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Original article

Extracellular signal-regulated kinase activation during cardiac hypertrophy reduces sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) transcription

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ABSTRACT

Pathologic cardiac hypertrophy can lead to heart failure, but the mechanisms involved are poorly understood. SERCA2 is critical for normal cardiac calcium handling and function and SERCA2 mRNA and protein levels are reduced by cardiac hypertrophy. We hypothesized that extracellular signal-regulated kinase (ERK) 1/2 activation during hypertrophy reduced SERCA2 transcription. Using a neonatal rat ventricular myocyte model of hypertrophy, we found that pharmacologic inhibitors of ERK activation preserve SERCA2 mRNA levels during hypertrophy. ERK activation is sufficient to reduce SERCA2 mRNA. We determined that ERK represses SERCA2 transcription via nuclear factor-kappaB (NFkB), and activation of NFkB is sufficient to reduce SERCA2 mRNA in cardiomyocytes. This work establishes novel connections between ERK, NFkB, and SERCA2 repression during cardiac hypertrophy. This mechanism may have implications for the progression of hypertrophy to heart failure.

1. Introduction

Pathologic cardiac hypertrophy, caused by excessive adrenergic stimulation or pressure overload, can lead to heart failure. Numerous pathways have been implicated, but the mechanisms of the transition from compensated hypertrophy to heart failure are still poorly understood [1]. Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) pumps cytosolic calcium into the sarcoplasmic reticulum, which is critical for normal cardiac calcium handling. SERCA2 mRNA and protein levels are reduced in cardiac hypertrophy, and reduced SERCA2 is thought to have an important role in both systolic and diastolic heart failure pathophysiology [2,3]. Increasing expression of SERCA2 is a promising strategy to treat heart failure [4,5].

Neonatal rat ventricular myocytes (NRVM) treated with phenylephrine (an alpha-adrenergic receptor agonist) are a well-established model of cardiac hypertrophy [6]. Phenylephrine induces hypertrophy in NRVM and reduces SERCA2 mRNA and protein levels [7]. Alpha-adrenergic receptor stimulation activates extracellular signal-regulated kinases (ERK)1/2, which are serine/threonine kinases with diverse functions in mammalian cells. At baseline, it appears that ERK1/2 has some low level of activity in cardiomyocytes, and ERK activation can protect cultured ventricular myocytes from apoptosis [8]. However, chronic ERK activation appears to be involved in the pathophysiology of hypertrophy and heart failure. Transgenic ERK1/2 activation causes cardiac hypertrophy in vivo [9]. ERK is upregulated in human heart failure from both ischemic and non-ischemic causes [10]. ERK is also activated in the hearts of diet-induced obesity wild-type mice and the LMNA mutant mouse model of cardiomyopathy, and pharmacologic ERK inhibition improves cardiac function in the transgenic model [11,12]. Further, ERK2^{T188S} (which functions as a dominant negative for both ERK1 and ERK2) transgenic mice have a reduced hypertrophic response [13].

There has been speculation that ERK activation may decrease SERCA2 expression, suggested by the fact that overexpressing Ras (which activates ERK) in NRVM reduces SERCA2 [14]. However, Ras activates other pathways besides ERK. There has been no direct evidence demonstrating that ERK activation decreases cardiac SERCA2 transcription. Furthermore, other cell types may have the opposite relationship between ERK activation and SERCA2 expression. Macrophage from ob/ob mice have less ERK activation and less SERCA2 mRNA, which is rescued by MEK adenovirus-induced activation of ERK [15]. Thus, it is unclear if activating ERK in cardiomyocytes increases or decreases SERCA2 expression. We hypothesized that ERK activation during hypertrophy reduced SERCA transcription and sought to determine the downstream mediators.

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2. Materials and methods

2.1. Animal care

Animal protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were carried out in accordance with the NIH guidelines for the care and use of laboratory animals. Pregnant Sprague–Dawley rats were purchased from Harlan Laboratories. Neonatal rat ventricular myocytes (NRVM) were isolated from 1 to 3 day old pups using techniques described previously [16].

2.2. Cell culture

NRVM were cultured in MEM with 10% FBS overnight, then in MEM without serum plus phenylephrine 100 μmol/L and propranolol 5 μmol/L for 3 days as previously described [17]. Media was changed daily. H9c2 cells were purchased from ATCC.

2.3. Chemicals and viruses

U0126 and PD98059 were from Cell Signaling and were used at final concentrations of 10 μM and 25 μM, respectively. Phenylephrine and propranolol were purchased from Sigma. The MEK adenovirus (constitutively active, ADV-119) was purchased from Cellbiolabs. The IKKBeta adenovirus (#1487) was purchased from Vector Biolabs. The GFP virus was a generous gift from Dr. Konstantinos Drosatos. Viral transduction was performed according to the manufacturer's instructions.

