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Elevated Expression of Lipoprotein-Associated Phospholipase A2 in Calcific Aortic Valve Disease

Implications for Valve Mineralization

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Objectives This study sought to document the presence and role of lipoprotein-associated phospholipase A2 (Lp-PLA2) in calcific aortic valve disease (CAVD).

Background CAVD is a chronic disorder characterized by pathological mineralization and remodeling. Studies have indicated that human CAVD tissues are infiltrated by lipids and that inflammation may play a role in the pathobiology. We hypothesized that Lp-PLA2 (encoded by the *PLA2G7* gene) is expressed in CAVD and may play a role in the mineralization of valve interstitial cells.

Methods We have documented the expression of the phospholipase A2 family of genes in aortic valves by using a transcriptomic assay. Messenger ribonucleic acid and protein expression were confirmed in aortic valves explanted from 60 patients by quantitative polymerase chain reaction and immunohistochemistry, respectively. The effect of lysophosphatidylcholine, the product of Lp-PLA2 activity, was documented on the mineralization of valve interstitial cell cultures.

Results Transcriptomic analyses of CAVD and control nonmineralized aortic valves revealed that Lp-PLA2 was increased by 4.2-fold in mineralized aortic valves. Higher expression of Lp-PLA2 in stenotic aortic valves was confirmed by quantitative polymerase chain reaction, immunohistochemistry, and enzymatic Lp-PLA2 activity. The number of Lp-PLA2 transcripts correlated with several indexes of tissue remodeling. In vitro, lysophosphatidylcholine increased the expression of alkaline phosphatase, the ectonucleotide pyrophosphatase/phosphodiesterase 1 enzyme, sodium-dependent phosphate cotransporter 1 (encoded by the *SLC20A1* gene), and osteopontin. We then showed that lysophosphatidylcholine-induced mineralization involved ectonucleotidase enzyme as well as apoptosis through a protein-kinase-A-dependent pathway.

Conclusions Together, these results demonstrated that Lp-PLA2 is highly expressed in CAVD, and it plays a role in the mineralization of valve interstitial cells. Further work is necessary to document whether Lp-PLA2 could be considered as a novel target in CAVD. (J Am Coll Cardiol 2014;63:460–9) © 2014 by the American College of Cardiology Foundation

Calcific aortic valve disease (CAVD) is a chronic and multifactorial disorder, which is characterized by an abnormal mineralization of aortic leaflets (1). Processes leading to the ectopic mineralization of the valvular tissue may involve lipid-derived factors (2,3).

Phospholipase A2 (PLA2), encoded by the *PLAG* family of genes, are important enzymes that hydrolyze

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the sn-2 ester bond of phospholipids and generate free fatty acids and lysophosphatidylcholine (LPC), which, in turn, modulate inflammation (4,5). Recently, LPC has been shown to promote mineralization of vascular smooth muscle cells (6). However, the mechanism whereby LPC promotes mineralization has not yet been clearly elucidated.

Pathological mineralization of valve interstitial cells (VIC), the main cellular component of the aortic valve, relies on the expression of ectonucleotidases, membrane-bound enzymes, that regulate the amount of nucleotides, inorganic phosphate (Pi), and inorganic pyrophosphate (PPi) (7). Pi-mediated mineralization and expression of ectonucleotidases, such as ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) and alkaline phosphatase (ALP), relies on the protein kinase A (PKA) pathway (8). In this work, we hypothesized that the PLA2 (*PLA2G*) family of genes is differentially regulated in human CAVD tissues and is associated with the remodeling and mineralization of the aortic valve.

Methods

Patients. We examined 40 aortic valves that were explanted from patients at the time of aortic valve replacement for CAVD. Control noncalcified aortic valves (n = 20) with normal echocardiographic analyses were obtained during heart transplant procedures. Patients with a history of rheumatic disease, endocarditis, and inflammatory diseases were excluded. Valves with moderate to severe aortic valve regurgitation (grade >2) were excluded. Patients with reduced left ventricular ejection fraction (<40%) were also excluded to eliminate potential patients with low-flow, low-gradient aortic stenosis in whom correlations with the gradient would not be reliable. All patients underwent a comprehensive Doppler echocardiographic examination pre-operatively. Doppler echocardiographic measurements were performed, including the left ventricular stroke volume and transvalvular gradients, using the modified Bernoulli equation. The protocol was approved by the local ethical committee, and informed consent was obtained from the subjects.

Tissue processing, microarray, and experiments. See the Supplemental Methods section in the Online Appendix.

