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MINI-FOCUS: AORTIC VALVE DISEASE

Lipoprotein(a) Induces Human Aortic Valve Interstitial Cell Calcification

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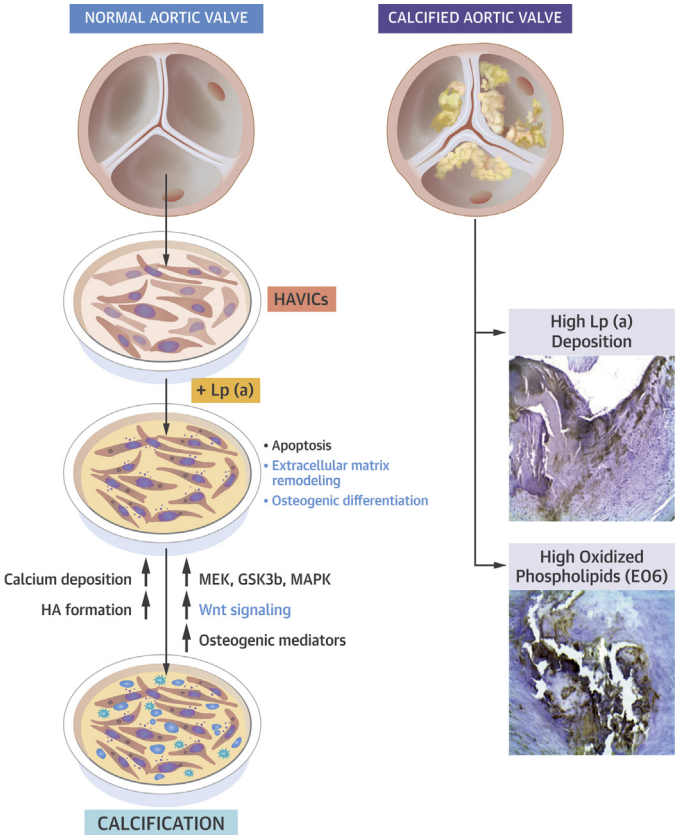
VISUAL ABSTRACT



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HIGHLIGHTS

- **Lp(a) significantly increased alkaline phosphatase activity, phosphate and calcium content, and matrix vesicle formation and induced apoptosis and calcification of normal human aortic valve interstitial cells.**
- **The type of minerals induced by Lp(a) resembles that seen in calcified human aortic valves as shown by Raman spectroscopy.**
- **Lp(a)-induced calcification of human aortic valve interstitial cells is mediated by activation of MAPK38, GSK3β, and Wnt signaling.**
- **Inhibition of GSK3β and MAPK38 significantly reduced lipoprotein(a)-induced aortic valve interstitial cell calcification.**
- **Lp(a)is abundant in calcified aortic valves, and lipoprotein(a) immunoreactivity colocalized with that of oxidized phospholipids.**

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

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SUMMARY

Lipoprotein(a), or Lp(a), significantly increased alkaline phosphatase activity, release of phosphate, calcium deposition, hydroxyapatite, cell apoptosis, matrix vesicle formation, and phosphorylation of signal transduction proteins; increased expression of chondro-osteogenic mediators; and decreased SOX9 and matrix Gla protein ($p < 0.001$). Inhibition of MAPK38 and GSK3β significantly reduced Lp(a)-induced calcification of human aortic valve interstitial cells ($p < 0.001$). There was abundant presence of Lp(a) and E06 immunoreactivity in diseased human aortic valves. The present study demonstrates a causal effect for Lp(a) in aortic valve calcification and suggests that interfering with the Lp(a)pathway could provide a novel therapeutic approach in the management of this debilitating disease. (J Am Coll Cardiol Basic Trans Science 2017;2:358–71) © 2017 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ABBREVIATIONS AND ACRONYMS

ALP = alkaline phosphatase
apo(a) = apolipoprotein(a)
BMP = bone morphogenetic protein
FWHM = full width half maximum
HAVIC = human aortic valve interstitial cell
LDL = low-density lipoprotein
Lp(a) = lipoprotein(a)
LOX-1 = oxidized LDL receptor 1
MAPK = mitogen-activated protein kinase
MGP = matrix Gla protein
mRNA = messenger ribonucleic acid
OxPL = oxidized phospholipid

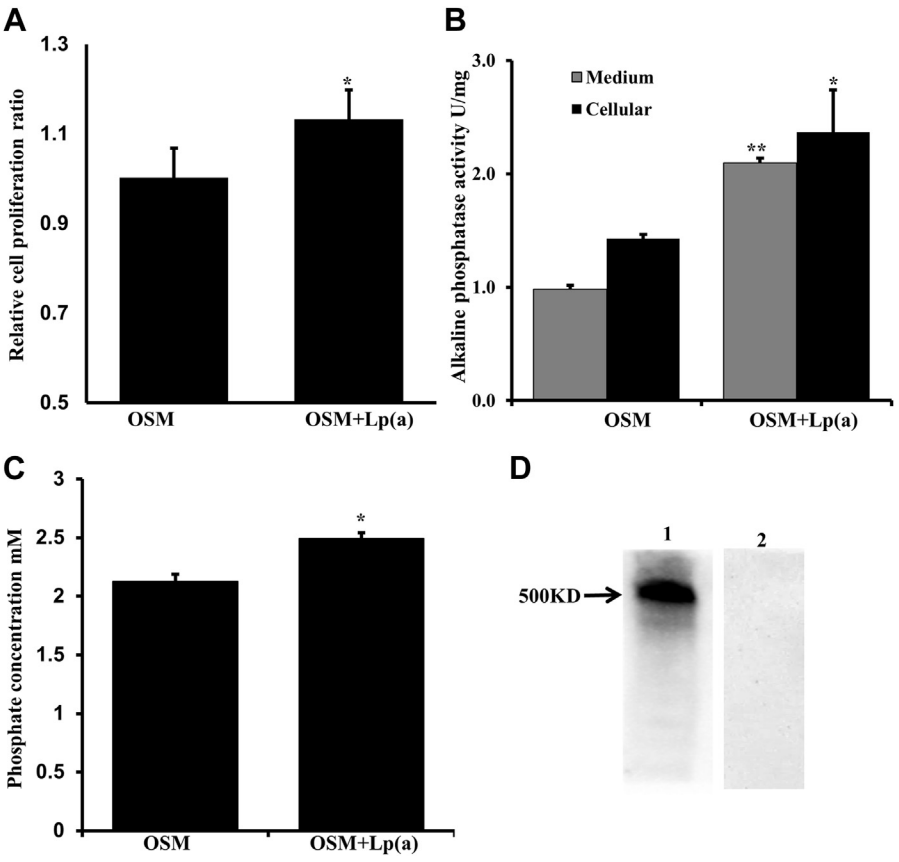
Aortic valve stenosis is a debilitating and relatively common condition for which no medical therapy exists. Classically thought to result from degeneration of the aortic valve, a number of novel contributing mechanisms have been identified, including the role of mechanical stress, age-related changes in valvular cells and extracellular matrix, the regulation of calcium flux in both physiological and cardiovascular calcification (1), and the role played by the renin-angiotensin system in promoting valve remodeling (2,3). Despite the similarities between aortic valve stenosis and coronary artery disease, both in terms of clinical risk factors and histopathological manifestations, statins do not have any effect on the progression of aortic valve stenosis (4,5), and it is increasingly evident that the pathogenesis of the 2 conditions is different.

