Nexilin Regulates Rat Ventricular Cardiomyocyte Contractility via Sarcomere Arrangement

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Abstract

Nexilin, an F-actin binding protein, localized at Z-disk in cardiomyocytes. Mutations on nexilin are related to hypertrophic cardiomyopathy characterized by impaired contraction. Here, we investigated the relationship between nexilin and cell contractility in normal or hypertrophic cardiomyocytes. In this study, we found that nexilin was upregulated in hypertrophic cardiomyocytes. To investigate nexilin functional role in cardiomyocyte, adenovirus carrying full length cDNA of nexilin or green fluorescence protein or shRNA target to nexilin were generated and expressed in cardiomyocytes. We found that contractility of hypertrophic cardiomyocytes increased compared with normal cardiomyocytes; Contractility of normal cardiomyocytes with Nexilin overexpression showed the similar results with that of hypertrophic cardiomyocytes. Nexilin knockdown in hypertrophic cardiomyocytes decreased contractility with disordered sarcomere. More over, through staining of actin filament, we found that hypertrophic cardiomyocytes and nexilin overexpression cardiomyocytes maintain normal sarcomere arrangement. On the contrary, nexilin knockdown disordered the sarcomere arrangement of hypertrophic cardiomyocyte and normal cardiomyocytes. These results demonstrated that nexilin is essential for cardiomyocyte sarcomere arrangement and contractility regulation. Nexilin was involved in cardiac hypertrophy progress.

Keywords: nexilin; cardiomyocyte hypertrophy; cell contractility

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1. Introduction

Heart failure has been becoming a major worldwide health problem, which is characterized by abnormal myocardial structure that impairs the contractility of heart. Cardiac hypertrophy is the major risk factor for heart failure. Cardiac hypertrophy can be induced by agonists such as endothelin-1 (ET-1) (Drawnel, Archer, & Roderick; X. Li et al., 2009). Large amounts of ET-1 and other peptides were secreted by cardiac myocytes when heart suffered from mechanical stress such as pressure overload. ET-1, a polypeptide containing 21 amino acids, induces cardiomyocyte hypertrophy characterized by increases in sarcomere organization, protein synthesis and cell surface area. ET-1 also causes a positive inotropic contractility of the normal separation of the heart or cardiomyocytes (Drawnel et al.; Zolk, Munzel, & Eschenhagen, 2004). Sarcomere organization is closely with the force generation, the heart to maintain normal blood circulation, if long-term maintenance of such a compensatory function, the heart chamber will expand and shrink force reduction, and ultimately heart failure (van Heerebeek et al., 2006). Nexilin was identified as an actin filament binding protein, interacted with α-actin, β-actin and α-actinin, and located at Z-disk in heart and skeletal muscle (Hassel et al., 2009; Ohtsuka et al., 1998). Tissue distribution analysis indicated that nexilin is highly expressed in heart and skeletal muscle in human (Hassel et al., 2009; Zhao, Wei, Cao, & Ding, 2001).

Nexilin protein was composed of two actin binding domains at N-terminal, a coiled coil domain, and an immunoglobulin superfamily class domain (Zhao et al., 2001). Nexilin mutations at immunoglobulin domain leaded to hypertrophic or dilated cardiomyopathy (Hassel et al., 2009; Wang et al.). However, the more detail mechanism how nexilin regulated cardiomyocytes contractility in hypertrophic cardiac on cellular level is still unclear. In the present study, to investigate the effects of nexilin on cardiomyocyte contractility on cellular level, nexilin was transiently overexpressed or knocked down in cultured rat cardiomyocytes with recombinant adenovirus carrying rat nexilin or shRNA target for nexilin. We studied the effects of nexilin on cardiomyocytes sarcomere organization, mechanical property in normal or hypertrophic cardiomyocytes.
2. Materials & Methods

2.1. Neonatal Rat Cardiomyocyte Culture

All protocols and animals in this study were approved by the Animal Care Committee of Fuwai Cardiovascular Hospital. This investigation conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication NO.85-23, revised 1996). Neonatal rat cardiomyocytes were prepared as previously described (Kubasiak, Hernandez, Bishopric, & Webster, 2002). Briefly, neonatal rat hearts from 1- to 3-day-old Sprague-Dawley rats were digested with 0.04% trypsin (Hyclone) and 0.03% collagenase II (Invitrogen). To purify cardiomyocytes, fibroblasts were removed by differential adhesion for 1h 20 min in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) with 10% fetal bovine serum (FBS) (PAA). Cardiomyocytes were cultured in dishes (Nunc) in DMEM supplemented with 10% FBS, 0.1 mM/L 5-bromo-2-deoxyuridine (BrDU (Sigma), 100 U/ml penicillin, 100 U/ml streptomycin, for 48~72h. Before experiments, cardiomyocytes were cultured in serum-free DMEM for 24 hours. In this study, to induce cardiomyocyte hypertrophy, endothelin-1 (ET-1) (Sigma) was added to a final concentration of 100 nM/L in the serum free medium for 24 hours.