Results

Transcriptomic of phospholipase A2 genes in calcific aortic valves. A tissue-based microarray experiment was conducted to explore the gene expression pattern of phospholipase A2 (PLA2/*PLA2G*) family of genes. Two control valves failed quality control and were discarded from subsequent analyses. Figure 1A shows a heat map of normalized expression values for PLA2/*PLA2G* genes for each sample. Fifteen probe sets were available to study the expression of *PLA2G3, PLA2G4A, PLA2G5, PLA2G6, PLA2G7, PLA2G10, PLA2G12A,* and *PLA2G12B* genes. The expression of *PLA2G4A* and *PLA2G5* was significantly down-regulated in stenotic valves. In contrast, the expression of *PLA2G7* (lipoprotein-associated phospholipase A2 [Lp-PLA2]) was increased by 4.2-fold in stenotic aortic valves (Fig. 1A). *PLA2G4A* and *PLA2G5,* respectively, are cytosolic and secreted enzymes that hydrolyze membrane phospholipids. On the other hand, Lp-PLA2 (*PLA2G7*) is a cellular enzyme that is also secreted, which can metabolize oxidized low-density lipoprotein (ox-LDL) into LPC, a powerful inflammatory metabolite. The result of the microarray experiment was then confirmed with quantitative polymerase chain reaction analyses in a larger group of patients (Table 1). Compared with control noncalcified aortic valves, the amount of Lp-PLA2 transcript was increased in stenotic aortic valves by 2.3-fold (Fig. 1B). Considering the age differences between patients with CAVD and control subjects, we have repeated the analyses after matching 20 patients for age (±1 year) (Table 2). In this analysis, we found in age-matched groups that patients with CAVD, when compared with control subjects, had a 3.5-fold increase in messenger ribonucleic acid encoding for Lp-PLA2 (control: 15.3 ± 3.9 copies/cyclophilin A vs. CAVD: 53.6 ± 17.0 copies/cyclophilin A; p = 0.03). Similarly, the activity of the Lp-PLA2 enzyme was increased by 1.9-fold in stenotic aortic valve tissues versus control nonmineralized valves (Fig. 1C). Within the blood plasma, the activity of Lp-PLA2 was significantly elevated in CAVD patients versus control subjects (Fig. 1D). In the age-matched subjects, the plasma Lp-PLA2 activity was also significantly elevated in patients with CAVD (control: 25.7 ± 1.6 nmol/min/ml vs. CAVD 33.7 ± 1.6 nmol/min/ml; p = 0.001). However, enzyme activity in aortic valve and blood plasma did not correlate significantly (r = −0.10; p = 0.75). We then documented by thin layer chromatography that LPC, the product of Lp-PLA2 activity, is present in stenotic valves (mean concentration: 177 ± 23 ng/g of tissue) (Fig. 1E).

Relationships between the expression of Lp-PLA2 and ox-LDL. Immunohistochemistry studies confirmed a higher expression of Lp-PLA2 within stenotic valves (Figs. 2A and 2B). We found that immunostaining of Lp-PLA2 in control nonmineralized aortic valves was faint or non-detectable (Fig. 2A), whereas there was a strong staining in stenotic aortic valves (Figs. 2B and 2C). Two distinct

Abbreviations and Acronyms

ALP = alkaline phosphatase
cAMP = cyclic adenosine monophosphate
CAVD = calcific aortic valve disease
ENPP1 = ectonucleotide pyrophosphatase/phosphodiesterase 1
LPC = lysophosphatidylcholine
Lp-PLA2 = lipoprotein-associated phospholipase A2 (encoded by *PLA2G7* gene)
OPN = osteopontin
ox-LDL = oxidized low-density lipoprotein
Pi = inorganic phosphate
Pit-1 = sodium-dependent phosphate cotransporter 1 (encoded by *SLC20A1* gene)
PKA = protein kinase A
PPi = inorganic pyrophosphate
VIC = valve interstitial cells

