecules being elevated in patients with increased oxidative stress (38). Increased plasma levels of various lipid peroxidation products and marked LDL oxidative modification have been reported in thalassemic patients (33). Thus a contributory role in the enhanced secretion of Lp-PLA₂ from monocytes/macrophages observed in our patients may be played by proinflammatory phospholipids such as PAF and structurally related oxidized phospholipids formed under conditions of increased oxidative stress, a hypothesis that needs further investigation (Fig 5). Some of the β-thalassemic patients exhibit liver disease or hypothyroidism that might have influenced the plasma Lp-PLA₂ levels. However when we excluded these patients from the statistical analysis, the differences in Lp-PLA₂ levels among studied groups were not altered (data not shown). Finally, differences in genetic expression of Lp-PLA₂ between patients with β-thalassemia and controls that may lead to increased Lp-PLA₂ expression and secretion from patient monocytes may not be excluded (Fig 5).

An important observation of our study is that the specific activity of Lp-PLA₂, ie the activity expressed per enzyme mass, is lower in our patients compared with controls. Since the enzyme is susceptible to oxidative inactivation (reviewed in Ref. 1), the decreased Lp-PLA₂ specific activity could be attributed to the increased oxidative stress observed in these patients. Hence, we may suggest that the enhanced production and secretion of new active enzyme from monocytes/macrophages could counteract the
oxidative stress-induced partial enzyme inhibition, thereby resulting in an overall increase in plasma Lp-PLA\textsubscript{2} activity.

Similarly to the total plasma Lp-PLA\textsubscript{2}, the HDL-Lp-PLA\textsubscript{2} is also elevated in both patient groups, despite the low HDL-cholesterol levels and it could also be primarily attributed to the enhanced enzyme secretion from monocytes/macrophages. Furthermore, the specific HDL-Lp-PLA\textsubscript{2} activity is decreased possibly due to its partial inactivation from the increased oxidative stress observed in these patients. The increased oxidative stress could also be responsible for the reduced PON1 activities observed in our patients. Indeed like Lp-PLA\textsubscript{2}, PON1 is also susceptible to oxidative inactivation (39).

The findings of the present study may be clinically important since patients with β-thalassemia exhibit endothelial dysfunction, premature carotid atherosclerosis (16) and high incidence of stroke despite their very uncommon rates of coronary artery disease (13, 16). Lp-PLA\textsubscript{2} is independently associated with endothelial dysfunction (40) and is highly expressed in carotid artery plaques (41). The significant correlation of plasma Lp-PLA\textsubscript{2} concentrations with carotid artery IMT values found in our β-TM patients, implies that this enzyme may be implicated in premature carotid atherosclerosis observed in β-thalassemia. Clinical studies have demonstrated that increased plasma Lp-PLA\textsubscript{2} levels are associated with high incidence of stroke (42). Our β-thalassemic patients exhibit high plasma Lp-PLA\textsubscript{2} levels but low LDL-cholesterol levels and low atherogenic LDL-cholesterol / HDL-cholesterol ratio. They are also presented with high levels of the antiatherogenic HDL-Lp-PLA\textsubscript{2} and the ratio of HDL-Lp-PLA\textsubscript{2} to total plasma enzyme, which could be a potential antiatherogenic marker in patients with dyslipidemia (18). Considering that dyslipidemias have long been associated with coronary artery disease,
but not with cerebrovascular disease (43), and that carotid artery disease could be a possible cause of stroke in cardiac disease-free patients with β-thalassemia (44), we may suggest that the high plasma Lp-PLA₂ levels in the presence of a relatively antiatherogenic cholesterol profile may contribute to premature carotid atherosclerosis and high incidence of stroke observed in patients with β-thalassemia (13, 16) but not to coronary artery disease (Fig. 5).