Recently, a genome-wide association study in 3 large cohorts revealed that a single-nucleotide polymorphism in the apolipoprotein(a) (*LPA*) locus was significantly associated with the presence of aortic valve calcification (6,7). This variant was also shown to associate with clinical aortic valve stenosis and valve replacement in several longitudinal cohorts, and current genetic evidence using the concept of Mendelian randomization suggests a probable causal role for circulating lipoprotein(a) [Lp(a)]. *LPA* encodes apolipoprotein(a) [apo(a)], an evolutionarily novel protein expressed only in primates, which is highly homologous with plasminogen, suggesting a possible origin via gene duplication. LPA is predominately expressed in hepatocytes (8–10); once secreted, apo(a) binds low-density lipoprotein (LDL) via disulfide bridging and stearic interaction (11), forming Lp(a). The latter has long been recognized as a risk factor for cardiac disease, but its exact role in vivo has remained contentious (12).

Nonetheless, several lines of evidence suggest that Lp(a) could play a fundamental role in the pathogenesis of aortic valve stenosis and its precursor lesion, aortic valve sclerosis. High levels of circulating Lp(a) have been associated with an increased incidence of aortic valve stenosis (13) and aortic valve sclerosis (14) in diverse human populations. Lp(a) scavenges oxidized phospholipid (OxPL) in human plasma (15), binding it covalently at a lysine binding site in kringle domain IV type 10 (KIV10) of Lp(a) that is thought to contribute to the pathological activity of Lp(a) (16). The association between high levels of Lp(a) and calcific aortic valve disease, in contrast, could result from either direct effects of Lp(a) or the higher serum OxPL content seen in patients with high Lp(a) levels. Although a previous study showed that Lp(a) enhanced atherosclerosis and vascular calcification in LDL receptor-defective Watanabe heritable hyperlipidemic transgenic rabbits (17), the effects of Lp(a) on human aortic valve interstitial cells (HAVICs) remain to be established.

Here, we hypothesized that exposure of cultured HAVICs isolated from noncalcified valves to Lp(a) would induce their phenotypic differentiation via activation of pro-osteogenic pathways. Therefore, we tested the effect of Lp(a) on HAVIC proliferation, apoptosis, differentiation, and gene and protein expression profile. We also determined what types of phosphate and calcium minerals are induced by Lp(a) in HAVICs using Raman spectroscopy. Furthermore, we sought to determine the effect of Lp(a) on phosphorylation of mitogen-activated protein kinases (MAPKs). Finally, we documented the tissue presence of Lp(a) and OxPLs in a large number of calcified and normal aortic valves.

FIGURE 1 Effects of Lp(a) Treatment on HAVICs



HAVICs were incubated in OSM in the presence or absence of 50 μg/ml purified Lp(a) (Athens Research & Technology, Inc., Athens, Georgia) for 48 h. **(A)** Cell proliferation measured using an MTT protocol and presented as relative cell proliferation rate. *p = 0.013 vs. OSM control. **(B)** Medium and cellular ALP activity measured with an ALP activity colorimetric assay kit and normalized to total protein concentration. Lp(a)-treated cells showed higher ALP activity in medium (**p = 0.0001) and cell lysate (*p = 0.003) than OSM control. **(C)** Medium phosphate release measured using a phosphate colorimetric assay kit (BioVision, Inc., Milpitas, California). *p = 0.0001 vs. OSM (control). **(D)** No detectable OxPLs were seen on purified Lp(a) using anti-OxPL E06 antibody. Purified human Lp(a) (1 μg) was loaded on 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1: Lp(a) was detected using anti-Lp(a) antibody (diluted at 1:10,000; Novus Biologicals, LLC, Littleton, Colorado); lane 2: no OxPLs were detected in purified Lp(a) using E06 antibody (diluted at 1:1,000; Avanti Polar Lipids, Inc., Alabaster, Alabama). ALP = alkaline phosphatase; HAVICS = human aortic valve interstitial cells; Lp(a) = lipoprotein(a); OSM = osteogenic medium; OxPL = oxidized phospholipid.

