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Differential Aortic and Mitral Valve Interstitial Cell Mineralization and the Induction of Mineralization by Lysophosphatidylcholine *In Vitro*

Dena C. Wiltz^a, Richard I. Han^{a,b}, Reid L. Wilson^{a,b}, Aditya Kumar^a, Joel D. Morrisett^b, and K. Jane Grande-Allen^a

^aRice University, Department of Bioengineering, Houston, TX

^bBaylor College of Medicine, Departments of Medicine and Biochemistry, Houston, TX

Abstract

Purpose—Calcific aortic valve disease (CAVD) is a serious condition with vast uncertainty regarding the precise mechanism leading to valve calcification. This study was undertaken to examine the role of the lipid lysophosphatidylcholine (LPC) in a comparison of aortic and mitral valve cellular mineralization.

Methods—The proportion of LPC in differentially calcified regions of diseased aortic valves was determined using thin layer chromatography (TLC). Next, porcine valvular interstitial cells (pVICs) from the aortic (paVICs) and mitral valve (pmVICs) were cultured with LPC (10^{-1} – 10^5 nM) and analyzed for cellular mineralization, alkaline phosphatase activity (ALPa), proliferation, and apoptosis.

Results—TLC showed a higher percentage of LPC in calcified regions of tissue compared to non-calcified regions. In pVIC cultures, with the exception of 10^5 nM LPC, increasing concentrations of LPC led to an increase in phosphate mineralization. Increased levels of calcium content were exhibited at 10^4 nM LPC application compared to baseline controls. Compared to pmVIC cultures, paVIC cultures had greater total phosphate mineralization, ALPa, calcium content, and apoptosis, under both a baseline control and LPC-treated conditions.

Address for correspondence: K. Jane Grande-Allen, PhD Department of Bioengineering Rice University 6100 Main St., MS 142 Houston, TX 77005, USA Phone: 1-713-348-3704 Fax: 1-713-348-5877 grande@rice.edu.

Human Subjects Declaration

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Animal Studies Declaration

No animal studies were carried out by the authors for this article. Animal tissues were purchased from a commercial abattoir.

Conflict of Interest Declaration

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Conclusions—This study showed that LPC has the capacity to promote pVIC calcification. Also, paVICs have a greater propensity for mineralization than pmVICs. LPC may be a key factor in the transition of the aortic valve from a healthy to diseased state. In addition, there are intrinsic differences that exist between VICs from different valves that may play a key role in heart valve pathology.

Keywords

aortic valve; calcification; lipid; mitral valve

INTRODUCTION

Lipids have been shown to play an active role in blood vessel disease,² but their role in valvular diseases, such as calcific aortic valve disease (CAVD), is not fully understood. CAVD is a condition characterized by narrowing of the valve orifice and thickening of the valve leaflets, and is prevalent in 2–3% of the population over the age of 75 years.^{13,39,47} It is the primary cause of aortic stenosis (AS), which, if left untreated, can lead to heart failure.^{28,35} Currently, the most widely used treatment for AS is heart valve replacement.³⁷ There is no ideal pharmacological treatment to stop or reverse the process of CAVD^{8,37}, largely due to a lack of knowledge regarding the initiation and progression of this disease. Therefore, it is essential to gain a better understanding of CAVD in order to prevent and perhaps reverse this disease.

Several factors, such as oxidative stress, extracellular matrix remodeling, mechanical stimulation, and altered renin-angiotensin signaling, are being investigated to determine their contribution to the progression of valve calcification.⁴² There is also substantial evidence suggesting that lipids play a prominent role in the transformation of the valve from a healthy to a diseased state. Indeed, a characteristic feature of stenotic aortic valves is the significant prevalence of lipid and protein deposition in the lesions and adjacent fibrotic areas of the tissues.³⁸ Some investigators hypothesize that it is possible for the binding of lipids by subendothelial glycosaminoglycans to promote lipid retention in the valve tissues, with subsequent lipid oxidation and other downstream effects; this view is similar to the hypothesis that retention of lipoproteins in the arteries is a main factor leading to atherosclerosis.^{14,48} The role of one lipid in particular, lysophosphatidylcholine (LPC), which is formed during the lipolysis of oxidized phosphatidylcholine by lipoprotein phospholipase A₂,²⁷ warrants investigation since it has been shown to induce inflammation, cell proliferation and migration, to increase intracellular calcium, and to upregulate osteogenic genes.^{1,4,7,31,55,61} Although the effects of LPC have been studied in a variety of cell types, its role in valve cell mineralization has yet to be elucidated. Furthermore, there has been less investigation of endogenous cell/tissue components such as lipids as drivers of *in vitro* mineralization by valve cells compared to non-endogenous or even synthetic factors such as beta-glycerophosphate and dexamethasone.^{26,50,60}

