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Original article

Nogo-A knockdown inhibits hypoxia/reoxygenation-induced activation of mitochondrial-dependent apoptosis in cardiomyocytes

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ABSTRACT

Programmed cell death of cardiomyocytes following myocardial ischemia increases biomechanical stress on the remaining myocardium, leading to myocardial dysfunction that may result in congestive heart failure or sudden death. Nogo-A is well characterized as a potent inhibitor of axonal regeneration and plasticity in the central nervous system, however, the role of Nogo-A in non-nervous tissues is essentially unknown. In this study, Nogo-A expression was shown to be significantly increased in cardiac tissue from patients with dilated cardiomyopathy and from patients who have experienced an ischemic event. Nogo-A expression was clearly associated with cardiomyocytes in culture and was localized predominantly in the endoplasmic reticulum. In agreement with the findings from human tissue, Nogo-A expression was significantly increased in cultured neonatal rat cardiomyocytes subjected to hypoxia/reoxygenation. Knockdown of Nogo-A in cardiomyocytes markedly attenuated hypoxia/reoxygenation-induced apoptosis, as indicated by the significant reduction of DNA fragmentation, phosphatidylserine translocation, and caspase-3 cleavage, by a mechanism involving the preservation of mitochondrial membrane potential, the inhibition of ROS accumulation, and the improvement of intracellular calcium regulation. Together, these data demonstrate that knockdown of Nogo-A may serve as a novel therapeutic strategy to prevent the loss of cardiomyocytes following ischemic/hypoxic injury.

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1. Introduction

Cardiomyocyte loss following myocardial ischemia results in increased biomechanical stress on the remaining myocardium, leading to contractile dysfunction, left ventricular remodeling, and congestive heart failure [1–3]. While initial cardiomyocyte loss is primarily due to necrosis, apoptosis plays a key role in the damage of tissue bordering and distant from the infarcted myocardium subsequent to reperfusion [4–7]. Apoptosis is a highly regulated program of cell death that contributes significantly to the gradual decline of left ventricular function following ischemia/reperfusion (I/R) injury [8,9]. Cardiomyocyte apoptosis following I/R can be mediated by activation of the death receptor pathway (extrinsic pathway), the mitochondria-dependent pathway (intrinsic pathway), or endoplasmic reticulum (ER) stress [10–15]. While death receptor-mediated apoptosis and ER stress are known to play a role in I/R injury, the mitochondria-dependent pathway serves as the critical integration site for a number of pro-apoptotic signals in the cardiomyocyte [16]. Following the sudden availability of oxygen during reperfusion, there is a burst of reactive oxygen species (ROS) production, increased mitochondrial permeability transition, decreased mitochondrial membrane potential ($\Delta\Psi_m$), and the release of pro-apoptotic factors, such as cytochrome *c*, ultimately leading to the activation of caspase-3 and apoptosis [17–20]. Protection of mitochondrial function is therefore a promising therapeutic strategy for the treatment of myocardial infarction.

Members of the reticulon (RTN) family of proteins contain a C-terminal reticulon-homology domain and are highly enriched in the membranes of the endoplasmic reticulum [21]. The largest of the RTN proteins, Nogo-A (RTN4-A), contains well-characterized regions essential for inhibiting neurite outgrowth and plasticity in the central nervous system (CNS) [22–26]. Following CNS injury, axonal regeneration, neuronal plasticity, and functional recovery are promoted using antibodies that neutralize Nogo-A function [27–29].

In addition to being highly expressed in the brain and spinal cord, Nogo-A is clearly detectable in other tissues, including the heart, where its function remains largely unclear despite evidence that it may play a role in normal cardiac development [22,30–32]. Nogo-A

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expression in the CNS has been shown to be significantly up-regulated in several models of injury, including hypoxia/ischemia [33–38]. Interestingly, Nogo-A has been proposed as a potential indicator of heart failure due to its elevated expression in genetic models of dilated cardiomyopathy (DCM) and its increased mRNA expression in end-stage heart failure in humans [39,40].

To better determine the relationship between Nogo-A up-regulation and heart failure, we evaluated Nogo-A protein expression in human left ventricular tissue from DCM and ischemic hearts and determined, *in vitro*, which cell type is primarily responsible for its expression. Nogo-A expression was found to be significantly increased in left ventricular tissue from DCM and ischemic hearts, as well as in cultured cardiomyocytes subjected to H-R injury. The pathophysiological significance of Nogo-A up-regulation in H-R-induced cardiomyocyte apoptosis was determined by knocking down its expression. Knockdown of Nogo-A in cardiomyocytes was found to significantly inhibit H-R-induced activation of the intrinsic pathway of apoptosis. Based on these findings, we propose that Nogo-A may serve as novel therapeutic target in the treatment of ischemic/hypoxic-related cardiovascular injury.

2. Materials and methods

2.1. Human tissue

Left ventricular (LV) tissue from patients with dilated, ischemic, or non-failing hearts was obtained from the Cardiovascular Institute Tissue Bank at Loyola University Medical Center. Acquired LV tissue was frozen in liquid nitrogen and immediately stored at −80 °C.

All surgical procedures and tissue harvesting were approved by the Loyola University Medical Center Institutional Review Board and were conducted in accordance with NIH guidelines.