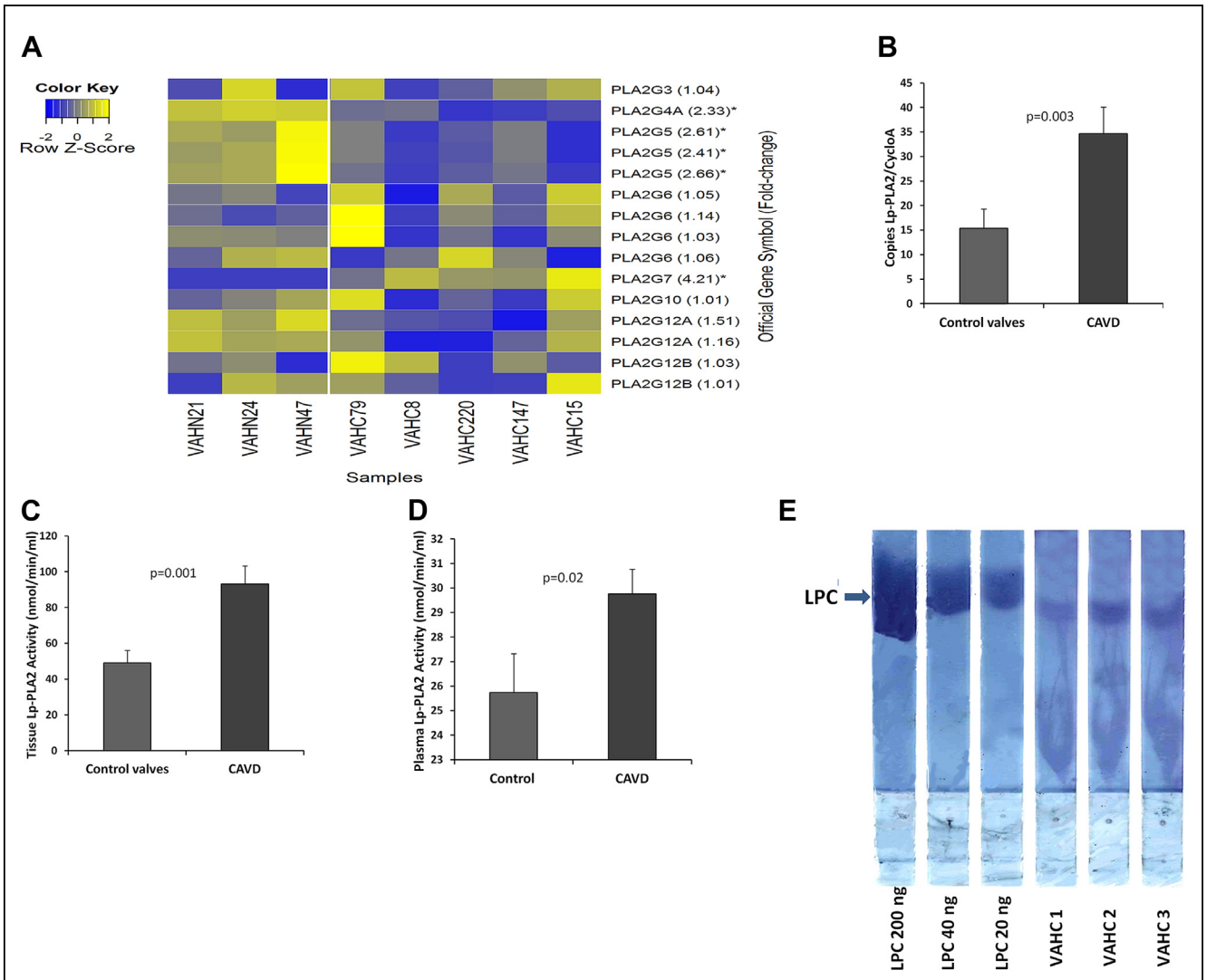

Figure 1 **Expression of Lp-PLA2 in CAVD**

(A) Gene expression profiles of the phospholipase A2 (PLA2)/*PLA2G* family of genes in control, noncalcified aortic valves (VAHN21, VAHN24, and VAHN47) and stenotic aortic valves (VAHC79, VAHC8, VAHC220, VAHC147, and VAHC15). Each **row** represents a different probe set tagging a specific enzyme, indicated at the **right**. The fold-change comparing the expression of calcific aortic valve disease (CAVD) versus control, noncalcified aortic valves is indicated in parentheses. The **asterisks** represent probe sets that are significant based on the whole microarray experiment. **(B, C)** In a larger group of patients both the number of lipoprotein-associated phospholipase A2 ([Lp-PLA2] encoded by the *PLA2G7* gene) transcripts and enzyme activity were increased in CAVD tissues versus tissues from control valves. **(D)** Lp-PLA2 activity was increased in the blood plasma of subjects with CAVD. **(E)** A thin layer chromatography showed the presence of lysophosphatidylcholine (LPC) in CAVD valves (VAHC1-3). CycloA = cyclophilin A.

patterns of expression were observed for Lp-PLA2 in stenotic valves. First, Lp-PLA2 was expressed in cellular-rich inflammatory infiltrates, which are abundant in areas of tissue remodeling and mineralization (Fig. 2B). Second, Lp-PLA2 expression appeared as a diffuse immunostaining (Fig. 2C), which colocalized with ox-LDL (Fig. 2D). Ox-LDL is a powerful promoter of Lp-PLA2 expression as well as a substrate for the enzyme. We then measured the blood profile of patients and correlated the results with the amount of Lp-PLA2 transcript within the aortic valve. Among the different blood lipid variables, the number of Lp-PLA2 transcripts within stenotic aortic valves significantly correlated with the blood plasma level of ox-LDL ($r = 0.33$; $p = 0.01$) and low-density lipoprotein (LDL) levels ($r = 0.29$; $p = 0.02$) (Table 3).

Expression of Lp-PLA2 within the aortic valve is related to tissue remodeling and the hemodynamic severity of aortic stenosis. We next documented the relationships between Lp-PLA2 and indexes of CAVD disease activity and severity. The number of Lp-PLA2 transcripts increased with the severity of the remodeling score of stenotic aortic valves (p analysis of variance = 0.002) (Fig. 3A). Also, the number of Lp-PLA2 transcripts correlated with the weight of the aortic valve, a marker of active remodeling that is also related to the severity of aortic stenosis ($r = 0.56$; $p = 0.0009$) (Fig. 3B). In addition, the level of Lp-PLA2 transcripts also correlated with the peak transaortic gradient ($r = 0.56$;

Table 1 Clinical Characteristics of Patients

	Control Valves	CAVD	p Value
Age, yrs	51 ± 3	70 ± 1	<0.0001
Male	78	56	NS
Smoking	11	7	NS
Hypertension	33	67	0.04
Diabetes	11	26	NS
Coronary heart disease	64	49	0.01
Bicuspid aortic valves	0	30	<0.0001
BMI, kg/m^2	27.9 ± 1.5	27.6 ± 0.5	NS
Statins	73	61	NS
Ang II receptor blockers	11	16	NS
Aortic valve area, cm^2	—	0.77 ± 0.03	—
Aortic mean gradient, mm Hg	—	43 ± 2	—
Triglycerides, mmol/l	1.39 ± 0.15	1.40 ± 0.08	NS
LDL, mmol/l	2.42 ± 0.28	2.26 ± 0.12	NS
HDL, mmol/l	1.66 ± 0.37	1.33 ± 0.04	NS
Creatinine, mmol/l	92.2 ± 9.5	92.2 ± 3.42	NS
Creatinine clearance, ml/min	88.7 ± 9.05	63.1 ± 2.8	0.01

Values are mean ± SD or %.
Ang II = angiotensin II; BMI = body mass index; CAVD = calcific aortic valve disease; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NS = not significant.