The aortic and mitral valves clearly show differences in remodeling in their most common disease states. The aortic valve tends to exhibit a more bone-like calcification, whereas the mitral valve tends to exhibit a more cartilage-like change.³ Although the annulus of the

aging mitral valve does become more calcified with age,^{40,41} a histological analysis of valves from 200 patients demonstrated that the significant accumulation of calcium within the mitral valve leaflets appears approximately 10 years later than comparable changes in the aortic valve.⁴⁴ This study addresses these differences by comparing the concentrations of LPC in calcified and non-calcified regions of human aortic valves and the *in vitro* mineralization of interstitial cells from porcine aortic and mitral valves treated with LPC.

METHODS

Tissue Procurement

Human aortic valve tissues were collected from patients undergoing heart valve replacement surgeries at the Houston Methodist Hospital. The aortic valve tissues were immediately immersed in PBS:glycerol (50:50) and kept at -20°C before use. Five aortic valve tissue samples were selected. The selection criteria were: 1) each aortic valve had three intact leaflets so that the bicuspid valve could be excluded and 2) the combined leaflet area contained roughly equal amounts of normal area and calcific area in 1:1 ratio on the fibrotic side. This study fulfilled both institutional ethical guidelines with approval from the Houston Methodist Hospital, Baylor College of Medicine, and Rice University, and the full consent of the patients.

Lipid Extraction from Aortic Valve Tissue

In order to remove the glycerol, the valve tissue was rinsed in PBS three times, for 30 min on a shaker at 4°C . After dabbing dry, the tissue was carefully dissected into normal, non-calcified areas and calcified areas with a teasing needle. The dissected tissue was weighed and then homogenized (Brinkmann Polytron, Westbury, NY) in the presence of 3 ml of Folch reagent (2:1 chloroform:methanol) on ice. The homogenate was centrifuged at 2500 rpm for 25 min and the lower organic phase was collected. To achieve complete lipid extraction, an additional two rounds of extractions were carried out, using 2 mL of the reagent added to the residual pellet, followed by centrifugation at 2500 rpm for 25 min. The collected organic phases were pooled together and then evaporated using a stream of nitrogen and a heated sand bath.

Thin Layer Chromatography

The dried extract residue was re-dissolved in 0.5 ml of chloroform/methanol (9:1) solution. After a further 1:5 dilution in the same solution, 10 μl of the lipid extract was loaded onto a thin layer chromatography plate (silica gel 60A, 250 μm thickness, 20 $\times$ 20 cm, Watman, England) along with L- α -lysophosphatidylcholine standards (from egg yolk, Sigma L4129, St. Louis, MO). The lipids on the plate were first separated in a polar solvent (65:25:4 chloroform:methanol:water) for 12 min. After drying, the lipids on the plate were separated in a non-polar solvent (75:35:1 hexane:diethyl ether/acetic acid) for 30 min. The plate was thinly sprayed with 0.05% primuline (Sigma, St. Louis, MO) in 80% acetone. The band detection was performed under fluorescence mode in a Storm imaging system (GE Healthcare, CT). The band analysis and quantification was based on integration of band density using ImageQuant TL software (GE Healthcare, CT). The amount of LPC in each

sample group was extrapolated from the standards and reported as a percentage of the total tissue weight.

Cell Isolation

Aortic and mitral valves were harvested from adult porcine hearts obtained from a local abattoir (Fisher Ham & Meat Company, Incorporated, Spring, TX). For each cell isolation, valves from three to five hearts were pooled. Porcine aortic and mitral valve interstitial cells (paVICs and pmVICs, respectively) were isolated from the aortic and mitral valve leaflets via collagenase digestion similar to a previously published protocol.⁴⁶ Briefly, the leaflets underwent a digestion in collagenase II for 30 minutes at 37° Celsius in a rotating shaker (Barnstead Lab-Line, Melrose Park, IL) set to 150 rpm. The tissues were swabbed to remove the outer layer of endothelial cells, minced, and placed in a collagenase III digestion solution for 4 hours at 37° Celsius in a rotating shaker set to 150 rpm. After 4 hours, the cells were strained from any remaining tissue using a 70 µm filter. The following experiments were performed with cells at passage 2.

