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Functionally redundant control of cardiac hypertrophic signaling by inositol 1,4,5-trisphosphate receptors

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ABSTRACT

Calcium plays an integral role to many cellular processes including contraction, energy metabolism, gene expression, and cell death. The inositol 1, 4, 5-trisphosphate receptor (IP_3R) is a calcium channel expressed in cardiac tissue. There are three IP_3R isoforms encoded by separate genes. In the heart, the IP_3R-2 isoform is reported to being most predominant with regards to expression levels and functional significance. The functional roles of IP_3R-1 and IP_3R-3 in the heart are essentially unexplored despite measureable expression levels. Here we show that all three IP_3Rs isoforms are expressed in both neonatal and adult rat ventricular cardiomyocytes, and in human heart tissue. The three IP_3R proteins are expressed throughout the cardiomyocyte sarcoplasmic reticulum. Using isoform specific siRNA, we found that expression of all three IP_3R isoforms are required for hypertrophic signaling downstream of endothelin-1 stimulation. Mechanistically, IP_3Rs specifically contribute to activation of the hypertrophic program by mediating the positive inotropic effects of endothelin-1 and leading to downstream activation of nuclear factor of activated T-cells. Our findings highlight previously unidentified functions for IP_3R isoforms in the heart with specific implications for hypertrophic signaling in animal models and in human disease.

1. Introduction

In the heart calcium is an essential modulator of a wide variety of cellular functions including cardiomyocyte excitation-contraction coupling (ECC) and gene expression. Cardiomyocyte function can be modulated by neuro-hormonal agonists to accommodate cardiac demand. One example is endothelin-1 (ET-1), which is a potent vasoconstrictor that plays an important role in modulating muscle contractility, vascular tone, cardiomyocyte growth, and survival [1,2]. Plasma levels of ET-1 are also increased during pathological conditions such as chronic heart failure, myocardial infarction, cardiac hypertrophy and in hypertension [3,4]. As such, ET-1 has been linked to pathological remodeling of the heart [1,5]. ET-1 signaling is initiated by ET-1 binding to G-protein coupled receptors at the plasma membrane leading to the activation of phospholipase C (PLC). PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) which leads to increased production of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 then acts as a second messenger that binds inositol 1,4,5-trisphosphate receptors (IP_3Rs), activating IP_3-induced calcium release (IICR).

IP_3Rs are a family of calcium channels involved in a variety of cellular functions. There are three different IP_3R isoforms encoded by separate genes. The three IP_3Rs share a high degree of sequence homology and are found in a variety of tissues including the heart [6,7]. Cardiac IP_3Rs are implicated in regulating the progression of cardiac hypertrophy [8,9]. Within the cardiomyocyte, IP_3Rs are known to localize in the dyadic cleft, sarcoplasmic reticulum and at the outer/inner nuclear membrane [1,8–10]. Several lines of evidence have also implicated nuclear calcium transients as a significant contributor to cardiomyocyte hypertrophy. Nuclear or perinuclear IP_3Rs may promote nuclear-restricted calcium release events that initiate gene transcription [10]. Nuclear calcium transients are involved in the activation of transcription factors such as histone deacetylase 5 (HDAC5) [1,11]. However the mechanism by which cardiomyocytes can discriminate between calcium signals from ECC and calcium signals that target gene transcription it still unclear, as calcium release events mediated by ECC

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are also efficiently transmitted to the nuclear matrix. Hypertrophic agents such as ET-1 also increase contractility [9,12], which may afford a mechanism for decoding IP_3-dependent signals without requiring subcellular compartment-specific IP_3R activation.

The IP_3R-2 isoform is considered the predominant isoform in the heart [13,14]. However, transgenic IP_3R-2 rodent models have either supported [15,16], or contradicted [17,18] the role of IP_3R-2 channel in cardiac hypertrophy. As such, it is still unclear whether IP_3R channels are significant contributors to cardiac physiology and pathologic remodeling such as hypertrophy [19]. It has been shown that all three IP_3R isoforms, at least at the mRNA level, are expressed in the heart of humans and mice [8,20]. This opens the question of whether IP_3R-1 and − 3 are able to functionally compensate for IP_3R-2 deficiencies in these models.

We now show that all three IP_3R isoforms are expressed in cardiomyocytes and that they are essential for the progression of ventricular hypertrophy induced by ET-1. IP_3R-dependent activation of the hypertrophic program was not dependent upon nuclear-specific calcium transients, but instead was mediated by increased contractility induced by ET-1. Lastly, these results were independent of increased IP_3R expression both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Antibodies, expression constructs, and reagents

Rabbit polyclonal antibody against type-1 IP_3R was developed in-house and is specific for the type-1 isoform [21]. The rabbit polyclonal antibody against type-2 IP_3R have been described elsewhere [6] and was kindly provided by Dr. Richard Wojcikiewicz (SUNY Upstate). Mouse monoclonal anti-IP_3R type-3 was purchased from BD Bioscience. Mouse monoclonal anti-α-actinin and anti-ryanodine receptor antibody was purchased from Sigma-Aldrich. Mouse anti-SERCA2 antibody was from Thermo Fisher and rabbit anti-ANP was purchased from Abcam. Anti-alpha-fodrin was purchased from EMD Millipore. Secondary antibodies conjugated to Alexa-488 and Alexa-555 were from Molecular Probes, and peroxidase-conjugated antibodies were from Jackson ImmunoResearch. Expression constructs 9 × NFAT-apha-MHC-Luc was a gift from Jeffery Molkentin (Addgene plasmid # 51941), pGP-CMV-GCaMP6s was a gift from Douglas Kim ([22]; Addgene plasmid # 40753) and Tol2-elavl3-H2B-GCaMP6s was a gift from Misha Ahrens (Addgene plasmid # 59530). Endothelin-1 was purchased from Bachem. Silencer pre-design siRNAs were purchased from Ambion. Fura-2 AM was purchased from Molecular Probes, and the dual luciferase reporter assay kit was from Promega. All other reagents were purchased from Sigma-Aldrich.

