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Inhibition of ectonucleotidase with ARL67156 prevents the development of calcific aortic valve disease in warfarin-treated rats

Nancy Côté [a], Diala El Husseini [a], Andrée Pépin [a], Céline Bouvet [c], Liz-Ann Gilbert [c], Audrey Audet [a], Dominique Fournier [a], Philippe Pibarot [b], Pierre Moreau [c], Patrick Mathieu [a,*]

[a] Laboratoire d'Études Moléculaires des Valvulopathies (LEMV), Groupe de Recherche en Valvulopathies (GRV), Quebec Heart and Lung Institute/Research Center, Department of Surgery, Laval University, Quebec, Canada
[b] Institut universitaire de cardiologie et de pneumologie de Québec, Québec, Canada
[c] Faculty of Pharmacy, University of Montreal, Québec, Canada

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ABSTRACT

Calcific aortic valve disease is the most common heart valve disorder. So far, there is no medical treatment for calcific aortic valve disease. The expression of ectonucleotidases, which metabolize nucleotides into phosphate products, may influence the calcification of the aortic valve. In this study, we investigated if the administration of an ectonucleotidase inhibitor, ARL67156 (6-*N*,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt), may prevent the calcification of the aortic valve in the warfarin-induced mineralization rat model. Male Wistar rats were treated with warfarin or warfarin + ARL67156 for 28 days. All rats had comprehensive Doppler-echocardiographic studies at 28 day. A gene profiling of ectonucleotidases expressed in aortas of rats was documented by quantitative real-time PCR. The amount of calcium was determined by quantitative method and von Kossa staining. *Ex vivo* cultures of rat aortas were also used to further assess the effect of ARL67156 on the calcifying process and Akt signaling. Mineralization of the aorta/aortic valve was documented in warfarin-treated rats and was accompanied by the development of aortic stenosis. These changes were paralleled by an increased of ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1). Administration of the ectonucleotidase inhibitor, ARL67156 prevented the development of aortic stenosis by lowering the level of apoptosis and mineralization of the aortic valve/aorta. In addition, ARL67156 normalized the level of pAkt, an important kinase involved in the survival pathway. Inhibition of ectonucleotidase activity prevented the development of calcific aortic valve disease in a rat model. On that account, ectonucleotidase may represent a novel target in the treatment of calcific aortic valve disease.

1. Introduction

In developed countries, aortic stenosis or also called calcific aortic valve disease is the most frequent cause of valvular heart disorder, affecting up to 3% of the population older than 65 years old (Freeman and Otto, 2005). The culprit process is an abnormal calcification of the aortic valve. The incidence of calcific aortic valve disease increases with age, smoking, diabetes, dyslipidemia and hypertension (Mathieu et al., 2009). In this regard, calcification of the aortic valve is rarely isolated and is often accompanied by the mineralization of the aorta resulting in aortic stiffness, systolic hypertension and increased pulse wave velocity, an independent marker of cardiovascular mortality (Garcia et al., 2007; Izzo and Shykoff, 2001). Calcific aortic valve disease is characterized by a long asymptomatic period, which may last for several years before symptoms developed and surgery is required. Calcific aortic valve disease can be diagnosed during the early phase with the help of cardiac auscultation and Doppler-echocardiography, offering a time-window opportunity to initiate a medical treatment. However, effective drug therapy to inhibit the progression of calcific aortic valve disease has not yet been developed (Rajamannan et al., 2011).

The ectonucleotidase family of genes represents a group of membrane-bound enzymes, which includes ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs), ectonucleoside triphosphate diphosphohydrolases (ENTPDs), 5′-nucleotidase (NT5E), and alkaline phosphatase (ALPL) (Goding et al., 2003). Ectonucleotidases are known regulators of phosphate (Pi) and nucleotide metabolism (Yegutkin, 2008; Orriss et al., 2007). On the other hand, Pi and nucleotides are potent mediators controlling the pathologic mineralization process, which has been shown to be largely dependent on apoptosis (Son et al., 2007). To this

* Corresponding author. Tel.: +418 656 4717; fax: +418 656 4707.
E-mail address: patrick.mathieu@chg.ulaval.ca (P. Mathieu).

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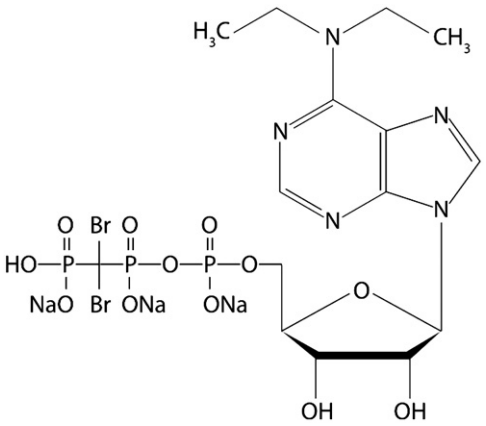

Fig. 1. Chemical structure of ARL67156 (6-*N*,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt).

effect, studies indicate that ectonucleotidase enzyme modulate apoptosis and, in doing so, mineralization (Johnson et al., 2001a). Hence, expression of ectonucleotidases and their modulation may exert an influence on the calcification of the aortic valve/aorta. In this work we hypothesized that level of ectonucleotidase may be modulated by the mineralizing process. In a rat model of aortic valve/aorta mineralization, we have thus administered the ectonucleotidase inhibitor, ARL67156 (6-***N***,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt) (Fig. 1), in order to document its effect on the calcifying process.