2.4. Real-time PCR

Cells were homogenized and RNA was then purified using a Qiagen RNeasy kit (#74104). cDNA was synthesized using the Applied Biosystems high capacity RNA to cDNA kit (#4387406) and diluted to 10 ng/μL for use as a template (20 ng template was used for each 20 μL reaction). Real-time PCR was performed using an Applied Biosystems StepOne Plus Real-Time PCR system with StepOne Software v2.0 and inventoried primers from Applied Biosystems. PCR was performed for 40 cycles with automated detection of crossing threshold; the ΔΔCT method was used for relative quantification. PCR reactions were performed with duplicate wells and with the housekeeping gene ribosomal 18S as a reference.

2.5. Promoter-luciferase vectors, transfection, and mutagenesis

The mouse SERCA2 proximal promoter (3 kB region upstream from the start of transcription) was digested from a BAC template (bacpac.chori.org) using native Mlu1 and Sma1 restriction sites and ligated into the pGL3 basic vector (Promega). The human SERCA2 proximal promoter was cloned by PCR from a BAC template. Constructs were verified by DNA sequencing. Restriction enzymes and ligase were from New England Biolabs. Taq and dNTPs were from Invitrogen. The NFkB reporter luciferase construct is from Agilent technologies (#219077). The CREB1 expression vector (#22395), ERK1 expression vector (#12656), ERK2 expression vector (#8974), PKD expression vectors (#10808, 10812), NFAT expression vectors (11790, 11788), the MEK expression vector (40809), and the p50 and p65 expression vectors (#21965, #21966) were purchased from Addgene.

Vectors were co-transfected into rat cardiomyocyte H9c2 cells with a beta-gal reporter vector as a transfection control using Lipofectamine (Invitrogen). We transfected 200 ng of luciferase construct and 100 ng of protein expression vector per well; control transfection wells received empty vector pcDNA3 to equalize the amount of DNA. Standard 12-well tissue culture plates were used. Cells lysates were harvested 36–48 h after transfection. After cell lysis, luciferase substrate (Promega, E1501) was added to lysates in a 96-well plate and luminescence was measured with a Tecan Infinite 200 plate reader.

Mutagenesis to eliminate the NFkB binding site in the SERCA2 promoter was performed with the Agilent kit (#210515) following the manufacturer's protocol. Mutagenesis primers were designed to substitute two base pairs in the middle of binding site to disrupt the NFkB binding sequence, as shown in the supplement.

2.6. Microscopy

Cells on glass coverslips were fixed with 4% paraformaldehyde and were visualized using a phase-contrast microscope (Nikon Eclipse Ti) with a digital camera (Photometrics Coolsnap HQ2). Surface area was measured using ImageJ software.

2.7. Bioinformatics

Transcription factor binding sites were predicted using Matinspector (http://www.genomatix.de/cgi-bin/matinspector).

2.8. Statistical analysis

Results are presented as mean ± SEM. The unpaired *t*-test was used for comparisons of means; a 2-tailed value of $P < 0.05$ was considered statistically significant. For groups of 2 or more ANOVA was used with post-hoc testing (Prism v5, GraphPad Software).

3. Results

3.1. Phenylephrine-induced hypertrophy reduces SERCA2 mRNA and protein

We used a well-established model of hypertrophy, NRVM treated with phenylephrine (PE) [17,18]. NRVM with PE-induced hypertrophy had larger surface area and greater expression of the hypertrophy marker Nppa (aka atrial natriuretic peptide), indicating that hypertrophy was induced (Fig. 1). As previously reported, hypertrophy resulted in a significant decrease in SERCA2 mRNA (Fig. 1D) and protein (Fig. 1E).

3.2. Inhibition of ERK activation preserves SERCA2 mRNA, and ERK activation reduces SERCA2 mRNA

PE causes a rapid activation of ERK in NRVM, as indicated by phosphorylation, (Fig. 2A). ERK phosphorylation status causes a conformational change and has been found to correlate with activity [19,20]. To determine if ERK activation is responsible for reducing SERCA2 mRNA during hypertrophy, we inhibited MEK, the upstream kinase that activates ERK1/2, with two different pharmacologic inhibitors, PD98059 and U0126 (Fig. 2B). Both MEK inhibitors were able to preserve SERCA2 mRNA levels during hypertrophy, indicating that ERK1/2 activation is necessary for the reduction in SERCA2 mRNA. In addition, pharmacologic inhibition of MEK preserves SERCA2 protein levels during hypertrophy (Fig. 2E). In the absence of hypertrophic stimulation, treating NRVM with PD does not change the native SERCA2 mRNA levels, and U0126 by itself causes a decrease (Fig. 2C). This shows that the ability of these drugs to rescue native SERCA2 mRNA during hypertrophy is a specific effect. The inhibitor PD98059 is relatively specific for MEK1 (whereas U0126 inhibits MEK 1 and 2) suggesting that MEK1 is the critical upstream kinase in this pathway.

To determine if ERK activation is sufficient to reduce SERCA2 mRNA, we transduced NRVM with a constitutively active form of MEK, which is the most selective method for activating ERK. This resulted in a significant reduction in SERCA2 mRNA, to 37% of control viral transduction levels (Fig. 2D). Thus, pharmacologic inhibition of ERK activation rescues SERCA2 mRNA levels during hypertrophy, and specific activation of ERK is sufficient to reduce SERCA2 mRNA levels in cardiac myocytes.