Journal of Molecular and Cellular Cardiology 113 (2017) 51–62

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

TFEB activation protects against cardiac proteotoxicity via increasing autophagic flux

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ARTICLE INFO

Keywords:
TFEB
mTOR
The autophagic-lysosomal pathway
Proteotoxicity
Misfolded proteins
Cardiomyocytes

ABSTRACT

Insufficient lysosomal removal of autophagic cargoes in cardiomyocytes has been suggested as a main cause for the impairment of the autophagic-lysosomal pathway (ALP) in many forms of heart disease including cardiac proteinopathy and may play an important pathogenic role; however, the molecular basis and the correcting strategy for the cardiac ALP insufficiency require further investigation. The present study was sought to determine whether myocardial expression and activity of TFEB, the recently identified ALP master regulator, are impaired in a cardiac proteinopathy mouse model and to determine the effect of genetic manipulation of TFEB expression on autophagy and proteotoxicity in a cardiomyocyte model of proteinopathy. We found that increased myocardial TFEB mRNA levels and a TFEB protein isoform switch were associated with marked decreases in the mRNA levels of representative TFEB target genes and increased mTORC1 activation, in mice with cardiac transgenic expression of a missense (R120G) mutant αB-crystallin (CryAB^{R120G}), a well-established model of cardiac proteinopathy. Using neonatal rat ventricular cardiomyocyte cultures, we demonstrated that downregulation of TFEB decreased autophagic flux in cardiomyocytes both at baseline and during CryAB^{R120G} overexpression and increased CryAB^{R120G} protein aggregates. Conversely, forced TFEB overexpression increased autophagic flux and remarkably attenuated the CryABR^{120G} overexpression-induced accumulation of ubiquitinated proteins, caspase 3 cleavage, LDH leakage, and decreases in cell viability. Moreover, these protective effects of TFEB were dramatically diminished by inhibiting autophagy. We conclude that myocardial TFEB signaling is impaired in cardiac proteinopathy and forced TFEB overexpression protects against proteotoxicity in cardiomyocytes through improving ALP activity.

1. Introduction

Protein quality control (PQC) acts to keep the level of misfolded proteins low and to minimize the toxicity of misfolded proteins in the cell, indispensable to protein homeostasis and cell survival and functions. As indicated by increases in the abundance of total ubiquitinated proteins and pre-amyloid oligomers in the vast majority of human hearts with end-stage heart failure, increased proteotoxicity and inadequate PQC may contribute to the genesis of and the progression from a large subset of heart diseases to heart failure [1,2]. Hence, it is conceivable that improving cardiac PQC has the potential to become a new therapeutic strategy for heart failure, a leading cause of disability and mortality in humans.

PQC is accomplished by intricate collaboration between molecular chaperones and targeted protein degradation; the latter is carried out primarily by the ubiquitin-proteasome system (UPS) and the autophagic-lysosomal pathway (ALP) [3]. It is generally accepted that all abnormal cellular proteins are degraded by the UPS but the proteasome can only degrade protein molecules one at a time. By contrast, the ALP degrades cellular content in a bulky fashion and thereby plays an important role in the quality control of not only proteins but also organelles such as mitochondria. Terminally misfolded proteins, when escaped from or overwhelmed the surveillance of chaperones and the UPS, undergo aggregation via hydrophobic interaction and form aberrant aggregates which are inaccessible by the proteasome and can only be degraded by macroautophagy [3,4]. Hence, the ALP plays a critical role in PQC.

The lysosomal degradation of cell's own components, or autophagy, takes three different forms: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy, among which macroautophagy is most extensively studied [3]. In microautophagy, invagination of lysosomal membrane captures directly a small amount of soluble cytoplasmic content into the lumen of the lysosome. By CMA, the lysosome selectively uptakes specific individual protein molecules one at a time

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http://dx.doi.org/10.1016/j.yjmcc.2017.10.003
Received 10 June 2017; Received in revised form 25 September 2017; Accepted 5 October 2017
Available online 07 October 2017
0022-2828/

with the help of heat shock proteins (e.g., HSC70) and through a translocating complex formed by lysosome associated membrane protein 2A (LAMP2A). Proteins that are targeted for CMA degradation harbor the KFERQ motif [5]. Neither microautophagy nor CMA is well studied in the heart. Macroautophagy (hereafter referred to as autophagy for simplicity) segregates a portion of cytoplasm through formation of a double membraned vacuole known as an autophagosome for fusion with and degradation by lysosomes. It is increasingly evidenced that the ALP capacity of removing its cargos becomes inadequate in cardiomyocytes under many diseased conditions including cardiac proteinopathy [6]; however, the mechanism underlying the cardiac ALP insufficiency remains largely unknown and, as result, measures tested to improve cardiac ALP are often non-specific, flaunted with unwanted off-target effects [3]. Thus, a better understanding of ALP insufficiency and search for specific strategies to coordinately enhance both the formation and the removal of autophagosomes, i.e., the entire ALP, are urgently needed.

Recent advances in cell biology identify the transcription factor EB (TFEB) as a master regulator of lysosome biogenesis. TFEB is a basic helix-loop-helix- leucine-zipper (bHLH-Zip) transcription factor, belonging to the microphthalmia family (MiT family) [7]. Similar to other bHLH-Zip transcription factors, TFEB recognizes and binds the palindromic E box (i.e., CACGTG). A common 10-base *E*-box like palindromic sequence, referred to as the coordinated lysosomal expression and regulation (CLEAR), has been identified in lysosomal genes via promoter analysis. The network of genes harboring the CLEAR motif is referred to as the CLEAR network. By directly binding to the CLEAR element, TFEB can activate all genes of the CLEAR network [8,9], increase lysosome numbers and lysosomal enzyme levels, and thereby promote lysosomal catabolic function [9]. More recently, it has been further shown that TFEB also regulates the expression of many autophagy-related genes to orchestrate autophagosome formation and lysosomal degradation [10]. Overexpression of TFEB was shown to enhance the clearance of misfolded or aggregation-prone proteins in neurons [9,11–16], hepatocytes and lung epithelia [17,18]; however, this has not been demonstrated in cardiomyocytes expressing a human disease-linked bona fide misfolded protein. The present study was sought to fill this gap.

