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Metformin ameliorates TGF-β1–induced osteoblastic differentiation of human aortic valve interstitial cells by inhibiting β-catenin signaling

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ABSTRACT

Osteoblastic differentiation of aortic valve interstitial cells (AVICs) is the central process in the development of calcific aortic valve disease (CAVD). Metformin is a widely used first-line antidiabetic drug, and recently, pleiotropic benefits of metformin beyond hypoglycemia have been reported in the cardiovascular system. Here, we examined the effect of metformin on the osteoblastic differentiation of human AVICs. Our results showed that metformin ameliorated TGF-β1–induced production of osteogenic proteins Runx2 and osteopontin as well as calcium deposition in the cultured human AVICs. Experiments using AICAR, Compound C and AMPKα siRNA showed that the beneficial effect of metformin on TGF-β1–induced osteoblastic differentiation of human AVICs was mediated by AMPKα. Moreover, metformin inhibited the TGF-β1–induced activation of β-catenin, and β-catenin siRNA blocked TGF-β1–induced osteoblastic differentiation of AVICs. Smad2/3 and JNK were phosphorylated to promote the TGF-β1–induced activation of β-catenin and osteoblastic differentiation of AVICs, and metformin also alleviated TGF-β1–induced activation of Smad2/3 and JNK. In conclusion, our results suggest a beneficial effect of metformin based on the prevention of osteoblastic differentiation of human AVICs via inhibition of β-catenin, which indicates the therapeutic potential of metformin for CAVD.

1. Introduction

Calcific aortic valve disease (CAVD) has been the most common heart valve disease among people over the age of 65 in western countries. However, there are no available pharmacological interventions to prevent this disease, and aortic valve replacement is the only effective treatment for CAVD patients [1].

Although the pathologic mechanism of CAVD remains incompletely understood, CAVD is now recognized as an active and multifactor regulated pathological process, rather than simply a result of aging and degeneration [2,3]. Aortic valve interstitial cells (AVICs) are the most prevalent cell type in aortic valves, and the osteoblastic differentiation of AVICs plays a pivotal role in aortic valve calcification [4,5]. Such differentiation of AVICs can be activated by many mediators including oxidized-low density lipoprotein (ox-LDL) and pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and transforming growth factor- β1 (TGF-β1) [5,6]. The resulting osteoblast-like cells express osteogenic transcription factors Runx2, MSX2, SOX9, etc., which further increases the production of bone matrix proteins such as osteocalcin, osteopontin and bone sialoprotein and promotes the synthesis of collagens and alkaline phosphate kinase (ALP) activity, all of which ultimately lead to the deposition of calcium nodules within aortic valve leaflets [3,4,7].

Metformin is widely used to lower blood glucose in patients with type 2 diabetes mellitus (T2DM), and activation of 5′-adenosine monophosphate-activated protein kinase (AMPK) has been identified as a major mechanism by which metformin exerts its anti-T2DM effect [8,9]. Recent studies demonstrated that metformin also has protective effects in cardiovascular diseases and cancer beyond lowering blood glucose levels, and these effects include the inhibition of vascular calcification in aortic smooth muscle cells

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(SMCs) [10,11]. Interestingly, activation of AMPK was shown to negatively regulate the differentiation of chondrocytes and osteoblasts [12,13]. Additional studies revealed that ablation or inactivation of AMPK promotes aortic arterial calcification in vivo, and treatment with metformin inhibited arterial calcification in vivo and in vitro [14,15]. However, it is unknown whether metformin attenuates the osteoblastic differentiation of human AVICs. Here, we investigated the effect of metformin on the osteoblastic differentiation of human AVICs induced by TGF-β1 and further explored the associated signaling mechanisms.

2. Materials and methods

2.1. Human aortic valve collection

Relatively healthy aortic valve leaflets were obtained intraoperatively from five patients undergoing Bentall operation due to acute type I aortic dissection in the Department of Cardiovascular Surgery of Wuhan Union Hospital through a protocol that conformed to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Union Hospital and Tongji Medical College. The five patients, whose ages ranged from 39 to 53 years, were all males with no history of diabetes, infective endocarditis, rheumatic heart disease, or a congenital bicuspid aortic valve. Valves were obtained in a sterile environment, preserved in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) pre-chilled to 4 °C, and transported to the laboratory on ice.

2.2. Cell culture and treatments

Human AVICs were isolated and characterized as previously described [16,17]. AVICs from passages 3–5 were used in all experiments. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS; Gibco, New Zealand), 100 U/ml penicillin, and 100 U/mL streptomycin (Gibco, USA), in a humidified incubator at 37 °C. When the cultured AVICs reached 70%–80% confluency, the medium was changed to DMEM containing only 2% FBS overnight, and cells were treated with various concentrations of recombinant human TGF-β1 (R&D Systems, USA) for various periods. As appropriate, pharmacological reagents, including metformin, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), Compound C, and SP600125 (all purchased from Selleck Chemicals, USA) were added to the culture medium 2 h before the addition of TGF-β1.