Culture in Osteogenic Differentiation Media

To characterize the calcification of VICs *in vitro*, cells were grown in 2D atop tissue culture polystyrene for 8 days without passaging as previously described.^{29,30,59} paVICs and pmVICs were seeded at a density of 50,000 cells/cm² in Dulbecco's Modified Eagle Medium. (1 g/L glucose, L-glutamine, and sodium pyruvate) mixed with Ham's F-12 nutrient mixture, 1.1% HEPES buffer, 1% penicillin-streptomycin-fungicide, and 1% bovine growth serum (BGS). On day 1, the cell cultures were treated with unmodified media or media containing 10 mM β-glycerophosphate (βGP) ± 10 nM dexamethasone. Both of these compounds are common components of media used for osteogenic differentiation of mesenchymal cells and have been used at these and other concentrations to induce calcification in various cell types.^{26,45,53} All cells were cultured for 8 days at 37°C, 5% CO₂ with changes of the treatment medium occurring on days 3, 5, and 7. Two runs of four wells per treatment condition were conducted and the data from each run was pooled (eight individual wells total per group) to conduct statistical analysis.

LPC Application

To characterize the response to LPC, VICs were cultured for 8 days as described in the previous section. Serum was applied at a low concentration (following the approach of Monzack et al.³⁰) in order to assess the effects of LPC on cell proliferation. On day 1, the cell cultures were treated with medium containing L-α-LPC (Sigma-Aldrich, Inc., St. Louis, MO) at concentrations ranging from 10⁻¹ – 10⁵ nM. A positive control group was treated with medium containing 10 mM βGP (positive control) but no LPC. There was an additional control group that experienced no LPC or βGP (baseline control). For each of the following assays conducted to assess the study outcomes, there were two runs conducted with five wells from each group in each run, and the data from each run was pooled (ten individual wells total per group) to conduct statistical analysis.

Alizarin Red S Staining

Mineralization in cell cultures treated with osteoblastic differentiation media was visualized using Alizarin Red S (ARS) histological staining.^{15,21} After removing the medium, the cells were washed three times with phosphate buffered saline (PBS). The cells were fixed overnight with 10% formalin at 4°C. The formalin was then removed, and the cell cultures were rinsed three times with deionized distilled H₂O (ddH₂O). Next, a 500 µL aliquot of 40 mM ARS was added to each well for 30 minutes. The ARS solution was then removed and well was washed three times with ddH₂O.

The stained nodules were imaged using brightfield microscopy with a charge-coupled device camera (ProgRes C5, Jenoptik, Easthampton, MA) mounted to a Zeiss Axiovert 135 inverted microscope (Zeiss, Thornwood, NY). The areas of the nodules were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Four representative images were taken from each well and the total calcified area per well was determined by summing the individual areas of each nodule in all four images and normalizing that value to full well area.

Von Kossa Staining

Mineralization in cell cultures treated with LPC (including the concurrent positive and negative controls) was visualized using von Kossa histological staining. The cell cultures were prepared and washed in the same manner described in the ARS staining section. Next, a 500 µL aliquot of 1% silver nitrate in ddH₂O (Sigma-Aldrich Inc., St. Louis, MO) solution was added to each well before incubating for 30 minutes under a 100 Watt ultraviolet lamp (UVP, LLC, Upland, CA). After removing the silver nitrate solution, the cells were washed three times with ddH₂O then incubated with 500 µL of 5% sodium thiosulfate in ddH₂O (Fisher Scientific, Fair Lawn, NJ) for 5 min at room temperature to stop the reaction. The sodium thiosulfate solution was removed and each well was washed two times with ddH₂O.

The stained nodules were imaged and analyzed as described in the ARS staining section. Every nodule in each well was imaged. To determine average nodule size, the nodule areas were averaged for each well. Nodule number was determined by counting all the nodules in a given well. The total calcified area for each well was determined by summing the individual areas for each nodule in a given well.

Alkaline Phosphatase Activity

Alkaline phosphatase is an important enzyme that provides free phosphate¹⁰. Alkaline phosphatase activity (ALPa) was measured using the SensoLyte[®] FDP Alkaline Phosphatase Assay Kit *Fluorimetric* (AnaSpec, Inc., Fremont, CA) per kit instructions. Briefly, cells were lysed using 200 µL of a lysis buffer containing Triton X-100 and then centrifuged. 50 µL of the supernatant was removed and added to a 96-well plate. Next, 50 µL of 3,6-fluorescein diphosphate reaction mixture was added to each well, and the wells were incubated away from light at 37°C, 5% CO₂ for 30 min. Afterwards, a stop solution was added to each well to stop the reaction for end-point readings. The wells were read on a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA), measured in

relative fluorescent units (RFU), and normalized to an average (for each group) total protein content.