In conclusion, patients with β-thalassemia exhibit high plasma Lp-PLA₂ levels, which could be primarily attributed to increased enzyme production and secretion from monocytes/macrophages as well as to the predominance of sdLDL particles in plasma. This phenomenon may be a feature characteristic of β-thalassemia since patients with other types of anemia such as sickle cell anemia have similar with controls Lp-PLA₂ activity (44). The high plasma Lp-PLA₂ levels in the presence of a relatively antiatherogenic cholesterol profile may contribute to premature carotid atherosclerosis but not to coronary artery disease in patients with β-thalassemia, a hypothesis that needs further investigation.

References


associated phospholipase A(2) in carotid artery plaques predicts long-term cardiac outcome. *Eur Heart J* **30**: 2930-2938.


Figure Legends

Figure 1. Correlations between serum ferritin levels and Lp-PLA<sub>2</sub> activity (A) or mass (B) as well as between serum LDL-cholesterol levels and Lp-PLA<sub>2</sub> activity (C) or mass (D) in patients with β-thalassemia.

Figure 2. Bar graphs illustrating the Lp-PLA<sub>2</sub> activity (A) and mass (B) associated with apoB-containing lipoprotein subspecies in patients with β-thalassemia. Lipoprotein subspecies were fractionated by isopycnic density gradient ultracentrifugation. Enzymatic activity was determined by the trichloroacetic acid precipitation procedure and enzyme mass by use of a dual monoclonal antibody immunoassay standardized to recombinant Lp-PLA<sub>2</sub>. Values represent the mean ± SD. *p<0.005 compared with controls and #p<0.02 compared with β-TI patients.

Figure 3. A. Representative flow cytometric dot plot illustrating the PMPs in plasma of β-TM patients. PMPs were labeled with annexin-V-PE, and anti-CD41a-FITC. B. Lp-PLA<sub>2</sub> activity associated with PMPs in plasma of patients with β-thalassemia. ELISA plates were coated with the monoclonal antibody anti-CD61 or the isotype-matched mouse monoclonal IgG. Coating was performed overnight at 4°C. One hundred μl of the plasma-containing microparticles, were placed into the wells and incubated for 2 h at 37°C. The plate was washed three-times with a HEPES buffer supplemented with 0.01% EDTA (Lp-PLA<sub>2</sub> assay buffer) and then Lp-PLA<sub>2</sub> assay was performed in each well using the trichloroacetic acid precipitation procedure. Data represent the mean ± SD.
Figure 4. Increased spontaneous production and secretion of Lp-PLA$_2$ activity from peripheral blood monocytes in culture, in patients with β-thalassemia. Peripheral blood monocytes from each participant in the present study were cultured and grown as described in the materials and methods section. Cultures were performed in duplicate for each donor. Total (A) and secreted (B) Lp-PLA$_2$ activity was determined at 48 hours of culture by the trichloroacetic acid precipitation procedure. Lines represent the mean from all donors of each studied group.

