Mechanical strain induces the production of spheroid mineralized microparticles in the aortic valve through a RhoA/ROCK-dependent mechanism

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Abstract

Calcific aortic valve disease (CAVD) is a chronic disorder characterized by an abnormal mineralization of the leaflets, which is accelerated in bicuspid aortic valve (BAV). It is suspected that mechanical strain may promote/ enhance mineralization of the aortic valve. However, the effect of mechanical strain and the involved pathways during mineralization of the aortic valve remains largely unknown. Valve interstitial cells (VICs) were isolated and studied under strain conditions. Human bicuspid aortic valves were examined as a model relevant to increased mechanical strain such as in bicuspid aortic valve (BAV) [8]. Hence, abnormal mechanical cues applied to valve interstitial cells (VICs), the main cellular component of the aortic valve, may play a role in triggering mineralization [9,10]. Expression of ecto-nucleotidase enzymes including ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) plays a crucial role in controlling pathological mineralization [11]. Recently, a high level of ENPP1 has been detected in stenotic aortic valves. Furthermore, it has been documented that a high level of ENPP1 promotes apoptosis-mediated mineralization of valve interstitial cells (VICs) [12]. A previous study has shown that ectonucleotidase enzyme activity is promoted by mechanical strain in tendon cells [13]. In this work, we provide evidence that VICs under mechanical strain produce spheroid mineralized microparticles, which are liberated at the cellular surface during apoptosis. Furthermore, we found that mechanical

1. Introduction

Calcific aortic valve disease (CAVD) is the most common heart valve disorder and there is, so far, no medical treatment for this condition [1]. Mineralization of the aortic valve is the major culprit leading to the development of CAVD [2]. In the recent years, a growing number of papers have explored the mechanisms whereby mineralization of the aortic valve is triggered [3,4]. However, the key molecular processes remain to be investigated [5]. A study using electron microscope and energy dispersive X-ray (EDX) analyses has recently identified that dense spherical particles are present in explanted human stenotic aortic valves [6]. The composition of these particles is compatible with hydroxyapatite of calcium (HAC). However, the origin and the mechanism of production of these particles remain to be determined [7].

Mineralization of the aortic valve is accelerated in condition of increased mechanical strain such as in bicuspid aortic valve (BAV) [8]. Hence, abnormal mechanical cues applied to valve interstitial cells (VICs), the main cellular component of the aortic valve, may play a role in triggering mineralization [9,10]. Expression of ecto-nucleotidase enzymes including ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) plays a crucial role in controlling pathological mineralization [11]. Recently, a high level of ENPP1 has been detected in stenotic aortic valves. Furthermore, it has been documented that a high level of ENPP1 promotes apoptosis-mediated mineralization of valve interstitial cells (VICs) [12]. A previous study has shown that ectonucleotidase enzyme activity is promoted by mechanical strain in tendon cells [13]. In this work, we provide evidence that VICs under mechanical strain produce spheroid mineralized microparticles, which are liberated at the cellular surface during apoptosis. Furthermore, we found that mechanical
strain-induced apoptosis and mineralization of VICs relied largely on the expression of ENPP1 in a RhoA dependent manner.

2. Methods

2.1. Procurement of tissues for analyses and cell culture

We examined 54 bicuspid and 19 tricuspid stenotic aortic valves that were explanted from patients at the time of aortic valve replacement. The protocol was approved by the local ethical committee and informed consent was obtained from the subjects.

2.2. Aortic valve processing

Each valve excised at the time of surgery was placed in a container filled with HEPES. One cusp was also placed in RNA later (Ambion Inc., TX, USA) for posterior mRNA quantification by microarray or quantitative real-time PCR. One cusp was decalcified in Cal-Ex (Fisher, ON, Canada) for 24 h for histological analyses.

2.3. Valve interstitial cell isolation, culture and mineralizing media

Human (non-mineralized aortic valves obtained from heart transplant recipients) and mouse (C57BL/6) (Jackson Laboratory, ME, USA) valve interstitial cells (VICs) were isolated by collagenase digestion. Aortic valve leaflets were incubated for 30 min at 37 °C with agitation. In 1 mg/ml collagenase solution, washed with HEPES and incubated for 30 min at 37 °C with agitation in 4.5 mg/ml collagenase solution, and after washing, tissues were seeded in complete growth medium. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS); in mineralization assay cells were incubated in pro-calcifying medium: DMEM containing 5% FBS, 10−7 M insulin, 50 μg/ml ascorbic acid and 2 mM NaH2PO4.

