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Original article

RalGDS-dependent cardiomyocyte autophagy is required for load-induced ventricular hypertrophy

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ABSTRACT

Recent work has demonstrated that autophagy, a phylogenetically conserved, lysosome-mediated pathway of protein degradation, is a key participant in pathological cardiac remodeling. One common feature of cell growth and autophagy is membrane biogenesis and processing. The exocyst, an octomeric protein complex involved in vesicle trafficking, is implicated in numerous cellular processes, yet its role in cardiomyocyte plasticity is unknown. Here, we set out to explore the role of small G protein-dependent control of exocyst function and membrane trafficking in stress-induced cardiomyocyte remodeling and autophagy. First, we tested in cultured neonatal rat cardiomyocytes (NRCMs) two isoforms of Ral (RalA, RalB) whose actions are mediated by the exocyst. In these experiments, mTOR inhibition in response to starvation or Torin1 was preserved despite RalA or RalB knockdown; however, activation of autophagy was suppressed only in NRCMs depleted of RalB, implicating RalB as being required for mTOR-dependent cardiomyocyte autophagy. To define further the role of RalB in cardiomyocyte autophagy, we analyzed hearts from mice lacking RalGDS (*Ralgds*$^{-/-}$), a guanine exchange factor (GEF) for the Ral family of small GTPases. RalGDS-null hearts were similar to wild-type (WT) littermates in terms of ventricular structure, contractile performance, and gene expression. However, *Ralgds*$^{-/-}$ hearts manifested a blunted growth response ($p < 0.05$) to TAC-mediated pressure-overload stress. Ventricular chamber size and contractile performance were preserved in response to TAC in *Ralgds*$^{-/-}$ mice, and load-induced cardiomyocyte autophagy was suppressed. Interestingly, TAC-induced activation of the fetal gene program was similar in both genotypes despite the relative lack of hypertrophic growth in mutant hearts. Together, these data implicate RalGDS-mediated induction of autophagy and exocyst function as a critical feature of load-induced cardiac hypertrophy.

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1. Introduction

In response to pathological stress such as neurohormonal activation, hypertension, or myocardial injury, the heart is capable of robust changes in cardiac mass [1]. From a teleological perspective, the early phases of cardiac hypertrophy may be a compensatory response to increased workload, serving to normalize wall stress and thereby minimize oxygen consumption. In the long run, however, pathological hypertrophy is a milestone in the pathogenesis of heart failure [2]. Numerous signaling pathways have been implicated in the regulation of cardiac hypertrophy [3]. More recently, autophagy has emerged as a critical process involved in cardiac hypertrophy [4].

Autophagy, or more precisely, macroautophagy, is an evolutionarily conserved, near-ubiquitous mechanism for the degradation of long-lived proteins and clearance of organelles [5]. Autophagy is involved in numerous disease processes, including neurodegeneration, cancer, and infectious disease [6]. Our group and others have demonstrated a role for autophagy in a variety of forms of cardiovascular disease, as well. A major question that remains largely unanswered about autophagy in the heart is whether autophagy is an adaptive or maladaptive response to stress [7,8]. Our findings point to a maladaptive role of robust activation of autophagy in the setting of severe pressure overload stress [9]. Conversely, inactivation of Atg5, a gene required for autophagy, points to an adaptive role for autophagy [10]. These seemingly contradicting results imply that autophagic activity exists on a continuum, where too little or too much autophagy in response to stress is maladaptive, but a minimum amount of constitutive autophagic activity is required for cell survival.

The dichotomous roles of cardiac hypertrophy and cardiomyocyte autophagy led us to consider a common feature of the two processes: membrane biogenesis and processing. A plausible nexus for these two processes lies in the exocyst, an octomeric protein complex involved in vesicle trafficking. This complex serves a necessary role in the targeting of Golgi-derived vesicles to the basolateral membrane of polarized epithelial cells and to the growth cones of differentiating PC12

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cells [11–13]. Recent work has demonstrated a role for the exocyst in autophagosome assembly as well; the small G protein, RalB, and an Exo84-dependent subcomplex of the exocyst were demonstrated to be critical for nutrient starvation and pathogen-induced autophagosome formation [14]. While many small GTPases have critical effects on cardiac plasticity [15], the role of small G protein-dependent membrane trafficking in stress-induced cardiomyocyte remodeling and autophagy remains largely unexplored.

Whereas previous studies have suggested a role for the Ral family of small GTPases in cardiac hypertrophy [16], underlying mechanisms remain unknown. Here, we set out to test the role of these enzymes and their associated guanine exchange factor (GEF), RalDGS (Ral GDP dissociation stimulator), in the control of membrane trafficking in stress-induced cardiomyocyte remodeling and autophagy.

2. Materials and methods

2.1. Animal models and echocardiography

Male C57/BL6 mice (8–10 weeks old) were subjected to thoracic aortic constriction (TAC) [17] for 3 weeks as previously described [18]. Control animals underwent sham operations. The Animal Care and Use Committee of the University of Texas Southwestern Medical Center approved all animal care and procedures. Echocardiograms were performed on conscious, gently restrained mice using a Vevo 2100 system with a MS400C scanhead. LVEDD and LVESD were measured from M-mode recordings. Fractional shortening (FS) was calculated as (LVEDD − LVESD) / LVEDD and expressed as a percentage. All measurements were made at the level of the papillary muscles.