Calcium Content

Calcium content was measured using the Calcium Arsenazo III Reagent Set (Pointe Scientific, Inc., Canton, MI). On day 8 of culture, a sample aliquot of the media from each well was removed and 1 mL of the Arsenazo III reagent was added to each sample. The media-Arsenazo III solution was mixed and incubated at room temperature for one min. The absorbance of the solution (optical density, OD) was read at 650 nm on a spectrophotometer (SpectraMax M2).

Total Protein

Total protein was measured using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. Briefly, cells from identically seeded wells of 48-well plates were lifted using 100 µL of 0.25% trypsin-EDTA (Mediatech, Manassas, VA) at 37°C for 5 minutes. 100 µL of media containing 10% BGS was added to trypsinized cells. The cells were then centrifuged, the supernatant was removed, and the cells were resuspended in ddH₂O. Next, 25 µL of the suspension was added to a 96-well plate, mixed with a cupric sulfate solution, and incubated for 30 min at 37°C. The wells were cooled to room temperature, and absorbance was measured on a spectrophotometer (SpectraMax M2) at 562 nm. An average of total protein for each group was computed and used to normalize ALPa and calcium content data.

Cell Proliferation and Apoptosis

Cell proliferation was approximated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). Briefly, 500 µL of medium was added to each well, followed by the addition of 50 µL of MTT (5 mg/mL in ddH₂O). The wells were incubated for 4 hours at 37°C, 5% CO₂. Next, the solution was aspirated from each well and 500 µL of 0.1N hydrochloric acid in anhydrous isopropanol was added. 100 µL volumes of the resulting solution were transferred to a 96-well plate and absorbance was read at 570 and 690 nm using a spectrophotometer (SpectraMax M2). Absorbance at 690 nm was subtracted from the absorbance measured at 570 nm and readings were expressed in terms of optical density (OD).

Apoptosis was measured using the Caspase-Glo® 3/7 Assay (Promega Corporation, Madison, WI) per kit instructions and normalized to DNA content using the Quant-iT PicoGreen® dsDNA Reagent Kit (Invitrogen, Eugene, Oregon). Caspases -3 and -7 are known to play key roles in apoptosis.^{22,57} Briefly, cells were detached from 24-well plates with the addition of 200 µL of 0.25% trypsin-EDTA (Mediatech, Manassas, VA) at 37°C for 5 minutes. 200 µL of media containing 10% BGS was added to trypsinized cells. The cells were then centrifuged, the supernatant was removed, and the cells were resuspended in 1 mL of medium containing 10% BGS. 100 µL of each cell suspension was added to a well of a 96-well plate. After mixing the picogreen reagent directly into Caspase-Glo® reagent at a ratio of 1:200, a 100 µL volume of the combined reagent mixture was added to each well prior to incubation at room temperature. After 30 minutes of incubation, the luminescence

(measure of caspase -3 and -7 activity) was assessed using a spectrophotometer (SpectraMax M2). Next, the wells were incubated for an additional 30 minutes and the fluorescence (measure of DNA content) was assessed using a spectrophotometer (SpectraMax M2) with sample excitation at 480 nm and emission intensity measured at 520 nm. The measurements of caspase -3 and -7 normalized to DNA were expressed in relative luminescence units/relative fluorescence units (RLU/RFU).

Statistical Analysis

A paired t-test was conducted, using Prism software (GraphPad Software, Incorporated, La Jolla, CA), to compare the percent of LPC content in calcified and non-calcified regions of calcified aortic valves. To compare the effects of LPC on all measures of cellular mineralization, a one-way ANOVA followed by Tukey's post-hoc testing was conducted using JMP statistical software (SAS, Cary, NC) for each cell type (paVICs and pmVICs). A Student's t-test was conducted to compare differences between paVICs and pmVICs. Statistical significance was determined as $p < 0.05$.

Results

Calcified regions contain more LPC than non-calcified regions

In the non-calcified regions, LPC accounted for 0.06–0.20% of the total tissue weight. In the calcified areas, LPC accounted for 0.39–0.83% of the total tissue weight, at least a 2 fold increase (Figure 1).