2.4. Inhibitors

The following inhibitors were used in this study: ARL67156 (50 μM, Tocris, CA, USA), an ecto-ATPase inhibitor, HA1100 (30 μM, Tocris, CA, USA), Y27632 (8 μM, Selleckchem, TX, USA), Rho kinase inhibitor, HA1100 (30 μM, Tocris, CA, USA), Y27632 (8 μM, Selleckchem, TX, USA) inhibitors of the Rho kinase and Z-VAD-FMK (100 μM, R&D Systems, MN, USA), a pan caspase inhibitor.

2.5. Mechanical stress

Cells were cultured in Flexcell I flexible membrane dishes coated with Collagen I (Flexcell International, NC, USA). Strain was applied to VICs with the Flexcell 5000 system (Flexcell International, NC, USA), which allows application of precise deformation of culture plates via a vacuum pump under constant pressure monitoring. Cells were subjected to cyclic stretch produced by a computer-controlled station. Sineoidal waveforms at 1 Hz and forces that generated 10 or 15% stretch were applied for 24 h or 3 days.

2.6. Determination of calcium concentrations

Calcium content was determined by the Arsenazo III method (Synermed, CA, USA), which relies on the specific reaction of Arsenazo III with calcium to produce a blue complex. Results are measured at 650 nm on the Modular P800 Elesys of Roche Diagnostics apparatus (Roche Diagnostics, QC, Canada). This reaction is specific for calcium. Magnesium is prevented from forming a complex with the reactive. Results were reported as percent changes.

2.7. Real-time PCR

RNA was extracted from explanted valves of 73 patients and from cells during in vitro experiments. Total RNA was isolated with RNasy micro kit from Qiaegen (Qiagen, ON, Canada). The RNA extraction protocol was performed according to manufacturer’s instructions using 100 mg of tissues or 2 × 105 cells. The quality of total RNA was monitored by capillary electrophoresis (Experior, Biorad, ON, Canada). One microgram of RNA was reverse transcribed using the Quantitect Reverse Transcription Kit from Qiaegen. Quantitative real-time PCR (q-PCR) was performed with Quantitect SYBR Green PCR kit from Qiaegen on the Rotor-Gene 6000 system (Corbett Robotics Inc., San Francisco, CA, USA). Primers were obtained from Invitrogen (ON, Canada). The expression of the 18S gene was used as a reference gene to normalize the results for cell experiments and HPRT for human tissues.

2.8. Measurement of phosphodiesterase activity

Cells were subjected to mechanical stress for 24 h as described previously. Cells were washed three times with PBS 1× collected in harvesting buffer (95 mM NaCl, 45 mM Tris–HCl pH7.5, 0.1 mM PMFS, aprotenin 10 mg/ml) and sonicated. Extracts were centrifuged at 4 °C for 5 min at 300 g. Supernatants were used in the enzymatic assay. Para-nitrophenyl thymidine 50-monophosphate (pnp-TMP, Sigma-Aldrich, MO, USA) was used as a substrate in the assay. Briefly, the substrate was incubated along with cell extracts in the reaction buffer (1 mM CaCl2, 140 mM NaCl, 5 mM KCl, 50 mM Tris–HCl pH8.5) at 37 °C for 60 min and OD at 405 nm was measured [14]. A sample of cell extracts was quantified to account for protein amount.

2.9. Flow cytometer assay

Cells were stretched for three days and the level of VIC apoptosis was tested by FAC Scan Flowcytometer (BD Bioscience Pharmingen, CA, USA) by using annexin V-FITC labeling kit (BD Bioscience Pharmingen, CA, USA). One thousand cells were examined and the apoptosis rate was recorded as the percentage of apoptotic cells with annexin V positive. Cells were also labeled with ENPP1 antibody (Santa Cruz Biotechnologies, CA, USA) in 0.5% BSA in PBS 1× for 15 min at 4 °C. Cells were next washed three times with 0.5% BSA in PBS 1× followed with 15 minute incubation with alexa568-conjugated rabbit antibody. Positive cells were quantified with flow cytometry.

2.10. Determination of ATP level

VICs were subjected to mechanical stress for 3 days. The level of ATP released by cells in supernatants was measured using the ATP SL luminescent kit from BioThema (Cedarlane, ON, Canada) according to manufacturer instructions.