2. Materials and methods

2.1. Animal study

Animal experiments were approved by the Animal Care and Use Committee of Laval University and University of Montreal. Male Wistar rats (initial weight 200 g) were obtained from Charles River Breeding Laboratories (St Constant, QC, Canada). The animals received warfarin (20 mg/kg/day in drinking water) and vitamin K (phylloquinone) (to prevent bleeding) (15 mg/kg/day subcutaneous injection) ($n=10$) at day 1, 3, 5, 7, 14, 21, and 28. Dosages were adjusted every second day. Controls consisted of age-matched untreated rats ($n=7$). In another group of warfarin rats, ARL67156(6-*N*,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt) was administered at 1.1 μg/kg/day ($n=10$) during 28 days with osmotic pumps implanted subcutaneously.

2.2. Tissue analysis

At day 28, animals were anesthetized with pentobarbital (65 mg/kg) for blood plasma sampling and the harvesting of aorta and heart. The heart including the aortic root and valve was fixed in formaldehyde 10% for histological processing and immunohistology. Transversal section 5 μm of the aortic root was stained with hematoxylin and eosin (H&E) and von Kossa (for calcium). Morphometric analyses of the aortic root (at the level of aortic valve and sinuses of Valsalva) were carried out using the Image-Pro Plus 6.1 (MediaCybernetics, Bethesda, MD, USA) image analysis software to quantify the calcified areas (with the von Kossa staining) normalized to the total surface area of the section.

2.3. Hemodynamic parameters

Doppler-echocardiographic analyses (Sonos 7500, Philips Medical) with the use of high frequency (12 MHz) imaging probe and rodent imaging analysis package were performed for the measurement of aortic transvalvular velocity at the beginning of the protocol and at the end (28 day). Pulse wave velocity (PWV) was measured as previously described (Essalihi et al., 2003).

2.4. Immunostaining and histologic analyses

Immunostaining analyses were performed with anti-ENPP1antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Slides were then incubated with EnVision Dual Link System-HRP, followed by AEC substrate (Dako, Carpinteria, CA, USA). Nonimmunized rabbit serum was used as a negative control in all immunohistology experiments. Mineralization was detected with von Kossa staining. Morphometric analyses were carried out using the Image-Pro Plus 6.1 (MediaCybernetics, Bethesda, MD, USA) image analysis software.

2.5. Real-time PCR

RNA was extracted from aortas explanted from rats. Total RNA was isolated with RNeasy micro kit from Qiagen (Qiagen, Mississauga, ON, Canada). The RNA extraction protocol was performed according to manufacturer's instructions using 100 mg of tissue. The quality of total RNA was monitored by capillary electrophoresis (Experion, Biorad, Mississauga, ON, Canada). 4 μg of RNA was reverse transcribed using the Quantitec Reverse Transcription Kit from Qiagen. Quantitative real-time PCR (q-PCR) was performed with Quantitec SYBR Green PCR kit from Qiagen on the Rotor-Gene 6000 system (Corbett Robotics Inc, San Francisco, CA, USA). Primers for the following transcripts were obtained from Invitrogen (Burlington, ON, Canada): ENPP1, ENPP2, ENPP3, ENTPD1, ENTPD2, ENTPD3, NT5E, and ALPL. The expression of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (Invitrogen, Burlington, ON, Canada) was used as a reference gene to normalize the results.

2.6. Detection of apoptosis

Apoptosis was documented in rat paraffin slides by TUNEL assay using Apoptag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) and quantitative morphometric analysis was performed. The apoptotic cells were counted on the whole section. Then, the area of each valve was determined using the Image-Pro Plus Version 6.1 image analysis software and the percentage of apoptotic cells on the total area was calculated.

2.7. Ex vivo model of mineralization in rat thoracic aortas

Under general anesthesia thoracic aortas were retrieved from male Wistar rats (Charles River Breeding Laboratories St Constant, QC, Canada). They were then rinsed with PBS and incubated for 4 days with a pro-calcifying medium containing: DMEM + 5% FBS, 10^{-7} M insulin, 50 μg/ml ascorbic acid, warfarin (10 μM) NaH_2PO_4 at 2.9 mM. Some aortas were treated with ARL67156(6-*N*,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt) (100 μM). These experiments were conducted in triplicates. After 4 days aortas were retrieved and calcium concentration were determined as detailed in 2.8. Also, immunoblotting were performed for Akt and pAkt. Ratios of pAkt/Akt were determined by quantitative measurement of band density.

2.8. Determination of calcium concentrations

A segment of aorta tissue was kept in liquid nitrogen until determination of the calcium content. Aortas were treated with HCl 6N at 90 °C during 24 h. Treated tissues were then centrifuged at 4400 RPM during 30 min and supernatants were collected.