2.2 Analysis of Gene Expression

Cardiomyocytes was lysed with TRIzol as described previously (Rio, Ares, Hannon, & Nilsen). First-strand cDNA was synthesized using superscript III transcript kit (Invitrogen) and purified with QIAquick PCR Purification kit (Qiagen). 50 ng cDNA was used as a template for amplification. Changes in mRNA expression of nexilin in cardiomyocytes were determined using SYBR Green I (Invitrogen) quantitative real-time polymerase chain reaction (real-time PCR) and fluorescence was measured with DNA Engine Opticon 2 real-time PCR Detection system (Bio-Rad). GAPDH was used as internal control for normalization. The following primers were used: 5' AAAGTGAACATGAAAGCCAGATT-3' (forward) and 5' AGCCGTAAACAATGCTACCTTC-3' (reverse) for nexilin and 5' CAACGACCCCTCATGCATTGACCT-3' (forward) and 5' CAGTAGACTCCACGACATACTC-3' (reverse) for GAPDH. Data was analyzed using the $2^{\Delta\Delta C_t}$ method. Where $\Delta\Delta C_t = [(C_{t \text{ target gene}} - C_{t \text{ GAPDH}})_{\text{time x}} - (C_{t \text{ target gene}} - C_{t \text{ GAPDH}})_{\text{time 0}}]$. 
2.3 Preparation of Adenoviruses

shRNA sequences targeting rat nexilin were screened previously (Hui Wang, 2010). For adenoviral packaging, AdMax™ adenoviral vector system was used to generate recombinant adenovirus vectors. The most efficient shRNAs was subcloned into pShuttle vector to generate Ad-shRNA. Amplification was performed in 293A cells. Virus titer was determined using plaque assay method. Adenovirus carrying full length cDNA of nexilin (Ad-nexilin) was a gift from Professor Cao HuiQing (Department of Human Population Genetics, Institute of Molecular Medicine, Peking University, Peking, China). For control, adenoviruses carrying GFP (Ad-GFP) was purchased from Vector Gene Technology Company Limited (VGTC). Adenovirus amplification and titer were determined as above. Cardiomyocytes were infected with adenovirus at varying multiplicity of infection (MOI) as required for 48h in DMEM.

2.4 Immunoblotting Analysis

Protein samples were extracted from cells using a lyses buffer containing 1% NP-40, 150mM/L NaCl, 20mM/L sodium fluoride, 2mM/L EDTA, 2mM/L EGTA, 0.2mM/L PMSF, 2mM/L NaVO₄, 10mM/L Tris, and cocktail protease inhibitor (Roche) at pH 7.4. Total protein concentrations were determined using BCA Protein Assay kit (Applygen, China). Samples equally loaded (about 40 µg) were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (BioRad) and blocked in 5% non-fat milk. Antibodies including anti-nexilin (Sigma) and anti-GAPDH (Zhongshan Goldbridge Biotechnology co. Ltd) were used for immunoblotting analysis. The specific bands were detected by enhanced chemiluminescence (ECL reagent, Applygen). The immunoreactive bands were analyzed using image J software to estimate the density.