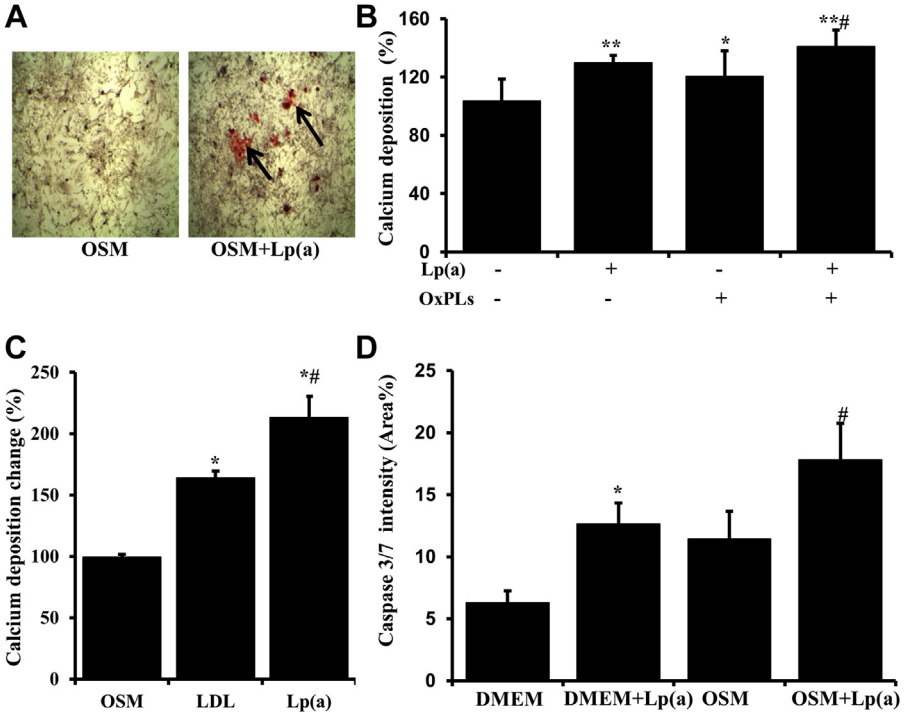
METHODS

A detailed Methods section is provided in the Supplemental Appendix. Briefly, primary human HAVIC cell lines were generated as described previously (18,19), with cultured HAVICs showing positive staining of α-smooth muscle actin, indicating myofibroblast phenotype, after 2 passages. HAVICs at passages 3 to 5 were used for all experiments. To induce calcification, HAVICs were cultured in osteogenic medium (full Dulbecco's modified Eagle's medium plus 2 mmol/l buffered phosphate, pH 7.4). The study was approved by the McGill University Health Centre Ethics Committee, and written consents were obtained from all participating patients.

RESULTS

EFFECTS OF Lp(a) ON HAVICs. Although previous studies have delineated the effect of Lp(a) on vascular cells (10), its effect on valve interstitial cells remains to be elucidated. Here, we found that short-term (up to 48 h) incubation of HAVICs isolated from noncalcified human aortic valves with 50 μg/ml Lp(a) significantly increased cell proliferation (p = 0.013) (Figure 1A), cellular (p = 0.003) and

FIGURE 2 Effects of Lp(a) Treatment on HAVIC Calcification and Apoptosis



(A) Prolonged Lp(a) treatment for 3 weeks at 50 μg/ml in OSM increased calcification nodule formation in HAVICs **(arrows)**. **(B)** Calcium deposition measured after 2 weeks of Lp(a) incubation. *p = 0.03, **p = 0.001 vs. control; #p = 0.04 vs. Lp(a)-treated cells. **(C)** Lp(a) demonstrated a greater effect on calcium deposition than LDL (Alfa Aesar, Tewksbury, Massachusetts) after 2 weeks of incubation in OSM. *p < 0.00001 vs. OSM control; #p = 0.006, Lp(a) treated vs. LDL treated. **(D)** Lp(a) effects on HAVIC apoptosis assessed using caspase-3/7 activity. *p = 0.0003, Lp(a)-treated vs. DMEM; #p = 0.007 Lp(a)-treated vs. OSM. DMEM = Dulbecco's modified Eagle's medium; LDL = low-density lipoprotein; other abbreviations as in Figure 1.

medium (p = 0.0001) alkaline phosphatase (ALP) activity (Figure 1B), and release of phosphate (p = 0.0001) (Figure 1C). OxPLs were not detected using mouse anti-OxPL antibody EO6 in the purified human Lp(a) (Figure 1D). Prolonged incubation (3 weeks) of HAVICs with Lp(a) significantly increased calcification nodules (Figure 2A), resulted in higher calcium deposition (Figures 2B and 2C) and caspase-3 and caspase-7 activity (Figure 2D), and decreased cellular proliferation as demonstrated by MTT assay (19.63 ± 4.12%; p < 0.01) (Supplemental Figure 1). Incubation of HAVICs with Lp(a) plus OxPLs significantly augmented the effects of Lp(a) on calcium deposition (Figure 2B). The effect of Lp(a) on HAVIC mineralization was significantly higher than that of LDL (p = 0.006) (Figure 2C). To characterize the type of minerals induced by Lp(a) treatment, we used Raman spectroscopy, which revealed that the spectra collected on HAVICs incubated in osteogenic medium in the presence of Lp(a) for 3 weeks showed a ν_1PO_4 peak centered at 960 cm^{-1} (Figure 3A), which is indicative of hydroxyapatite. A similar peak was found on the spectra of synthetic hydroxyapatite, bone, and enamel, as well as HAVICs collected from calcified heart valves (Figure 3A). HAVICs incubated in osteogenic medium for the same period but without addition of Lp(a) did not show a significant presence of phosphate-containing minerals. A complete list of peak assignments for these spectra is presented in Supplemental Appendix.

By integrating the area of the ν_1 phosphate peak and dividing this value by the area of the peak at 1,440 cm^{-1}, which is relative to the stretching of CH_2/CH_3 groups, we estimated the amount of inorganic deposits relative to the amount of organic material found in HAVICs isolated from calcified heart valves and HAVICs incubated in osteogenic medium for 3 weeks, with or without the presence of Lp(a) (Figure 3B). HAVICs treated with Lp(a) showed more abundant mineral deposition than nontreated cells (p = 0.002) or HAVICs collected from calcified aortic valves (p = 0.03).