2.2. Cell culture

This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals,* published by the National Institutes of Health, using protocols approved by the Institutional Animal Care and Use Committee. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1 to 2 day old Sprague–Dawley rats as previously described [41], plated on gelatin-coated 60 mm^2 dishes (2.5×10^6–3.0×10^6 cells/60 mm^2) in serum-free PC-1 medium (Lonza, Walkersville, MD), and allowed to attach for 14–18 h. Cells were subsequently maintained in (4:1) DMEM/medium 199 (HyClone Laboratories Inc., Logan, UT) containing (100 U/mL) penicillin/(100 μg/mL) streptomycin (Invitrogen, Carlsbad, CA). Adult rabbit cardiomyocytes were prepared as previously described [42]. Neonatal rat fibroblasts were isolated via a pre-plating procedure during the preparation of NRVMs. Adherent cells, highly enriched in fibroblasts, were maintained in DMEM containing (100 U/mL) penicillin/(100 μg/mL) streptomycin and 10% FBS (HyClone Laboratories Inc., Logan, UT) and used after 4 passages.

2.3. Adenovirus preparation and infection

Nogo-A siRNA, targeting nucleotides 856–874 of the rat Nogo-A cDNA sequence, was designed as previously described [43]. Nogo-A sense and anti-sense oligonucleotides (Integrated DNA Technologies, Inc., Coralville, IA, USA), flanked by *Bam*HI and *Hind*III restriction endonuclease sites, were annealed, subcloned into GenScript pRNA-H1.1/Adeno, and sequenced. The shNogo-A adenovirus, empty vector adenovirus control, and Nogo-A adenovirus containing the sequence for full-length human Nogo-A were constructed using AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). Nogo-A cDNA containing plasmid was generously provided by Dr. Martin Schwab. Adenovirus expressing scrambled shRNA sequence was obtained from Vector Biolabs (Philadelphia, PA). Adenoviruses were amplified, purified, and titered as previously described [41]. NRVMs were infected after 24 h of culture at a multiplicity of viral infection (MOI) of 50 (Ad-shScr and Ad-shN) or 10 (Ad-Con and Ad-Nogo-A) and allowed 48 h to express the transgene prior to hypoxia/reoxygenation (H-R).

2.4. Hypoxia/reoxygenation injury

NRVMs were subjected to hypoxia using GasPak EZ Anaerobe Pouch System (Becton, Dickinson, and Company, Sparks, MD). Culture dishes were placed inside pouches containing gas generating sachets and incubated at 37 °C for 24 h, unless noted otherwise. Following hypoxia, the media was changed with (4:1) DMEM/medium 199 and culture dishes were reoxygenated for 2 h. Normoxic controls were subjected to 24 h normoxia followed by a change of media and 2 h of additional normoxia. Cells subjected to ischemia were treated in the same manner, except that culture media was changed to standard ischemic medium [1.13 mM $CaCl_2$, 0.5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 128 mM NaCl, 4 mM $NaHCO_3$, and 10 mM HEPES] during hypoxia and control medium [ischemic medium supplemented with 0.3 mM Na_2PO_4 and 10 mM D-glucose] during reoxygenation.

2.5. Immunofluorescence staining

NRVMs were cultured on 4-well Lab-Tek Chamber Slides (Nalge Nunc International Corp., Naperville, IL) at a density of 3×10^5 cells/well. Cells were fixed with 4% paraformaldehyde (4 °C) for 3 min, followed by 100% methanol (−20 °C) for 1 min, and rinsed briefly with phosphate buffered saline (PBS). Slides were incubated at room temperature (rt) in PBS/ 0.5% Triton X-100 for 15 min, washed twice with PBS/0.1% Triton X-100 for 10 min, then blocked for 1 h with blocking buffer (PBS/0.1% Triton X-100/1% normal goat serum). Slides were incubated with 11 μg/mL mouse anti-Nogo-A antibody (11c7), 1/200 rabbit anti-SERCA2a (Badrilla Ltd., Leeds, UK), or 1/1000 rabbit anti-VDAC (BioVision, Mountain View, CA) in blocking buffer overnight at 4 °C. Cells were washed three times with PBS/0.1% Triton X-100, then incubated with 1/200 anti-mouse IgG-Alexa Fluor 488, 1/200 anti-rabbit IgG-Texas Red (Invitrogen, Carisbad, CA), and 50 ng/mL Hoechst 33342 (Sigma Aldrich, St. Louis, MO) in blocking buffer for 1 h at rt. Cells were washed with PBS/0.1% Triton X-100, mounted with Vectasheild (Vector Laboratories, Inc., Burlingame, CA), and imaged with a Zeiss Axiovert 100 microscope (Carl Zeiss Microimaging, LLC, Thornwood, NY).

2.6. Immunoblots

Cells were harvested in ice cold lysis buffer [44] with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO), sonicated, and centrifuged at 16,000*g* for 5 min at 4 °C to remove cellular debris. Extracted proteins were separated on 10% or 12% SDS-polyacrylamide gels, electrophoretically transferred onto nitrocellulose membrane, and incubated overnight with 3 μg/mL anti-Nogo-A (11c7), 1/1000 anti-Nogo-A/B (Imgenex, San Diego, CA), 1/1000 anti-Grp94, 1/000 anti-Grp78, 1/1000 anti-PDI (Assay Designs, Ann Arbor, MI), 1/1000 anti-CHOP, 1/500 anti-cleaved Caspase-3, 1/1000 anti-Bcl-2, 1/1000 anti-Bcl-xL (Cell Signaling Technology, Beverly, MA), or 1/5000 anti-GAPDH (Research Diagnostics, Inc., Flanders, NJ) in 3% non-fat milk/tris-buffered saline/0.1%Tween-20. Membranes were then incubated for 30 min with 1/5000 anti-mouse IgG, 1/5000 anti-rabbit IgG (Cell Signaling Technology, Beverly, MA), or 1/5000 anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and detected with enhanced chemiluminescence (Pierce, Rockford, IL).

2.7. TUNEL assay

NRVMs were cultured on 10 mm^2 glass cover slips, infected with adenovirus, and subjected to H-R or normoxia as above. Following H-R,