2.5 Adult Rat Ventricular Cardiomyocytes Culture

Adult rat ventricular myocytes were isolated by cardiac retrograde aortic perfusion as described previously (Kim et al., 2008). Briefly, heart was quickly isolated and retrogradely perfused with an oxygenated calcium free Tyrode buffer [140 mM/L NaCl, 5.4 mM/L KCl, 1.0 mM/L MgCl₂, 10 mM/L glucose, and 10 mM/L HEPES (pH 7.4)] for 5 min. Then the heart was perfused at 37°C with an oxygenated Tyrode buffer containing 0.06% collagenase II (Roche) for additional 15 to 20 min.
The left ventricular was separated and split into single myocytes in Kraft-Bruhe (K-B) buffer [80 mM/L KOH, 40 mM/L KCl, 25 mM/L KH$_2$PO$_4$, 3 mM/L MgSO$_4$, 50mM/L L-Glutamic, 20mM/L Taurine, 1 mM/L EGTA, 10 mM/L D-Glucose, and 10 mM/L HEPES (pH 7.2)]. After standing for 1~2 hours, isolated ventricular myocytes were cultured at a density of 1 × 10$^5$ cells/ml in M199 medium supplemented with 0.1 µM/L insulin, 2 mg/ml BSA, 5 mM/L taurine, 2 mM/L L-carnitine, 5mM/L creatine and 100 U/ml penicillin/streptomycine. To induce adult rat cardiomyocyte hypertrophy, endothelin-1 (ET-1) (Sigma) was added to a final concentration of 100 nM/L in the culture medium for 24 hours.

2.6 Actin Staining

Cardiomyocytes sarcomere is mainly composed with actin. Thus we indicate sarcomere organization using acting staining. Adult rat cardiomyocytes were cultured on collagen coated glass coverslips and treated with ET-1 or infected with Ad-GFP, Ad-nexilin, or Ad-nexilin-RNAi for 24 hours. After fixed with 3.7% formaldehyde, Cardiomyocytes were permeabilized with 0.1% Triton X-100, then stained with phalloidine-Tetramethylrhodamine B isothiocyanate (phalloidine-TRICT) [2ug/ml] (Sigma) for 1~2h at room temperature in dark.

2.7 Cardiomyocyte Mechanical Property Measurements

Adult rat ventricular myocytes infected with different virus were incubated for 24 hours. To determine the mechanical properties of adult rat ventricular myocytes, an IonOptix Myocam system (IonOptix Inc) was used as previous describe (Gupta, Aberle, Ren, & Sharma, 2005). Cells were placed into a chamber mounted on the stage of an inverted microscope (Nikon), standing for 5 min, field-stimulated at a frequency of 1 Hz, 15 V, and superfused with a buffer containing: 140 mM/L NaCl, 5.4 mM/L KCl, 1.0 mM/L MgCl$_2$, 1.8 mM/L CaCl$_2$, 10 mM/L glucose, and 10 mM/L HEPES (pH 7.4). Cell contractility were traced using Ionwizrd software version 5.0 (IonOptix) and assessed using cell shortening, maximal rate of contraction (-dL/dt) (contraction velocity), and maximal rate of relaxation (+dL/dt) (relaxation velocity).
2.8 Statistical Analysis

Values are given as means ± SEM. Comparisons of group means were performed by one-way ANOVA. Values with $P<0.05$ were considered statistically significant.

3. Results

3.1 Nexilin is Upregulated in ET-1 Induced Cardiomyocyte Hypertrophy

To investigate whether nexilin take part in the progress of hypertrophy, we determined the mRNA expression level of nexilin in hypertrophic cardiomyocyte induced by ET-1 using quantified real-time PCR analysis. As shown in Figure 1, nexilin mRNA expression level is continuously upregulated significantly from 1 hour to 24 hours. Nexilin mRNA expression increased $2.46±0.069$ fold ($P<0.01$) at 1 hour, $2.72±0.029$ fold at 3 hour ($P<0.01$), $2.47±0.042$ fold at 6 hour ($P<0.01$), $2.91±0.065$ fold ($P<0.01$) at 12 hour and $3.36±0.0094$ fold ($P<0.01$) at 24 hour. These results suggested that nexilin mRNA expression is upregulated in ET-1 induced hypertrophic cardiomyocyte.

![Figure 1: Quantitative Analysis of Nexilin mRNA Expression during Endothelin-1 (ET-1)-Induced Hypertrophy](image)

Serum-starved neonatal rat cardiomyocytes were treated with 100 nmol / L ET-1 for different durations of time.
The relative expression of nexilin was analysed by quantitative real-time reverse transcription polymerase chain reaction and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. White bars indicate groups not treated with ET-1 (control). Black bars indicate groups treated with ET-1. All values are shown as means ± SEM (n = 6). *P < 0.01 versus control.

3.2 Adenovirus Transfection in Neonatal Rat Cardiomyocyte

To determine reasonable transfection dose, neonatal rat cardiomyocytes were infected with Ad-nexilin or Ad-nexilin-RNAi at vary multiplicity of infection (MOI) for 48 hours. Expression of nexilin in cardiomyocytes was determined using western blot with a specific antibody. Figure 2A showed that expression of nexilin in cardiomyocytes infected with Ad-nexilin rose in a dose-dependent manner. On the contrary, as shown in Figure 2B, endogenic expression of nexilin can be efficiently knocked down at 40 MOI. To lower the impact of virus dose difference on cardiomyocytes, an MOI of 40 was chosen for the following study.

