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BMP2/Smad signaling pathway is involved in the inhibition function of fibroblast growth factor 21 on vascular calcification

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ABSTRACT

Vascular calcification is extremely common and associated with major adverse cardiovascular events. Fibroblast growth factor (FGF) 21 has been identified as a potent metabolic regulator and a protector of the cardiovascular system. In this study, we aimed to investigate the effect of FGF21 on calcification of vascular smooth muscle cell (VSMC) and its mechanism. FGF21 inhibited beta-glycerophosphate (BGP) induced mineralization in VSMCs as determined by calcium concentration and Alizarin Red S. FGF21 suppressed BGP-induced BMP2/Smad signaling pathway components as well as osteoblast differentiation markers. FGF21 and Noggin could synergistically inhibit BGP-induced BMP2/Smad pathway expressions and calcification. Taken together, FGF21 inhibits vascular calcification in vitro by modulating BMP2/Smad signaling pathway.

1. Introduction

Vascular calcification is closely connected with high morbidity and mortality of cardiovascular disease [1]. It is prevailing in the patients with aging, diabetes mellitus, hypertension, coronary artery disease (CAD) and chronic kidney disease (CKD). Vascular calcification is now recognized as an active regulation process which involves differentiation of smooth muscle cells (SMCs) or pericytes into osteoblast-like cells [2]. These processes are believed to associate with apoptosis, increased SMC oxidant or endoplasmic reticulum stress, loss of calcification inhibitors, disorders of calcium-phosphate homeostasis, and DNA damage response signaling [3].

FGF21 is a member of the FGF subfamily that is produced mainly in the liver [4]. FGF21 acts through FGF receptors (FGFR1-4) in the presence of its co-receptor β-Klotho. FGFR1c was reported to be the preferred isoform of FGF21 [5]. FGF21 has been determined to be a potent metabolic regulator in glucose and lipid metabolism [6]. It has been shown to have multiple beneficial effects on hyperlipidemia, obesity, and diabetes. These conditions are all considered to be major cardiovascular risk factors [7–9]. The cardiovascular protection of FGF21 is supported by several studies, that it may improve endothelial function at the early stage of atherosclerosis and protect cardiomyocytes from myocardial ischemia/reperfusion injury [10–12]. Our previous studies have shown that FGF21 inhibits vascular calcification through the OPG/RANKL system [13,14]. Although OPG knockdown impaired the inhibition of FGF21 on runt-related transcription factor-2(Runx2) mRNA and protein expression, FGF21 significantly down-regulated the expression of Runx2 and alkaline phosphatase (ALP) that are markers and positive regulatory factors of osteoblast differentiation [15,16]. Therefore, we infer that other signaling pathways are related to the inhibitory effect of FGF21 on osteoblast differentiation.

Bone morphogenetic proteins (BMPs) are known to be members of the transforming growth factor-β(TGF-β) family. BMP2 is a potent osteogenic protein required for osteoblast differentiation and bone formation that has been involved in vascular calcification [17,18]. Our previous study showed that FGF21 significantly suppressed BMP2 mRNA expression [13,14]. Accordingly, we speculate that FGF21 inhibits vascular calcification via BMP2/Smad signaling pathway. In this study, we detected BMP2/Smad signaling pathway and osteoblast differentiation markers in VSMCs treated with FGF21, investigated the role of BMP2/Smad signaling pathway in

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the inhibition of vascular calcification by FGF21.

2. Materials and methods

2.1. Cell culture and calcification in vitro

We obtained four-week-old Sprague Dawley(SD) rats (weighing 150–180 g) from SPF Bioscience Company, Beijing, China. All animal procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Primary VSMCs were isolated enzymatically from SD rat aorta and identified by immunofluorescence for α-smooth muscle actin (Abcam plc, Cat. No.ab5694, Fig. S1). The control cells were cultured with growth medium(GM).To induce calcification, We treated VSMCs for 9 days in calcifying medium (CM), which is composed of GM adding 10 mM BGP (Sigma,Cat.No.G9422), 10 mM sodium pyruvate, 50 μg/ml ascorbic acid, 4.5 g/L glucose, 10^{-7} mol/L insulin. Recombinant FGF21(R&D Systems, Cat. No. 2539 -FG) at 50 ng/ml has been added to the culture medium 48 h before the detection time points.10 μM SU5402(Sigma,Cat.No.SML0443),Noggin (R&D Systems, Cat. No. 1967-NG - 025) at 200 ng/ml were inhibitors targeting FGFR1and BMPs respectively.

2.2. Calcification assay

To determine calcium deposition, we fixed VSMCs with 95% ethanol for 15 min at room temperature. VSMCs were stained with 1% alizarin resolution (Sigma, Cat.No.A5533) for 5 min. The cells were then photographed with a microscope. Calcium content was measured by Calcium Colorimetric Assay Kit(Nanjing Jiancheng Bioengineering Institute).