2.11. Transfection of valvar interstitial cells with siRNAs

VICs were cultured into 6-well plates, at a density of 1 × 105 cells per well, for determination of calcification. VICs were grown in a volume of 2 ml and allowed to adhere overnight in serum-containing antimicrobial-DMEM (5% CO2 and 37 °C). The next day, VICs were transfected by using HiPerfect reagent mixed with 300 ng of scramble or ENPP1-specific siRNA (Qiagen, ON, Canada). After two days, the medium was replaced with calcifying medium and a second transfection was performed before starting mechanical stress. Reduction of the target gene was measured by real-time PCR and enzymatic activity.
2.12. Immuno-fluorescence studies

2.12.1. Tissues

Frozen tissue slices were fixed in acetone (60%) and methanol (40%), washed 3 times with TBS1×, quenched in 50 mM NH4Cl for 30 min and blocked with 1% BSA in TBS1× for 30 min. Slides were then incubated with rabbit polyclonal ENPP1 antibody (Santa Cruz Biotechnologies, CA, USA) overnight at 4 °C. Slides were washed with TBS1× and incubated with an alexa568-conjugated anti-rabbit secondary antibody. Images were acquired using a 20× objective with an Olympus BX51 microscope (Olympus, ON, Canada), mounted with an Evolution QEi camera (Media Cybernetics, MD, USA) driven by Image-Pro Plus 7.0 (Olympus, ON, Canada).

2.12.2. Cells

Cells were fixed in 3.7% formaldehyde in PBS1× for 30 min at 37 °C. Cells were treated for 15 min with a 50 mM NH4Cl solution, washed with PBS1× and incubated in 4% BSA and 0.4% saponin in a PBS1× solution. Cells were washed with 0.4% saponin in PBS1× and then incubated with an alexa568 conjugated anti-rabbit secondary antibody and alexa488-conjugated phalloidin. Cells were mounted in DAPI-containing mounting medium. Images were acquired using a 40× objective with an Olympus BX51 microscope (Olympus, ON, Canada), mounted with an Evolution QEi camera (Media Cybernetics, MD, USA) driven by Image-Pro Plus 7.0 (Olympus, ON, Canada).

2.12.3. F-actin labeling

Cells were fixed in 3.7% formaldehyde in PBS1× for 30 min at 37 °C. Cells were treated for 15 min with a 50 mM NH4Cl solution, washed with PBS1× and incubated in 4% BSA in PBS1× containing alexa488 labeled phalloidin for 30 min. Cells were mounted in DAPI-containing mounting medium. Images were acquired using a 40× objective with an Olympus BX51 microscope (Olympus, ON, Canada), mounted with an Evolution QEi camera (Media Cybernetics, MD, USA) driven by Image-Pro Plus 7.0 (Olympus, ON, Canada). Brightness and contrast were adjusted using ImageJ 1.47g (NIH, Bethesda, USA).

2.13. Transmission electron microscopy

VICs were fixed overnight at 4 °C by immersion in 2.5% glutaraldehyde and 2.5% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.4) and washed in sodium cacodylate buffer for further 30 min. The samples were post-fixed in 1% osmium tetroxide for 1 h at 4 °C. Samples were then dehydrated through graded alcohol and embedded in Epon 812 resin. Ultrathin sections of 70 nm were cut, contrasted with uranyl acetate and lead citrate and examined at 70 kV with a JEOL electron microscope (JOEL, MA, USA). Images were captured digitally by Mega View III camera (Olympus, ON, Canada).

2.14. Scanning electron microscopy

Samples were fixed for 2 h in 2.5% glutaraldehyde at 4 °C then washed in 0.1 M sodium cacodylate before being post-fixed with osmium tetroxide 1% for 1 h at 4 °C. Dehydration was then performed in increasing ethanol concentrations up to the critical point of drying with...
hexamethyldisilazane overnight. Dried samples were sputtered with palladium (Nanotech, USA) and observed with SEM (Quanta 3D FEG, FEI, USA). Quantification of cell area was performed by using ImageJ 1.43 (NIH, Bethesda, USA).

2.15. Energy dispersive X-ray microanalysis EDX

EDX was used to compare the calcific deposits on cell surface [15]. The operating program identified peaks by position in the spectrum and shape of count distribution, matching these to a stored reference library of elemental peak spectra and their derivatives. Accurate quantification depended on the identification of every peak present in the spectrum: count data were collected for all detected peaks and calcium and phosphorus quantified from the proportions present in the region of interest relative to the other elemental species present.