**Figure 2:** Effects of nexilin overexpression or knockdown in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were infected with Ad-nexilin or Ad-nexilin-RNAi for 48 hours at different multiplicity of infection (MOI). Cell lysates were probed with anti-nexilin antibody. GAPDH was used to equal sample loading.

3.3 Nexilin Regulates Sarcomere Organization

To study the effects of nexilin on sarcomere, normal or hypertrophic adult rat cardiomyocytes were infected with Ad-nexilin, Ad-nexilin-RNAi or Ad-GFP for 24 hours.
As shown in Figure 3, normal or hypertrophic cardiomyocytes infected with Ad-nexilin had normal sarcomere while Ad-nexilin-RNAi disordered sarcomere organization in cardiomyocytes. These data suggested that nexilin plays an important role in sarcomere organization.

![Figure 3: Effects of Nexilin on Sarcomere Organization](image)

Rat adult cardiomyocytes were infected with adenovirus carrying green fluorescent protein (Ad-GFP) or adenovirus carrying nexlin (Ad-nexilin) or adenovirus carrying shRNA for nexilin knockdown (Ad-nexilin-RNAi) were treated with endothelin-1 (ET-1) and then incubated for 24 h before staining with phalloidin-TRICT. Ad-nexilin-RNAi disordered sarcomere organization in cardiomyocytes compared with Ad-GFP or Ad-nexilin infected myocytes.
3.4 Nexilin Altering Adult Rat Cardiomyocyte Mechanical Property

The sarcomere of cardiomyocyte was essential for heart contraction. To further demonstrate effects of nexilin on cardiomyocytes contractility, we measured adult rat cardiomyocytes contractility using a video-based edge detection system (Ion Optix). Isolated adult rat ventricular cardiomyocytes or hypertrophic cardiomyocytes were infected with Ad-GFP or Ad-nexilin or Ad-nexilin-RNAi and incubated for 24 hours. Cell shortening traces from representative adult rat cardiomyocytes infected with Ad-GFP or Ad-nexilin or Ad-nexilin-RNAi were displayed in Figure 4A-B. Cell contractility was measured in infected cardiomyocytes as described in methods. As shown in Figure 4C-E, in normal cardiomyocytes, compared with Ad-GFP infected cardiomyocytes, Ad-nexilin overexpression induced significant increases in cell shortening (4.68 ± 1.28%, $n=20$ vs. 1.91±0.38%, $n=20$; $p<0.01$), in maximal rate of contraction (-dL/dt) (94.82±8.12µm/s, $n=20$ vs. 41.48±4.24µm/s, $n=20$; $p<0.01$) and in maximal rate of relaxation (+dL/dt) (69.82±7.51µm/s, $n=20$ vs. 22.90±1.67µm/s, $n=20$; $p<0.01$); While Ad-nexilin-RNAi induced significant decreases in cell shortening(1.17±0.45%, $n=20$ vs. 1.91±0.38%, $n=20$; $p<0.05$), but failed to induced statistically significant differences in maximal rate of contraction and in maximal rate of relaxation.

In hypertrophic cardiomyocytes, compared with Ad-GFP infected hypertrophic cardiomyocytes, Ad-nexilin overexpression induced significant increases in cell shortening (6.59 ± 0.83%, $n=20$ vs. 3.03±0.25%, $n=20$; $p<0.01$), in maximal rate of contraction (-dL/dt) (124.9±12.93µm/s, $n=20$ vs. 44.47±3.57µm/s, $n=20$; $p<0.01$) and in maximal rate of relaxation (+dL/dt) (93.02±13.51µm/s, $n=20$ vs. 14.09±2.28µm/s, $n=20$; $p<0.01$); While Ad-nexilin-RNAi induced significant decreases in cell shortening(1.17±0.45%, $n=20$ vs. 1.91±0.38%, $n=20$; $p<0.05$), but failed to induced statistically significant differences in maximal rate of contraction and in maximal rate of relaxation. Compared with normal cardiomyocytes infected with Ad-nexilin, hypertrophic cardiomyocytes infected with Ad-nexilin induced significant increases in cell shortening (6.59±0.83%, $n=20$ vs. 4.68±0.45%, $n=20$; $p<0.01$), in maximal rate of contraction (-dL/dt) (124.91±12.9µm/s, $n=20$ vs. 94.82±8.12µm/s, $n=20$; $p<0.01$) and in maximal rate of relaxation (+dL/dt) (93.02±13.5µm/s, $n=20$ vs. 69.82±7.51µm/s, $n=20$; $p<0.01$). Compared with normal cardiomyocytes infected with Ad-nexilin-RNAi, hypertrophic cardiomyocytes infected with Ad-nexilin-RNAi exhibited no significant changes in contractility.
These results suggest that overexpression nexilin positive regulates contractility of cardiomyocytes.

