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Original article

Carbonic anhydrase XII in valve interstitial cells promotes the regression of calcific aortic valve stenosis

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ABSTRACT

Aims: Calcific aortic valve stenosis (CAVS) is the most common heart valve disease. In the present work we sought to determine the reversibility of mineralization in the aortic valve.
Methods and results: By using in vitro analyses we found that valve interstitial cells (VICs) have the ability to resorb minerals. We documented that agonist of P2Y2 receptor (P2Y2R) promoted the expression of carbonic anhydrase XII (CAXII) at the cell membrane of VICs, whereby minerals are resorbed. P2Y2R-mediated mineral resorption was corroborated by using mouse VICs isolated from wild type and $P2Y2R^{-/-}$ mice. Measurements of extracellular pH (pHe) by using core–shell nanosensors revealed that P2Y2R-mediated CAXII export to the cell membrane led to an acidification of extracellular space, whereby minerals are resorbed. In vivo, we next treated $LDLR^{-/-}/ApoB^{100/100}$/IGF2 mice, which had developed CAVS under a high-fat/high-sucrose diet for 8 months, with 2-thioUTP (a P2Y2R agonist) or saline for the next 2 months. The administration of 2-thioUTP (2 mg/kg/day i.p.) reduced the mineral volume in the aortic valve measured with serial microCT analyses, which improved hemodynamics and reduced left ventricular hypertrophy (LVH). Examination of leaflets at necropsy confirmed a lower level of mineralization and fibrosis along with higher levels of CAXII in mice under 2-thioUTP. In another series of experiment, the administration of acetazolamide (a CA inhibitor) prevented the acidification of leaflets and the regression of CAVS induced by 2-thioUTP in $LDLR^{-/-}/ApoB^{100/100}$/IGF2 mice.
Conclusion: P2Y2R-mediated expression of CAXII by VICs acidifies the extracellular space and promotes the regression of CAVS.

1. Introduction

Calcific aortic valve stenosis (CAVS) is a chronic disorder characterized by an abnormal mineralization of aortic leaflets [1]. Although CAVS can be diagnosed in its early stage with echocardiographic examination, there is no medical treatment that can prevent the progressive calcification of leaflets, which ultimately results in a severe stenosis [2]. Hence, the only treatment of CAVS is thus a surgical intervention, which consists in the replacement of the aortic valve by using either an open heart surgery or a percutaneous intervention. However, these interventions are invasive and are associated with a significant morbidity/mortality as well as with elevated cost.

Bone mineralization is the result of a delicate balance between deposition and resorption of minerals [3]. If applied to pathologic mineralization this concept suggests that it could be possible to promote the resorption of ectopic calcium deposit. Emerging evidence suggests that the expression of carbonic anhydrase (CA) during vascular calcification may promote the resorption of minerals [4]. In the reversa $LDLR^{-/-}$ $ApoB^{100/100}$ mice the normalization of cholesterol level after 6 months of western type diet led to a significant reduction of mineral content in the aortic valve [5]. These data suggest that ectopic mineralization of the aortic valve is potentially reversible. However, the molecular mechanisms that may promote the regression of aortic valve mineralization are presently unknown. The purinergic system exerts an important control over pathologic mineralization of the aortic valve [6]. In CAVS, the overexpression of ectonucleotidase enzymes,

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which use nucleotides as substrates, contribute to deplete the extracellular level of nucleotides and to decrease purinergic signaling delivered to valve interstitial cells (VICs), the main cellular component of the aortic valve. P2Y2 receptor (P2Y2R) inhibits the osteogenic transition of VICs and the blockade of ectonucleotidases prevents the development of CAVS [7,8]. However, whether a purinergic receptor-mediated process could promote the regression of CAVS remains to be explored. Herein, we report a novel function for VICs, which have the intrinsic capacity to resorb minerals. We show through several lines of evidence that stimulation of P2Y2R promotes the transport of carbonic anhydrase XII to the cell membrane, whereby pathologic mineralization of the aortic valve is resorbed.

2. Material and methods

Expanded material and method sections are in the online supplementary material.

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies/Thermo Fisher Scientific (ON, Canada). Concentrations used were: ARL67156 (25–100 μM), (ectonucleotidases inhibitor/Tocris Bioscience, MI, USA), 2-thioUTP (2–20 μM regression) ($P2Y_2$ receptor agonist/ Tocris Bioscience, MI, USA or Tri-Link BioTechnologies, CA, USA), ATPγS (50–100 μM) (non-hydrolyzable ATP/Tocris Bioscience, MI, USA), MRS2365 (2 nM) (selective $P2Y_1$ receptor agonist/Tocris Bioscience, MI, USA), acetazolamide (20–40 μM) (carbonic anhydrase inhibitor/Sigma-Aldrich, ON, Canada), SITS (50 μM) (4-Acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid disodium salt hydrate) (anion transport inhibitor/Sigma-Aldrich, ON, Canada).