Table 2 Clinical Characteristics of Age-Matched Patients

	Control Valves	CAVD	p Value
Age, yrs	52 ± 2	53 ± 3	NS
Male	70	70	NS
Smoking	10	20	NS
Hypertension	30	40	NS
Diabetes	10	0	NS
Coronary heart disease	70	20	0.02
Bicuspid aortic valves	0	70	<0.0001
BMI, kg/m^2	27.2 ± 1.5	25.8 ± 0.9	NS
Statins	80	30	0.07
Ang II receptor blockers	10	20	NS
Aortic valve area, cm^2	—	0.80 ± 0.10	—
Aortic mean gradient, mm Hg	—	46 ± 3	—
Triglycerides, mmol/l	1.32 ± 0.16	1.60 ± 0.27	NS
LDL, mmol/l	2.36 ± 0.25	2.42 ± 0.29	NS
HDL, mmol/l	1.55 ± 0.34	1.31 ± 0.13	NS
Creatinine, mmol/l	90.3 ± 8.7	84.4 ± 7.6	NS
Creatinine clearance, ml/min	88.8 ± 8.0	87.5 ± 8.4	NS

Values are mean ± SD or %.
Abbreviations as in Table 1.

p = 0.0004) (Fig. 3C). In the same line, the level of messenger ribonucleic acid transcript encoding Lp-PLA2 was significantly correlated with the amount of calcium measured within CAVD valves (r = 0.27; p = 0.03). However, the enzymatic activity of Lp-PLA2 in CAVD tissues had a better correlation with the concentration of calcium (r = 0.56; p = 0.01) (Fig. 3D).

Lysophosphatidylcholine induces mineralization of the aortic valve through a PKA pathway. LPC is among the main products derived from ox-LDL, following the action

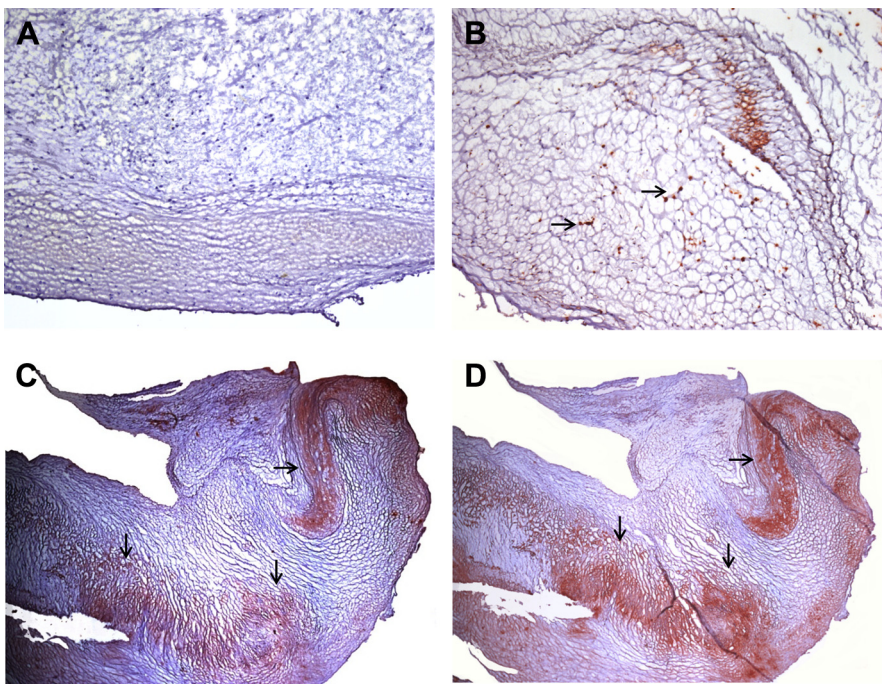

Figure 2 Immunodetection of Lp-PLA2 and Ox-LDL

Immunohistochemistry studies showed that in control nonmineralized aortic valves **(A),** Lp-PLA2 was not expressed, whereas there was a strong immunostaining in CAVD, which appears as cellular marking **(arrows) (B)** or as a diffuse staining **(arrows) (C)**. In areas of diffuse staining, Lp-PLA2 colocalized with oxidized low-density lipoprotein (ox-LDL) **(arrows) (D)**. Abbreviations as in Figure 1.

Table 3 Correlation Matrix Between the Number of Lp-PLA2 Transcripts and Clinical Data

Variables	R	p Value
Age	−0.22	0.09
Cholesterol	0.26	0.04
LDL	0.29	0.02
HDL	0.03	0.79
Triglycerides	0.04	0.74
ox-LDL	0.33	0.01

Lp-PLA2 = lipoprotein-associated phospholipase A2; ox-LDL = oxidized low-density lipoprotein; other abbreviations as in Table 1.

of Lp-PLA2, and present within CAVD tissues. We tested the effect of LPC on the mineralization of VIC cultures. We found that a small amount of LPC (1 nmol/l) increased mineralization of VIC cultures treated with the mineralizing medium (PO_4) (Fig. 4A). LPC is normally bound to lipoproteins and its content is elevated in ox-LDL (9). Furthermore, it has been shown that the high content of LPC in ox-LDL is largely dependent on Lp-PLA2 activity (10). We thus treated VIC with ox-LDL. In this experiment, similarly to LPC, ox-LDL increased mineralization of VIC treated with the mineralizing medium by 2.6-fold (Fig. 4B). In isolated VIC, LPC elevated the expression of ENPP1, ALP, the sodium-dependent phosphate cotransporter-1 ([Pit-1] encoded by the *SLC20A1* gene), and osteopontin (OPN) (Fig. 4C). The enzyme activity of ALP and ENPP1 were also increased following a treatment of VIC cultures with LPC (1 nmol/l) (Figs. 4D and 4E). We have previously shown that a high expression of the ectonucleotidase enzymes contribute to the mineralization of VIC cultures by elevating phosphate levels, whereby apoptosis-mediated mineralization is promoted. We then treated VIC cultures with ARL67156, an ectonucleotidase inhibitor. ARL67156 is an inhibitor of ENPP1, ENTPD1, and ENTPD3, and only ENPP1 is expressed significantly by human VIC (7). In this experiment, ARL67156 prevented the mineralization induced by the mineralizing medium and LPC (Fig. 4F), suggesting that LPC-mediated mineralization relies on the expression of ENPP1, which, in turn, promotes mineralization. We then hypothesized that apoptosis might be implicated in LPC-induced mineralization. First, by using the detection of activated caspase 3/7 assay, we found that LPC induced the activation of the effector caspases (Fig. 5A). Also, on exposure to LPC, there was a loss of the mitochondrial membrane potential in the VIC culture, indicating that the mitochondrial-dependent pathway is involved in LPC-mediated apoptosis (Figs. 5B and 5C). Correspondingly, we found that LPC promoted cytochrome c release within the cytosol of VIC (Fig. 5D). To further document the role of apoptosis in LPC-mediated mineralization of VIC, we have used the general caspase