2.16. Immunogold labeling

Strain was applied for three days and cells were washed once with PBS 1× and fixed in 3.7% formaldehyde for 30 min at 37 °C. Cells were treated for 15 min with a 50 mM NH₄Cl solution and were incubated in PBS 1× containing 4% BSA for 1 h at room temperature. Incubation with rabbit polyclonal anti-ENPP1 (Santa Cruz Biotechnologies, CA, USA) was performed in the same solution overnight at 4 °C. Cells were washed four times with a PBS 1× solution, followed with one hour incubation with nanogold-conjugated rabbit antibody. Cells were fixed for 2 h in 2.5% glutaraldehyde at 4 °C and washed in 0.1 M sodium cacodylate before being post-fixed with osmium tetroxide 1% for 1 h at 4 °C. Dehydration was then performed in increasing ethanol concentrations up to the critical point of drying with hexamethyldisilazane overnight. Dried samples were sputtered with carbon (Nanotech, USA) and observed with SEM (Quanta 3D FEG, FEI, USA).

2.17. Rho kinase (ROCK) activity assay

Cells were subjected to mechanical strain in the presence of mineralizing medium for 30 or 60 min. Cells were collected in harvesting buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM 2-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na₂VO₄, 0.1 mM PMSF and 10 μg/ml aprotinin) and sonicated. Following lysis, samples were centrifuged at 4 °C and supernatants were used for the ROCK assay. The assay was performed with the 96-well ROCK Activity Assay Kit (Cell Biolabs, Inc., CA, USA) according to manufacturer instructions.

2.18. Promoter luciferase assay

VICS were transfected with a pCMV vector encoding for the ENPP1 promoter (kind gift of Dr Nan E. Hatch, University of Michigan) [16] fused to firefly luciferase along with a vector encoding for the renilla luciferase (Promega, WI, USA) as an internal control. The cells were...
transfected overnight and then, calciﬁcation was added as well as the ROCK inhibitor (HA1100) at a ﬁnal concentration of 30 μM 1 h before strain application for 24 h. Cells were lysed and luciferase activity was quantiﬁed using the Dual-luciferase reporter assay, according to manufacturer instructions (Promega, WI, USA).

2.19. Cell fractionation

VICs were submitted to a cyclic mechanical strain for 24 h as described previously. Cells were washed with cold PBS 1× and were disrupted and homogenized using a Polytron device in 1 ml of ice cold extraction buffer-A containing (mmol/L): HEPES 20 (pH = 7.5); EDTA 5; EGTA 5; NaF 20; Na3VO4 0.2; β-glycerophosphate 20; benzamidine 10; AEBSF 0.5; leupeptine 25 μg/ml; DTT 5. Homogenates were centrifuged at 48 000 rpm for 30 min at 4 °C. The supernatant corresponding to the cytosolic fraction was collected. The pellet was resuspended in buffer-A supplemented with 1% TritonX100 (volume/volume) and incubated for 30 min on ice. Ultracentrifugation was again performed at 48 000 rpm. Supernatants were used in the enzymatic assay. Enzymatic activity was subtracted from total activity to obtain membrane activity.

2.20. Statistical analyses

For the comparisons of groups the results were expressed as means ± SEM. For continuous data, values were compared between groups with Student t-test or ANOVA when two or more than two groups were compared, respectively. Post hoc Tukey analyses were done when the p value of the ANOVA was < 0.05. A p value < 0.05 was considered as statistically signiﬁcant. Statistical analyses were performed with a commercially available software package JMP IN 8.1.