FIGURE 3 Effects of Lp(a) on Inorganic Mineralization in HAVICs

(A) Representative Raman spectra. Noncalcified HAVICs were incubated in OSM in the absence (a) or presence (b) of Lp(a) for 3 weeks; calcified HAVICs were cultured in DMEM medium (c) and bone (d), enamel (e), and standard HA (f). n = 3 for each sample. **(B)** Inorganic to organic ratio measured by dividing the area of the ν1PO4 peak (inorganic marker) at 960 cm^{-1} and the area of the CH2/CH3 (organic compounds containing -CH2/-CH3 groups) stretching at 1,440 cm^{-1} (n = 3 for each sample). Noncalcified HAVICs were incubated in OSM for 3 weeks in the absence or presence of Lp(a), and stenotic HAVICs were cultured in DMEM for 3 weeks. Lp(a)-treated HAVICs and calcified HAVICs showed significantly higher inorganic-organic ratios than nontreated OSM control, with *p = 0.002 and p = 0.0001, respectively. Lp(a)-treated HAVICs showed a higher ratio against calcified HAVICs (#p = 0.03). **(C)** FWHM measured on the ν1PO4 peak at 960 cm^{-1} for the spectra of HAVICs from calcified heart valve, noncalcified HAVICs incubated with Lp(a) in OSM for 3 weeks, enamel, bone, and HA. *Significant differences: Lp(a)-treated HAVICs vs. calcified AV, $p < 0.001$; vs. enamel, bone, and HA, $p < 0.0001$. AV = aortic valve; FWHM = full width at half maximum; HA = hydroxyapatite; other abbreviations as in Figures 1 and 2.

To further characterize the type of calcific nodules induced by Lp(a) compared with diseased aortic valves, we estimated the degree of crystallinity of the hydroxyapatite deposits by measuring the full width at half maximum (FWHM) of the ν_1 phosphate peak. Wider peaks indicate a less crystalline material, whereas sharper peaks show a higher crystallinity. Clearly, the peak of synthetic hydroxyapatite is much sharper than any of those found in biological samples [bone, enamel, calcified heart valves, and Lp(a)-treated HAVICs] (Figure 3A). We compared the FWHM measured on all the spectra (Figure 3C). There was no difference in the FWHM measured for deposits found on HAVICs treated with Lp(a) (24.4 ± 0.6) compared with HAVICs from calcified heart valves (22.5 ± 0.5), which indicates somewhat similar crystalline deposits on Lp(a)-treated cells (Figure 3C). Significantly lower FWHMs ($p < 0.0001$) were found on synthetic hydroxyapatite (5.3 ± 0.1) and natural samples such as bone (18.1 ± 0.2) and enamel (11.7 ± 0.3) (Figure 3C), which indicates that less crystalline hydroxyapatite is deposited in ectopic calcification in heart valves than in physiological hard tissues.

To assess the morphological characteristics of Lp(a)-induced calcification in HAVICs, we used fluorescence microscopy, which revealed the presence of numerous calcium-containing membrane budding vesicles (Figure 4A). Scanning electron microscopy confirmed the presence of various-sized microvesicles on HAVICs (Figure 4B). Extracellular vesicles released in the culture medium were characterized with quantification techniques such as nanoparticle tracking analysis. Medium vesicle concentrations of Lp(a)-treated HAVICs were found to be significantly higher than those of nontreated cells in serum-free osteogenic medium at 7 days, and there was different size profiling between 50 and 500 nm in response to Lp(a) treatment (Figures 4C and 4D).

FIGURE 4 Presence of Extracellular Vesicles in HAVICs

(A) HAVICs exhibited calcium-containing cellular vesicles. 1: Phase-contrast image; 2: membrane lipophilic Vybrant DiO dye stains (Thermo Fisher Scientific, Carlsbad, California) of vesicles of various sizes **(green)**; 3: high-calcium-containing vesicles (arrows) stained with Calcium Orange AM (Thermo Fisher Scientific) **(red)**; 4: superimposed image of 1, 3, and nuclear **(blue)** stained with NucBlue reagent (Thermo Fisher Scientific). **(B)** Cluster of extracellular vesicles on HAVICs observed using scanning electron microscopy **(arrows)**. **(C)** HAVICs were treated with 50 μg/ml purified Lp(a) for 7 days in serum-free DMEM. The medium was freshly changed at days 3 and 5. The harvested media (days 3 and 7) were subjected to extracellular vesicle profiling using NanoSight NS500 (Malvern Instruments, Malvern, United Kingdom). Lp(a)-treated cells show higher particle concentration than nontreated, with *$p = 0.04$ at day 3 in DMEM, and **$p = 0.0001$ at day 7 in OSM; $n = 3$ for each group. **(D)** Extracellular vesicle size profiling shows different particle concentration vs. particle size profiling of Lp(a)-treated HAVICs at 7 days. Each condition was sampled 5 times for size profiling using NanoSight NS500. Data were combined and presented as average particle concentration vs. particle size (mean ± SEM). Abbreviations as in Figures 1 and 2.

To examine the effects of Lp(a) on the inflammatory state of HAVICs, profiling of cytokine release was tested with a Proteome Profiler of human cytokine array, panel A (R&D Systems Inc., Minneapolis, Minnesota). Incubation of HAVICs with 50 μg/ml Lp(a) for 48 h did not affect the release of inflammatory cytokines or chemokines, including interleukin-6, interleukin-8, monocyte chemoattractant protein-1, plasminogen activator inhibitor-1, macrophage migration inhibitory factor, and GROα (Figure 5A). Conversely, phospho-MAPK profiling assayed with a Proteome Profiler of human phospho-MAPK array found that Lp(a) significantly increased phosphorylation of MAPK38, MAPK kinase 3/6 (MKK3/6), MSK2, and GSK3α/β (Figures 5B and 5C). Inhibition of MAPK38 with SB203580 or GSK3α/β with CHIR99021 significantly reduced ALP activity and calcium deposition (Figure 5D). PD184352 significantly increased calcium deposition (Figure 5D).