Figure 5. Possible mechanisms underlining the increase of plasma Lp-PLA$_2$ in patients with β-thalassemia. Relationship to atherosclerotic diseases. β-thalassemia is characterized by increased inflammation and oxidative stress, increased plasma levels of ferritin and sdLDL as well as by increased hemolysis. The increased oxidative stress induces (possibly through the generation of PAF and oxidized phospholipids) platelet and monocyte/macrophage activation. The later may be also attributed to the increased ferritin levels and possibly to unknown yet genetic factors. The activated monocyte/macrophages as well the increased levels of sdLDL but not the oxidative stress induces platelet activation or the increased hemolysis may account for the increased plasma levels of Lp-PLA$_2$ observed in β-thalassemia despite the relatively antiatherogenic cholesterol profile (low LDL-cholesterol levels and low ratio of LDL-cholesterol/HDL-cholesterol). The high plasma Lp-PLA$_2$ levels in the presence of a relatively antiatherogenic cholesterol profile in patients with β-thalassemia may contribute to premature carotid atherosclerosis and cerebrovascular disease but not to coronary artery disease.
Table 1. Clinical and biochemical parameters of the study groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=30)</th>
<th>β-TI (n=25)</th>
<th>β-TM (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>33±9</td>
<td>36±12</td>
<td>27±7</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>16 (53)</td>
<td>16 (64)</td>
<td>17 (49)</td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td>1.71±0.19</td>
<td>1.67±0.16</td>
<td>1.66±0.18</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>111±12</td>
<td>111±20</td>
<td>106±13</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>67±8</td>
<td>67±12</td>
<td>68±7</td>
</tr>
<tr>
<td>Heart rate (beats/minute)</td>
<td>74±10</td>
<td>80±8</td>
<td>74±10</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>14 (40)</td>
<td>13 (52)</td>
<td>14 (40)</td>
</tr>
<tr>
<td>Hb, pre-transfusional (mg/dl)</td>
<td>13.6±1.4</td>
<td>9.3±1.0*</td>
<td>10.3±0.9*</td>
</tr>
<tr>
<td>Serum ferritin over the last year (ng/ml)</td>
<td>122±65$^§$</td>
<td>1568±1343*</td>
<td>1975±1635*</td>
</tr>
<tr>
<td>Splenectomy, n (%)</td>
<td>0</td>
<td>10 (40)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>Hepatitis C, active or healed, n (%)</td>
<td>0</td>
<td>4 (16)</td>
<td>12 (34)</td>
</tr>
<tr>
<td>History of hypothyroidism, n (%)</td>
<td>0</td>
<td>12 (48)</td>
<td>15 (42)</td>
</tr>
<tr>
<td>History of hypoparathyroidism, n (%)</td>
<td>0</td>
<td>8 (32)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>History of hypogonadism, n (%)</td>
<td>0</td>
<td>9 (36)</td>
<td>14 (40)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>94±14</td>
<td>98±12</td>
<td>99±13</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/l)</td>
<td>19±6</td>
<td>31±11**</td>
<td>41±22***#</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/l)</td>
<td>21±11</td>
<td>44±21**</td>
<td>54±39***#</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>196±41</td>
<td>144±49*</td>
<td>120±34*</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dl)</td>
<td>130±40</td>
<td>70±36*</td>
<td>61±27*</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>51±14</td>
<td>31±11*</td>
<td>32±10*</td>
</tr>
<tr>
<td>LDL-Cholesterol / HDL-Cholesterol</td>
<td>2.5±0.4</td>
<td>2.2±0.3*</td>
<td>1.9±0.2*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>82±39</td>
<td>204±80*</td>
<td>155±61*</td>
</tr>
<tr>
<td>Lp(a) (mg/dl)</td>
<td>8 (1-24)</td>
<td>10 (4-28)</td>
<td>6 (1-26)</td>
</tr>
<tr>
<td></td>
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<tr>
<td>--------------------------</td>
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<td>-------</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.3±0.9</td>
<td>2.4±0.8**</td>
<td>3.3±1.5**#</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>1.8±0.7</td>
<td>2.6±0.9**</td>
<td>3.5±1.5**#</td>
</tr>
<tr>
<td>Total plasma Lp-PLA₂ activity (nmol/ml/min)</td>
<td>43±5</td>
<td>88±17*</td>
<td>113±20*#</td>
</tr>
<tr>
<td>Total plasma Lp-PLA₂ mass (ng/ml)</td>
<td>250±53</td>
<td>665±99*</td>
<td>869±96*#</td>
</tr>
<tr>
<td>HDL-Lp-PLA₂ activity (nmol/ml/min)</td>
<td>3.8±0.8</td>
<td>12.9±4.9*</td>
<td>18.1±4.8*#</td>
</tr>
<tr>
<td>HDL-Lp-PLA₂ mass (ng/ml)</td>
<td>84±21</td>
<td>331±36*</td>
<td>476±45*#</td>
</tr>
</tbody>
</table>

Values are means ± SD, except for Lp(a) which represent the median (range). *p<0.001 and **p<0.01 compared with controls, #p<0.01 compared with β-TI patients.