3. Results

3.1. Mechanical strain induces mineralization of VICs and expression of ENPP1

Human primary valve interstitial cells (VICs) were submitted to a cyclic mechanical strain and mineralization was quantiﬁed following exposure to a mineralizing medium (PO4). First, we documented in VIC cultures that mineralizing medium did not induce a signiﬁcant increase in mineralization neither in cells without strain nor in cells under a 10% strain over a 24 hour period. However, in VIC cultures submitted to a strain of 15%, we documented at 24 h that mineralization increased by 120-fold when compared to control cells not under strain condition (Fig. 1A). The 15% strain condition is considered to be pathologic and compatible with excess strain as seen for instance during high blood pressure. We thus used the 15% strain rate in our protocol, which was compared to control grown in mineralizing medium in collagen coated plates without mechanical strain. In mineralizing media, when compared to control VIC cultures, cells under cyclic strain had higher level of transcripts encoding for the phosphate co-transporter Pit-1 (1.5-fold), bone morphogenetic protein 2 (BMP2) (3.7-fold), osteonectin (1.8-fold) and ENPP1 (3-fold) (Fig. 1B). Similarly to human cells, in mouse VICs (MVICs) the expression of osteoblastic genes was up-regulated following the exposition to strain (Fig. 1C). Expression of ENPP1 was then conﬁrmed by measuring enzyme speciﬁc activity. In this assay mechanical strain of 24 h increased ENPP speciﬁc activity by 8-fold, whereas mechanical strain combined with mineralizing medium increased enzyme activity by 12.5-fold (Fig. 1D). ENPP1 has been shown to promote apoptosis-mediated mineralization of VIC cultures when overexpressed. To assess the level of apoptosis in VIC cultures we determined the expression of annexin-V by ﬂow cytometry and using mouse VICs (MVICs). MVICs exhibit a similar response to strain when compared to human VICs (Fig. 1C) and were used for ﬂow cytometry
analyses as they are readily available in large amount. In mineralizing medium, we found that mechanical strain increased apoptosis in MVIcs by 1.9-fold (Figs. 2A and B). Mechanical strain-induced apoptosis was next confirmed with transmission electron microscope (TEM) analyses, which showed the presence of pycnotic nucleus as well as membrane blebbing in human VICs submitted to mechanical strain (Fig. 2C). Moreover, we determined that during strain-induced apoptosis a large proportion of cells (75%) co-expressed annexin-V and ENPP1 (Figs. 2A and D) indicating that ENPP1 expression is closely associated with apoptosis in VICs. This result was next confirmed by using confocal microscope analysis. This experiment showed that apoptotic cells, characterized by nuclear chromatin condensation, overexpressed ENPP1 (Fig. 2E). ENPP1 uses nucleotide triphosphate such as adenosine triphosphate (ATP) as a substrate and ATP delivers crucial survival signals to VICs in preventing apoptosis. We next measured the level of ATP in supernatants of cells exposed to mechanical strain. In this experiment, we found that extracellular levels of ATP are depleted following exposure to mechanical strain and that inhibition of ENPP1 by ARL67156 restored the level of extracellular ATP (Fig. 3A). Also, a siRNA against ENPP1, which reduced significantly both mRNA level and enzyme activity (Figs. 3B and C), prevented strain-induced depletion of ATP in supernatants (Fig. 3A). Also, inhibition of ENPP1 with ARL67156 or with a siRNA decreased significantly strain-induced apoptosis of MVIcs as evaluated with the expression of annexin-V in flow cytometry (Fig. 3D). We next evaluated the role of apoptosis in strain-induced mineralization. In this experiment inhibition of caspases with ZVAD-fmk reduced mineralization significantly (Fig. 3E). In the same line, ARL67156 or a siRNA targeting ENPP1 significantly reduced strain-induced mineralization of cell cultures (Fig. 3F). When taken together these findings suggest that mechanical strain enhances the expression of ENPP1, which, in turn, promotes mineralization of VIC cultures.

3.2. Bicuspid aortic valve as a model of increased mechanical strain

BAV by its abnormal valve configuration is known to increase mechanical strain on valvular tissues, particularly in the area of conjoined cusps [17,18]. We thus hypothesized that expression of ENPP1 may be increased in human BAV compared to tricuspid aortic valves (TAV). To verify this hypothesis, the amount of ENPP1 transcript was measured in CAVD tissues (n = 73) and levels were compared according to the anatomy (BAV vs. TAV). When compared to TAV, BAV tissues had a higher level of transcript encoding for ENPP1 (Fig. 4A). Immunofluorescence studies revealed that ENPP1 was highly expressed in the area of conjoined leaflets, where the mechanical strain is maximal (Figs. 4B and C). We next used scanning electron microscope (SEM) combined with energy dispersive X-ray (EDX) analyses to document the presence of mineralized areas in BAV. At the surface of BAV, where ENPP1 was highly expressed we documented that electron dense particles ranging from 1 to 3 μm. (Fig. 4B and E). The presence of electron dense diffuse structures was also observed. (G) Energy dispersive X-ray analyses of this material showed that it was composed of calcium and phosphorus with a Ca/P ratio compatible with hydroxyapatite of calcium.

![Figure 4](https://example.com/figure4.png)
in size from 1 to 3 μm were present and often formed aggregates (~10 μm) (Figs. 4D and E). In some regions we observed at the surface of aortic valve the presence of electron dense diffuse structures (Fig. 4F). EDX analyses of this material revealed that it was composed of calcium and phosphorus (Fig. 4G). The Ca/P ratio (1.86) was compatible with hydroxyapatite of calcium (HAC). These findings thus suggest that mineralization of the aortic valve is associated with the production of mineralized spheroid particles. However, the mechanism leading to the formation of mineralized microparticles in the aortic valve remains largely unresolved.

