Biochimica et Biophysica Acta 1852 (2015) 1709–1718

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

# Salusin-β contributes to vascular remodeling associated with hypertension via promoting vascular smooth muscle cell proliferation and vascular fibrosis

Hai-Jian Sun [a], Tong-Yan Liu [a], Feng Zhang [a], Xiao-Qing Xiong [a], Jue-Jin Wang [a], Qi Chen [b], Yue-Hua Li [b], Yu-Ming Kang [c], Ye-Bo Zhou [a], Ying Han [a], Xing-Ya Gao [a], Guo-Qing Zhu [a,*]

[a] Key Laboratory of Cardiovascular Disease and Molecular Intervention, Department of Physiology, Nanjing Medical University, Nanjing, Jiangsu 210029, China
[b] Department of Pathophysiology, Nanjing Medical University, Nanjing, Jiangsu 210029, China
[c] Department of Physiology and Pathophysiology, Cardiovascular Research Center, Xi'an Jiaotong University School of Medicine, Xi'an 710061, China

ARTICLE INFO

*Article history:*
Received 19 December 2014
Received in revised form 27 April 2015
Accepted 12 May 2015
Available online 19 May 2015

*Keywords:*
Salusin
Vascular remodeling
Hypertension
Proliferation
Fibrosis

ABSTRACT

Vascular smooth muscle cell (VSMC) proliferation and vascular fibrosis are closely linked with hypertension and atherosclerosis. Salusin-β is a bioactive peptide involved in the pathogenesis of atherosclerosis. However, it is still largely undefined whether salusin-β is a potential candidate in the VSMC proliferation and vascular fibrosis. Experiments were carried out in human vascular smooth muscle cells (VSMCs) and in rats with intravenous injection of lentivirus expressing salusin-β. In vitro, salusin-β promoted VSMCs proliferation, which was attenuated by adenylate cyclase inhibitor SQ22536, PKA inhibitor Rp-cAMP, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478, ERK inhibitor U0126 or cAMP response element binding protein (CREB) inhibitor KG501. It promoted the phosphorylation of ERK1/2, CREB and EGFR, which were abolished by SQ22536 or Rp-cAMP. Furthermore, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478 diminished the salusin-β-evoked ERK1/2 and CREB phosphorylation. On the other hand, salusin-β increased collagen-I, collagen-III, fibronectin and connective tissue growth factor (CTGF) mRNA and phosphorylation of Smad2/3, which were prevented by ALK5 inhibitor A83-01. In vivo, salusin-β overexpression increased the media thickness, media/lumen ratio coupled with ERK1/2, CREB, EGFR and Smad2/3 phosphorylation, as well as the mRNA of collagen-I, collagen-III, fibronectin, transforming growth factor-β1 (TGF-β1) and CTGF in arteries. Moreover, salusin-β overexpression in rats caused severe hypertension. Intravenous injection of salusin-β dose-relatedly increased blood pressure, but excessive salusin-β decreased blood pressure and heart rate. These results indicate that salusin-β promotes VSMC proliferation via cAMP-PKA-EGFR-CREB/ERK pathway and vascular fibrosis via TGF-β1-Smad pathway. Increased salusin-β contributes to vascular remodeling and hypertension.



## 1. Introduction

Proliferation of vascular smooth muscle cells (VSMCs) and vascular fibrosis are closely linked with many clinical diseases including hypertension, atherosclerosis and diabetes and their target organ damage [1,2]. Excess synthesis and accumulation of extracellular matrix (ECM) mainly in the vascular wall responses to chronically elevated blood pressure induces vascular stiffening, which reinforce the development of hypertension, thus reflecting a vicious circle [3,4]. The indices of small resistance artery structure, such as the tunica media to internal lumen ratio may have a strong prognostic significance in hypertensive and diabetic patients [5,6].

