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Activated p300 acetyltransferase activity modulates aortic valvular calcification with osteogenic transdifferentiation and downregulation of Klotho

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ABSTRACT

Background: The calcific aortic valve (AV) disease is a common disease with the unclear mechanism, and optimal pharmacological treatment remains unavailable. Epigenetic modulation by histone acetyltransferase (HAT) plays a critical role in osteogenic transdifferentiation and atherosclerosis. The purposes of this study were to investigate whether HAT contributes to the pathophysiology of AV calcification and assess the therapeutic potential of HAT inhibition.

Methods: Porcine valvular interstitial cells (VICs) were treated with osteogenic medium (10 ng/mL of tumor necrosis factor-α and 4 mmol/L of high phosphate) for 7 days. We analyzed the RNA and protein expression of myofibroblastic (α-SMA, vimentin, collagen 1A1, collagen 3, Egr-1, MMP2, MMP9) and osteoblastic markers (osteocalcin and alkaline phosphatase) in VICs, and studied the effects of a p300 inhibitor (C646, 10 μmol/L) on calcification (Alizarin Red S staining), osteogenesis, HAT activity, the mitogen-activated protein kinase (MAPK) and Akt pathway, and Klotho expression on VICs.

Results: Osteogenic medium treated VICs had higher expressions of osteocalcin, alkaline phosphatase and acetylated lysine-9 of histone *H*3 (ac-H3K9) than control cells. C646 attenuated osteogenesis of VICs with simultaneous inhibition of the HAT activity of p300. There was neither significant increase of p300 protein nor p300 transcript during the osteogenesis process. Additionally, osteogenic medium treated VICs decreased the expression of Klotho, which is attenuated by C646.

Conclusions: Activated HAT activity of p300 modulates AV calcification through osteogenic transdifferentiation of VICs with Klotho modulation. P300 inhibition is a potential therapeutic target for AV calcification.

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1. Introduction

Calcific aortic valve (AV) disease (CAVD) is a leading valvular heart disease in older adults and has a poor prognosis [1]. Diseased AV leaflets finally form a restricted opening of the outflow tract of the left ventricle, which leads to congestive heart failure or sudden cardiac death. CAVD arises from an active disease process with interactions between fibrosis and calcification through the mediation of extracellular matrix (ECM) remodeling, inflammation, cell apoptosis, and dystrophic calcification. Currently, no effective medication can prevent or reverse the disease progression of CAVD [2]. Invasive surgical procedures with AV replacement or transcatheter AV implantation are the gold standard treatment of CAVD, but both are associated with variable risks. Moreover, the crucial pathophysiological process of CAVD continually damages the implanted bioprosthesis even after the native valve is exenterated [3].

Abbreviations: ac-H3K9, histone acetylation at histone *H*3 Lys 9; AV, aortic valve; CAVD, calcific aortic valve disease; ECM, extracellular matrix; Egr-1, early growth response-1; ERK, extracellular signal-regulated kinase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HP, high phosphate; IκB, inhibitor of kappa B; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p300, EP300 or E1A binding protein p300 (adenovirus early region 1A); CBP or CREBBP, CREB (cAMP response element-binding protein) -binding protein; Pi, phosphate; TGF, transforming growth factor; TNAP, tissue non-specific alkaline phosphatases; TNF, tumor necrosis factor; RXR, retinoid-x receptor; VIC, valve interstitial cell; VDR, vitamin D (1,25-dihydroxy vitamin D3) receptor; VDRE, vitamin D response element.

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In patients with renal insufficiency or on dialysis, the prevalence of CAVD (ranging 28% –85%) and severe aortic stenosis (6% –13%) is significantly higher than in the general population [4,5]. High circulating phosphate (Pi) and tumor necrosis factor (TNF)-α are common in these patients, and both factors contribute to the genesis of additional vascular calcification [6]. In bone homeostasis, TNF-α can induce osteogenesis through the p38 mitogen-activated protein kinases (MAPK) signaling pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) plus the SMAD pathway, and NF-κB plus the Wnt/β-catenin pathway [7,8]. High phosphates induce valvular interstitial cell (VIC) calcification with activation of Akt signaling [9,10].

The histone acetyltransferase (HAT) p300 is a transcriptional coactivator, which participates in gene expression regulation and protein acetylation. p300 regulates gene expressions through scaffolding, bridging, or intrinsic HAT activity. The HAT domain of p300 can catalyze histone acetylation and vitamin D (1,25-dihydroxy vitamin D3) receptor (VDR)-associated osteocalcin transcription [11,12]. Moreover, HAT plays a critical role in the pathophysiology of atherosclerosis. TNF-α upregulates the coactivators p300/CBP to induce transcription of osteogenic and fibrogenic genes. With inflammatory cytokines such as TNF-α, valvular calcification can be induced by activation of NF-κB and induction of histone *H*3 phosphorylation [13]. Accordingly, the HAT signaling pathway in VICs may cooperate to regulate the progression of inflammatory-mediated AV calcification. The purposes of this study were to investigate whether HAT or HAT inhibition modulates the pathophysiology of AV calcification and evaluate the potential mechanisms.