§ Single measurement for each control subject

β-TI; β-thalassemia intermedia, β-TM; β-thalassemia major, IL-6; interleukin-6, LDL; low-density lipoprotein, HDL; high-density lipoprotein, Lp-PLA₂; lipoprotein-associated phospholipase A₂, HDL-Lp-PLA₂; HDL-associated Lp-PLA₂, TNF-α; tumor necrosis factor-α
**Table 2.** ApoB-containing lipoprotein subclasses in patients with β-thalassemia and controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>β-TI</th>
<th>β-TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL-cholesterol, mmol/l</td>
<td>1.2 ± 0.2</td>
<td>1.6 ± 0.3*</td>
<td>1.7 ± 0.3*</td>
</tr>
<tr>
<td>IDL-cholesterol, mmol/l</td>
<td>1.7 ± 0.3</td>
<td>1.3 ± 0.2*</td>
<td>1.2 ± 0.3*</td>
</tr>
<tr>
<td>Buoyant LDL-cholesterol, mmol/l</td>
<td>3.6 ± 0.7</td>
<td>2.8 ± 0.8*</td>
<td>2.6 ± 0.9*</td>
</tr>
<tr>
<td>sdLDL-cholesterol, mmol/l</td>
<td>0.20 ± 0.08</td>
<td>0.43 ± 0.10§</td>
<td>0.52 ± 0.30§</td>
</tr>
<tr>
<td>sdLDL proportion, %</td>
<td>3.6 ± 0.9</td>
<td>9.5 ± 2.1§</td>
<td>12.0 ± 4.1§</td>
</tr>
<tr>
<td>Mean LDL size, Å</td>
<td>269 ± 4</td>
<td>259 ± 3§</td>
<td>258 ± 4§</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD values. *p<0.01 and §p<0.001 compared with control values.

VLDL; very low-density lipoprotein, IDL; intermediate-density lipoprotein, LDL; low-density lipoprotein, sdLDL; small-dense LDL
Table 3. PON1 activities and 8-epiPGF2a levels in the study population

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>β-TI</th>
<th>β-TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1 activity towards paraoxon (U/l)</td>
<td>77.4±35.1</td>
<td>37.8±29.1*</td>
<td>51.7±28.7*</td>
</tr>
<tr>
<td>PON1 activity towards phenylacetate (U/ml)</td>
<td>66.6±7.3</td>
<td>39.4±3.6*</td>
<td>54.1±8.7*</td>
</tr>
<tr>
<td>Serum 8-epiPGF2a (pg/ml)</td>
<td>75.2±27.3</td>
<td>98.1±30.2*</td>
<td>125.0±35.8§#</td>
</tr>
<tr>
<td>Urine 8-epiPGF2a (ng/mg creatinine)</td>
<td>0.48±0.16</td>
<td>0.65±0.22*</td>
<td>0.80±0.29§#</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD values. *p<0.01 and §p<0.001 compared with control values. #p<0.001 compared with β-TI values.
FIGURE 1

A  $r = 0.521, p < 0.01$

B  $r = 0.596, p < 0.01$

C  $r = 0.440, p < 0.01$

D  $r = 0.463, p < 0.01$
FIGURE 2

A

![Graph showing Lp-PLA₂ Activity (nmol/mg Protein/min)]

- Control
- β-TI
- β-TM

B

![Graph showing Lp-PLA₂ Mass (ng/mg Protein)]

- Control
- β-TI
- β-TM
FIGURE 3

Lp-PLA₂ Activity
(nmol / ml / h)

Control
β-TI
β-TM

CD41a-FITC
Annexin-V-PE

A

B
FIGURE 5

β-Thalassemia

- Inflammation
- Oxidative stress
- Ferritin
- Genetic determinants
- sdLDL
- Haemolysis

↑ Ferritin
↑ Oxidative stress
↑ PAF, ↑ Oxidized phospholipids

Monocytes/Macrophages

Platelets

↑ Lp-PLA₂
↑ Plasma Lp-PLA₂, ↑ HDL-Lp-PLA₂,
↑ HDL-Lp-PLA₂/Plasma Lp-PLA₂

Antiatherogenic cholesterol profile
Other Factors?

Low rate

Progression

Low rate

Carotid Atherosclerosis

Cerebrovascular disease

Cardiovascular disease