Figure 4: Effects of nexilin on adult rat cardiomyocyte contractility. A, Representative traces of cell shortening from cardiomyocytes infected with Ad-GFP or Ad-nexilin or Ad-nexilin-RNAi; B, Representative traces of cell shortening from cardiomyocytes treated with ET-1 and infected with Ad-GFP or Ad-nexilin or Ad-nexilin-RNAi; C, Contraction amplitude (%); D, Contraction velocity; E, relaxation velocity. All values are represented as mean ± SEM, n=20 per data group, *p<0.05 vs Ad-GFP.
4. Discussion

To the best of our knowledge, for the first time, we observed that nexilin is highly expressed in ET-1 induced cardiomyocytes hypertrophy. It suggests that nexilin might take part in the formation of cardiac hypertrophy. Thus, we tested the effects of nexilin overexpression or knocked down on cardiomyocyte hypertrophy. We found that nexilin overexpression or knockdown had no significant influence on cardiomyocyte hypertrophy including cardiac fetal gene expression, cell surface area and protein incorporation (data not show here). These data suggested that nexilin took part in the progress of hypertrophy but overexpressing nexilin is not sufficient for hypertrophy. Nexilin might be a marker of hypertrophy. ET-1 mediates biological function via heterotrimeric Gaq protein-coupled receptor. Two types of ET-1 receptor exist in cardiomyocytes, ET$_A$ receptor and ET$_B$ receptor. It has been reported that contractility of cardiomyocytes from rat, rabbit and human can be improved by ET-1 (Kelly et al., 1990; Kusumoto, Fujiwara, Ikeda, Watanabe, & Fujino, 1996; K. Li, Stewart, & Rouleau, 1991; Takanashi & Endoh, 1991). MacCarthy et al found that ET-1 positively regulated normal cardiomyocytes contractility through ET$_A$ receptor activating PKC signal pathway (MacCarthy, Grocott-Mason, Prendergast, & Shah, 2000). Thus, we suppose that ET-1 might regulate nexilin expression and increase cardiomyocyte contractility via PKC signaling pathway activation. The potential mechanism need to be confirmed future.

Hypertrophic cardiac myopathy (HCM) is a disease that is characterized by thickening of the heart muscle, smaller ventricular champ and impaired diastole function. Dilated cardiac myopathy (DCM) is characterized by thinner wall of the heart muscle, dilated ventricular champ and impaired systolic function. A second interesting phenomenon, in our study, is that either nexilin overexpression in normal cardiomyocytes or in hypertrophic cardiomyocytes increased the cardiomyocytes contractility; on the contrary, knocking down nexilin in normal cardiomyocytes or in hypertrophic cardiomyocytes decreased the cardiomyocytes contractility. These data suggested that nexilin might be a candidate gene for HCM or DCM. Hassel et al found that mutations including G650del, Y652C and P611T on C terminus of nexilin are cause of dilated cardiomyopathy (Hassel et al., 2009). The mechanism might be that mutations on C terminus, an immune globin domain, decreased the actin binding ability of nexilin, disordered sarcomere organization and lower the heart contractility.
A recent study also showed that Q131E mutation on nexilin resulted in hypertrophic cardiomyopathy (Wang et al.). The underline mechanism, they found, is that Q131E mutation, occurred in F-actin binding domain, had nexilin lose the F-actin binding ability and down regulated the heart contraction. Altogether, all the reports are consistent with our results. Taken together, the results presented here suggested that nexilin is upregulated in ET-1 induced hypertrophic cardiomyocytes; nexilin overexpression or knockdown in vitro is not sufficient for cardiomyocyte hypertrophy; nexilin regulates cardiomyocytes contractility in normal or hypertrophic cardiomyocytes. It provides a novel treatment target for hypertrophic cardiomyopathy and dilated cardiomyopathy.

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5. References