2. Materials and methods

2.1. Isolation and culture of VICs

Porcine hearts (from 6-month-old animals) were purchased from a commercial slaughterhouse (Yahsen Frozen Foods, Taoyuan, Taiwan) and shipped in ice-cold saline within 3 h of sacrifice. Aortic valves were digested with collagenase I (250 U/mL, Sigma, St. Louis, MO, USA) for 30 min with gentle shaking at 37 °C to remove endothelial cells, and then were subjected to a second digestion for 12 h with collagenase I (250 U/mL) under gentle rocking at 37 °C. Isolated VICs were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 medium containing 10% heat inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, and antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 2 days, and the VICs were seeded at a density of 2×10^4 cells/cm^2 in 6-well plates. Aortic VICs from passage 2 or 3 were used for further studies.

2.2. Induction of osteoblast-like differentiation of aortic VICs

For calcification induction, aortic VICs at 80% confluence were cultured in osteogenic medium with TNF-α (10 ng/mL, Sigma) and HP (4 mmol/L Na_2HPO_4/NaH_2PO_4 mixture, pH 7.4) for 7 days, which has been shown to significantly induce cultured cells calcifications [14]. To identify the signaling involved in osteogenesis, VICs were treated with a HAT inhibitor (C646, 10 μmol/L; TOCRIS, Bristol, UK), with and without osteogenic medium for 7 days, and then harvested for further experiments.

2.3. Assessment of calcification

Calcium deposition was measured by Alizarin Red-S (ARS) staining. After osteogenic induction, VICs were fixed with 4% paraformaldehyde for 15 min, and then rinsed and incubated with a 2% Alizarin Red S solution (pH 4.2) for 30 min. Excessive dye was removed by washing with distilled water. Alizarin Red S stained cells were photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan). For quantitative analysis, the Alizarin Red S stain was bleached with 10% acetic acid at 85 °C for 10 min, and then the optical density of supernatants was measured at an absorbance of 450 nm after mixing with 10% ammonium hydroxide.

2.4. Western blot

VICs were lysed in protein extraction reagent (Thermo Scientific, Waltham, MA, USA) with protease inhibitor cocktails (Sigma). Proteins were separated in gradient sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrophoretically transferred to an equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). The primary antibodies used in this study were summarized in Table 1. Blots were probed with primary antibodies against osteocalcin, ac-H3K9, phosphorylated (p)-ERK, ERK, p-Akt, Akt, p38, p-p38, c-Jun N-terminal kinase (JNK), p-JNK, collagen 1A1, matrix metalloproteinase (MMP)2, MMP9, RUNX2, early growth response (Egr)-1, p300, NF-κB (p65), IκB-α, p-IκB-α, cleaved caspase3, Pit-1, Pit-2, tissue-nonspecific alkaline phosphatases (TNAP), collagen 3, α-SMA, VDR, Vimentin, Klotho, and secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected with an enhance chemiluminescence (ECL) detection system (Merck Millipore, Billerica, MA, USA) and analyzed with Image-Pro Plus software. Targeted bands were normalized to β-actin to confirm equal protein loading.

2.5. Assessment of histone acetyltransferase (HAT) activity

HAT activities were assayed using a HAT Activity Fluorometric Assay Kit (BioVision, Milpitas, CA, USA, catalog no. K334-100) according to the manufacturer's instructions. Briefly, equal amounts of nuclear extracts (5 μg) were incubated with reaction reagents containing *H*3 peptide and acetyl-CoA, then the fluorescence (ex/em = 535/587 nm) was read in kinetic mode at 25 °C for 60 min. HAT activities (mU/mg)

Table 1
Primary antibodies used for Western blot.

Target	Catalog Number of Antibodies	Dilutions	Host	Company
Osteocalcin	AB10911	1:1000	Rabbit	Merck Millipore
Ac-H3K9	#06–942	1:500	Rabbit	
p-ERK	sc-101,761	1:1000	Rabbit	Cell Signaling Technology
ERK	#9102	1:1000	Rabbit	
p-Akt	#4060	1:1000	Rabbit	
Akt	#4685	1:1000	Rabbit	
p-p38	#9211	1:1000	Rabbit	
p38	#9212	1:1000	Rabbit	
p-JNK	#9255	1:2000	Mouse	
JNK	#9258	1:1000	Rabbit	
Collagen 1A1	sc-25,974	1:1000	Goat	Santa Cruz
MMP2	sc-10,736	1:1000	Rabbit	
MMP9	sc-6841	1:500	Rabbit	
RUNX2	sc-10,758	1:1000	Rabbit	
Egr-1	sc-189	1:200	Rabbit	
P300	sc-585	1:3000	Rabbit	
NF-κB (p65)	sc-372	1:100	Rabbit	
IκB-α	sc-371	1:1000	Rabbit	
p-IkB-α	sc-101,713	1:1000	Rabbit	
PiT-1	sc-442	1:1000	Rabbit	
TNAP	sc-23,430	1:1000	Goat	
cleaved caspase3	ab13847	1:500	Rabbit	Abcam
Collagen 3	ab6310	1:2000	Mouse	
α-SMA	ab7817	1:1000	Mouse	
VDR	ab-3508	1:100	Rabbit	
Vimentin	#2707-1	1:200	Rabbit	Epitomics
Klotho	LS-C145689	1:1000	Rabbit	LifeSpan Biosciences
PiT-2	NBP1-69702	1:2000	Rabbit	Novus Biologicals
β-actin		1:7000	Mouse	

Egr-1, early growth response-1. TNAP, tissue non-specific alkaline phosphatases.

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