10^5 nM LPC treatment resulted in cell death

All cell culture treatment groups were successfully cultured for 8 days, with the exception of the highest concentration of LPC. Within 24 hours of 10^5 nM LPC application, significant cell detachment occurred. After 8 days, by visual inspection, all cells treated with 10^5 nM LPC were completely detached from the wells. Cells treated with this high concentration were excluded from all further analyses, including statistical analysis.

Dexamethasone application increased levels of insoluble calcium in paVIC cultures

Addition of 10 mM β GP to paVICs and pmVICs resulted in a slight but statistically insignificant increase in total nodule area (Figure 3). Cultures of paVICs treated with a combination of 10 mM β GP and 10 nM dexamethasone demonstrated greater total mineralization relative to both baseline (1206% increase) and 10 mM β GP conditions (904% increase). In contrast, pmVIC cultures exhibited a muted response to application of both β GP and dexamethasone.

Increased LPC application resulted in increased phosphate content, but did not affect alkaline phosphatase activity

Von Kossa stained nodules (representative nodule shown in Figure 2) were observed within all groups at day 8 (Figure 4). There was no significant difference in average nodule size between groups; mean nodule size ranged from approximately 1692 to 1763 μm^2 .

There was, however, a steady increase in nodule number with increasing LPC concentration, with a significant increase from baseline control in groups containing 10^2 nM - 10^4 nM (paVICs) and 10 nM - 10^4 nM (pmVICs) LPC (Figure 4A and 4C). At 10^2 nM and 10^4 nM LPC, paVICs had 44% and 94% more nodules, respectively, than the baseline control. At 10 nM and 10^4 nM LPC, pmVICs had 51% and 162% more nodules, respectively, than the baseline control.

For both paVICs and pmVICs, there was a significant increase in total mineralization in groups containing 10^1 nM - 10^4 nM LPC compared to baseline controls (Figure 4B and 4D). paVICs had 36% and 92% greater total mineralization for groups containing 10^1 nM and 10^4 nM LPC, respectively, compared to baseline controls. pmVICs had 52% and 164% greater total mineralization for groups containing 10^1 nM and 10^4 nM LPC, respectively, compared to baseline controls.

Likewise, β GP also exhibited no significant difference in nodule size but did significantly increase nodule number (44%) for the paVICs compared to baseline controls. In addition, paVICs and pmVICs had 42% and 50% higher total mineralization, respectively, in β GP treated groups compared to baseline controls (Figure 4).

There were no significant differences in ALPa between baseline control and LPC treatment groups for paVICs or for pmVICs (data not shown). Application of β GP had no significant effect on ALPa compared to baseline control.

Certain levels of LPC increased calcium content

A significant increase in calcium content was observed with the application of 10^{-1} and 10^4 nM LPC in paVICs and with the application of 10^{-1} and $10^3 - 10^4$ in pmVICs compared to baseline controls ($p < 0.05$). Compared to the baseline control calcium concentration of 3.80 ± 0.47 OD/mg protein, paVICs had 62% and 82% higher calcium content with the application of 10^{-1} LPC (6.15 ± 0.80 OD/mg protein) and 10^4 nM LPC (6.90 ± 2.36 OD/mg protein), respectively. Compared to the baseline control calcium concentration of 1.52 ± 0.62 OD/mg protein, pmVICs had more than double the calcium content with the application of 10^{-1} LPC (3.23 ± 0.90 OD/mg protein), 10^3 LPC (3.46 ± 0.71 OD/mg protein), and 10^4 nM LPC (3.73 ± 0.53 OD/mg protein).

Application of β GP increased calcium content by 79% and 156% compared to baseline controls in paVICs (6.79 ± 1.15 vs. 3.80 ± 0.47 OD/mg protein) and pmVICs (3.90 ± 1.40 vs. 1.52 ± 0.62 OD/g protein), respectively.

LPC dose-dependently increased proliferation

LPC caused proliferation to increase in a dose-dependent manner at days 2, 5, and 8 for both paVICs and pmVICs. At the highest viable dose of LPC (10^4 nM), paVICs had 54%, 119%, and 98% higher proliferation compared to baseline controls at day 2 (not shown), 5, and 8, respectively (Figures 5A, 5C). Similarly, pmVICs treated with 10^4 nM LPC had 56%, 113%, and 81% higher proliferation compared to baseline controls at day 2 (not shown), 5, and 8, respectively (Figures 5B, 5D).

The positive control, β GP treated paVICs and pmVICs, also showed greater proliferation (27–39% greater than baseline control) on days 2, 5, and 8 (Figure 5).

