Experimental Cell Research 347 (2016) 261–273

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research Article

NMNAT3 is involved in the protective effect of SIRT3 in Ang II-induced cardiac hypertrophy

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ARTICLE INFO

Article history:
Received 14 February 2016
Received in revised form
29 June 2016
Accepted 12 July 2016
Available online 14 July 2016

Keywords:
SIRT3
NMNAT3
Cardiac hypertrophy

ABSTRACT

Pathological cardiac hypertrophy is a maladaptive response in a variety of organic heart disease (OHD), which is characterized by mitochondrial dysfunction that results from disturbed energy metabolism. SIRT3, a mitochondria-localized sirtuin, regulates global mitochondrial lysine acetylation and preserves mitochondrial function. However, the mechanisms by which SIRT3 regulates cardiac hypertrophy remains to be further elucidated. In this study, we firstly demonstrated that expression of SIRT3 was decreased in Angiotension II (Ang II)-treated cardiomyocytes and in hearts of Ang II-induced cardiac hypertrophic mice. In addition, SIRT3 overexpression protected myocytes from hypertrophy, whereas SIRT3 silencing exacerbated Ang II-induced cardiomyocyte hypertrophy. In particular, SIRT3-KO mice exhibited significant cardiac hypertrophy. Mechanistically, we identified NMNAT3 (nicotinamide mononucleotide adenylyltransferase 3), the rate-limiting enzyme for mitochondrial NAD biosynthesis, as a new target and binding partner of SIRT3. Specifically, SIRT3 physically interacts with and deacetylates NMNAT3, thereby enhancing the enzyme activity of NMNAT3 and contributing to SIRT3-mediated anti-hypertrophic effects. Moreover, NMNAT3 regulates the activity of SIRT3 via synthesis of mitochondria NAD. Taken together, these findings provide mechanistic insights into the negative regulatory role of SIRT3 in cardiac hypertrophy.

1. Introduction

Cardiac hypertrophy is characterized by re-activation of the fetal gene program, increase in protein synthesis, and subsequent increase in the size of the cardiomyocytes [1,2]. Cardiac hypertrophy is initially regarded as a compensatory response of the heart to various physiological or pathological stimuli. However, prolonged hypertrophy of the heart ultimately impairs the cardiac function and results in heart failure [3]. The incidence of cardiac hypertrophy is dramatically increased with age [4], implying that aging-associated mechanisms are involved in the pathogenesis of cardiac hypertrophy.

Sirtuins, a family of evolutionarily conserved histone deacetylases (HDACs), play pivotal roles in the regulation of aging-related pathophysiology. Distinguished from other HDAC classes, sirtuins require nicotinamide adenine dinucleotide (NAD) for their activation, and thus are defined as class III HDACs [5,6]. Collective evidence has indicated that sirtuins are critical regulators of many

Abbreviations: SIRT, sirtuin; NMNAT, nicotinamide mononucleotide adenylyltransferase; NAMPT, nicotinamide phosphoribosyltransferase; NAD, nicotinamide adenine dinucleotide; Ang II, angiotensin II; ANF, atrial natriuretic factor; BNP, brain natriuretic polypeptide; NRCMs, neonatal rat cardiomyocytes; NAM, nicotinamide; FOXO3a, forkhead box O3a; LKB1, liver kinase B1; ROS, reactive oxygen species; HDACs, histone deacetylases; UPR, unfolded protein response; COX-IV, Cytochrome C oxidase IV

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http://dx.doi.org/10.1016/j.yexcr.2016.07.006
0014-4827/

cellular cascades, including stress responses, cell growth, energy metabolism and apoptosis [7–9]. Seven subtypes of sirtuins, named as SIRT1–7, have been identified in mammals. SIRT3, located in the mitochondria, is considered as the major mitochondrial deacetylase [10]. SIRT3 is involved in the regulation of multiple mitochondrial metabolic events, such as fatty acid oxidation, tricarboxylic acid (TCA) cycle, the electron transport chain, and the urea cycle [11–13]. SIRT3 is abundantly expressed in organs with highly metabolic turnover, such as the heart [14]. SIRT3 depletion impairs mitochondrial and contractile function in the heart, due to increased acetylation of various energy metabolic proteins and subsequent myocardial energy depletion [15]. Coincidently, SIRT3 preserves heart function and capillary density in the setting of obesity [16]. SIRT3 protects cardiomyocytes from stress-mediated cell death and preserves contractile function in response to a chronic increase in workload via FOXO3a-mediated suppression of oxidative stress [17,18]. Furthermore, lack of SIRT3 facilitates the opening of the mitochondrial permeability transition pore by increasing acetylation and spatial dislocation of cyclophilin D [19]. Considering there are many downstream substrates of SIRT3 in mitochondria, the mechanisms by which SIRT3 regulates cardiac function and cardiac hypertrophy still need to be further elucidated.

