Lipoprotein(a) Associated Molecules are Prominent Components in Plasma and Valve Leaflets in Calcific Aortic Valve Stenosis

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METHODS

Materials

Synthetic standards 1,2-dinonanoyl-sn-glycero-3-phosphocholine (DNPC), 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), 1-Palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC), 1-palmitoyl-2-(9-oxo)nonanoyl-sn-glycero-3-phosphocholine (PONPC), 1-heptadecanoyl-snglycero-3-phosphate (17:0 LysoPA), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate ,1-stearoyl-2-hydroxy-sn-glycero-3-phosphate and 1-arachidonoyl-2-hydroxy-sn-glycero-3-phosphate were obtained from Avanti Polar Lipids (Alabaster, AL). 1-(palmitoyl)-2-(5-keto-6-octene-dioyl)-3-phosphocholine (KOdiAPC), 1-palmitoyl-2-(4-keto-dodec-3-ene-dioyl)-sn-glycero-3-phosphocholine (KDdiAPC) were purchased from Cayman Chemicals (Ann Arbor, MI). All solvents were HPLC grade.

Antibodies to Lp(a), Autotaxin and Oxidation-Specific Epitopes

LPA4 is a murine monoclonal IgG antibody to apo(a) that was generated by immunizing mice with the apo(a) sequence TRNYCRNPDAEIRP. This sequence is present as one copy on KIV_5, KIV_7, and KIV_8 of apolipoprotein(a), and does not cross-react with plasminogen (1). MB47 is a murine IgG monoclonal antibody that binds to apoB-100 (2). E06 is a monoclonal IgM natural murine antibody that binds to the phosphocholine head group of oxidized, but not native, phospholipids (3). MDA2 is a murine IgG monoclonal antibody that recognizes the malondialdehyde(MDA)-lysine epitopes on modified proteins and lipid adducts (4,5). All of these antibodies have been previously used in immunostaining murine, rabbit and human atherosclerotic tissues (6-8). An alkaline phosphatase labeled goat anti-human autotaxin was purchased from Life Technologies (Carlsbad, CA).

Novel Chemiluminescent ELISA to Detect Lipoprotein-Associated Autotaxin

A sensitive and quantitative sandwich-based chemiluminescent enzyme-linked immunosorbent assay (ELISA) was used to measure autotaxin associated with plasma lipoproteins containing apoB-100 (ATX-apoB) and Lp(a) (ATX-apo(a)). 96-well microtiter plates were coated overnight at 4°C with antibodies MB47 to bind apoB-100 and LPA4 to bind Lp(a) (40µl of antibody at 5µg/ml were added to each well). Excess material was washed off and the plates blocked with 1% tris buffered saline/bovine serum albumin (TBS/BSA) for 45 minutes. Conditions were established so that the amount of patient plasma added to each well was sufficient to ensure that a saturating and equal amount of each lipoprotein was captured in

each well. After the plates were washed, EDTA plasma was added at 1:50 dilution (40 µl/well) for 75 minutes to bind apoB-100, Lp(a) and HDL, respectively. After the plates were again washed, goat anti-human autotaxin antibody at 1 µg/ml, 40 µl/well, was incubated with the plates for 60 minutes. After washing excess material off the plates, alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma, St Louis, MO) (40 µl/well) was added for 60 min. After a final washing, Lumi-phos 530 (Lumigen, Inc., Southfield, Michigan) (25 µl/well) was added for 75 minutes and luminescence read on a Dynex luminometer (Chantilly, Virginia). The results are reported as relative light units (RLU) in 100 milliseconds after the background (TBS/BSA) RLU counts were subtracted. High and low values were added to each 96-well plates as internal controls.

Oxidized Phospholipids

Oxidized phospholipids on apoB (OxPapoB) and OxPL on apolipoprotein(a) (OxPL-apo(a) were measured as previously described (9-11).