High concentrations of LPC increased apoptosis

There were no significant differences between any groups on day 2. On day 5, application of 10^4 nM LPC resulted in 35% and 40% higher levels of activity of caspases -3 and -7 for paVICs and pmVICs, respectively, compared to baseline controls (Figure 6A and 6B). On day 8, treatment of paVICs and pmVICs with 10^4 nM LPC increased activity of caspases -3 and -7 (33% and 37%, respectively) compared to baseline control (Figure 6C and 6D)..

Application of β GP significantly increased (17%) caspases -3 and -7 activities compared to baseline control on day 8 in pmVIC cultures; no other significant differences in caspase activity were seen with the application of β GP compared to baseline controls (Figure 6).

paVICs had greater mineralization compared to pmVICs

The paVIC and pmVIC results were directly compared in two ways. To evaluate the inherent potential for mineralization between cells from the two different valves, the baseline control groups were compared. To compare the cells' differential response to mineralization promoting factors, all groups (baseline controls, LPC-treated [excluding 10^5 nM LPC], and β GP-treated) were pooled together for each cell type. For the proliferation and apoptosis assays, days were grouped together, and then similar comparisons (comparing baseline control groups only or all groups pooled together) were conducted.

Compared to pmVICs, paVICs had 65% and 39% greater phosphate mineralization as shown by von Kossa staining for controls and all groups pooled together, respectively (Figure 7A). With respect to ALPa, paVICs had 2 and 3 times greater ALPa compared to pmVICs for controls and all groups pooled together, respectively (Figure 7B). paVICs had 150% and 73% greater calcium content compared to pmVICs for controls and all groups pooled together, respectively (Figure 7C). There was no significant difference in proliferation between paVIC and pmVIC controls, although paVICs had slightly (9%) greater proliferation than pmVICs when all groups were pooled together (Figure 7D). For caspases -3 and -7, paVICs had 26% and 23% greater activity compared to that in pmVICs for controls and all groups pooled together, respectively (Figure 7E).

DISCUSSION

Lipid accumulation is a hallmark of calcified, stenotic aortic valves and is a significant driver of remodeling in other cardiovascular diseases ^{2,19,38}. The phospholipid LPC, in particular, has been shown to accumulate in atherosclerotic lesions, is a major component of oxidized low density lipoprotein, and promotes mineralization of vascular smooth muscle cells *in vitro* ^{43,55}. This study has shown that LPC is present in greater abundance within calcified regions of human aortic valves, and that this phospholipid dose-dependently affects the behavior of valvular interstitial cells, namely causing increased phosphate deposition, increased calcium content at 10^4 nm LPC application, increased caspase 3/7 activity at later timepoints, and a general increase in cellular proliferation. In addition, 10^5 nM LPC was toxic to the VICs. This work was the first to show the concentration dependent effects of

LPC on the mineralization of VICs. Furthermore, it was observed that the inherent and induced mineralization capabilities were different between VICs derived from the aortic and mitral valves. In the future, it will be important to elucidate the effects of LPC on VIC mineralization by examining signaling pathways where LPC has a demonstrated role.

There are multiple forms of calcium that may exist in calcified aortic valves, including the calcium salts calcium phosphate and hydroxyapatite.²³ Therefore, it is pertinent to conduct a wide variety of tests that may highlight all forms of calcium that may be present. This study used von Kossa staining to visualize phosphate deposition, which may be present in calcium salts, in the VIC cultures treated with LPC. The increase in total phosphate mineralization with increasing applications of LPC indicates a possible rise in the presence of calcium salts in the VIC cultures with LPC application.

LPC has been shown to affect the calcium levels in a variety of cell types by directly affecting calcium channels. One method in which calcium levels are altered by LPC is through activation of the ryanodine receptor (RyR) channels, a calcium release channel.¹¹ In rabbit ventricular muscles, application of 5 μ M LPC caused an increase in mean open time and membrane capacitance of the channel although no significant change in closing time was observed.³³ It is possible that within our study, exogenous LPC was incorporated into the lipid membrane bilayer and affected RyR channels allowing greater mobilization of calcium within the cells and ultimately the production of mineralized nodules. LPC also affects another calcium channel, L-type, causing increased calcium current levels through these channels in rabbit portal vein smooth muscle cells.¹⁸ It would be very compelling to investigate whether LPC enhances activity of RyR and L-type calcium channels in VICs.

