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Myofibril growth during cardiac hypertrophy is regulated through dual phosphorylation and acetylation of the actin capping protein CapZ

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ABSTRACT

The mechanotransduction signaling pathways initiated in heart muscle by increased mechanical loading are known to lead to long-term transcriptional changes and hypertrophy, but the rapid events for adaptation at the sarcomeric level are not fully understood. The goal of this study was to test the hypothesis that actin filament assembly during cardiomyocyte growth is regulated by post-translational modifications (PTMs) of CapZβ1. In rapidly hypertrophying neonatal rat ventricular myocytes (NRVMs) stimulated by phenylephrine (PE), two-dimensional gel electrophoresis (2DGE) of CapZβ1 revealed a shift toward more negative charge. Consistent with this, mass spectrometry identified CapZβ1 phosphorylation on serine-204 and acetylation on lysine-199, two residues which are near the actin binding surface of CapZβ1. Ectopic expression of dominant negative PKCε (dnPKCε) in NRVMs blunted the PE-induced increase in CapZ dynamics, as evidenced by the kinetic constant (Kfrap) of fluorescence recovery after photobleaching (FRAP), and concomitantly reduced phosphorylation and acetylation of CapZβ1. Furthermore, inhibition of class I histone deacetylases (HDACs) increased lysine-199 acetylation on CapZβ1, which increased K_{frap} of CapZ and stimulated actin dynamics. Finally, we show that PE treatment of NRVMs results in decreased binding of HDAC3 to myofibrils, suggesting a signal-dependent mechanism for the regulation of sarcomere-associated CapZβ1 acetylation. Taken together, this dual regulation through phosphorylation and acetylation of CapZβ1 provides a novel model for the regulation of myofibril growth during cardiac hypertrophy.

1. Introduction

Transient physiological demands in mechanical loading induce hypertrophic growth of cardiomyocytes, increased contractility, and tissue remodeling. The mechanotransduction signaling pathways initiated by loading result in well studied long-term transcriptional changes but also in rapid transient modifications at the protein level. Here we studied the rapid post-translational modifications (PTMs) of phosphorylation and acetylation of CapZβ1, a capping protein known to regulate the assembly of myofibrils, which underlie cardiac hypertrophy. The actin capping protein is a mushroom-like heterodimeric protein (α and β subunits) that binds to the barbed ends of the actin filaments and slows down their assembly [1]. In muscle cells, this complex was named CapZ because of its localization to the *Z*-disc [2]. Actin capping and uncapping by CapZ are highly regulated by several binding proteins and polyphosphoinositides [3,4,5]. In myocytes, both CapZ and actin dynamics are increased very rapidly by mechanical strain, suggesting the possible involvement of PTMs in actin filament assembly [4,6].

Phosphorylation is a common PTM, with protein kinase C (PKC) isoforms being crucial in cardiac contraction and hypertrophy [7,8,9]. The PKCε isoform is abundantly expressed in human and rodent hearts [10,11]. Activation of PKCε enhances cardiac contractility and remodeling [12,13], and has a cardio-protective function in ischemic preconditioning [14,15,16]. Transgenic mice that overexpress a constitutively active PKCε enzyme develop cardiac hypertrophy, leading to dilated cardiomyopathy after many months [17,18]. In cardiomyocytes stimulated by mechanical strain, PKCε translocates to the *Z*-disc and modifies CapZ, leading to increased CapZ dynamics. This provides a novel mechanism for PKCε-mediated cardiomyocyte growth [19].

Acetylation of lysine residues is another major post-translational event. Acetyl groups are transferred to lysines by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs) [20]. The most well characterized function for acetylation is in epigenetic control of gene expression through acetylation/deacetylation of nucleosomal histone tails. However, proteomic studies have revealed that thousands of non-histone proteins also undergo reversible acetylation [21,22,23,24]. The functional consequences of non-histone protein acetylation in the heart remain poorly characterized.

The goal of the present study was to test the hypothesis that actin filament assembly is regulated by post-translational modifications of

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CapZβ1, which are mediated by PKCε and HDACs. In hypertrophying neonatal rat ventricular myocytes (NRVMs) stimulated by phenylephrine (PE), variations of PKCε and HDACs by activators or inhibitors were applied to test whether phosphorylation or acetylation alter CapZ and actin dynamics. The coordination of phosphorylation and acetylation on CapZβ1 capping was approached by manipulating both pathways, individually and simultaneously, to study PTMs of CapZβ1 in rapidly hypertrophying NRVMs. The data reveal novel functions for PKCε and class I HDACs in the control of CapZβ1 activity and myofibril formation during cardiac hypertrophy.

2. Materials and methods

2.1. Cell culture

Primary heart cultures were obtained from neonatal rats according to Institutional Animal Care and Use Committee and NIH guidelines for the care and use of laboratory animals. Hearts were removed and cells isolated from 1 to 2 days old neonatal Sprague-Dawley rats with collagenase (Worthington) as previously described [25]. The cells were re-suspended, filtered through a metal sieve to remove large material and plated at high density (200,000/cm^2) in PC-1 medium (Biowhittaker/Cambrex) on fibronectin coated 3.5 cm dishes. Cells were left undisturbed for 24 h in a 5% CO_2 incubator when the unattached cells were removed by aspiration and the PC-1 media was replenished. Cells were incubated for another 24 h prior to the experiment.