Patients with CAVS

The first group was comprised of 68 patients undergoing AVR for symptomatic CAVS with prospective collection of aortic valve leaflets. This cohort of subjects and the protocols for valve collection was recently described (12). Concomitant blood samples were not available in these patients. Briefly, formalin-fixed non-rheumatic aortic valves with varying degrees of macroscopic disease were analyzed for the presence of apolipoprotein(a), autotaxin, oxidation-specific epitopes and macrophages (6,8). The second group was comprised of 14 patients with mild-moderate CAVS who had available EDTA plasma samples that had been stored frozen at -70°C. They were derived from the ASTRONOMER trial (10) and were chosen randomly for measurement of lipoprotein-associated autotaxin levels. The third group was comprised of 4 patients who underwent AVR and who were recruited for analysis of valve leaflets for the presence of specific species of OxPL and LysoPA by mass spectrometry. All patients gave written informed consent and the local ethics committees at each institution gave approval for use of human biological samples. Echocardiography was used to calculate peak aortic jet velocity (V_{peak}), from which was derived mean and peak gradients and aortic valve area, as previously described (10,13).

Histological and Immunohistochemical Analysis of Valve Leaflets

Although all patients had clinical diagnosis of CAVS, there was pathological variability in the extent of involvement of aortic valve leaflets. For this reason, valve leaflets were classified pathologically as Grade 1 to 4, with a pathological classification as previously described (12,14). Leaflets were sectioned vertically for histological and immunohistochemical analysis. Paraffin-embedded specimens were stained with Elastica van Gieson (Merck Millipore) to illustrate the layered architectural pattern and with alizarin red S to determine calcific deposition of the chosen cusp areas (12). Immunohistochemistry was performed using the Dako REAL En Vision Detection System, rabbit/mouse kit (K5007, Dako Corporation). Serial sections of 3 µm thick sections of paraffin-embedded aortic valve leaflets were deparaffinized and treated with 0.3 % Peroxidase Block (Dako) to block endogenous peroxidase activity, and then blocked with host serum of secondary antibody. After blocking, slides were incubated with primary mouse

monoclonal antibodies LPA4 (IgG, dilution 1:500), EO6 (IgM, dilution 1:400), MDA2 (IgG, dilution 1:1000), ATX (IgG, dilution 1:50) and CD68 (IgG, dilution 1:100) for 60 min. Antigen retrieval of CD68 was achieved by heating the sections in target retrieval solution (Dako) in a steamer for 20 minutes. Application of the primary antibody was followed by the secondary antibody for 30 minutes and the reaction products were revealed by immersing the slides in diaminobenzidine tetrachloride (DAB) to give a brown reaction product. Finally, the slides were counterstained with hematoxylin (Merck Millipore) and mounted. An established scoring system was adopted for visual interpretation of the serial slices to allow semi-quantitative analysis of the data (15). The proportion of the areas stained for EO6 relative to the overlapping area stained for LPA4 (designated as 100%) was estimated and assigned to 1 of 5 scores: 0, <5%; 1, 6% to 25%; 2, 26% to 50%; 3, 51% to 75%; or 4, 76% to 100%. using similar methodology as previously described (12).

Lipid Extraction

Four patients with CAVS underwent AVR. The valve leaflets were immediately placed in an ice-cold solution containing EDTA/PBS and stored at -80°C until analysis. Lipids were extracted from stenotic aortic tissue using a modification of the procedure described by Folch et al. (16,17). Briefly, tissue was pulverized in liquid nitrogen and 100 mg was weighed into a glass extraction tube. Lipids were extracted with 2:1 (v/v) chloroform:methanol (CM) containing 0.01% butylated hydroxytoluene (BHT) and PBS (pH 7.4). The internal standard mixture spiked into each sample prior to extraction consisted of DNPC and 17:0 LysoPA. Sample extraction and centrifugation (3500 rpm at 4°C for 5 min) was performed in triplicate and the combined chloroform extracts were dried under an atmosphere of N_2 (g) and stored in 2:1 CM at -80°C also under N_2 (g).