NAD, a classic coenzyme for cellular redox reactions, has been identified as a substrate for sirtuins [20,21]. In mammalians, NAD can be synthesized through both salvage and *de novo* pathways [22]. Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme for NAD salvage from nicotinamide. Previous studies demonstrate that overexpression of NAMPT significantly increases NAD biosynthesis in primary cultured cardiomyocytes [23]. Nicotinamide mononucleotide adenylyltransferase (NMNAT) is another central enzyme for NAD biosynthesis, which catalyzes both *de novo* and salvage pathways [24]. Currently, three NMNAT isoforms have been identified, which varied in subcellular localization and tissue distribution [25]. Our previous study showed that NMNAT2 attenuates Ang II (angiotensin II) induced cardiomyocyte hypertrophy via increasing NAD biosynthesis [26]. NMNAT3 is localized in the mitochondrial matrix and is the only known enzyme of NAD synthesis residing within the organelles [27]. A recent study has reported that exogenous NAD could suppress the agonist-induced cardiac hypertrophy through activating SIRT3, rather than SIRT1 [28]. Thus, it is plausible that SIRT3 interacts withNMNAT3 in the mitochondria of cardiomyocytes. The present study was designed to investigate this potential interaction and the co-regulations of these enzymes in cardiac hypertrophy.

2. Materials and methods

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 4′, 6-diamidino-2-phenylindole (DAPI), penicillin/streptomycin, and Lipofectamine 2000 were purchased from Life technologies/ Invitrogen (Carlsbad, CA, USA). Anti-NMNAT3 antibody was obtained from Santa Cruz Biotechnology (CA, USA). Anti-acetyl-lysine (anti-AC-K), anti-SIRT3, anti-COX-IV, anti-Flag antibodies, and normal rabbit immunoglobulin G were all bought from Cell Signaling Technology (Boston, MA, USA). Anti-α-tubulin antibody, Anti-NAMPT antibody, and Angiotensin II (Ang II) were provided by Sigma-Aldrich (St. Louis, MO, USA), Proteintech (Wuhan, China), and Merck Millipore (Billerica, MA, USA), respectively. SIRT3 genetic knockout mice (strain#129-Sirt3$^{tm1.1Fwa}$/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Age-, sex-, and background-matched wild type mice were purchased from Shanghai Biomodel Organism Science & Technology Development Company (Shanghai, China). The adult male mice (8 weeks old) were used in the study. Wild type and knockout mice were infused with Ang II (Sigma, 3 mg/kg/d dissolved in 0.9% NaCl), with saline-infused mice serving as vehicle controls. All mice were infused with Ang II by implanted osmotic minipumps (Alzet model 1002, Alza Corp.) for 2 weeks [29]. After 14 days, mice were sacrificed and subjected to hypertrophic analysis.

Additional materials and methods are listed in Supplementary Materials. Data were presented as means ± standard error of the mean (SEM). Statistical analysis was performed by SPSS statistic software13.0. Mean difference between two groups was tested by Student's *t*-test. Statistical analysis among the various groups was performed by One-way analysis of variance (ANOVA) with *Bonferroni post hoc* test. In all cases, difference between groups was considered statistically significant at $P < 0.05$.

3. Results

3.1. SIRT3 was downregulated in Ang II-induced cardiac hypertrophy

We first determined the cellular localization of SIRT3 in cardiomyocytes. Our data showed that SIRT3 was present in the mitochondria (Fig. 1), in line with previous reports [10]. In response to Ang II (100 nM), a neurohumoral factor that stimulates cardiac hypertrophy, the mRNA and protein levels of SIRT3 in neonatal rat cardiomyocytes (NRCMs) were decreased in a time-dependent manner (Figs. 1 and 2A and B). Moreover, the enzyme activity of SIRT3 was downregulated by Ang II treatment (Fig. 2C). In vivo, we also observed that the protein level and enzyme activity of SIRT3 were also decreased in the mouse myocardium treated with Ang II (3 mg/kg/d) for 2 weeks (Fig. 2D and E).

3.2. SIRT3 protected cardiomyocytes against Ang II-induced hypertrophy

To investigate the potential effect of SIRT3 on cardiomyocyte hypertrophy, NRCMs were transfected with plasmids encoding Flag-tagged wild-type SIRT3, or the catalytically inactive mutant H248Y-HA. Expression of SIRT3 was augmented after SIRT3-Flag or H248Y-HA transfection (Fig. 3A), whereas, the enzyme activity of SIRT3 was increased by SIRT3-Flag, rather than H248Y-HA mutant (Fig. 3B). After treatment with 100 nM Ang II for 24 h, the expression of hypertrophic biomarkers (atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP)), as well as the cell surface area, were significantly elevated (Fig. 3C and D). However, transfection with SIRT3-Flag, rather than H248Y-HA, reversed Ang II-induced increase of hypertrophic biomarkers and cell surface area (Fig. 3C and D).

By contrast, endogenous SIRT3 in cardiomyocytes was depleted by RNA interference. Quantitative RT-PCR was performed to evaluate the efficiency of three independent siRNAs, marked as si001, si002, and si003, respectively. The si003 significantly decreased the mRNA and protein level of SIRT3 (Fig. S1A), but had no significant effects on SIRT4 and SIRT5 (Fig. 4A), suggesting the specificity of siRNA. Therefore, si003 was renamed as siSIRT3 hereafter and used in the following experiments. Transfection with siSIRT3 significantly increased the mRNA levels of ANF and BNP, as well as the cell surface area (Fig. 4B and C). Additionally, silencing of endogenous SIRT3 further augmented Ang II-induced hypertrophic response (Fig. 4B and C). In line with the observations in in vitro studies, genetic knockout of SIRT3 in mice significantly increased the cardiac expression of hypertrophic biomarkers. Additionally, SIRT3 depletion could further exacerbate Ang II-induced cardiac hypertrophy (Fig. 4D).