2.3. Cell viability

To determine cytotoxic effects of FGF21 and CM, we detected cell viability with MTT assay (Cat. No.M2128; Sigma). VSMCs were treated with BGP and different concentrations of FGF21 for 48 h. We also measured the cell viability of FGF21 group and CM group at different time points. Cell viability was evaluated by incubation with MTT assay for 4 h at 37 °C. The formazan crystals were dissolved in dimethyl sulfoxide(DMSO). We measured the absorbance at 570 nm with a microplate reader (BioTek, Winooski, VT, USA).

2.4. Real-time RT-PCR analysis

Total RNA was isolated from VSMCs with TRIzol reagent (Tiangen Biotech, Cat. No.DP 405–02). The first-strand cDNA was synthesized by using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Cat. No. RR047B). RT-PCR was performed using the Ex Taq SYBR Green Supermix (TaKaRa, Cat.No. RR82L R). The expression levels of all the tested genes were analyzed using ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). Relative mRNA expression was normalized to18srRNA. The primer sequences of PCR were shown in the Supplemental Table 1.

2.5. Western-blot

Total protein was extracted from VSMCs with radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology) for 30min. Bicinchoninic acid (BCA) Protein assay kit was used to measure protein concentration. Subsequently, proteins were resolved by SDS-PAGE and transferred to PVDF membrane. The membranes were then incubated with primary antibodies against SM22 alpha (Abcam plc,Cat.No.ab14106), Beta-klotho (Santa, Cat.No.Sc-74343), BMP2(Abcam plc,Cat. No.ab14933), RUNX2 (CST, Cat.No.8486S), ALP (Abcam plc,Cat. No.ab95462), Smad1 (Santa, Cat.No.Sc-7965), Samd5(D4G2) (CST, Cat.No.12534S), Phospho-Smad1(Ser 463/465)/Smad5(Ser463/465)/Smad9(Ser465/467) (D5B10) (CST, Cat.No.13820S).Afterwards, gel documentation system (Bio-tanon, Shanghai, China) was used to visualize bands.

2.6. Statistical analyses

Data were presented as mean ± SEM. One-way ANOVA was performed for the comparison. The software was provided by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ were considered statistically significant. GraphPad Prism version 6.01 was used to draw histograms.

3. Results

3.1. FGF21 suppressed BGP-induced mineralization in VSMCs

It is well established that incubation of VSMCs with medium containing BGP induces mineral deposition and phenotypic changes in VSMCs [19]. To identify the effect of BGP in vascular mineralization, we applied Alizarin Red S staining in VSMCs. The amount of mineral deposition increased with time after the treatment of CM (Fig. 1A). Meanwhile, the calcium concentration in VSMCs exhibited similar increase (***$P < 0.001$, respectively) (Fig. 1B).

Our previous study has determined the experimental concentration of FGF21 at 50 ng/ml [14]. The formation of mineralized nodules of VSMCs cultured in CM was notably inhibited by FGF21 at 50 ng/ml(Fig. 1A). FGF21 at 50 ng/ml significantly decreased calcium concentration induced by BGP in the 3, 6, and 9 days of culture (**$P < 0.01$, ***$P < 0.001$, respectively) (Fig. 1B).

To exclude the inhibitory effect of FGF 21 on calcification due to cytotoxicity, we detected the effect of FGF21 on the viability of VSMCs. The cell viability of VSMCs treated with CM was reduced compared with the cells in GM. However, FGF21 at different concentrations did not influence the cell viability (Fig. 1C). FGF21 at 50 ng/ml had no cytotoxicity on VSMCs in CM (Fig. 1D).

3.2. FGF21 inhibited BGP-induced osteoblast differentiation in VSMCs

BMP2, Runx2, and ALP are representative markers of osteoblast differentiation. In this study, we first confirmed that BGP induced osteoblast differentiation in VSMCs. The protein expressions of ALP, Runx2, and BMP2 were increased at 3, 6 and 9 days when GM was switched into CM (***$P < 0.001$) (Fig. 2A–C).

Next, we detected whether FGF21 could regulate bone transformation related proteins in calcified VSMCs. The ALP protein expression was induced by CM in VSMCs, while the induction was suppressed by the co-treatment with FGF21 (**$P < 0.01$, ***$P < 0.001$, respectively) (Fig. 2A). Up-regulations of Runx2 and BMP2 induced by CM in the course of time were also observed. However, FGF21 inhibited the increases (***$P < 0.001$) (Fig. 2B–C).

Our study also showed that BGP treatment down-regulated SM22αprotein expression. However, the decrease was reversed by FGF21 treatment (***$P < 0.001$) (Fig. 2D).

3.3. FGF21 opposed calcification partly via FGFR1

In our previous study, FGF21 opposed calcification in VSMCs by promoting the expression of several receptors, including β-Klotho, FGFR1 and FGFR3 [14].To investigate the detailed mechanism,

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