Liquid Chromatography, Tandem Mass Spectrometry

The separation of PC-containing OxPL (PC-OxPL) was carried out in reverse-phase (RP) chromatography as reported previously (6). Extracted valves were reconstituted in reverse phase (RP) eluent consisting of 60:40 acetonitrile:water, 10 mM ammonium formate and 0.1% formic acid immediately prior to injection. Thirty microliters of the sample was injected onto an Ascentis Express C18 HPLC column (15cm × 2.1mm, 2.7µm; Supelco Analytical, Bellefonte, Pennsylvania, USA) with separation by a Prominence UFLC system from Shimadzu Corporation (Canby, Oregon, USA). Elution was performed using a linear gradient of solvent A (acetonitrile/water, 60:40 v/v) and solvent B (isopropanol/acetonitrile, 90:10, v/v) with both solvents containing 10 mM ammonium formate and 0.1% formic acid. The time program used was as follows: initial solvent B at 32% until 4.00 min; switched to 45% B; 5.00 min 52% B; 8.00 min 58% B; 11.00 min 66% B; 14.00 min 70% B; 18.00 min 75% B; 21.00 min 97% B; 25.00 min 97% B; 25.10 min 32% B. A flow rate of 260 µl/min was used for analysis, and the sample tray and column oven were held at 4 and 45˚C, respectively.