Salusins are identified to be peptides of 28 and 20 amino acids with peripheral hypotensive, bradycardic and mitogenic effects, and to be designated as salusin-α and salusin-β in 2003. They are translated from an alternatively spliced mRNA of TOR2A, a gene encoding a protein of the torsion dystonia family [7]. The initial 18 amino acids of human salusin-β have high homology with the estimated N-terminal sequence of rat salusin, but human salusin-α have a very big difference in the sequence compared with rat salusin [8]. Central salusin-β contributed to sympathetic activation, arginine vasopressin release and hypertension, and plasma salusin-β level was increased in renovascular hypertensive rats [9–12]. Plasma salusin-β levels in subjects with diabetes mellitus,

*Abbreviations:* AC, adenylate cyclase; ALK5, activin receptor-like kinase 5; Ang II, angiotensin II; CCK-8, cell counting kit-8 kits; CREB, cAMP response element binding protein; CTGF, connective tissue growth factor; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF-like growth factor; HR, heart rate; MAP, mean arterial pressure; PBS, phosphate-buffered saline; SBP, systolic blood pressure; TGF-β, transforming growth factor-β; Veh, vehicle; VSMCs, vascular smooth muscle cells

* Corresponding author at: Key Laboratory of Cardiovascular Disease and Molecular Intervention, Department of Physiology, Nanjing Medical University, 140 Hanzhong Road, Nanjing 210029, China. Tel./fax: +86 25 86862885.
*E-mail address:* gqzhucn@njmu.edu.cn (G.-Q. Zhu).

http://dx.doi.org/10.1016/j.bbadis.2015.05.008
0925-4439/

coronary artery disease, and cerebrovascular disease showed distinctly higher levels than healthy controls, and it may be taken as an indicator of systemic vascular diseases [13]. Salusin-β immunoreactivity was found to be dominant in the VSMCs and fibroblasts in coronary atherosclerotic lesions rather than salusin-α. Salusin-β promoted but salusin-α suppressed the formation of human macrophage foam cells in atherosclerosis. We hypothesized that salusin-β contributes to the VSMCs proliferation and vascular fibrosis. In this study, we determined the effects of salusin-β on VSMCs proliferation and vascular fibrosis and their downstream signal pathway in human VSMCs. Lentivirus vectors encoding salusin-β were constructed in rats to determine the effects of persistently increased salusin-β on the vascular remodeling and blood pressure.

## 2. Materials and methods

### 2.1. Cell culture

Human aortic vascular smooth muscle cells (VSMCs) were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in F12K Kaighn's modification medium supplemented with 10% fetal bovine serum (FBS) and 1 × Antibiotic-Antimycotic Solution at 37 °C, humidified atmosphere containing 5% $CO_2$. The growth medium was replaced every 3-4 day and the cells were seeded onto petridishes or multiwell plates at a ratio of 1 to 4 upon 80% confluence, and were starved for 24 h in serum-free medium prior to use [14].

### 2.2. Animals

Total 66 male Sprague-Dawley rats weighing between 250 and 300 g were used in the experiments. Procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011). The rats were housed on a 12-h light/dark cycle in a temperature-controlled room with standard chow and tap water ad libitum. Surgery for blood pressure recording was carried out under anesthesia with intraperitoneal injection of urethane (800 mg/kg) and a-chloralose (40 mg/kg). The depth of anesthesia was determined by the absence of corneal reflexes and paw withdrawal response to a noxious pinch [11]. At the end of the experiment, the rats were euthanized with an overdose of pentobarbital sodium (150 mg/kg, iv).

### 2.3. VSMCs proliferation assay

Cell counting kit-8 kits (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) were used for evaluation VSMC proliferation. According to the manufacturer's instructions, cell growth was arrested by incubation of the cells in serum-free medium for 24 h prior to be used. Then, VSMCs were seeded onto a 96-well cell culture plate at a density of $2 \times 10^3$ cells/well for 24 h at 37 °C, and treated with different concentrations of salusin-β for 24, 48 or 72 h. Finally, 10 μl of CCK-8 solution was added into each well, and incubated for 2 h at 37 °C. The absorbance was conducted at 450 nm using a microplate reader (ELX800, BioTek, Vermont, USA).