3.3. Mechanical strain-induced activation of RhoA promotes mineralization of VICs through the production of spheroid mineralized microparticles

By using scanning electronic microscopy (SEM), we next documented the expression of ENPP1 and changes in cell morphology that could be associated with strain-induced mineralization of isolated VICs. Immunogold labeling of ENPP1 showed in scanning electron microscopy (SEM) that following mechanical strain VICs expressed ENPP1 at the cell membrane and that the protein appears to form clusters in pitted areas between folds of the cell membrane (Figs. 5A–C). Furthermore, SEM studies revealed striking changes in cell morphology following exposure to mechanical strain. When compared to control cells non-exposed to mechanical strain (Fig. 5D), VICs submitted to mechanical strain appeared elongated (Fig. 5G) and vesicles were observed on the surface of cells undergoing membrane blebbing, typical of apoptosis. (Figs. 5E and F). These crystals were forming aggregates on the cell surface (diameter ~5–10 μm) and tended to form more diffuse mineralized structures (Fig. 5G). EDX analyses confirmed that these electron dense aggregates were composed of calcium and phosphorus. The ratio of calcium to phosphorus (Ca/P: 1.76) indicated that this material was mostly composed of HAC (Fig. 5H) [19]. Noteworthy, these modifications were not observed in control non-stretched VICs treated with the mineralizing medium for three days. Hence, these findings mirrored the observations made in explanted human stenotic BAVs and provide further insights as to the origin of mineralize microparticles. In this regard, it is likely that VICs produce these mineralized spheroid particles as a response to mechanical cues, which involves the expression of ENPP1.

Quantitative analyses of cell area revealed that mechanical strain significantly reduced cell area (Figs. 6A and B). To further assess modifications of cell morphology, we stained F-actin with alexa488 coupled phalloidin. In mineralizing media, cyclic strain induces a dramatic elongation of cells coupled to F-actin re-organization (Fig. 6C). Cells acquired an elongated shape and showed long cellular extension characterized by strong cortical actin staining (Fig. 6C). The small Rho GTPase, RhoA, is known to be activated upon exposure to mechanical forces and it works upstream of Rho kinase (ROCK) [20]. Therefore, we next studied the effect of ROCK inhibitors (HA1100 and Y27632) on stretch-induced morphological changes. Treatment with HA1100 and Y27632 completely abolished cell area reduction as well as cell elongation in response to mechanical strain (Fig. 6B). Cells were enlarged and they round up into a more circular shape (Fig. 6C). These findings suggest that RhoA could play an important role in delivering key signals during mineralization of VIC cultures in response to strain. Hence, ROCK activity was measured following a mechanical strain in VICs. In this experiment, we found that 30 min after initiation of mechanical strain ROCK activity was increased by 1.6-fold when compared to control VICs treated with the mineralizing medium only (Fig. 7A). Sixty minutes after starting mechanical strain the activity of ROCK was back to its initial value. We next verified if the activation of RhoA/ROCK was sufficient to impact on mineralization of VIC cultures. Inhibition of ROCK downstream of RhoA with HA1100 reduced significantly (44% reduction) mechanical strain-induced mineralization of VIC cultures (Fig. 7B).

**Fig. 5.** Electron microscopy analyses following strain application. (A) Image showing cell surface of ENPP1 immunogold labeling in backscatter-scanning electron microscopy and (B) in secondary electron microscopy showing ENPP1 organized into clusters (C) in pitted areas between folds of the cell membrane. (D) Scanning electron microscopy (SEM) analysis of a control cell. (E) Cell exposed to strain with the presence of spheroid particles on its surface (diameter of 1–5 μm). (F) Vesicles were also observed on the surface of a cell undergoing membrane blebbing typical of apoptosis. (G) Exposure to strain leads to cell elongation and spheroid crystal accumulation into a larger structure. (H) EDX analyses confirm that these electron dense aggregates were composed of calcium and phosphorus to a ratio compatible with hydroxyapatite of calcium.
3.4. RhoA/ROCK promotes ENPP1 export to the plasma membrane