Here we report that myocardial TFEB signaling is inhibited in mice with advanced cardiac proteinopathy induced by cardiomyocyte-restricted expression of a missense (R120G) mutant αB-crystallin ($CryAB^{R120G}$), a well-established animal model of cardiac proteotoxicity. Moreover, we demonstrate for the first time in cardiomyocytes that TFEB is required for sustaining ALP activity and forced TFEB expression is sufficient to facilitate ALP activity and thereby protects against misfolded protein-induced proteotoxicity. Our findings suggest that enhancing TFEB should be explored as a therapeutic strategy to ameliorate cardiac proteotoxic stress that is implicated in a large subset of heart disease during their progression to heart failure.

2. Methods

2.1. Animals

The protocol for the care and use of animals in this study was approved by University of South Dakota Institutional Animal Care and Use Committee. The creation and baseline characterization of the inbred FVB/N mice with transgenic (tg) overexpression of wild type CryAB ($CryAB^{WT}$) or $CryAB^{R120G}$ driven by the murine *Myh6*-promoter were previously described [19–22]. Mixed sex tg and non-tg (Ntg) littermate mice at 6 months of age were used. In-house bred Sprague-Dawley rats at postnatal day 2 were used for isolating ventricular cardiomyocytes for cell cultures.

2.2. Reagents

Phenylmethylsulfonyl Fluoride (PMSF), and 5-Bromo-2′-Deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO). 3-Methyladenine (3-MA) was purchased from ThermoFisher Scientific (Waltham, MA). Bafilomycin A1 (BFA) was from LC laboratory (Woburn, MA). DNase I and complete protease inhibitor cocktail was from Roche Applied Science (Indianapolis, IN).

2.3. Neonatal rat ventricular myocytes (NRVMs) culture and adenoviral infection

Primary NRVMs were isolated from the ventricles of 2-day old Sprague-Dawley rats and plated on 6-cm plates at a density of 2.0×10^6 cells in 10% FBS in DMEM as previously described [23]. The plated cells were then cultured in a 5% CO_2 incubator at 37 °C for at least 24 h before the medium was changed to meet the needs of the follow-up experiments. Forty-eight hours after plating, cells were infected with recombinant replication-deficient adenoviruses harboring desired transgenes for 4 h in DMEM media. Adenoviral constructs expressing β-galactosidase (Ad-β-gal) or human influenza hemagglutinin (HA) epitope tagged $CryAB^{R120G}$ (Ad-$CryAB^{R120G}$-HA) were previously described [21]. Ad-TFEB was custom made to harbor the expression cassette of human TFEB cDNA (Addgene plasmid # 38119) [24]. Post-infection cells were maintained in 2% FBS, 1% penicillin/streptomycin in DMEM until harvested or fixed.

2.4. Total protein extraction and western blot analysis

Proteins were extracted from ventricular myocardium or cultured NRVMs with 1 × sampling buffer (41 mM Tris-HCl, 1.2% SDS, 8% glycerol). The determination of protein concentration used bicinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL). Equal amounts of protein samples were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane using a Trans-blot apparatus (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature before being incubated with the primary antibodies overnight at 4 °C. The following primary antibodies were used: anti-ubiquitin (#3933, Cell Signaling; 1:1000), anti-GAPDH (G8795, Sigma-Aldrich; 1:1000), anti-LC3 (#2775, Cell Signaling; 1:1000), anti-HA (#3724, Cell Signaling; 1:1000), anti-mTOR (#2983, Cell Signaling; 1:1000), anti-Phospho-mTOR (Ser2481) (#2974, Cell Signaling; 1:1000), anti-p70 S6 kinase (#2708, Cell Signaling; 1:1000), anti-Phospho-p70 S6 kinase (Thr389) (#9234, Cell Signaling; 1:1000), anti-4E-BP1 (#9644, Cell Signaling; 1:1000), anti-Phospho-4E-BP1 (Thr37/46) (#2855, Cell Signaling; 1:1000), and anti-TFEB (A303-673A, BETHYL; 1:10,000). The corresponding horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or goat anti-guinea secondary antibodies (Santa Cruz Biotechnology) were used respectively for chemiluminescence-based western blot analyses. The bound secondary antibody signals were as detected using either enhanced chemiluminescence (ECL-Plus) reagents (GE Healthcare, Piscataway, NJ) or, for weak signals, ECL Advance Western Blotting Kit (GE Healthcare) and digitalized with a VersaDoc3000 or ChemiDoc MP imaging system (Bio-Rad). The digital signal was quantified with the Quantity One or Image Lab™ software (Bio-Rad). For some of the western blot analyses, the in-lane total protein content derived from the stain-free protein imaging technology was used for loading normalization [25].

2.5. Preparation of soluble and insoluble fractions of myocardial proteins

Frozen ventricular tissues were homogenized in cold phosphate-buffered saline (PBS) at pH 7.4 containing 2% Triton-× 100, 2.5 mM EDTA, 0.5 mM PMSF, and a cocktail of complete protease inhibitors and