2.2. Primary culture of neonatal cardiomyocytes, siRNA transfection and adenovirus infection

Neonatal rat cardiomyocytes (NRCMs) were isolated and cultured as described previously [19]. After 24 h, NRCMs were transfected with Silencer Select Pre-designed siRNA constructs (Ambion) using Lipofectamine RNAiMax (Invtirogen) in Optimem (Gibco) for 4 h, and then switched to basal media: DMEM supplemented with 3% fetal bovine serum (FBS), BrdU, and antibiotics. After 48 h, cells were infected with adenovirus (MOI 10) for 1 h in Optimem and then switched to basal media. Torin1 and Bafilomycin A1 (LC Laboratories) were used at a concentration of 50 nM.

2.3. Real-time RT-PCR

Total RNA was harvested from NRCMs or mouse left ventricles using TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed using SYBR green on an ABI 7000 Prism Sequence Detection System (Applied Biosystems). To confirm amplification specificity, the PCR products were subjected to melting curve analysis. Negative controls containing water instead of cDNA were run concomitantly. Data for each transcript were normalized to reactions performed with 18S rRNA primers, and fold change was determined using the comparative threshold method [20].

2.4. Immunoblot analysis

Tissues were either homogenized immediately or quick frozen in liquid nitrogen and stored at −80 °C for later use. To harvest protein, tissues were homogenized at 4 °C in M-PER® mammalian protein extraction reagent (Thermo Scientific) supplemented with protease inhibitors (Roche), and phosphatase inhibitors (Roche). Whole cell lysates from cultured neonatal myocytes were prepared by directly harvesting cells in M-PER®. Homogenates were passed over glass wool to remove DNA. Proteins were separated by SDS/PAGE, transferred to a supported nitrocellulose membrane, and immunoblotted. The following antibodies were used: rabbit anti-LC3 (described previously [9]); mouse anti-p62 (Abnova); RalA (BD Biosciences); mouse anti-RalB (provided by Larry Feig, Tufts University); mouse anti-S6 Ribosomal Protein (Cell Signaling); rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (Cell Signaling); mouse anti-mTOR (Cell Signaling); rabbit anti-Phospho-mTOR (Ser2448) (Cell Signaling); mouse monoclonal anti-gapdh (Fitgerald Industries Int.); rabbit anti-α-tubulin (Abcam); and mouse anti-α-tubulin (Sigma). Blots were scanned, and bands were quantified using an Odyssey Licor (version 3.0) imaging system. Immunoblots from a representative experiment are shown. The graphs shown indicate mean ± SD for 3 experiments.

2.5. Histology

All tissues were fixed in 4% paraformaldehyde overnight at 4 °C, rinsed, and transferred to PBS followed by paraffin embedding. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) staining for apoptotic cells was performed by labeling with fluorescein, and the sections were counterstained with propidium iodide.

2.6. Myocyte cross-sectional area

Images of tissues stained with wheat germ agglutinin were paraffin fixed (Vector Laboratories), and images were acquired on a confocal microscope (TCS SP5; Leica) with Leica LAS AF software. The following lenses were used: HC PL APO 20×/0.70, HCX PL APO 40×/1.25-0.75 oil, and HCX PL APO 63×/1.40-0.60 oil. All images were taken at room temperature and processed in ImageJ for CSA analysis. Occasionally, images were linearly rescaled to optimize brightness and contrast uniformly without altering, masking, or eliminating data.

2.7. RalB activity assay

Assays were performed according to the recommended protocol for the Active Ras Pull-Down and Detection Kit (Thermo Scientific). Briefly, 1 mg protein from flash frozen heart lysates was subjected to affinity purification with GST-RalBP1-RBD, a domain with affinity for the activated GTP-RalB. This purified aliquot was then subjected to immunoblot analysis as above.

2.8. Statistics

Data are presented as mean ± SD. The unpaired Student's t test was used for comparison between two groups, and ANOVA with Bonferroni correction was used for comparison among multiple groups. Values of $p < 0.05$ were considered significant.

3. Results

3.1. RalB is necessary for cardiomyocyte autophagy

To test whether Ral small GTPases are required for cardiomyocyte autophagy, we selectively depleted NRCMs of RalA or RalB using RNAi. The siRNA constructs were specific for their respective isoform and were able to selectively deplete their target protein 48 h after transfection and without a compensatory increase in the abundance of the other isoform (Fig. 1A). To evaluate autophagy, we incubated NRCMs in Earle's Balanced Salt Solution (EBSS) for 2 h, thereby simulating starvation, a powerful inducer of autophagy [5]. Nutrient deprivation led to increased levels of the faster migrating, lipidated form LC3-II, indicating an accumulation of autophagosomes (Fig. 1B). Concomitant treatment with the lysosomal inhibitor

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