2.2. Preparation of primary neonatal cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVM) were obtained from 1 to 2 day old Sprague-Dawley rat hearts as previously described, with minor modifications [23]. Cardiomyocytes were plated into fibronectin-coated culture dishes and incubated at 37 °C in 5% CO_2 incubator. Two days after plating, media was replaced with 50% Ham's F10–50% DMEM culture medium with β-D-arabinofuranoside (ARA-C; 1 μM) to inhibit growth of fibroblasts. NRVMs were transfected using Lipofectamine 3000 following manufacturer's instructions. All experiments were carried out 48 h after transfection unless otherwise stated. All vertebrate animal procedures were approved by the Animal Welfare Committee (AWC) at UTHealth.

2.3. Preparation of adult cardiomyocytes

Calcium-tolerant adult rat ventricular myocytes (ARVM) were isolated from hearts of wildtype Sprague-Dawley rats (300–320 g) as described by Louch et al. [24]. Briefly, animals were anesthetized with chloralhydrate (400 mg/kg b.w. i.p.) and heparinized (5000 U/kg b.w.) *via* direct injection into the vena cava inferior. The hearts were aseptically removed and directly placed in ice-cold Krebs-Henseleit (KH) buffer (133.5 mM NaCl, 4 mM KCl, 1.2 mM NaH2PO4, 10 mM HEPES, 1.2 mM MgSO4, 10 mM BDM) containing glucose (5.5 mM) before being perfused with a Langendorff preparation. Perfusion (3 min) with KH buffer at 37 °C lacking Ca2 + was followed by perfusion with recirculating KH buffer containing 2% BSA (wt/vol), 50 μM Ca2 + and type II collagenase. After 20 min of perfusion, hearts were minced, and undigested tissue was separated with a 230 μm mesh sieve. The cell suspension was allowed to settle with gravity within 5 to 7 min, and the cell pellet was re-suspended in KH containing 2% BSA (wt/vol), and calcium was slowly reintroduced to a final concentration of 1 mM. Cardiomyocytes were plated and culture under 5% carbon dioxide.

2.4. Immunofluorescence labeling of isolated cardiomyocytes

Adult and neonatal cardiomyocytes were plated at a density of 300 cells per mm^2 on glass coverslips coated with fibronectin. On day 4 the medium was exchanged and the cells were treated with 100 nM ET-1 for 48 h. Adult cardiomyocytes cells were fixed with 4% paraformaldehyde in PBS. Neonatal cardiomyocytes were fixed with 100% cold MeOH. Briefly, cells were incubated with either rabbit polyclonal anti-IP_3R-1 (1:250), rabbit polyclonal anti-IP_3R-2 (1:250), mouse monoclonal anti-IP_3R-3 (1:250), mouse monoclonal anti-α-actinin (1:250), mouse anti-SERCA2 (1:250), or mouse anti-ryanodine2 (1:250), overnight at 4 °C. Followed by incubation with secondary antibodies conjugated to Alexa-488 and Alexa-555 for 1 h.

2.5. Immunofluorescent labeling of human heart failure samples

Disease heart tissue was obtained from patients undergoing heart transplantation due to advanced heart failure. Immediately after explant the tissue is flash frozen with liquid nitrogen for future analyses. Control heart tissue was obtained from organs that were declined for transplantation due to non-cardiac reasons. Frozen left ventricular cardiac tissues were cryo-sectioned onto charged glass slides. The sections were fixed with 4% paraformaldehyde in PBS. Tissue was stained with anti-IP_3R-1 (1:100), anti-IP_3R-2 (1:100), anti-IP_3R-3 (1:100), or anti-α-actinin (1:100), for 1 h at 37 °C. Subsequently, slides were washed and incubated with secondary antibodies conjugated to Alexa-488 and Alexa-555 for 1 h. Total corrected tissue fluorescence was quantified using ImageJ exactly as described previously [25]. All experiments on human samples were approved by the Institutional Review Board (IRB) at the Houston Methodist Institute for Academic Medicine and the McGovern Medical School at UTHealth.

2.6. Cell size determination

NRVM were plated on glass coverslips. Two days after plating cells were transfected with control siRNA or triple IP_3R siRNA. Following transfection cells were treated with ET-1 for 48 h. After treatment cells were fixed with 4% paraformaldehyde in PBS. Subsequently, coverslips were stained with anti-α-actinin and secondary antibodies conjugated to Alexa-555. For measurement of cell area, at least 30 fields randomly chosen were analyzed in each coverslip. Cardiomyocytes area was measured in captured images using ImageJ software.

2.7. Western blotting

Cells were harvested by gently scraping plates with a cell scraper and washing once with cold PBS. Cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.8, 1% Triton X-100 and 1 mM EDTA) was added to the cell pellet. Samples were cleared of insoluble debris by centrifugation at 20,000*g* at 4 °C. Cell lysates were quenched with SDS sample buffer. Samples were resolved by SDS–PAGE and analyzed by