In addition to acting on calcium channels, LPC may be acting on the enzyme protein kinase C (PKC) to affect cellular mineralization. When LPC was applied at concentrations below 20 μ M to purified PKC, PKC activity was increased; whereas, when LPC was applied at concentration greater than 30 μ M, PKC activity was inhibited.³⁶ PKC is well known to promote lipid hydrolysis.³⁴ In fact, in human coronary endothelial cells, a PKC inhibitor was used to block an LPC-induced increase in activity of phospholipase D;⁹ phospholipase D hydrolyzes phosphatidylcholine to produce phosphatidic acid, which has been shown to alter intracellular calcium levels in rat cardiomyocytes.^{52,58} PKC can also influence inflammatory responses,³² which further links LPC with remodeling pathways involved in calcification.¹³ As LPC could contribute to mineralization by VICs through promoting PKC activity and thus enhancing lipid hydrolysis and inflammatory signaling, it will be important to investigate these interactions further, including the possible use of PKC inhibitors.

Apoptosis is another factor that contributes to mineralization, partly through the production of matrix vesicles which can contain factors, such as calcium binding proteins, promoting calcification.^{20,56} LPC induces apoptosis in a variety of cell types. When LPC was applied to rat pancreatic AR42J cells, there was an increase in DNA fragmentation as concentrations of LPC increased up to 25 μ M.²⁵ The application of 75 μ M LPC to human umbilical vein endothelial cells also caused apoptosis, presumably via the p38 mitogen-activated protein kinase pathway.⁵¹ Those reports are consistent with the results of this study in which there

was an increase in caspase 3/7 activity for the VICs treated with 10^4 nM LPC. The apoptosis seen in this study may play an important role in the mineralization of VIC cultures.

It was not surprising that at high concentrations ($10^4 - 10^5$ nM), LPC was either toxic to the VICs or caused significantly elevated levels of caspase activity, indicative of apoptosis. This range of concentrations should be examined further to determine the critical concentration of LPC that VICs can withstand without eliciting substantial dysfunctional behavior. In a study conducted on rat aorta vascular smooth muscle cells, LPC concentrations above 10000 nM increased lactate dehydrogenase levels in the culture supernatant.⁵ In addition, increased membrane permeability, indicating possible cell damage, occurred in rabbit aortic endothelial cells with 30 μ M LPC.²⁴ However, it has also been reported that bovine aortic endothelial cells treated with 50 μ M LPC for 30 minutes showed no significant change in lactate dehydrogenase release.⁴⁹ In general, LPC is believed to have a detergent action on lipid membranes through micelle formation and mechanical destabilization,¹⁶ but the critical concentration of LPC leading to undesired cell behavior may depend upon the cell type and length of treatment.

An interesting finding of this study was that VICs from the aortic valve showed greater propensity for mineralization than did VICs from the mitral valve. Cells from both sources, however, showed some degree of mineralization, both under baseline control conditions and when treated with LPC or β GP. The VIC response to β GP was statistically significant as shown by the von Kossa staining and especially the calcium assay, although not by the ARS staining, which may be due to differences in the analytical methods. As expected, the aortic VICs (but not the mitral VICs) showed a very strong degree of ARS-stained mineralization in response to the addition of dexamethasone. Dexamethasone is a synthetic glucocorticoid, not natively present within heart valves, that is often used to drive osteogenic differentiation of mesenchymal stem cells *in vitro*.⁵³ It is important to note that these VICs were all harvested under identical conditions from porcine hearts, and were used at early passage number (P2). This ability of VICs to form calcific nodules in a spontaneous, endogenous manner was originally reported by Mohler et al., who found that interstitial cells grown in explant culture from human and canine aortic valves could form nodules within 3–6 weeks of culture.²⁹ VICs enzymatically isolated from porcine or ovine tissues, when cultured for several days without passaging, typically show the formation of calcific nodules within several days, depending upon the degree of stimulation with cytokines, the culture substrate stiffness, or mechanical stretch.^{12,17,60} The differences between the mineralization capacities of the interstitial cells from different valves may play a role in the onset and degree of osteoblastic transformation that is observed in the diseased states of different valves, namely the more bone-like remodeling and early onset of calcification seen in the aortic valve compared to the mitral valve.^{3,41,44} The differences seen in the mineralization capacity between VICs from the aortic and mitral valves may arise from their slightly different embryonic origins, as the leaflets develop from distinctly located endocardial cushions.⁶ Although the results found in this research give insight into previous observations investigating the diseased states between the aortic and mitral valves, a recent genetic study investigating differences between human aortic VICs (haVICs) and human mitral VICs (hmVICs) reported that hmVICs demonstrated greater ARS intensity, ALP mRNA level,