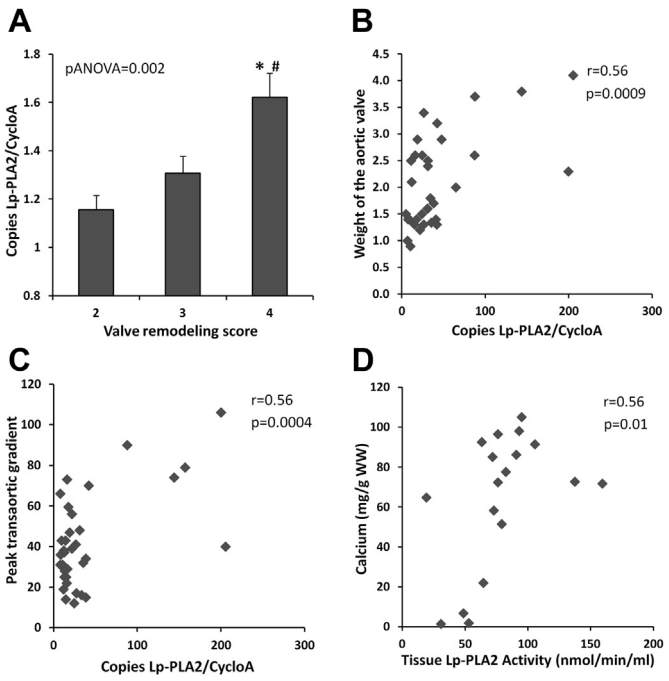

Figure 3 Lp-PLA2 and Indexes of CAVD Severity

(A) The number of Lp-PLA2 transcripts in CAVD valves increased significantly with the severity of the remodeling score of the aortic valve (to stabilize variances, the number of Lp-PLA2 transcripts has been log transformed). The number of Lp-PLA2 transcripts in CAVD valves correlated significantly with the weight of the aortic valve **(B)** and the peak transaortic gradient **(C)** (Spearman correlation). Within CAVD valves, enzyme activity of Lp-PLA2 correlated significantly with the amount of calcium (Spearman correlation) **(D)**. *$p < 0.05$ compared with remodeling score 2; #$p < 0.05$ compared with remodeling score 3. ANOVA = analysis of variance; other abbreviations as in Figure 1.

