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Original Article

CaMKIIδ meditates phenylephrine induced cardiomyocyte hypertrophy through store-operated Ca^{2+} entry☆

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ABSTRACT

Evidence suggests that store-operated Ca2+ entry (SOCE) is involved in the hypertrophy of cardiomyocytes. The signaling mechanisms of SOCE contributing to cardiac hypertrophy following phenylephrine (PE) stimulation are not fully understood. Ca^{2+}/calmodulin-dependent protein kinase II δ (CaMKIIδ) plays an important role in regulating intracellular Ca^{2+} hemostasis and function in the cardimyocytes. This study is aimed to determine the role of CaMKIIδ in regulating the PE-induced myocardial hypertrophy and the associated molecular signaling mechanisms. We used primary cultures of neonatal cardimyocytes isolated from the left ventricle of Sprague Dawley rats to investigate the effects of CaMKIIδ on myocardial hypertrophy and intracellular Ca^{2+} mobilization. We found that the expression of CaMKIIδ was enhanced in PE-induced hypertrophic cardiomyocytes. CaMKIIδ siRNA, CaMKII inhibitor KN93, and SOCE blocker BTP2 attenuated the increase in the expression of CaMKIIδ and normalized the hypertrophic markers, atrial natriuretic peptide and brain natriuretic peptide, and size of cardiomyocytes induced by PE stimulation. The protein level of stromal interaction molecule 1 and Orai1, the essential components of the SOCE, is also enhanced in hypertrophic cardiomyocytes, which were normalized by CaMKIIδ siRNA and KN93 treatment. Hypertrophic cardiomyocytes showed an increase in the peak of Ca^{2+} transient following store depletion, which was inhibited by SOCE blocker BTP2, CaMKIIδ siRNA, and KN93. The Ca^{2+} currents through Ca^{2+} release-activated Ca^{2+} channels were increased in PE-treated cardiomyocytes and were attenuated by CaMKIIδ siRNA and KN93. These data indicate that PE-induced myocardial hypertrophy requires a complex signaling pathway that involves activation of both CaMKIIδ and SOCE. In conclusion, these studies reveal that up-regulation of CaMKIIδ may contribute to the PE-induced myocardial hypertrophy through the activation of SOCE expressed in the cardiomyocytes.

1. Introduction

Cardiac hypertrophy is the enlargement of the heart, which is caused by cardiovascular diseases, including congestive heart failure (HF), valve disease, and hypertension [1]. Phenylephrine (PE), an α_1-adrenergic agonist and a hypertrophic agent, is involved in cardiac hypertrophy in cultured cardiac myocytes and in perfused hearts [2] involving activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [3]. CaMKII is one of the critical signaling pathways which contributes to maladaptive cardiac hypertrophy [4].

Increased activity of CaMKII has been suggested to contribute to the development of cardiac hypertrophy associated with HF in patients and in animal models [5–7]. CaMKII is a multifunctional signaling molecule that has been implicated in pathological cardiac remodeling in response to stress [8]. CaMKII has been known to be activated following Ca^{2+}-dependent activation of calmodulin through a variety of posttranslational modifications including autophosphorylation, oxidation, and O-linked glycosylation [9–11]. CaMKIIδ, one of the subtypes of CaMKII, is dominantly expressed in cardiomyocytes [12]. In response to the activation of G protein-coupled receptors by agonists such as PE, and to α_1 adrenergic receptor stimulation, CaMKII predominantly regulates RyR2 by phosphorylation of residue S2814 [7,13], possibly resulting in leak of Ca^{2+} from RyR2 on the sarcoplasmic reticulum (SR). In addition, CaMKII regulates Ca^{2+} dynamics through voltage-dependent L.-type Ca^{2+} channels (LTCC). CaMKII-dependent phosphorylation of LTCC potentiates Ca^{2+} inward currents and slows its inactivation [14]. As a result, LTCC blockers are commonly used for the treatment of cardiac hypertrophy in both animals and patients [15,16]. On the contrary, experimental evidence indicates that CaMKII decreased the expression of the LTCC pore-forming α1c-subunit and treatment with CaMKII inhibitor KN-93 increased LTCC current [17]. Moreover, LTCC blockers increase left

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ventricular pressure gradients [18]. Therefore, the mechanisms of how CaMKIIδ regulates Ca^{2+} homeostasis in cardiac hypertrophy are not completely understood.