We next hypothesized that activation of RhoA/ROCK could be involved in mechanical strain-induced expression of ENPP1. By using a luciferase promoter assay for ENPP1 we next evaluated the role of cyclic strain on the promoter activity with and without the ROCK inhibitor, HA110. In VIC cultures submitted to mechanical strain the ENPP1 promoter activity increased by 1.35-fold, whereas HA110 did not modify the activity of the promoter (Fig. 7C). Hence, we hypothesized that rather than acting on the transcriptional activity of ENPP1 RhoA/ROCK may be essential in promoting mineralization by exporting ENPP1 to the plasma membrane, where it exerts a control on extracellular levels of nucleotides and phosphate products. VICs were next submitted to mechanical strain with and without HA110, a ROCK inhibitor, and cell surface expression of ENPP1 documented by fluorescence flow cytometry analyses. In this experiment cell surface expression of ENPP1 increased by 1.8-fold following exposure to mechanical strain and inhibition of ROCK with HA110 prevented this rise (Fig. 8A). Immuno fluorescence studies in VICs for ENPP1 also stained with F-actin (alexa488 coupled phalloidin) showed that cyclic mechanical strain induced the translocation of ENPP1 to the cell periphery, while inhibition of ROCK prevented this transport (Fig. 8B). In cells treated with HA110, ENPP1 remained in the perinuclear region (Fig. 8B). Using cell fractionation we documented by using this technique that membrane-associated ENPP activity was undetectable in control VICs, whereas after exposition to strain the ratio of membrane/cytoplasmic activity increased by 2.4-fold. Of note, a treatment of VICs with HA110 prevented the rise of membrane-associated ENPP activity (Fig. 8C). Hence, measurement of membrane-associated ENPP1 activity confirmed immunofluorescence and flow cytometry analyses. Mechanical strain induced the activation of the RhoA pathway whereby it promoted export of ENPP1 to the plasma membrane, where mineralization is initiated by the production of calcified spheroid particles (Fig. 8D).

4. Discussion

In this work, we provide new mechanistic evidence that mineralization of the aortic valve is relying on apoptosis-mediated release of mineralized spheroid particles at the surface of VICs. Moreover, we found that mechanical strain leads to the expression of ENPP1 at the surface of VICs in a RhoA/ROCK dependent manner and, that it is an important mediator of mineralization.

4.1. Mechanical strain increases mineralization of VICs: role of spheroid mineralized microparticles

The present series of experiments highlighted that cyclic mechanical strain of VICs increased mineralization of cell culture by several-fold. These findings are in line with previous findings, which indicate that mechanical strain is a potent inducer of mineralization [21]. However, the processes whereby mineralization of the aortic valve is promoted remained up to this day elusive. However, the present findings provide some insights into this mechanism. The use of SEM combined with EDX provided novel understanding of the processes underlying mineralization of the aortic valve. In this regard, the use of this approach unraveled that during mineralization of cell cultures electron dense spherical particles (1–3 μm) appear at the surface of VICs and may aggregate and coalesce into larger structures. Of interest, we also detected spheroid
mineralized particles of similar size in explanted BAVs, which is reminiscent of the in vitro findings. Noteworthy, a recent investigation using SEM with focused ion beam (FIB) also showed in human stenotic aortic valves that dense spherical particles, similar in size to what we observed, were present at the surface of mineralized valves [6]. Furthermore, within diffuse plate-like calcified materials Bertazzo and colleagues documented following section with FIB that dense spheroid bodies were embedded in these structures, suggesting that mineralized spheroid particles may aggregate into larger structures. However, the source of these microparticles was not established. Our findings give support to the notion that mineralization of the aortic valve is promoted by mechanical cues delivered to VICs and that production of mineralized microparticles accounts for an effective process whereby calcification of the aortic valve is entrained. In this scheme of things, it is likely that apoptosis promotes the production of mineralized microparticles and may thus explain that in vivo the vast majority of mineralized matrix is observed in the absence of cells; mineralized microparticles would be the only remnants left after programmed cell death. In addition, we also found that osteoblastic transition occurs following exposition of VICs to strain. Hence, it is possible that both mechanisms, osteoblastic transition and apoptosis, play a role in strain-induced mineralization. Other source of cell vesicles may thus include the production of matrix vesicles, which have been described during the mineralization of osteoblast [22]. Taken together, these experiments and observations suggest that VICs are actively involved in the formation of mineralized microparticles and may well be the source of calcified spheroid particles observed in aortic valves by SEM and EDX.