FIGURE 5 Effects of Lp(a) on Cytokines and Phosphorylated MAPKs in HAVICs

(A) Effects of Lp(a) on release of cytokines and chemokines by HAVICs. Rectangular outlines indicate reference controls. **(B)** Effects of Lp(a) on phosphorylated MAPKs in HAVICs. Cells were treated with or without 50 μg/ml Lp(a) for 1 h, and total proteins were extracted and used for phosphorylated MAPK profiling using Proteomic Profiler of Human Phospho-MAPK Array (R&D Systems, Minneapolis, Minneapolis) according to the manufacturer's instructions. Blot images were taken using Chemidoc MP (Bio-Rad Laboratories, Hercules, California). Rectangular outlines indicate reference controls. **(C)** Densitometry of phosphorylated MAPKs. Intensities of individual molecules on blot **(B)** were first normalized to the 3 sets of control spots **(rectangle boxed spots)**, and calculated as ratio against non-Lp(a)-treated controls. Total intensities of molecules shown are presented as mean ± SEM. *Significance of Lp(a)-treated vs. nontreated controls, $p = 0.002$ for p38β/δ/y, $p = 0.001$ for MKK3/6, $p = 0.001$ for MSK2, and $p = 0.03$ for GSK-3α/β. **(D)** Effect of MAPK and GSK3b inhibition on HAVIC calcification. HAVICs were treated with Lp(a) in the presence or absence of the MAPKP38 inhibitor SB203580 (SB; 2 μmol/l, Sigma-Aldrich Co., LLC., St. Louis, Missouri), GSK3β inhibitor CHIR99021 (CH; 5 μmol/l, Sigma-Aldrich Co., LLC.) or the MEK1/2 inhibitor PD184352 (PD; 2 μmol/l, Sigma-Aldrich Co., LLC.) for 2 weeks in OSM. Total calcium deposition was measured using Alizarin Red S stain and 10% cetylpyridinium chloride extraction protocol, normalized with total protein concentration, and presented as percentage changes against OSM control. One-way ANOVA test showed *$p = 0.007$, Lp(a)-treated vs. nontreated control; #significant difference between Lp(a)-treated and Lp(a) plus inhibitors ($p < 0.00001$). MAPK = mitogen-activated protein kinase; other abbreviations as in Figure 1.

To assess the effects of Lp(a) on HAVIC function and remodeling, we determined its effects of gel contractility, nuclear activity of β-catenin, and expression of osteogenic mediators. Short-term (up to 48 h) incubation of HAVICs with Lp(a) significantly increased gel contractility, induction of β-catenin translocation (Figures 6A to 6C), messenger ribonucleic acid (mRNA) expression of osteocalcin, bone-specific transcription factor *SP7* (osterix), the transcriptional activator of osteoblast differentiation (*OSF2*), Runt-related factor 2 (*RUNX2*), Msh homeobox 2 (*MSX2*), cadherin-associated protein B1

FIGURE 6 Effects of Lp(a) on HAVIC Gel Contraction, β-Catenin Nuclear Translocation, and Lp(a) Uptake

(A) Effect of Lp(a) treatment on gel contraction. Lp(a)-treated vs. nontreated control: **p = 0.01 at 6 h, *p = 0.04 at 24 h; n = 6. **(B)** β-catenin nuclear translocation assessed with confocal microscopy. HAVICs were treated with or without 50 μg/ml Lp(a) for 48 h in OSM on CultureSlide (Falcon), stained with rabbit anti-CTNNB1 antibody and Alexa Fluor 488 donkey anti-rabbit IgG (Thermo Fisher Scientific), and stained with NucBlue (nuclear stain; ThermoFisher Scientific, Grand Island, New York). Tile images were taken with a Zeiss LSM780 confocal microscope (20× magnification). Nuclear masking and average integrated intensity were analyzed with Cellprofiler and are presented as mean ± SEM. Total number of nuclei analyzed: 30 min: Lp(a) n = 69, Control n = 76; 1 h: Lp(a) n = 59, Control n = 64; 4 h: Lp(a) n = 89, Control n = 70; 24 h: Lp(a) n = 74, Control n = 76. Lp(a)-treated vs. nontreated control: **p = 0.001 at 1 h and 6 h, *p = 0.04 at 24 h. **(C)** β-catenin nuclear translocation assessed with high content screening confocal imaging. HAVICs were treated with or without Lp(a) 50 μg/ml for 1 h in triplicates of 24-well glass-bottom optical culture plates and stained for CTNNB1 as described above. Tile images were taken using ImageXpress Micro XLS at 10× magnification. Average integrated nuclear CTNNB1 intensity was analyzed using MetaXpress as described above. *p = 0.003, Lp(a)-treated vs. nontreated control. N = total number of nuclei analyzed. **(D)** Time course of radiolabeled ^{3}H-chlosterol Lp(a) uptake in HAVICs; n = 3. HAVICs cultured in triplicates of 24-well plates were loaded with ^{3}H-chlosterol Lp(a) at ~15,000 cpm/ml in serum-free DMEM. Cell lipid extraction was used for liquid scintillation count. Cellular uptake of labeled Lp(a) at different time intervals was calculated as percentage against initial loading. Abbreviations as in Figures 1 and 2.