Recent studies have shown that store-operated Ca^{2+} entry (SOCE) through Ca^{2+} release activated Ca^{2+} (CRAC) channels in the plasma membrane modulates Ca^{2+} homeostasis [19]. The depletion of intracellular Ca^{2+} activates CRAC channels which allows influx of Ca^{2+} from the extracellular space and may be enhanced in pathological remodeling [20]. CRAC channels are voltage-independent Ca^{2+} channels which were found in nonexcitable cells as the major Ca^{2+} entry mechanism [21]. More recently CRAC channels were also found in excitable cells, such as cardiomyocytes and neurons, and play important roles in Ca^{2+} homeostasis [22,23]. CRAC channels contain two components including stromal interaction molecule 1 (STIM1) and Orai1. STIM1 located in the SR membrane is a sensor of Ca^{2+} in the SR. Ca^{2+} depletion leads to oligomerization of STIM1, which induces STIM1 to relocate to the junction of SR and the plasma membrane and interacts with Orai1, finally activating CRAC channels [24–26]. Given the evidence that the CRAC channel is a major cellular Ca^{2+} signaling pathway responsible for stressful challenges, CaMKII activation of RYR2 which induces SR Ca^{2+} down-regulation and STIM1 are expressed on the membrane of SR in cardiomyocytes; we hypothesized that the role of CaMKIIδ on hypertrophy of cardiomyocytes is mediated through activation of SOCE and CRAC current in cardiomyocytes after PE treatment.

2. Methods

2.1. Reagents

(PE), KN93, BTP2, and thapsigargin (TG) were purchased from Sigma–Aldrich Corporation (MO, USA). The final concentration of PE, KN93, BTP2, and TG were 100 μM, 20 μM, 5 μM, and 2 μM, respectively [27–30]. CaMkIIδ siRNA and PE hydrochloride were purchased by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals and myocyte isolation

All procedures and animal care were approved by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Ventricular cardiomyocytes from 1- to 2-day-old eight neonatal rat hearts (neonatal rat cardiomyocyte [NRCMs]) were prepared by trypsin digestion for each culture as described previously [31]. After 24 h, NRCMs were treated with PE, PE + KN93, PE + CaMkIIδ siRNA, or PE + BTP2. According to different treatments, NRCMs were divided into five groups, including control, PE, PE + KN93, PE + CaMkIIδ siRNA, and PE + BTP2.

2.3. Western blot analysis

After a 48-h treatment, NRCMs were collected for Western blot analysis. Equal amounts of protein (25 μg) were separated on an SDS–PAGE gel and transferred to a polyvinylidene fluoride membrane. The membrane was rinsed and blocked with 5% nonfat skim milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature. Then, the following primary and secondary antibodies were used: anti-STIM1 antibody (Abcam), anti-orai1 antibody (Abcam), anti-CamkIIδ antibody (Abcam), anti-β-Actin (Sigma), and horseradish peroxidase goat antirabbit IgG (Jackson Immuno). Densitometry was quantitated using Quantity One software (Bio-Rad). Data are given as relative intensity units normalized to the actin protein abundance.