4.2. Role of ENPP1 in mediating apoptosis and mineralization of the aortic valve

ENPP1 uses ATP as a substrate and produces pyrophosphate (PPi), a potent inhibitor of mineralization [23]. On the one hand, mice with defective ENPP1 develop extensive mineralization of tendons [24]. On the other hand, expression of ENPP1 is increased in calcified knee meniscal cells and transfection of ENPP1 increased mineralization of meniscal cells through an apoptotic mechanism [25]. Noteworthy, Côté et al. documented that single nucleotide polymorphisms (SNPs) of ENPP1 were significantly associated with CAVD [12]. Furthermore, it was found that ENPP1 was elevated in human stenotic valve and promoted apoptosis-mediated mineralization of VICs. Elevated expression of ENPP1 contributes to deplete the extracellular pool of ATP and, in doing so, decrease survival signals delivered through purinergic signaling [26]. It then follows that when ENPP1 is highly expressed apoptosis of VICs is triggered and initiates mineralization. These facts suggest that expression of ENPP1 must be tightly controlled in tissues in order to prevent ectopic mineralization. It should be underlined that the level of apoptosis in human stenotic aortic valve is elevated and that apoptotic VICs express ENPP1. Of interest, we documented in the present study that mechanical strain-induced mineralization was accompanied by an elevated expression and enzyme activity of ENPP1. Furthermore, we found a high level of apoptosis in VICs following mechanical strain and incidentally a high percentage (75%) of apoptotic cells expressed ENPP1. These findings were paralleled by a decrease level of ATP in supernatants. Of note, inhibition of ENPP1 reestablished the level of ATP in the growth medium and reduced significantly mechanical strain-induced mineralization of VIC cultures. Hence, these experiments indicate that mechanical strain promotes the expression of ENPP1 in VICs, whereby apoptosis-mediated mineralization is triggered.

4.3. RhoA/ROCK-dependent ENPP1 export to cell membrane

Cell synthesis and cargo vesicle export to organelles or plasma membrane are highly regulated processes, which determine function and cell fate. To this effect, in the present series of experiment we found that RhoA/Rock was actively involved in the export of ENPP1 to the plasma.
membrane. It is worth to point out that ENPP1 is a membrane bound enzyme, which has an active enzymatic site facing the extracellular space [27]. Hence, proper localization of ENPP1 ensures its biological effect, which is to control the extracellular level of nucleotides and phosphate-derived products. Hence, inhibition of RhoA significantly reduced mechanical strain-induced mineralization of VIC cultures. Similarly, Gu et al. documented that nodule formation in VIC cultures was associated with RhoA/ROCK activity [28]. RhoA/ROCK promotes reorganization and polymerization of F-actin, whereby cargo vesicle export is promoted [29]. In this regard, studies indicate that F-actin can mediate long-range vesicular export to the plasma membrane [30]. In the present investigation, the observation that following exposure of VICs to strain F-actin was re-organized with a strong cortical staining as well as with cell elongation, is consistent with the activation of RhoA/ROCK pathway. Hence, we found that strain-induced mineralization was coupled to a RhoA/ROCK-dependent ENPP1 export to cell membrane.

4.4. Clinical implications

Considering that there is no medical treatment for CAVD the present work gives further insights into the pathobiology of aortic valve mineralization, which may help the development of novel pharmaceutical treatments. In this regard, we found that mechanical cues deliver important intracellular signaling, which promotes the formation of mineralized microparticles. Mineralization of the aortic valve relied, at least in part, on the expression of ENPP1. Hence, inhibition of ectonucleotidase may represent a novel avenue for the treatment of CAVD [26]. However, further research is needed to develop pharmaceutical compounds to inhibit ectonucleotidase enzyme and to test these molecules in pre-clinical animal models [31].

4.5. Limitations

In this study, we examined stenotic aortic valves with an advanced state of mineralization. Further testing in aortic valves apparently healthy or sclerotic valves may give further insights as to whether the identified process is operating prior to macroscopic and advanced calcification. Nonetheless, by studying human stenotic aortic valves as well as with in-depth functional assays we identified a novel mechanism whereby VICs produce mineralized microparticles under mechanical strain.

4.6. Conclusions

This work identified that mineralization of the aortic valve is a highly coordinated process culminating by the production of mineralized microparticles. In addition, we found that export of ENPP1 to the plasma membrane through RhoA/ROCK pathway following mechanical strain...
is a critical event in driving mineralization of VICS. Upon inspection of human stenotic BAVs we found that ENPP1 is highly expressed in areas of elevated mechanical strain. Taken together, these findings suggest that mechanical strain is one potent factor involved in the mineralization of the aortic valve.

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Disclosures

P. Mathieu has patent applications for the use of ectonucleotidase inhibitors and purinergic agonists in the treatment of CAVD.

References