(*CTNNB1*), *WNT3a, WNT5b*, bone morphogenetic protein-2 (*BMP-2*), and *BMP-4* (Table 1). In addition, there was a significant increase in *collagen IA1, KLF4,* and myocardin and a significant decrease in α-smooth muscle actin. After 3 weeks of incubation with Lp(a), although there was still a significant increase in expression of *ALPL, BMP-2, WNT11,* oxidized LDL receptor 1 (*LOX-1*), collagen IA1, and myosin heavy chain 11 (*MYH11*), factors such as matrix Gla protein (*MGP*) and almost all other nuclear factors and osteogenic mediators were significantly reduced (Table 1).

Lp(a) UPTAKE BY HAVICs. To determine whether HAVICs are capable of uptake of circulating Lp(a), we determined Lp(a) internalization in vitro. Radiolabeled Lp(a) was rapidly internalized by HAVICs, thereby confirming the uptake of Lp(a) by aortic valves (Figure 6D).

EXPRESSION OF apo(a) mRNA AND PROTEIN IN AORTIC VALVES AND HAVICs. *LPA* mRNA expression was detected in some RNA samples from calcified valves (Figure 7A). Most of the 11 primary cultured HAVICs showed detectable *LPA* mRNA (Figure 7B), and real-time polymerase chain reaction product was

TABLE 1 Osteogenic-Related Genes Differentially Regulated in HAVICs Treated With Lp(a) in Osteogenic Medium

Gene Name	Relative Normalized Expression (Fold)
Day 2	
BGLAP	4.99
SP7	4.44
WNT3a	1.92
RUNX2	1.72
BMP2	1.69
MYOCD	1.66
CTNNB1	1.62
COL1A1	1.52
WNT5b	1.49
OSF2	1.46
MSX2	1.45
BMP4	1.36
KLF4	1.33
SOX9	0.66
MGP	0.64
αSMA	0.54
OPN	0.51
LOX1	0.27
3 Weeks	
MYH11	2.70
WNT11	2.60
LOX1	1.87
COL1A1	1.80
BMP2	1.50
ALPL	1.42
OSF2	0.70
MYOCD	0.64
MGP	0.60
CTNNB1	0.56
RUNX2	0.52
αSMA	0.49
WNT3a	0.37
SP7	0.26
OPN	0.24
BGLAP	0.05

HAVIC = human aortic valve interstitial cell; Lp(a) = lipoprotein(a).

sequenced to confirm the correct identity of *LPA* mRNA (Figure 7C). Western blot showed weak to strong apo(a) protein in calcified human aortic valves (Figure 7D, lanes 4 to 11). Apo(a) protein was also seen in cultured HAVICs (Figure 7D). Endogenous LPA expression was detected in cellular vesicles of various sizes and in endoplasmic reticulum in cultured HAVICs using mouse anti-LPA antibody (Figure 7E).

Lp(a) AND OxPL IMMUNOREACTIVITY IN CALCIFIED HUMAN AORTIC VALVES. Only weak and infrequent apo(a) immunoreactivity was seen in normal aortic valves or normal leaflets of stenotic aortic valves (Figure 8A). In contrast, strong immunoreactivity for apo(a) was seen in calcified aortic valves. The immunostaining was evident in areas of subendothelial, thickened fibrosa; calcification; activated myofibroblasts; and mononuclear inflammatory cells (Figures 8B, 8C, 8D, 8F, 8J, and 8M). Inflammatory cells were identified using anti-CD68 antibody (Figure 8E), and myofibroblast cells were identified using anti-αSMA antibody (Figure 8G).

Deposition of OxPLs in aortic valve tissues was detected with E06 monoclonal antibody. The latter is a murine monoclonal immunoglobulin M natural antibody that recognizes the phosphocholine head group of OxPLs and was developed using apolipoprotein E knockout mice fed a high-fat diet (20). Immunoreactivity for E06 was mainly seen in endothelial cells and subendothelium of normal leaflets of the human aortic valve. Strong immunostaining for E06 was seen in subendothelial, thickened fibrosa; calcification; lipid core; and mononuclear inflammatory cells in calcified leaflets of the human aortic valve (Figures 8H, 8I, 8K, and 8L). E06 immunoreaction was seen in similar regions to that of Lp(a) in diseased segments, but they exhibited differential immunostaining patterns, with some cases showing very strong E06 staining but minimal Lp(a) stain or vice versa (Figures 8I, 8J, 8L, and 8M). E06 exhibited a wider distribution and more cellular staining than Lp(a). Colocalization studies revealed the occasional presence of Lp(a) and E06 in the same cells. Negative control sections immunostained with nonimmune serum or pre-absorbed with the respective antigens did not reveal any staining (data not shown).

Mean ranks of Lp(a) and E06 immunostaining were significantly higher in calcified than in normal leaflets ($p < 0.0002$). There was a significant correlation between the immunostaining for Lp(a) and E06 ($r = 0.284$, $p = 0.001$). Also, there was a significant correlation between the immunostaining for Lp(a) and E06, respectively, and the presence of lipids (Lp(a): $r = 0.243$, $p < 0.005$; E06: $r = 0.527$, $p < 0.0001$) and calcification (Lp(a): $r = 0.343$, $p < 0.05$; E06: $r = 0.548$, $p < 0.0001$). Only E06, not Lp(a), immunoreactivity correlated with the presence of inflammation ($r = 0.30$, $p < 0.001$). Moreover, there was a significant correlation between E06 immunostaining and peak pressures (Pmean $r = 0.32$; Pmax $r = 0.326$, $p < 0.01$).