2.4. Quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated from cultured myocytes using an RNA isolation kit (Bio Teke) following the manufacturer's instructions. The integrity and purity of the extracted RNA were evaluated by the ratio of the absorbance at 260 nm to that at 280 nm. Single-stranded cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo). Quantitative real-time PCR was performed on cDNA using 20-μL reaction volumes with FastStart Universal SYBR Green Master (Roche) using ANALYTIK JENA AG qTower 2.2 up PCR System (Germany). The mixtures were heated to and held at 95 °C for 10 min followed by 36 cycles at 95 °C for 15 s, 60 °C for 30s, 72 °C for 30s and, lastly, melt for 6 s. All reactions were performed in triplicates. We carried out a study to analyze the relative quantity of the expression data based on the $2^{\Delta\Delta Ct}$ method. The oligonucleotide primer sequences were as follows: CaMKIIδ (forward: 5′ -CCTAAATGGCATAGTTCAC- 3′, reverse: 5′ -GGATCTTTACGTAGGACTTC- 3′); atrial natriuretic peptide (ANP) (forward: 5-GGGGGTAGGATTGACAGGAT-3, reverse: 5′ -GGATC TTTTGCGATCTGCTC- 3′); and brain natriuretic peptide (BNP) (forward: 5′- GCTGCTTTGGGCAGAAGATA- 3′, reverse: 5-‘ GGAGTCTGCAGCCAGG AGGT -3’) [32]; Quantitative PCR was done using Glyceraldehyde 3-phosphate dehydrogenase (Sangon Biotech) as control.

2.5. Small interfering RNA-based experiment

Three small interfering RNAs (siRNAs) targeting CaMkIIδ gene were designed and synthesized by Biomics (Nantong, China), and the effect of siRNA was identified by Western blot. The sequence of siRNA is as follows: siRNA1, 5′ -GCCACCCUGUAUUCCAAAUdTdT- 3′; siRNA2, 5′ – GGUGCCAUCUUGACAACUAdTdT- 3′ and siRNA3, 5′ -CCUACGAAAGA UCCUUAUdTdT- 3′. Scrambled RNA oligonucleotides were used as control. Twenty-four hours prior to transfection, cardiomyocytes were plated onto a 6-well plate (Corning Inc., Corning, NY, USA). For each well, 50 nM of the three oligos were transfected using LipoD293 (SignaGen) according to the manufacturer's instructions. The medium was replaced with Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum after 6 h.

2.6. Cardiomyocyte morphological analysis

Cardiomyocyte images were captured with a charge-coupled device camera fixed to an inverted microscope (Leica, Wetzlar, Germany). Cardiomyocytes were selected randomly and viewed at 200 magnification in the inverted phase contrast microscope to observe the dynamic growth of cardiomyocytes after 24 h, 48 h, and 72 h. Cell surface areas (CSAs) were calculated at 48 h. Cardiomyocytes were outlined, and CSA was measured with Image pro plus 6.0 (Media Cybernetics, USA).

2.7. Ca^{2+} imaging and SOCE measurements

Cardiomyocytes were loaded with 2-μM Fluo-4 AM (Invitrogen) in extracellular buffer (in mM): 140 NaCl, 5 KCl, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 Hepes, for 30 min at room temperature. Cells were washed with extracellular buffer and kept in this buffer until use. A standard protocol referred to as “Ca^{2+} re-addition” was used to trigger SOCE [33,34], in which cells were exposed to Ca^{2+}-free medium containing TG (2 μM) to deplete SR Ca^{2+} stores. Then, Ca^{2+} (1.8 mM) was restored to the medium, and Ca^{2+} influx was recorded. Full images were collected every 6 s. Fluo-4 fluorescence was excited at 488 nm, and data were expressed as normalized changes in background-corrected fluorescence emission (F/F_0). Data were acquired using leica SP5 confocal software (Leica Microsystems). Representative Ca^{2+} signals averaged from 6 to 10 individual cells are shown in the figures.

2.8. Electrophysiological measurement of CRAC channel

Electrophysiological experiments were performed at 20–24 °C, using the patch-clamp technique in the whole-cell recording configuration. For CRAC current measurements, voltage ramps were applied every 5 s from a holding potential of −60 mV, covering a range of −100 to +20 mV over 1 s. The internal pipette solution for passive store