Detection of PC-OxPL compounds was carried out by mass spectrometry in positive polarity mode. MRM scans were performed on 6 transitions using a product ion of 184.3 m/z, corresponding to the cleaved phosphocholine moiety. Six commercially available standards of PONPC, POVPC, PGPC, PAzPC, KOdiAPC, and KDdiAPC were injected and accurate peak assignments were based upon retention times and mass transitions. The mass spectrometry

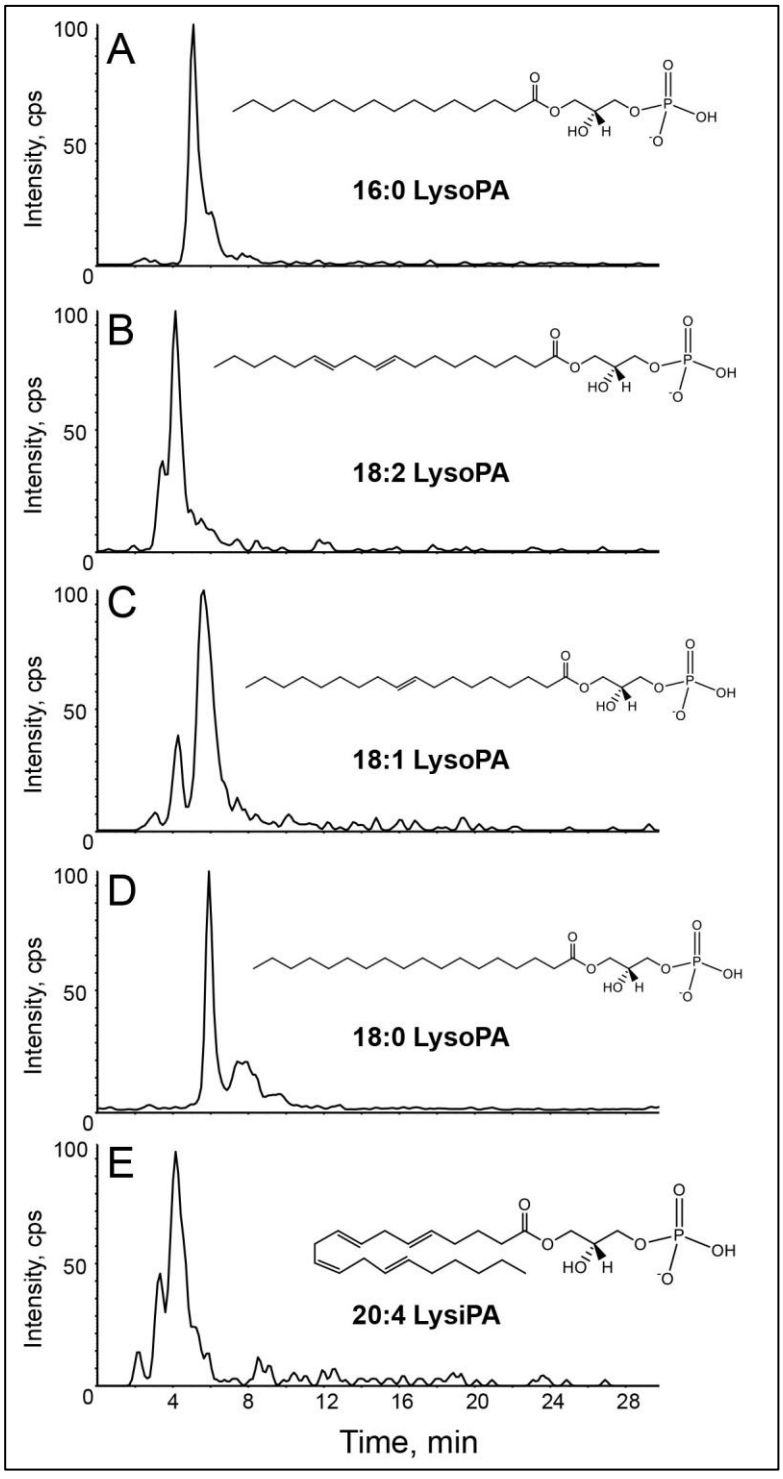
settings were as follows: curtain gas, 26 psi; collision gas, medium; ion spray voltage, 5500 V; temperature, 500.0°C; ion source gas 1, 40.0 psi; ion source gas 2, 30.0 psi; declustering potential, 125 V, entrance potential, 10 V; collision energy, 53 V; collision cell exit potential, 9 V; and dwell time, 50 msec. External mass calibration was performed at regular intervals. For quantitation, multiple reaction monitoring (MRM) calibration curves were made for each of the 6 commercially available PC-OxPL standards and peaks were normalized based on their relative responses. 10 ng of internal standard was added to all samples during extraction. A 4000 QTRAP® triple quadrupole mass spectrometer system with a Turbo V electrospray ion source from AB Sciex (Framingham, Massachusetts, USA) was coupled to the liquid chromatography system.