### 2.4. Cell cycle analysis and EdU incorporation assay

The distribution of various phases of the cell cycle was used to evaluate the VSMCs proliferation with flow cytometry. Simply, VSMCs were plated in six-well plates at a density of $1 \times 10^5$/well and were synchronized with 24 h of serum starvation. The medium was changed to serum-free basal medium for the next 24 hours. VSMCs were then incubated with PBS or salusin-β for 48 hours. The cells were washed twice with phosphate-buffered saline (PBS) and were digested with trypsin. After collection by centrifugation, the samples were mixed in ice-cold 70% ethanol overnight at -20 °C. Before measurement, cells were washed again and re-suspended in PBS containing 50 μg/ml RNase A and 50 μg/ml propidium iodide for 30 minutes at room temperature in the dark. The intracellular fluorescence intensity in each sample was measured using a flow cytometer (Becton-Dickinson Biosciences, San Jose, CA, USA) and the results were expressed as the percentage of cells in each cell cycle phase for cell cycle analysis.

EdU incorporation assay was employed to determine VSMC proliferation with In Vitro Imaging Kit (Guangzhou RiboBio, Guangzhou, China). The DNA synthesis of VSMCs was measured using a Cell-Light™ EdU Apollo®488. The EdU positive cells were counted and normalized by the total number of Hoechst 33342 stained cells.

### 2.5. Measurement of salusin-β

Commercial ELISA kit was used for the measurement of salusin-β (Uscn Life Science, Houston, TX, USA). As we previously reported [10], the standards or sample diluent were added and incubated in the appropriate well of specific antibody pre-coated microtiterplate. Conjugate was added and incubated for 1 h at 37 °C and then washed. The reactions were stopped with stop solution and read at 450 nm by using a microtiter plate reader (ELX800, BioTek, Vermont, USA).

### 2.6. Western blot analysis

The phosphorylation of ERK1/2, JNK, p38, PKA, histone H3, cAMP response element binding protein (CREB), epidermal growth factor receptor (EGFR) and Smad2/3 were determined with Western blot. Briefly, VSMCs or tissues were sonicated in RIPA lysis buffer and homogenized. The debris was removed and supernatant was obtained by centrifugation at 12,000 g for 10 min at 4 °C. Total protein concentration was determined to ensure that the equal loading of proteins was separated on 8% or 10% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with 5% non-fat milk in TBST and then probed with the desired primary antibody at 4 °C overnight followed by incubation with appropriate HRP-linked secondary antibody. The protein expressions were visualized by enhanced chemiluminescence. GAPDH protein served as a loading control.

### 2.7. Real-time quantitative PCR analysis

The mRNA of collagen-I, collagen-III, fibronectin, transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) were analyzed by real-time quantitative PCR. The total RNA was isolated by using Trizol reagent according to the manufacturer's instructions. The mRNA concentration in each sample was measured and 0.5 μg of total RNA was reverse transcribed to cDNA. Quantitative PCR with SYBR Premix Ex Taq ™ (Takara, Otsu, Shiga, Japan) was performed in triplicates by reacting with strand-specific primers, and the average cycle thresholds (Ct) were used to determine fold-change. The relative quantification of gene expression was shown as a relative quantity to the control value. The sequences of primers for human VSMCs and rats were listed in the supplemental tables (Tables S1 & S2, in the online-only Data Supplement).

### 2.8. Measurement of cAMP level

Samples were pre-incubated with 1 mM of 3-isobutyl-1-methylxanthine (IMBX), a phosphodiesterase inhibitor, for 30 min to prevent the degradation of accumulated cAMP. The intracellular cAMP levels were determined by an enzyme immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) following the manufacturer's instructions. Total protein concentration in the homogenate was measured with a protein assay kit (Santa Cruz, Dallas, TX, USA).