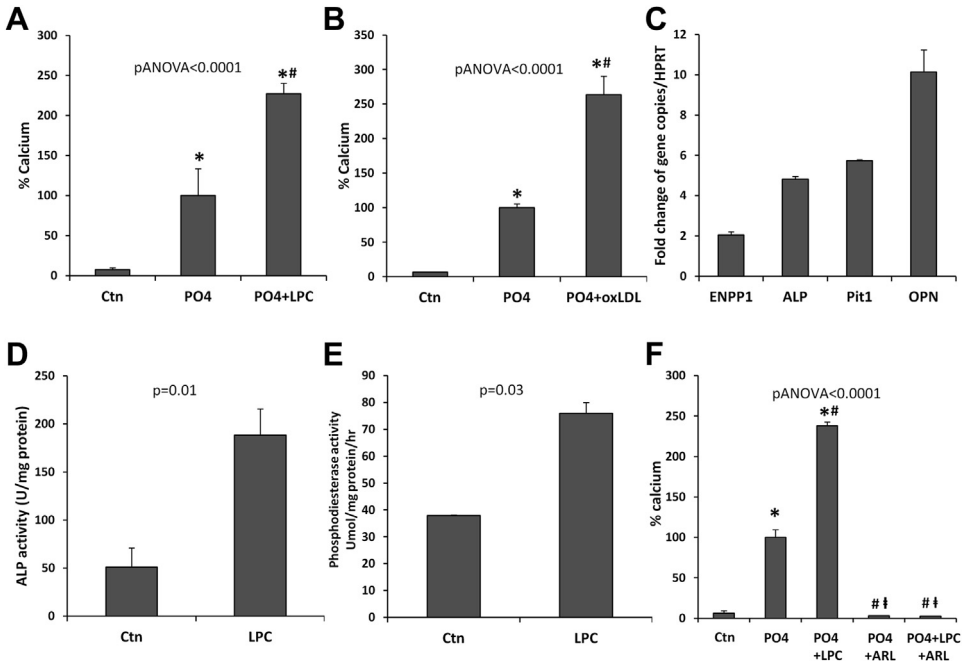

Figure 4 **LPC Induces Mineralization of VIC**

(A) In isolated valve interstitial cells (VIC), LPC exacerbated the mineralization induced by phosphate (PO_4). **(B)** In isolated VIC, medium ox-LDL exacerbated mineralization induced by PO_4. **(C)** In isolated VIC, exposure to LPC (1 nmol/l) increased the expression of ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), alkaline phosphatase (ALP), sodium-dependent phosphate cotransporter 1 (Pit-1), and osteopontin (OPN). In isolated VIC, LPC (1 nmol/l) increased enzyme activity of both ALP **(D)** and ENPP **(E).** **(F)** In isolated VIC, mineralization induced by both PO_4 and LPC was abrogated by a treatment with ARL67156 (ARL), a competitive inhibitor of ectonucleotidase enzymes. *$p < 0.05$ compared with control, noncalcified aortic valves (Ctn); # $p < 0.05$ compared with mineralizing medium (PO_4); †$p < 0.05$ compared with PO_4 + LPC. Abbreviations as in Figures 1, 2, and 3.

inhibitor ZVAD-fmk. ZVAD-fmk abolished the mineralization of VIC cultures induced by LPC and the calcifying medium (Fig. 6A). Expression of ectonucleotidases and mineralization of vascular smooth muscle cells has been previously shown to rely on the activation of the PKA pathway (8). Furthermore, studies have highlighted the role of PKA in apoptosis (11). We thus hypothesized that PKA could mediate LPC-induced mineralization of VIC cultures. In this regard, inhibition of PKA with PKA inhibitor fragment (6 to 22) amide prevented mineralization of VIC cultures induced by the mineralizing medium and LPC (Fig. 6B), suggesting that cyclic adenosine monophosphate (cAMP)-dependent activation of PKA is involved in LPC-mediated mineralization. LPC is a potent agonist of the G protein-coupled receptor G2A, which may signal through PKA (12). We have thus measured PKA activity in VIC and found that LPC increased PKA activity by 21%. Furthermore, this effect with LPC was synergistic with the mineralizing medium, increasing PKA activity by 40% (Fig. 6C). To further prove the implication of the PKA pathway in the mineralization of VIC cultures, we have used forskolin, an agent that increases adenylate cyclase activity and augments cell content in cAMP. To this effect, stimulation of cAMP production with forskolin treatment increased mineralization of VIC cultures induced by mineralizing medium and LPC (Fig. 6D). We next measured the level of cAMP in control and stenotic aortic valves to document the activation of the cAMP pathway in vivo. In control nonmineralized aortic valves, the amount of cAMP was very low and beyond detection level in 5 samples, whereas it was detected in 4 stenotic aortic valves from 5 patients (mean value 16.38 ± 8.75 pmol/mg protein), indicating activation of the cAMP/PKA pathway in stenotic aortic valve tissues.

Discussion

A recent report has documented that the mass of Lp-PLA2 within the blood plasma is elevated in patients with CAVD (13). In the present study, we identified for the first time that Lp-PLA2 is highly expressed within stenotic aortic valve tissues and that it contributes to valve mineralization by the production of LPC. We next showed that LPC-mediated mineralization of human VIC relies on ectonucleotidase enzyme and apoptosis through a PKA pathway.

Origin of Lp-PLA2 in CAVD. Lp-PLA2 is significantly expressed by platelets and macrophages. A significant fraction of Lp-PLA2 is bound to lipoproteins in the bloodstream (14). In this work, we documented that transcripts

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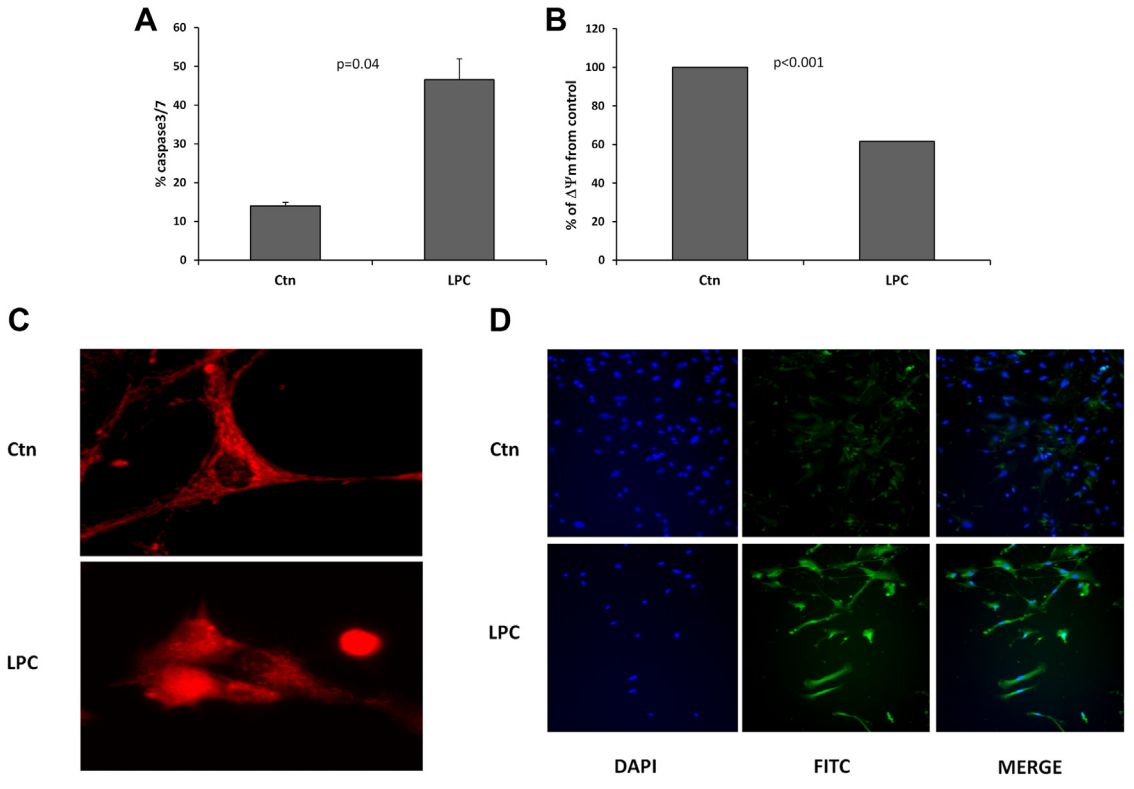

