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Exendin-4 promotes the vascular smooth muscle cell re-differentiation through AMPK/SIRT1/FOXO3a signaling pathways

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ABSTRACT

Background and aims: The phenotype switching of vascular smooth muscle cells (VSMCs) plays a key role during development and progression of vascular remodeling diseases. Recent studies show that GLP-1 can inhibit intima thickening to delay the progression of atherosclerotic plaques. The purpose of this study was to investigate the role of Exendin-4, a GLP-1 receptor agonist, in VSMCs phenotype switching and the related mechanisms.

Methods: Immunohistochemistry and Western blot were used to detect the effect of Exendin-4 on expression of markers of contractile VSMCs. Phalloidin staining was performed to observe the effect of Exendin-4 on morphology of VSMCs.

Results: Exendin-4 significantly increased the protein levels of contractile VSMCs markers like Calponin and SM22α. After treatment of Exendin-4, VSMCs showed more typical characteristic spindle shape. In addition, Exendin-4 significantly upregulated the phosphorylation of AMPK as well as the protein levels of Sirtuin1 (SIRT1) and FOXO3a in VSMCs. After inhibiting AMPK activity with compound C and SIRT1 activity with EX527, and knocking down FOXO3a expression through RNAi technique, Exendin-4 increased the protein levels of Calponin and SM22α and promoted the redifferentiation of VSMCs mainly through AMPK/SIRT1/FOXO3a signaling pathways.

Conclusions: Exendin-4 can regulate the phenotype switching of VSMCs and promote redifferentiation of VSMCs through AMPK/SIRT1/FOXO3a signaling pathways.

1. Introduction

Atherosclerosis (AS) is a chronic inflammatory disease and a major cause of death worldwide [1]. During the formation and progression of atherosclerotic plaques, the vascular smooth muscle cells (VSMCs) undergo phenotype switching and thus tend to proliferate and migrate, which is involved in remodeling of arterial wall [2]. In the normal arterial wall, contraction-type VSMCs are located in the medial layer, which are longspindle-shaped and rich in muscle fibers. Markers of contractile VSMCs include SM α-actin, SM22α, Calponin, and SM-MHC [3]. However, in response to various adverse stimuli, VSMCs undergo de-differentiation, from the quiescent, contractile type to the proliferative, synthetic type [4].

Exendin-4 is a 39-amino-acid polypeptide isolated from the saliva of Mexican lizards and has 53% structural homology with Glucagon-like peptide 1 (GLP-1) [5], therefore, it can activate receptors of GLP-1 in mammals and has extremely similar physiological functions to GLP-1 [6], [7]. Bioactive GLP-1 in body circulation is rapidly degraded and inactivated by dipeptidyl peptidase-4 (DPP-IV). However, Exendin-4 cannot be recognized by DPP-IV, thus avoiding the degradation by DPP-IV [7–9].

GLP-1 receptor (GLP-1R), which can be activated by both GLP-1 and Exendin-4, plays a key role in the metabolism of various organs. In the cardiovascular system, GLP-1R is widely expressed in VSMCs, cardiomyocytes and other cells [10]. The GLP-1R agonist Exendin-4 not only inhibited cell senescence induced by angiotensin II, but also inhibited proliferation and migration of rat VSMCs induced by PDGF [11,12]. Recent studies have shown that GLP-1 can regulate cell differentiation. GLP-1 promoted the differentiation of pluripotent stem cells into islet β cells [13,14]. GLP-1 was associated with the differentiation of neural stem cells [15,16]. In addition, GLP-1 also promoted the differentiation of endothelial progenitor

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cells by upregulating PDGF in the cardiovascular system [17].

Sirtuin1 (SIRT1) is an NAD^+-dependent class III histone deacetylase, a member of the sirtuins family [18]. In cells, AMPK and SIRT1 interact with each other and AMPK activates SIRT1 *via* LKB1 [19]. In addition, AMPK regulates SIRT1 activity by up-regulating the level of intracellular NAD^+ [20]. SIRT1 regulates cellular functions such as stress resistance, cell metabolism, differentiation and apoptosis through deacetylation of different substrates like FOXOs, p53, BCL6, Rb, etc [21]. In addition, compelling evidence has indicated that SIRT1 is an essential factor in modulating cell maturation and differentiation, but the role of SIRT1 in regulating the phenotype switching of VSMCs remains unclear.

In this study, we explored the effects of Exendin-4 on the re-differentiation of VSMCs and the related signaling pathways, to reveal the molecular mechanisms in the amelioration of atherosclerosis by Exendin-4 and to provide evidence for the potential therapeutic use of Exendin-4 in clinical treatment of cardiovascular diseases.