and ALP activity compared to haVICs when introduced to osteogenic differentiation media *in vitro*. In that study, there were no differences between the expression levels of genes encoding the osteogenic markers osteocalcin, osteopontin, and bone sialoprotein, as well as apoptosis associated gene CASPASE-3, in calcified hmVICs and calcified haVICs. There were, however, higher expression levels of osteoglycin and osteomodulin in normal (non-calcified) haVICs compared to normal hmVICs.⁵⁰ The higher levels of these genes support the idea that aortic VICs may have a predisposition for calcifying compared to mitral VICs. It was also recently reported that inflammatory stimulation with lipopolysaccharide caused increased production of BMP-2 by VICs from the aortic valve, but not by VICs from the mitral, pulmonary, or tricuspid valves.⁵⁴ The work reported here complements the recent genetic and inflammation studies by comparing the intrinsic ability of VICs from the aortic and mitral valve to calcify and comparing their calcifying capabilities in the presence of lipids. Further direct characterizations, as well as characterizations in the presence of a variety of calcification-inducing agents, may give greater insight into the differences between VICs from the aortic and mitral valves and their remodeling in diseased states.

In conclusion, the phospholipid LPC had significant effects on valvular interstitial cell mineralization in two-dimensional culture, affecting phosphate deposition, proliferation, and apoptosis. This work also showed that aortic and mitral VICs have different capacities for mineralization *in vitro*. These results may give insight into the distinct osteogenic versus chondrogenic transformations in disease between the aortic and mitral valve. Future studies of LPC in similar or unique culture models, such as three-dimensional engineered tissues or organ cultures, as well as in diseased human valves, are expected to provide further insight into the mechanisms involved in the formation of calcified nodules in CAVD.

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Abbreviations

ARS	Alizarin red S
ALPa	Alkaline phosphatase activity
AS	Aortic stenosis
βGP	Beta-glycerophosphate
CAVD	Calcific aortic valve disease
LPC	Lysophosphatidylcholine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
paVIC	Porcine aortic valve interstitial cell

PKC	Protein kinase C
pmVIC	Porcine mitral valve interstitial cell
RFU	Relative fluorescence units
RLU	Relative luminescence units
RyR	Ryanodine receptor
VIC	Valvular interstitial cell

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Fig. 1.

Regional weight percent of LPC, measured by thin layer chromatography, in calcified aortic valves shows a higher percentage of LPC in calcified areas of diseased aortic valves compared to non-calcified areas

Fig. 2.
Representative nodule observed from von Kossa staining shows the presence of mineralization in VIC cultures

Fig. 3. Total mineralization of paVIC (A) and pmVIC (B) cultures measured by Alizarin Red S staining showed total mineralized area increased with addition of osteogenic factors to culture media. Groups are represented by means and standard deviations. Within each chart, columns sharing a letter at the top represent groups that are not significantly different. Columns not sharing a letter represent groups that are significantly different ($p < 0.05$)

Fig. 4. von Kossa staining on nodule number (A and C) and total mineralization (B and D) in paVICs (A–B) and pmVICs (C–D) showed increasing nodule number and total mineralized area with increasing concentrations of LPC compared to baseline controls. Groups are represented by means and standard deviations. Within each chart, columns sharing a letter at the top represent groups that are not significantly different and vice versa ($p < 0.05$)

Fig. 5.

There was a general increase in MTT absorbance on day 5 (A and B), and day 8 (C and D) for paVICs (A and C) and pmVICs (B and D) with increasing LPC. Groups are represented by means and standard deviations. Within each chart, columns sharing a letter at the top represent groups that are not significantly different and vice versa ($p < 0.05$)

Fig. 6.

Caspase 3/7 activity on days 5 and 8 for paVICs (A and C, respectively) and pmVICs (B and D, respectively) increased with increasing concentrations of LPC. Groups are represented by means and standard deviations. Within each chart, columns sharing a letter at the top represent groups that are not significantly different and vice versa ($p < 0.05$)

Fig. 7.

Two types of comparisons between cell sources (■ paVICs or □ pmVICs): either normal controls only or all groups pooled together. paVICs showed greater total mineralized area (A), ALPa (B), calcium content (C) and caspase activity (E) compared to pmVICs for both control groups only and all groups pooled together. paVICs had increased proliferation compared to pmVICs when all groups were pooled together but not when comparing control groups only (D). Groups are represented by means and standard deviations. * $p < 0.05$