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Inhibition of Nogo-B promotes cardiac hypertrophy via endoplasmic reticulum stress

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ABSTRACT

Aims: Nogo-B is a key endoplasmic reticulum (ER) protein that regulates ER stress signaling. However, its role in cardiac hypertrophy remains poorly understood. ER stress is interrelated with autophagy in the process of cardiac hypertrophy. Therefore, we aimed to test the hypothesis that both ER stress and autophagy signaling mediate the function of Nogo-B in cardiac hypertrophy.

Main methods: Rat models of transverse aortic constriction (TAC), neonatal rat cardiomyocytes (NRCMs) stimulated with norepinephrine (Ne) and primary cardiac fibroblasts treated with transforming growth factor β1 (TGF-β1) were used in this study. The expression of Nogo-B and markers of ER stress were determined by quantitative RT-PCR, western blotting and immunofluorescence. Autophagy was measured by monitoring autophagic flux. Specific small interfering RNA (siRNA) of Nogo-B was transfected to investigate the role of Nogo-B in regulating cardiac hypertrophy.

Key findings: In TAC-induced hypertrophic heart tissues, Ne-treated hypertrophic cardiomyocytes and TGF-β1-stimulated cardiac fibroblasts, the expression of Nogo-B, and markers of ER stress were significantly elevated. Impairment of autophagic flux was observed in the activated cardiac fibroblasts. Down-regulation of Nogo-B by siRNA further exacerbated Ne-induced cardiomyocyte hypertrophy and TGF-β1-induced cardiac fibroblast activation. Gene silencing of Nogo-B promoted the activation of the ER stress pathway and the impairment of autophagic flux. Moreover, inhibition of Nogo-B activated the protein kinase RNA-like ER kinase (PERK)/activating transcriptional factor 4 (ATF4) and activating transcriptional factor 6 (ATF6) branches of ER stress pathways.

Significance: These findings suggest that inhibition of Nogo-B promotes cardiomyocyte hypertrophy and cardiac fibroblast activation by activating the PERK/ATF4 signaling pathway and defects of autophagic flux.

1. Introduction

Cardiac hypertrophy is a hallmark pathological feature that occurs in response to mechanical and/or pathological stresses [1]. This process includes cardiomyocyte hypertrophy, cardiac fibroblast activation, extracellular matrix (ECM) deposition and alterations of cardiac genes and protein expressions [2]. Different signaling pathways contribute to the elucidation of the molecular mechanisms of cardiac hypertrophy [3], in which endoplasmic reticulum (ER) stress is critically involved. Recently, evidence has shown that ER stress-induced cardiomyocyte death is implicated in the transition from cardiac hypertrophy to heart failure [4,5]. The alleviation of ER stress by 4-phenylbutyric acid (4-PBA) could attenuate both cardiomyocyte apoptosis and cardiac hypertrophy [6].

ER stress, i.e., the disruption of ER homeostasis, is initiated by three major signaling pathways: protein kinase RNA-like ER kinase (PERK)/activating transcriptional factor 4 (ATF4), inositol-requiring protein 1a (IRE1a)/X-box binding protein 1 (XBP1s) and activating transcriptional factor 6 (ATF6) [7]. ER homeostasis can be destroyed by some factors that cause damage to ER structure and function. Of particular note, proteins located in the ER membranes, such as Nogo-B, play critical roles in modulating ER stress [8].

Nogo-B is a conserved trans-membrane protein localized preferably to the curved membranes on ER tubules that maintain ER structure [9]. The highly conserved C-terminal sequence stretch named RHD is not only necessary but also sufficient for ER morphogenesis [10]. Importantly, accumulating evidence suggests that Nogo-B exerts a potential regulating effect in cardiovascular diseases, including ischemia,

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atherosclerosis and cardiac hypertrophy [11,12]. Nogo-B plays an important role in cardiovascular physiology and, more specifically, enhancing vascular Nogo-B expression reduces inflammation and vascular remodeling [13]. A previous study has shown that Nogo-B expression in cardiac tissues is associated with fibroblasts but not myocytes [12]. However, whether Nogo-B is expressed in the cardiomyocytes and its precise functions in cardiomyocyte hypertrophy remain poorly understood.

The dysregulation of autophagy also leads to cardiac hypertrophy [14]. Both ER stress and autophagy are essential for the response of cellular stresses in the pathogenesis of cardiac hypertrophy, and ER stress can either stimulate or inhibit autophagy [7]. Nogo-B is reported to modulate ER stress and autophagy during the process of hepatic stellate cell activation [15]. However, the association between ER stress and autophagy remains unclear, and the relationships between Nogo-B and ER stress and between Nogo-B and autophagy have not been studied in cardiac hypertrophy. Based on the aforementioned findings, we postulated that Nogo-B may regulate cardiac hypertrophy via the ER stress and autophagy pathways.

Accordingly, the present study was aimed to determine (1) whether Nogo-B expression is abnormal in cardiomyocyte hypertrophy and cardiac fibroblast activation; if so, (2) whether the signaling pathways of ER stress are down-stream effectors of Nogo-B in the process of cardiac hypertrophy; and (3) whether autophagy mediates the role of Nogo-B in cardiac hypertrophy.