DISCUSSION

Although several studies have suggested a potential role for Lp(a) in the pathogenesis of cardiovascular diseases, it remained unclear whether the *LPA* gene is locally expressed and whether Lp(a) could directly

FIGURE 7 LPA mRNA and Protein Expression Were Detected in Primarily Cultured HAVICs and Aortic Valve Tissues

(A) RT-PCR detection of *LPA* mRNA expression in primarily cultured HAVICs. Lane 0: DNA marker; lanes 2, 5, and 8 show positive signal at products 136 bp. **(B)** RT-PCR detection of *LPA* mRNA expression in stenotic aortic valves. Lane 0: DNA marker; lanes 1, 2, 5, 6, 7, 9, and 10 show positive signal at 136 bp. **(C)** Sequencing of gel purified RT-PCR product confirms the correct identity of *LPA* mRNA. **(D)** LPA protein expression in stenotic aortic valve tissues and HAVICs. **(Top)** Lanes 1 through 3, nonstenotic valves; lanes 4 through 11, stenotic valves; lane 12, human serum sample serving as loading marker. Lanes 8 and 9 show double bands indicating possible dual isoforms. **(Bottom)** LPA protein expression in 7 cultured HAVICs. Lane 1 show double bands indicating possible dual isoforms. **(E)** Immunofluorescence cytochemistry detection of LPA in HAVICs using mouse anti-LPA antibody and Alexa Fluor 488 donkey anti-mouse immunoglobulin G **(green)**; **solid arrows** show various-sized cellular vesicles. **Red** indicates pseudocolored F-actin stained with ActinRed reagent; **blue** indicates nuclei stained with NucBlue reagent. LPA = apolipoprotein(a) gene; mRNA = messenger ribonucleic acid; RT-PCR = real-time polymerase chain reaction; other abbreviations as in Figure 1.

induce calcification and remodeling of the human aortic valve. The present study demonstrates for the first time a causal relationship between Lp(a) and the induction of aortic valve calcification. Previous studies have shown increased plasma levels of Lp(a) in patients with aortic valve disease, and downstream signaling from Lp(a) such as autotaxin produced similar effects (13,14,21). A recent study showed that elevated levels of Lp(a) and OxPLs could predict the progression of aortic valve stenosis (22). Here, we systematically studied the distribution of Lp(a) and E06 in relation to disease severity in 112 cases of diseased aortic valves and demonstrated the presence of both molecules and their correlation with the presence of tissue lipid deposition and calcific nodules. We also observed a significant correlation between Lp(a) and OxPL immunoreactivities. E06 immunoreactivity, but not Lp(a), correlated with tissue inflammation and peak pressures. In 2 recently published articles, Nordestgaard and Langsted et al. (23,24) reported that increased plasma Lp(a) was not associated with increased inflammatory markers and eluded to the possibility that E06 merely recognizes apoB-100 on the Lp(a) particle. They also suggested that Lp(a) could cause aortic valve stenosis through a mechanism other than OxPL-dependent inflammation. Here, we demonstrate that Lp(a) along, without the presence of OxPLs induced an osteogenic differentiation of HAVICs.

Our data show that LPA is locally expressed in the stenotic aortic valve and can also be taken up by valve cells. We also demonstrated a significant increase in the number of matrix vesicles in response to Lp(a) treatment. Moreover, we have shown that Lp(a)

FIGURE 8 Immunostaining of LPA and OxPLs in Human Aortic Valve Tissues

(A) Normal valve shows no obvious LPA immunoreactivity; aorta side on top **(dashed arrow)**. High LPA immunoreactivity in thickened fibrosa **(B)**, calcified lesion **(C, J)**, monocytes in inflammatory region **(D)**, myofibroblast cells **(F)**, and lipid core **(M)**. **(E)** CD68 immunoreactivity in the same region of LPA staining **(D)**. **(G)** α-Smooth muscle actin staining shows the myofibroblast cells in the same region of LPA staining **(F)**. High E06 immunoreactivity in subendothelial, thickened fibrosa, and ventricularis **(H)**, calcified lesion **(I)**, angiogenic vessels **(K)**, and lipid core **(L)**. **(J)** LPA immunoreactivity in the calcified region of E06 staining **(I)**. **(M)** LPA immunoreactivity in the lipid core of E06 staining **(L)**. Abbreviations as in Figures 1 and 7.

induces osteogenic differentiation of HAVICs through induction of *ALPL* and pro-osteogenic mediators such as *osteocalcin, osterix, Runx2, BMP-2*, and *Wnts*. Furthermore, the signaling pathway of Lp(a)-mediated calcification involves activation of several MAPKs known to induce BMP-2 mediated osteoblast differentiation (25). These findings confirm a causal relationship between Lp(a) and aortic valve calcification.

Raman spectroscopy can be used to identify the calcium phosphate phases present in biological mineral deposits by analyzing the position of the peak that corresponds to the ν_1 stretching of PO_4 groups. The mineral deposits found on Lp(a)-treated HAVICs were composed of hydroxyapatite, the same mineral found both in hard physiological tissues (bone and teeth) and in ectopic mineralization. Although almost no minerals were deposited by HAVICs incubated in osteogenic medium for as long as 3 weeks, a large amount was found upon treatment with Lp(a). The relative amount of inorganic to organic material in Lp(a)-treated HAVICs was higher than that measured in HAVICs collected from calcified heart valves. This was somewhat expected, because cells isolated from calcified valves lose most of their calcium during isolation and subculturing. The crystallinity of the hydroxyapatite deposits found in Lp(a)-treated HAVICs was somewhat lower than that found in HAVICs collected from calcified heart valves, which could imply an earlier deposition stage in Lp(a)-treated samples. Ectopic calcifications are known to be less crystalline than physiological ones (26).

Our data showed that Lp(a)-induced HAVIC osteogenic differentiation and apoptosis were associated with increased phosphorylation of several kinases known to be involved in cellular remodeling and apoptosis, such as MAPK38 and GSK3β. Moreover, inhibition of MAPK38 or GSK3β resulted in a significant reduction of Lp(a)-induced HAVIC calcium deposition. Our findings are consistent with previous studies that demonstrated an important role for MAPK inhibition in the reduction of aortic valve interstitial cell calcification (1,27,28). On the other hand, inhibition of MEK1/2 with PD184352 resulted in a significant increase in calcium deposition. PD184352 is known to increase cell apoptosis, a process thought to play a pivotal role in valve calcification, and this explains the augmented effects on cell calcification. Importantly, the most profound decrease in calcium deposition was observed with the GSK3β inhibitor CHIR99021. Inactivation of GSK3β by phosphorylation attenuates degradation of β-catenin, and nuclear translocation of β-catenin is favored. The latter is known to drive the process of osteogenic differentiation through activation of Wnt and BMP-2 signaling pathway. However, CHIR99021 appears to be blocking the non-canonical Wnt-GSK3MT pathway, thereby offering an alternative way to mitigate Lp(a)-induced effects in aortic valve calcification.