2.2. Antibodies

Antibodies against CAXII (D-2) (monoclonal, sc-374314), CAXII (H-114) (polyclonal, sc-25601), CAII (G-2) (sc-48351) and calnexin (H-70) (sc-11397) were obtained from Santa Cruz Biotechnologies (TX, USA). Antibodies against α-actin (A2547), β-actin (A2228) and vimentin (V2258) were from Sigma-Aldrich (ON, Canada). Antibodies against von Willebrand factor (factor VIII) (250642) were from Abbiotec (CA, USA).

2.3. Valve interstitial cell isolation and in vitro analyses of calcification

Control non-calcified aortic valves, used for cell culture, were obtained during heart transplant procedure. Consents were obtained from patients; this protocol (2012–1984, 20770) is approved by the local ethics committee and was performed according to the declaration of Helsinki. Human and mouse (WT and $P2Y2R^{-/-}$, approved by Laval University animal ethics committee, protocol 2012173) (The Jackson Laboratory, USA) valve interstitial cells (VICs) were isolated by collagenase type I digestion (Invitrogen Life Technologies/Thermo Fisher Scientific, ON, Canada).

2.4. 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.

2.5. Mineral resorption studies in osteologic discs

Cells were seeded on osteologic discs (BD Biosciences, ON, Canada) or on OsteoAssay Surface (Corning, NY, USA) culture plates. VICs were cultured into 24-well plates containing osteologic discs for 21 days. The semi-quantitative morphometric analysis of pits formation was evaluated by Von Kossa staining of cultures or by scanning electron microscopy.

2.6. Scanning electron microscopy

Samples were fixed 2 h in 2.5% glutaraldehyde at 4 °C and washed in 0.1 M cacodylate before being post-fixed with osmium tetroxide 1% for 1 h at 4 °C. Dehydration was then performed with increasing ethanol concentrations up to the critical point of drying with hexamethyldisilazane overnight. Dried samples were sputtered with palladium (Nanotech, USA) and observed by scanning electron microscopy (SEM) at 30 kV (Quanta FEG 3D, FEI, USA).

2.7. Real-time PCR

Quantitative real-time PCR (q-PCR) was performed with QuantiTect SYBR Green PCR kit from Qiagen on the Rotor-Gene 6000 system (Corbett Robotics Inc, CA, USA).

2.8. Immunofluorescence of cells

Slides were mounted and analyzed with an Ultraview spinning disk confocal imaging system (objective 100× oil, 1.4 NA, PerkinElmer Life and Analytical Sciences, MA, USA) equipped with a cooled electron multiplying charge-coupled device camera at −50 °C (Hamamatsu Photonics K.K., Hamamatsu-shi, Japan) and driven by Volocity software, version 6.0.1 (PerkinElmer Life and Analytical Sciences).

2.9. Nanogold immunolabeling

Mouse VICs were seeded on glass coverslips. Before fixation, cells were left untreated or treated for 24 h with 2-thioUTP. Incubation with anti-CAXII (Santa Cruz Biotechnologies, TX, USA) was performed in the same solution overnight at 4 °C. Dried samples were sputtered with carbon (Nanotech, USA) and observed by scanning electron microscopy (SEM) at 30 kV (Quanta FEG 3D, FEI, USA).

2.10. Measurement of extracellular pH

The extracellular pH was measured by fluorescence ratio imaging of fluorescein-conjugated to core–shell nanobiosensors.

2.11. Measurement of cytosolic pH by flow cytometry

Cells were incubated with 2 μM BCECF-AM (Molecular Probes/ Thermo Fisher Scientific, ON, Canada) in DMEM medium at 37 °C for 30 min in the presence of 5% CO_2 in dark. Cells were then transferred into flow cytometry tubes for the measurement of fluorescence. FL1/FL2 ratios were calculated using the fluorescence mean values to create standard curve. The ratio was used to create the linear equation obtained from the standard curve to calculate pHi.

2.12. Animals

All animal protocols were conducted according to guidelines set out by the Laval University Animal Care and Handling Committee (protocol 2012175) and are conform to the NIH guidelines for the care and use of laboratory animals. $LDLR^{-/-}$ /$ApoB^{100/100}$ /IGF2 (on C57Bl/6J background) were generated from an established colony at the Heart and Lung Institute of Laval University from original founders kindly provided by Dr. SeppoYlä-Herttuala (University of Eastern Finland, Finland). Male mice were housed in a pathogen-free, temperature-controlled environment under a 12:12 hour light–dark cycle and fed ad libitum of a high fat, high sucrose, cholesterol diet (55% calories from fat, 28% from sucrose, 0.2% cholesterol) for 10 months starting at