Separation of the 8 LysoPA species was accomplished using the same chromatographic conditions as described above for PC-OxPL. LysoPA detection was carried out using a modified method of Wijesinghe et al (17). Briefly, detection was carried out in negative polarity mode by MRM of 8 transitions using a product ion of 153.0 m/z, Da, which corresponds to the cleaved glycerol phosphate group. The mass spectrometry settings were as follows: curtain gas, 26 psi; collision gas, medium; ion spray voltage, -4500 V; temperature, 500.0°C; ion source gas 1, 40.0 psi; ion source gas 2, 30.0 psi; declustering potential, -100 V, entrance potential, -10 V; collision energy, -30 V; collision cell exit potential, -20 V; and dwell time, 100 msec. MRM calibration curves for commercially available LysoPA species was used for quantitation. Data was collected utilizing Analyst® Software 1.6 (AB Sciex) and MultiQuant® Software 2.1 (AB Sciex) was used to compare peak areas of internal standards with unknown analytes for quantitation purposes.

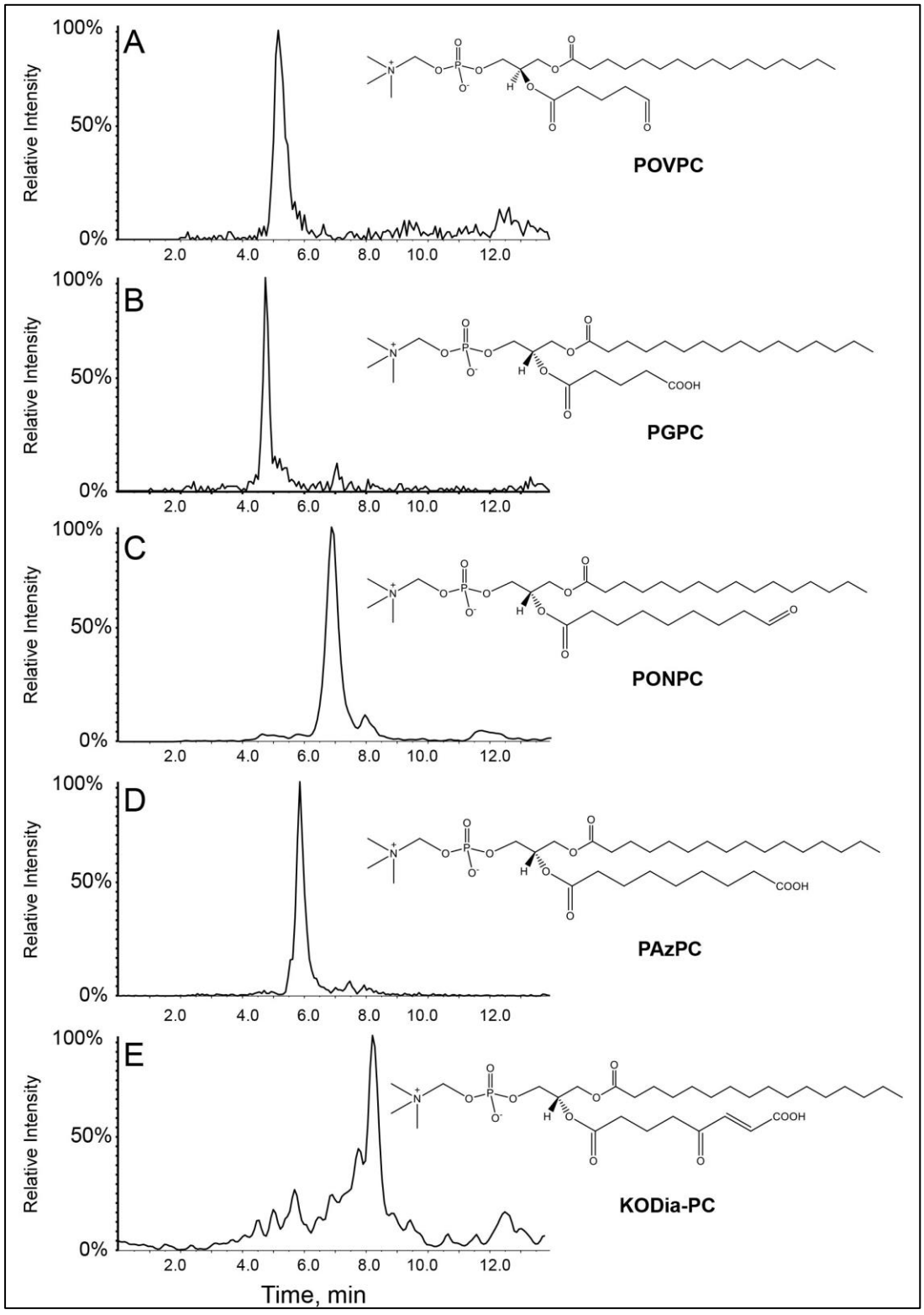
Statistical Analysis

Continuous variables are given as mean (SD) or median (interquartile range) and categorical variables as number (%). Statistical analyses of the immunohistochemical semiquantitative scoring system and of epitope measurement were performed by the Spearman correlation. Resulting p-values were adjusted for multiple testing by the Bonferroni-Holm procedure. Spearman's test was also used to test the correlation between ATX-apoB and ATX-apo(a). Statistical tests were two-sided and P-values <0.05 were considered statistically significant. All statistical tests were performed using Graph Pad Prism 5.04.

Supplementary Figure 1. Single ion MRM chromatogram of the lysophosphatidic acid (LysoPA) species extracted from human stenotic aortic valves. (A) 16:0 LysoPA, (B) 18:2 LysoPA, (C) 18:1 LysoPA, (D) 18:0 LysoPA, and (E) 20:4 LysoPA.



Supplementary Figure 2. Single ion MRM chromatogram of the most abundant fragmented PC-OxPL in human stenotic aortic valves based on known standards. (A) POVPC, (B) PGPC, (C) PONPC, (D) PAzPC and (E) KODiaPC.



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