HAVICs are thought to undergo a phenotypic switch in aortic valve disease that results in increased matrix deposition and calcification. Here, we have shown that Lp(a) increased gel contractility and expression of myocardin, KLF4, MYH11, and collagen, factors known to be involved in myofibroblast differentiation and matrix deposition. Moreover, in agreement with previous studies demonstrating the presence of a number of osteogenic mediators in calcified valves (29), we have demonstrated increased ALP activity in response to Lp(a) treatment. The increase in ALP activity was associated with increased calcific nodules, calcium, and phosphate deposition. In addition, incubation of HAVICs with Lp(a) induced the expression of a number of nuclear transcription factors such as CTNNB1 (β-catenin) and osterix, known to play an important role in valve calcification (30). It is well established that in the presence of Wnt ligand, CTNNB1 is not ubiquitinated and accumulates in the nucleus, where it acts as a coactivator for transcription factors of the TCF/LEF (T-cell factor/lymphoid enhancer-binding factor) family, thereby inducing Wnt responsive genes (27). Moreover, BMP-2, which has been detected in valve interstitial cells isolated from the aortic valve of aged rats, is thought to stimulate calcification by activating Wnt/β-catenin signaling, thereby increasing the expression of alkaline phosphatase and upregulating expression of the osteochondrogenic transcription factor *MSX2* (27,31). We found that both *BMP-2* and *MSX2* were increased by Lp(a). Indeed, MSX2 has been identified in valves from experimental models of aortic valve calcification, where it was localized to areas of calcification (32). These signaling pathways converge to induce expression of the master osteoblast transcription factor RUNX2 (33), another factor that was induced by Lp(a). Once RUNX2 is expressed, cells are committed to an osteoblast lineage, upregulate expression of calcification-related proteins (including osteocalcin), and undergo calcification (34). Indeed, our study showed a significant increase in osteocalcin. Taken together, these findings indicate that Lp(a) is capable of driving HAVICs to an osteoblast-like phenotype in which these cells express all of the markers of functional osteoblasts, elaborate bone matrix proteins, and mineralize to form calcific nodules typical of aortic valve calcification.

Downstream of the BMP-Smad and BMP-Wnt/β-catenin signaling pathways is the transcription factor osterix. This transcription factor has also been shown to be necessary for bone formation and has been detected in activated valve interstitial cells and inflammatory cells in calcified human aortic valves (33,35). Here, we showed that incubation of HAVICs with Lp(a) significantly increased the expression of osterix and Wnts such as *WNT3a* and *WNT11*. Wnt proteins belong to a family of secreted lipid-modified polypeptide ligands that bind to receptor complexes

of frizzled protein/lipoprotein receptor-related protein 5 or 6, which leads to an accumulation of β-catenin in the nucleus. Activation of this signaling pathway in experimental models of aortic valve calcification and explanted human valves has been confirmed by demonstrating the expression of *Wnt3a*, lipoprotein receptor-related protein 5, and nuclear β-catenin in calcified valve tissue (28,36).

MGP and osteocalcin are both calcium-binding proteins that are thought to participate in the organization of bone tissue. Both have glutamate residues that are post-translationally carboxylated by the enzyme gamma-glutamyl carboxylase in a reaction that requires vitamin K hydroquinone. MGP prevents calcification by inhibiting BMP signaling (37). Interestingly, our data show that Lp(a) has reciprocal effects on *MGP* and *BMP-2* expression in HAVICs. *MGP* levels have been shown to be significantly lower in patients with aortic valve calcification than in individuals without valve disease (38). Experimental models of MGP deficiency develop early valve calcification, whereas transgenic mouse models that overexpress MGP are protected, even in the setting of hypercholesterolemia (37,39). These findings suggest that the Lp(a)-induced repression of *MGP* expression observed in our study might also contribute to the progression of aortic valve calcification. Another gene that showed bidirectional expression in response to Lp(a) was *LOX-1*. Lp(a) treatment significantly increased *LOX-1* expression by day 21. *LOX-1* is thought to be a receptor for Lp(a) and could be involved in the regulation of Fas-induced apoptosis. Of note, 10 and 21 days of treatment of HAVICs with Lp(a) significantly increased apoptosis and decreased *OPN*. In light of the above findings, effects of Lp(a) on *MGP*, *OPN*, and *LOX-1* warrant further investigation.

CONCLUSIONS

We used a variety of techniques to demonstrate for the first time that Lp(a) without the presence of OxPLs causes HAVIC remodeling and calcification. We also demonstrated the expression of the *LPA* gene in the diseased aortic valve. The development of PCSK9 inhibitors, known to decrease serum Lp(a), or Lp(a)-targeted drugs could therefore pave the way for new therapeutic possibilities to prevent or reverse Lp(a)-induced aortic valve calcification. Further studies are needed to address the in vivo effects of kinase and Wnt inhibitors in the prevention of aortic valve calcification.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: This study demonstrated that Lp(a) induced HAVIC remodeling and calcification, as well as the expression of extrahepatic LPA, in the diseased human aortic valve.

TRANSLATIONAL OUTLOOK: These findings may help to pave the way for new therapeutic possibilities to prevent or reverse Lp(a)-induced aortic valve calcification by targeting LPA or Lp(a)-lowering drugs such as the PCSK9 inhibitor.

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APPENDIX For an expanded Materials and Methods section as well as supplemental tables and a figure, please see the online version of this paper.