2.2. Neurohormonal stimulation of NRVM

The neurohormonal treatment times chosen were sufficient to induce hypertrophy [3,26] with phenylephrine (10 μM, Sigma-Aldrich) for 24 h prior to experiment analysis.

2.3. Cell size measurement

NRVMs were washed with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min, placed in cold 70% ethanol, and stored at −20 °C until immunostaining. Primary anti-α-actinin antibody (Catalog No. ab9465, mouse IgG; Abcam, Cambridge, MA) was diluted (1:200) in 1% BSA in PBS (with 0.1% Triton X-100) and incubated on a shaker table at 4 °C overnight. Cells were then rinsed in PBS at 25 °C and blocked in 1% BSA in PBS for 1 h at 25 °C. Secondary antibody (Molecular Probes) was diluted at a ratio of 1:500 in 1% BSA in PBS and incubated for 1 h at 25 °C. Cells were washed in PBS. Anti-fade reagent with DAPI (Molecular Probes) was added and cover slips were mounted on glass slides. For the measurement of hypertrophy, NRVM boundaries were visualized by α-actinin antibody staining and cell area was measured by Image J.

2.4. HDAC inhibitors

HDAC inhibitors were used at the indicated final concentrations and treatment time: trichostatin A (TSA) (5 μM, 5 h; Sigma), MGCD0103 (500 nM, 24 h; Selleck), theophylline (10 μM, 24 h; Sigma) and tubastatin A (1 μM, 24 h; Selleck).

2.5. Adenoviral and lentiviral constructs and infection

Recombinant adenoviruses for GFP-CapZβ1, constitutively active PKCε (caPKCε) and dominant negative PKCε (dnPKCε) were kindly provided by Dr. Allen Samarel (Loyola University Chicago Stritch School of Medicine, Maywood, IL) as previously described [3,27]. Two days after NRVM isolation, NRVMs were infected with CapZβ1 (MOI 20), caPKCε (MOI 100), or dnPKCε (MOI 250) for 60 min at 37 °C diluted in PC-1 medium. The viral medium was then replaced with virus-free medium, and cells were left undisturbed for 24 h. Lentiviruses encoding short-hairpin (sh) RNAs targeting HDAC1, HDAC2 and HDAC3 have been previously described [28].

2.6. Actin-GFP expression

Actin-GFP expression was induced by CellLight® Reagents *BacMam 2.0* actin-GFP (Invitrogen). Two days after NRVM isolation, the CellLight® Reagent (30 μL per 1,000,000 cells) was used as modified from the manufacturer's instructions. Infected NRVMs were returned to the incubator for at least 16 h.

2.7. Fluorescence recovery after photobleaching for CapZ and actin dynamics

Fluorescence recovery after photobleaching (FRAP) has yielded qualitative and quantitative information about the processes that regulate actin polymerization in living myocytes [29]. The methods and analysis for FRAP of actin-GFP were described by us [6]. Briefly, binding of CapZ to the actin filament has two binding states [30], so FRAP curves of CapZ were fit using non-linear regression in OriginPro (OriginLab, Northampton, MA):

$$I_{frap}(t) = 1 - C_1 e^{-Koff1\ t} - C_2 e^{-Koff2\ t}. \quad (1)$$

The average kinetic constant (K_{frap}) for dynamics was calculated using the following formula:

$$K_{frap} = C_1 K_{off1} + C_2 K_{off2}. \quad (2)$$

For FRAP of actin-GFP and actin-RFP, since actin binding activity has one-binding state [31], the equation for curve fitting using non-linear regression in OriginPro was:

$$I_{frap}(t) = 1 - C_1 e^{-Koff1\ t}. \quad (3)$$

The average kinetic constant (K_{frap}) was calculated using the following formula:

$$K_{frap} = C_1 K_{off1} \quad (4)$$

2.8. Co-immunoprecipitation

Cultured NRVM were infected with GFP-CapZβ1 adenovirus. After 24 h of infection, cells were washed twice in ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.1% SDS) plus phosphatase inhibitors (Sigma Aldrich, #P5726, P0044) for 1 h at 4 °C under constant agitation. Following protein extraction, protein lysates were precleared using 25 μL Protein A/G Plus-Agarose beads (Santa Cruz, #sc-2003) for 1 h at 4 °C. Precleared lysates were incubated 24 h with 2 μg of HDAC3 antibody (Cell Signaling Technology; 4668) at 4 °C, then immunocomplexes were isolated by adding Protein A/G Plus-Agarose beads overnight at 4 °C. Beads were washed three times in the binding buffer. SDS-PAGE and Western blotting were performed using 12% Mini-PROTEAN® TGX™ Gel (Bio-Rad Laboratories, #456-1044). Polyvinylidene difluoride (PVDF) membranes were incubated with GFP primary antibody (Enzo Life Sciences, ADI-SAB-500), followed by horseradish peroxidase (HRP) secondary antibody (anti-mouse) for 1 h at room temperature. Proteins were finally treated with an ECL Plus kit and visualized with the aid of ChemiDoc XRS + and analyzed with Image Lab (Bio-Rad Laboratories).