Figure 5 LPC Mediates Apoptosis of VIC

(A) In isolated VIC, treatment with LPC increased the activation of caspase 3/7. **(B, C)** In isolated VIC, LPC decreased the mitochondrial membrane potential (ΔΨm). **(D)** In isolated VIC, LPC promoted the release of cytochrome c in the cytosol. DAPI = 4,6-diamino-2-phenylindole; FITC = fluorescein isothiocyanate; other abbreviations as in Figures 1 and 4.

encoding for Lp-PLA2 were highly expressed in CAVD valves, indicating that the enzyme is locally synthesized. This is in line with the immunohistochemistry studies, which showed the expression of Lp-PLA2 by inflammatory cells. However, it should be pointed out that immunohistochemistry studies also showed a diffuse staining of Lp-PLA2 in CAVD tissues, suggesting that the enzyme is locally secreted and released by macrophages and/or transported by the lipoproteins within stenotic aortic valves.

We previously showed that the plasma level of ox-LDL is associated with the remodeling of stenotic aortic valves (15). In addition, we also documented that the proportion of circulating small, dense LDL is related to the amount of ox-LDL within CAVD tissues (2). Of interest, one study has found that a higher proportion of Lp-PLA2 is associated with small, dense LDL (16). Hence, it is possible that small, dense LDL, which have a higher oxidation rate, promote the accumulation ox-LDL, which in turn promotes the expression of Lp-PLA2 within the aortic valve during CAVD (17). Also, oxidized phospholipids are transported and sequestered in the blood plasma by lipoprotein (a) (14). To this effect, a recent work has highlighted that gene polymorphism of lipoprotein (a) is significantly associated with CAVD at the genomewide level (18).

Lp-PLA2–derived lysophosphatidylcholine and mineralization of the aortic valve. In this study, the transcript levels and enzyme activity of Lp-PLA2 correlated with several indexes of disease activity. More specifically, the number of transcripts was significantly correlated with the remodeling score and the weight of the aortic valve. The weight of the aortic valve, a simple and convenient measurement, is an independent predictor of the hemodynamic severity of CAVD (19,20). Mineralization of the aortic valve is certainly an important culprit, which is related to the severity of CAVD (21). To this effect, we identified that both the number of transcripts encoding for Lp-PLA2 and enzyme-specific activity correlated with the amount of calcium within CAVD. Lp-PLA2 activity better correlated ($r = 0.56$) with the valvular concentration of calcium than did the number of transcripts ($r = 0.27$). This suggests that tissue enzyme activity, which may take into account several factors, such as the total amount of Lp-PLA2 and differences in enzyme activity related to the genotype, is a better correlate of pathological mineralization of the aortic valve.

The hydrolysis of oxidized phospholipids generates LPC, a powerful proinflammatory and atherogenic compound (22). A recent study using vascular smooth muscle cells found that LPC induces the expression of ALP and mineralization of cell cultures (6). In the present study, we documented that

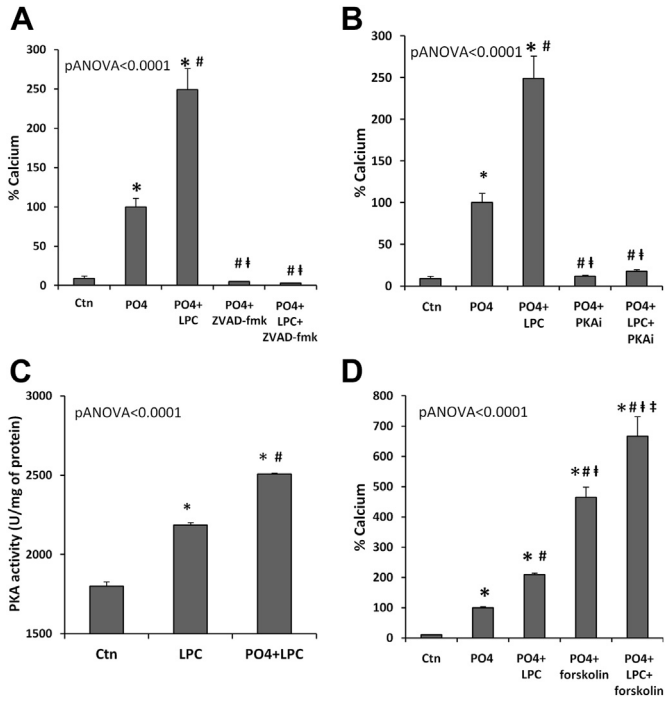

Figure 6 **LPC Promotes Apoptosis-Mediated Mineralization of VIC Through a PKA Pathway**

Mineralization of isolated VIC induced by PO_4 and LPC was prevented by ZVAD-fmk, a caspase inhibitor **(A),** and a protein kinase A (PKA) inhibitor (PKAi) **(B). (C)** In isolated VIC, LPC promoted PKA activity. **(D)** Mineralization of isolated VIC induced by PO_4 and LPC was amplified by a treatment with forskolin. *$p < 0.05$ compared with Ctn; #$p < 0.05$ compared with LPC **(C)** and mineralizing medium (PO_4) **(A, B, D);** †$p < 0.05$ compared with PO_4 + LPC; ‡$p < 0.05$ compared with PO_4 + forskolin. Abbreviations as in Figures 1, 3, and 4.

