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Research Article

# Adiponectin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the AMPK/mTOR pathway

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ABSTRACT

Vascular calcification is common in patients with peripheral artery diseases and coronary artery diseases. The osteoblastic differentiation of vascular smooth muscle cells (VSMCs) contributes significantly to vascular calcification. Adiponectin has been demonstrated to exert a protective effect in osteoblastic differentiation of VSMCs through regulating mTOR activity. However, the upstream and downstream signaling molecules of adiponectin-regulated mTOR signaling have not been identified in VSMCs with osteoblastic differentiation. In this study, the VSMC differentiation model was established by beta-glycerophosphate (β-GP) induction. The mineralization was identified by Alizarin Red S staining. Protein expression and phosphorylation were detected by Western blot or immunofluorescence. Adiponectin attenuated osteoblastic differentiation and mineralization of β-GP-treated VSMCs. Adiponectin inhibited osteoblastic differentiation of VSMCs through increasing the level of p-AMPKα. Pretreatment of VSMCs with AMPK inhibitor blocked while AMPK activator enhanced the effect of adiponectin on osteoblastic differentiation of VSMCs. Adiponectin upregulated TSC2 expression and downregulated mTOR and S6K1 phosphorylation in β-GP-treated VSMCs. Adiponectin treatment significantly attenuates the osteoblastic differentiation and calcification of VSMCs through modulation of AMPK–TSC2–mTOR–S6K1 signal pathway.



## Introduction

Vascular calcification is widespread in patients with peripheral artery diseases and coronary artery diseases [1]. It was previously considered a degenerative and passive consequence of aging. However, the accumulated evidence suggests that vascular calcification is an active biological process similar to bone formation [2,3]. The transformation of vascular smooth muscle cells (VSMCs) to osteoblast-like cells significantly contributes to vascular calcification [4], but the mechanisms driving this process remain to be fully elucidated. Recently, several osteoblastic phenotype genes have been demonstrated to be overexpressed during vascular calcification such as core binding factor α1 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OC) [5]. However, how these genes' expression is regulated remains unclear.

Adiponectin is a plasma protein encoded by the apM1 gene. The apM1 gene is highly expressed in adipocytes. The Adiponectin protein mainly exists in the form of a polymer, such as a trimer,

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hexamer, or multimer. The polypeptide of adiponectin has 244 amino acids and its structure is homologous to collagen VIII, X, and C1q [6]. In healthy people, the circulating level of adiponectin ranges from 3 to 30 μg/ml [7]. Recent studies have revealed that adiponectin exerts anti-atherogenic and anti-inflammatory effects in vascular diseases [8]. Low adiponectin levels may increase cardiovascular disease risk and late in-stent restenosis [9]. The beneficial roles of adiponectin signaling in some cells such as prostate cancer cells [10] and endothelial cells [11] have been demonstrated, but only a few studies have assessed the role of adiponectin in osteoblastic differentiation of VSMCs. For example, Luo et al. [12] study has demonstrated that adiponectin regulates vascular calcification and stimulates the differentiation of human osteoblasts via activation of p38 mitogen-activated protein kinase (MAPK), a serine/threonine kinase that is involved in cell differentiation.

AMP-activated protein kinase (AMPK) is also a serine/threonine kinase activated by adiponectin [13]. AMPK consists of one catalytic α subunit and two non-catalytic subunits (β and γ) [14]. Each of these three subunits exerts a specific role in both the stability and activity of AMPK. AMPK becomes activated when phosphorylation takes place at the threonine-172 residue by an upstream AMPK kinase [15]. AMPK activity has been successfully regulated by a synthetic activator, such as 5-aminoimidazole-4-carboxyamide ribonucleotide (AICAR) [16] or an inhibitor such as Compound C [17]. In addition, AMPK directly phosphorylates the tuberous sclerosis complex 2 (TSC2), an upstream negative effector of mammalian target of rapamycin (mTOR) to inhibit mTOR activity [18].

mTOR forms two complexes, mTOR complex 1 (mTOR C1) and mTOR complex 2 (mTOR C2), and is a central regulator in multiple cellular processes such as the cell cycle, cell growth, and autophagy [19]. Our previous study showed that adiponectin could inhibit osteoblastic differentiation of VSMCs via inhibiting mTOR C1 and ribosomal protein S6 kinase (S6K1), one of the best characterized effectors of mTOR [20]. Although our results suggest that mTOR signaling is involved in osteoblastic differentiation of VSMCs and is regulated by adiponectin, the upstream and downstream signaling cascades of mTOR signaling during this process have not been identified.

Adiponectin can achieve many of its actions via activation of AMPK in other cells such as mesangial cells [13]. However, the role of AMPK signaling in the active transformation of VSMCs into osteoblast-like cells has not been reported. Since our previous study revealed that the mTOR signaling pathway is involved in osteoblastic differentiation of VSMCs, we hypothesized that adiponectin may inhibit osteoblastic differentiation of VSMCs through the AMPK/TSC2/mTOR pathway. In this study, we investigated the effects of adiponectin in osteoblastic differentiation of VSMCs in a beta-glycerophosphate (β-GP)-induced VSMC differentiation model [21].

## Materials and methods

### Reagents

Human recombinant adiponectin was purchased from R&D Systems (Minneapolis, MI, USA). AICAR and Compound C were purchased from Calbiochem (San Diego, CA, USA). Antibodies for AMPKα, phosphorylated AMPKα (p-AMPKα $Thr^{172}$), phosphorylated TSC2, mTOR, phosphorylated mTOR (p-mTOR $Ser^{2448}$, p-mTOR $Ser^{2481}$, and p-mTOR $Thr^{2446}$), S6K1, phosphorylated S6K1 (p-S6K1 $Thr^{389}$), ALP, Runx, OC, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

### Cell culture and in vitro calcification

Human VSMCs were isolated from femoral arteries using a previously established method [22]. Written informed consent was obtained from patients. Approval was granted by the University Ethics Review Board of Second Xiangya Hospital, Central South University. Human VSMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37 °C under a humidified atmosphere containing 5% $CO_2$. Calcification was induced by 10 mM of β-GP as previously described [21].

### Mineralization assay

The extent of matrix mineralization in cultured VSMCs was determined by Alizarin Red staining. Cells were fixed with 4% formaldehyde for 10 min at room temperature and exposed to 2% Alizarin Red S for 5 min. Cells were then washed with PBS to remove excess dye. The stained matrix was photographed by a digital microscope.

### Osteoblastic differentiation

Human VSMCs were fixed in 4% paraformaldehyde, washed with Tris–Tween buffer, and permeabilized for 20 min in 0.1% triton-X. After blocking for 1 h, cells were incubated with primary antibodies overnight at 4 °C, followed by secondary antibody for 30 min. Images were obtained with a Olympus FV1000 laser-scanning confocal microscope.

### Western blot analysis

Protein concentration was measured using a BCA Protein Assay kit (Beyotime, Shanghai, China). 20-μg of total protein was loaded onto a 12% SDS-PAGE gel and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk for 1 h, membranes were incubated with primary antibody overnight at 4 °C and subsequently incubated with HRP-labeled secondary antibody (1:2000 dilution) for 2 h at room temperature. Reactive proteins were detected using chemiluminescent reagents (Pierce, Rockford, IL, USA). To control for loading efficiency, the blots were stripped and re-probed with GAPDH antibody.

### Statistical analysis

Data were presented as mean±standard deviation (SD) and analyzed using Statistical Product and Service Solutions (SPSS, version 17.0). Differences between two groups were analyzed using Student's t-test. $P<0.05$ was considered statistically significant.