2.3. Western blotting

Total protein or nuclear protein lysates were extracted from cultured AVICs using commercial sample buffers (Thermo Fisher, USA) according to the manufacturer's instructions. Protein samples were electrophoretically separated on a 10% acrylamide gel and blotted onto polyvinylidene difluoride membranes. Nonspecific binding was blocked by incubating the membrane in 5% non-fat milk in a TBS-T solution at room temperature for 1 h. Membranes were then incubated with appropriate primary antibodies overnight at 4 °C. Specific binding was detected via the use of horseradish peroxidase (HRP)-conjugated secondary antibodies and an enhanced chemiluminescence (ECL) Western Blotting Kit (Thermo, USA). The density of each band was analyzed using ImageJ software (NIH, USA). The primary antibodies used in this study were: anti-Runx2 (ab23981,1:500), anti-osteopontin (ab8448, 1:500), anti-vimentin (ab8978,1:50) and anti-alpha smooth muscle actin (αSMA; ab5694, 1:100) from Abcam (UK) along with anti-phospho-p38 (Thr180/Tyr182) (4511, 1:1000), anti-P38 (#8690, 1:1000), anti-phospho-p44/42 mitogen-activated protein kinase(ERK1/2)(Thr202/Tyr204) (#4377, 1:1000), anti-ERK (#4695, 1:1000), anti-phospho-c-Jun N-terminal kinase (JNK; Thr183/Tyr185) (#4668, 1:1000), anti-JNK (#9252, 1:1000), anti-phospho-glycogen synthase kinase 3β (GSK3β)(Ser9) (#5558, 1:1000), anti-GSK3β (#12456, 1:1000), anti-phospho-AMPKα(Thr172) (#2535, 1:1000), anti-AMPKα (#5832, 1:1000), anti-phospho-Smad2(Ser465/467)/Smad3(Ser423/425)(#9510, 1:1000), anti-Smad2/3(#8685, 1:1000), anti-non-phospho(active)β-catenin(Ser33/37/Thr41) (#8814, 1:1000), and anti-β-catenin (#8480, 1:1000) from Cell Signaling Technology (USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 60004-1-Ig, 1:5000) was purchased from Proteintech (China).

2.4. Cell viability

AVICs cultured in 96-well plates were treated with various concentrations of metformin (0.2, 0.5, 1, 2 or 5 mM) for 72 h, and the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, yellowtetrazole (MTT) assay was conducted to measure cell viability as previously described [18].

2.5. Immunofluorescence staining

AVICs were cultured in 3.5-mm glass-bottomed dishes (NEST, China), and TGF-β1 was added to the culture medium for 12 h with or without pretreatment with metformin. After treatment, cells were stained as previously described [17].

2.6. Gene knock-down

To silence AMPKα, β-catenin, or Smad2/3, cultured AVICs at 70%–80% confluency were transfected with specific siRNA (100 nM) or scramble siRNA (100 nM) using the Lipofectamine 3000 Transfection Reagent (Life Technologies, USA) according to the manufacturer's recommendations. The AMPKα siRNA consisted of three distinct RNA sequences (si-AMPKα #1 CACAGAAG GAUUUAAAUAUUGAGGG, si-AMPKα #2 CCCAUCCUGAAAGA-GUACCAUUCUU and si-AMPKα #3 ACCAUGAUUGAUGAAGCCUUAA) all purchased from Life Technologies (1299001). The β-catenin siRNA (sc-29209) and Smad2/3 siRNA (sc-37238) were purchased from Santa Cruz Biotechnology (USA). After transfection with siRNA for 6 h, the culture medium was changed and AVICs were cultured for an additional 48 h before being lysed for validation of knock-down or stimulated with TGF-β1 with or without metformin pretreatment.

2.7. Alizarin Red S staining

AVICs were cultured in 24-well plates, and cells were treated with different interventions upon reaching 70%–80% confluency in the calcific conditioning medium (DMEM with 2% FBS, 10 mmol/L β-glycerophosphate and 8 mmol/L $CaCl_2$) for 21 days. The medium was exchanged every 3 days during this culture period. Alizarin Red S staining was then conducted using a commercial Alizarin Red S staining kit (ScienCell, #0223, USA) as previously described [17].

2.8. Statistical analysis

All experiments were independently replicated in cells from at least three different human aortic valves, and the results are presented as mean ± standard deviation (SD). Kolmogorov-Smirnov test was used to confirm that all variables were normally distributed. Differences between two groups were evaluated via student's t-test. $P < 0.05$ were considered statistically significant.

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