LPC-induced mineralization relied on the expression of phosphate-related genes, including ENPP1, ALP, Pit-1, and OPN. Whereas ENPP1 and ALP use nucleotides to generate PPi and Pi, Pit-1 is a cell membrane Pi-transporter that plays an important role in the mineralization of the aortic valve (23). Expression of ectonucleotidase genes, Pit-1, and OPN is highly regulated by the concentration of extracellular phosphate (24). In the present study, we found that ARL67156, which inhibits ENPP1, abrogated LPC-induced mineralization. A complete knockdown of ENPP1 is associated with ectopic mineralization by lowering the mineralization inhibitor PPi to exceptionally low levels (25). For instance, the tiptoe-walking mice with invalidation of the ENPP1 gene develop extensive mineralization of ligament (26). On the other hand, overexpression of ENPP1, such as during pathologic mineralization, promotes calcification by several mechanisms (27). In this regard, highly-expressed ENPP1 contributes to elevate the Pi/PPi ratio whereby mineralization is promoted (7). In addition, it should be pointed out that ALP activity was increased following LPC treatment. ALP is a potent enzyme that transforms PPi into Pi (28). Hence, it is possible that ENPP1 and ALP work in tandem and generate an important amount of Pi and, in doing so, promote LPC-mediated mineralization.

Phosphate is a strong promoter of mineralization through programmed cell death (29). LPC induced the mitochondrial-dependent apoptosis of VIC cultures. Hence, the present findings suggest that LPC induced the expression of phosphate-related genes, such as ENPP1, ALP, and Pit-1, which, in turn, promoted apoptosis-mediated mineralization of VIC cultures. Phosphate-induced expression of ENPP1 and ALP as well as mineralization of vascular smooth muscle cells have been shown to be dependent on the PKA pathway (8). In the present work, we documented that LPC-induced mineralization was abrogated by PKA inhibition, whereas a treatment with forskolin, an activator of adenylate cyclase, exacerbated the process. Furthermore, LPC promoted a strong increase in PKA activity in VIC. These facts suggest that LPC induced the expression of phosphate-generating enzymes and promoted mineralization through a PKA-dependent pathway.

Clinical implications. There is actually no medical treatment to prevent the progression of CAVD. Statins have been used in randomized trials and have been shown to be inefficient to prevent the progression of aortic stenosis (30). Although statins reduce the level of circulating Lp-PLA2 by decreasing LDL concentration, they do not prevent the de novo synthesis and secretion by macrophages (31). Hence, it is likely that statins, although reducing the blood plasma level of LDL, do not affect local secretion of Lp-PLA2 within the aortic valve. Accordingly, in this study, we

documented that Lp-PLA2 activity was elevated in stenotic aortic valves and that tissue activity did not correlate with blood plasma enzyme activity. These findings are in accordance with a previous study (32), which showed that enzyme activity of Lp-PLA2 in carotid endarterectomy specimen was not associated with blood plasma activity. More recently, a human study showed that an Lp-PLA2 mass in the blood plasma is not increased following an inflammatory stimulus. Instead, Lp-PLA2 secretion is increased by several-fold in macrophages exposed to a lipid load. Together, these findings suggest that Lp-PLA2 is preferentially secreted by tissue macrophages rather than by circulating leukocytes (33). Also, it should be pointed out that the proinflammatory effect of Lp-PLA2 is controversial. To this effect, by using platelet-activating factor and ox-LDL as substrates, Lp-PLA2 may lower oxidative stress (34). But, it generates lysophospholipids, which have proinflammatory activity and, as shown in the present study, promote mineralization of VIC.

Lp-PLA2 is considered a potential target in the treatment of atherosclerosis. Darapladib is a potent inhibitor of Lp-PLA2 that is under investigation in patients with coronary artery disease and has been shown in a swine model to decrease atherosclerotic plaque volume and to decrease the lipid core content (5). Although CAVD shares some features with atherosclerosis, such as lipid deposition and inflammation, it is a distinct pathobiological process. Hence, findings in atherosclerosis cannot be transposed directly to CAVD before proper investigations have been performed.

Study limitations. We have examined CAVD with advanced pathological mineralization. The present study cannot necessarily be transposed to nascent disease process. Nevertheless, the present study documented several points suggesting that Lp-PLA2 is possibly involved during the development of CAVD.

Conclusions

Lp-PLA2 is highly expressed in human CAVD. Several lines of evidence documented in this work suggest that Lp-PLA2 activity may contribute to the pathological mineralization of the aortic valve. CAVD is a highly prevalent condition, and there is, so far, no medical treatment to prevent its progression. The present study gives impetus to realize further study to document whether Lp-PLA2 could be considered as a novel therapeutic target for CAVD.

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Key Words: apoptosis ■ calcific aortic valve disease ■ ectonucleotide pyrophosphatase/phosphodiesterase 1 ■ lipoprotein-associated phospholipase A2 ■ lysophosphatidylcholine ■ protein kinase A ■ valve interstitial cells.

APPENDIX

For a supplemental methods section, please see the online version of this article.