2. Materials and methods

2.1. Chemicals, reagents, and antibodies

Exendin-4 was from Sigma Aldrich Inc. (St. Louis, MO, USA). Antibody against SIRT1 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against SM22α, Calponin, phospho-AMPK (p-AMPK) were from Abcam (Cambridge, UK). The antibody against FOXO3a is from Cell Signaling Technology (Boston, MA, USA). NAD^+/NADH Quantification Colorimetric Kit was from BioVision (Milpitas, USA). Compound C (AMPK inhibitor) and EX527 (SIRT1 inhibitor) were from Selleck Chemicals (Houston, TX, USA). FOXO3a siRNA was from GenePharma (Shanghai, CN).

2.2. Cell culture

Rat aortic VSMCs were obtained from SD male rats (100 g), as described previously [5], and cultured in 10% FBS DMEM at 37 °C in a humidified atmosphere of 5% CO_2. VSMCs at passages 3–5 were used for all experiments.

2.3. Rat carotid-artery balloon-injury model

Rats (350–400 g) were randomized into 3 groups: Control, Injured or Exendin-4. After anesthetization with chloral hydrate (300 mg/kg) intraperitoneally, balloon injury surgeries were performed on rats of Injured and Exendin-4 groups as previously described [22]. Three days after surgery, rats of the Exendin-4 group were treated with 24 nmol kg^{-1} day^{-1} of Exendin-4 (Sigma) through intraperitoneal injection, while those of the Control and Injured groups were injected with an equal volume of vehicles (PBS containing 0.1% BSA). The treatment lasted for 14 days. Animals were euthanized subsequently for further investigations. All animal care and experimental protocols followed the guidelines for the Care and Use Committee of Peking University (Beijing, CN).

2.4. Immunohistochemical staining

The left carotid arteries were embedded into paraffin and cross-sections were prepared for immunohistochemistry staining, as described previously [23]. Briefly, maldehyde-fixed paraffin sections were incubated with the primary antibody for Calponin, SM22α, p-AMPK, SIRT1, FOXO3a overnight, then with the secondary antibody. The sections were incubated with 3,3-diaminobenzidine (DAB) solution to visualize peroxidase activity. Images were obtained under an inverted fluorescence microscope.

2.5. Western blot analysis

As described previously [5], proteins were extracted and the concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). Total protein at an equal amount was subject to SDS-PAGE. Membranes were incubated with specific antibodies against Calponin, SM22α, p-AMPK, SIRT1, FOXO3a, or β-actin overnight at 4 °C, followed by incubation with horseradish peroxidase-labeled secondary antibodies. The protein immunoblots were detected with the ChemiDoc XRS system (Bio-Rad).

2.6. siRNA transfection

Small interfering RNA (siRNA) against rat *FOXO3a* and scramble siRNA were synthesized by GenePharma (Suzhou, CN). As previously described [14], VSMCs were transfected with siRNA (40 nM) *via* Lipofectamine 2000 (Invitrogen, UK) according to the manufacturer's protocols before incubation with indicated agents, and harvested for further investigation.

2.7. Immunofluorescence staining

As previously described [24], cultured VSMCs were incubated with the antibody against SIRT1, followed by the secondary Alexa Fluor 555-conjugated donkey anti-rabbit IgG antibody. For actin staining, VSMCs were incubated with rhodamine phalloidin, and nuclei were stained with DAPI (Roche Diagnostics) for 3 min. Images were captured using Leica TCS SP8 laser scanning confocal microscopy (Leica).

2.8. NAD^+/NADH assay

NAD^+ and NADH were analyzed using NAD^+/NADH Quantification Colorimetric Kit. To detect total NAD (NADt, including NADH and NAD^+), NAD^+ needed to be decomposed before the reaction. NAD Cycling Mix was prepared for each reaction. Each well of NADH Standard and the samples were reacted and measured at 450 nm. The amount of NADt or NADH in the sample wells was calculated according to the NADH standard curve.

2.9. Statistical analysis

Quantitative data are expressed as means ± S.E.M. Differences were analyzed *via* one-way ANOVA with *post-hoc* Newman-Keuls test. $p < 0.05$ was considered to be significant. Non-quantitative results are representative of at least three independent experiments.

3. Results

3.1. Exendin-4 regulated phenotype switching of VSMCs and promoted the re-differentiation of VSMCs

To identify the role of Exendin-4 in phenotype switching of VSMCs, we first detected the levels of SM22α and Calponin in VSMCs *in vivo*. Immunohistochemistry of the carotid artery sections showed that levels of Calponin and SM22α were significantly increased in the Injured/Exendin-4 group, compared with the Injured/PBS group (Fig. 1). Accordingly, protein levels of markers of contractile VSMCs, i.e. Calponin and SM22α, were significantly up-regulated by Exendin-4, both time-dependently and dose-dependently *in vitro* (Fig. 2A–D). Furthermore, after treatment with Exendin-4, VSMCs showed a more typical characteristic