2. Materials and methods

2.1. Animal model of transverse aortic constriction (TAC)

All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animal (NIH Pub. No. 85-23, Revised 1996), and all animal experiments in this study were approved by the Medical Ethics Committee of the West China Hospital, Sichuan University (#2014003 A). Transverse aortic constriction (TAC) in rats is characterized as a model for pressure overload-induced cardiac hypertrophy [16]. Twenty three-month-old Sprague-Dawley rats (male, body weight 300 ± 10 g) were provided by the Laboratory Animal Institute, Sichuan Provincial Medical Academy (Chengdu, China). The TAC- and sham-operated groups were anesthetized i.p. with 10% chloral hydrate (400 mg/kg body weight). TAC surgery was performed by ligating the transverse abdominal aorta with surgical sutures according to previous reports [17,18]. Before the operation's effects were evaluated by echocardiography, animals were fed for 4 weeks after the surgery.

2.2. Histological analysis and immunohistochemistry

After echocardiography detection, the left ventricles were isolated from the rat hearts, fixed with 4% paraformaldehyde, embedded in paraffin and transversally sectioned. After rehydration, the sections (4–5 μm) were used to perform hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) for Nogo-B. The cross-sectional areas of the cardiomyocytes were determined via a quantitative digital analysis system (ImageJ, National Institutes of Health, Bethesda, MD, USA) as a marker of cardiac hypertrophy [16].

For IHC of Nogo-B, after deparaffinization and antigen-retrieval, the tissue sections were incubated with Nogo-B antibody (1:100) at room temperature for 2 h. Then, secondary antibody was incubated, and a DAB detection system was used to develop antibody staining. Tissues were subjected to hematoxylin counterstaining before determination via a quantitative digital analysis system.

2.3. Primary cell culture and treatments

Primary neonatal rat cardiomyocytes (NRCMs) and cardiac fibroblasts (CFs) were obtained from the myocardium of Sprague-Dawley neonatal rats at 0–3 d after birth by collagenase II-trypsin digestion according to previous studies [16,19]. NRCMs were identified via immunofluorescence of the specific marker protein of cardiomyocytes (α-sarcomeric actin, α-SCA). Activation of cardiac fibroblasts was identified by evaluating the expression of the specific marker α-smooth muscle actin (α-SMA). Norepinephrine (Ne, 10^{-5} M), angiotensin II (AngII, 10^{-6} M) or transforming growth factor β1 (TGF-β1, 10 ng/mL), the known stimulators that induce cardiomyocyte hypertrophy or cardiac fibroblast activation, was used to induce the hypertrophic phenotype of NRCMs or the activation of cardiac fibroblasts.

2.4. H9c2 cell culture and treatments

H9c2 cells, a cardiac myoblast cell line, have been widely used to study cardiomyocyte hypertrophy [20,21]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, BI, Israel). H9c2 cells were incubated with 1% bovine serum albumin (BSA, Gibco, USA) for 6 h before being treated with Ne for 24 h.

2.5. Small interfering RNA (siRNA) transfection

Cells were seeded on 6-well plates at 50–60% confluence before transfection. siRNAs for Nogo-B (siNogo-B) were transfected into NRCMs or CFs for 24 h using Lipofectamine® 3000 (Invitrogen, USA) before stimulation with Ne or TGF-β1 for 24 h. Individual siRNAs (100 nM, RiboBio™, China), lipofectamine 3000 and DMEM were mixed, incubated at room temperature for 10–15 min and then added to the cell cultures. All siRNA sequences are shown in Table 1.

2.6. Real-time polymerase chain reaction (qRT-PCR) analysis

Gene expression was measured by qRT-PCR, as reported previously [22,23]. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized using a reverse transcription (RT) kit (Toyobo, Japan). qRT-PCR was carried out on the CFX96™ Real-Time PCR Detection System (BIO-RAD, USA) with fluorescence dye EvaGreen (EvaGreen Supermix Kit, Bio-Rad, USA). The primer sequences are shown in Table 2. Gene expression was normalized by comparing the expression of β-actin for each corresponding sample.

2.7. Western-blot analysis

Total proteins were extracted from cells or rat heart tissues with radio-immunoprecipitation assay (RIPA) lysis buffer. Tissue or cell lysates (25 μg) were separated by denaturing 10% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA) using a MiniProtein III system (Bio-Rad, USA). Membranes were incubated with the corresponding primary antibodies at 4 °C overnight and HRP-conjugated secondary antibodies at room temperature, followed by detection with Enhanced Chemiluminescence (ECL, Bio-Rad). The western blotting density was determined using the Bio-Rad Quantity One-D analysis software (Bio-Rad, USA). The protein levels were normalized to β-actin.

Table 1
siRNA sequences of Nogo-B.

Names	Sequences
siNogo-B-1	GTTCAATGGTCTGACACTA
siNogo-B-2	GGGCATATTTAGAATCTGA
siNogo-B-3	CTACTGGAGAGACATTAAG
Negative control (NC)	Supported by RiboBio™