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Increases of desmin and α -actinin in mouse cardiac myofibrils as a response to diastolic dysfunction

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Abstract

Up-regulation of desmin has been reported in cardiac hypertrophy and failure but the pathophysiological cause and significance remain to be investigated. By examining genetically modified mouse models representative for diastolic or systolic heart failure, we found significantly increased levels of desmin and α -actinin in the myofibrils of hearts with impaired diastolic function but not hearts with weakened systolic function. The increased desmin and α -actinin are mainly found in myofibrils at the Z-disks. Two weeks of transverse aortic constriction (TAC) induced increases of desmin and α -actinin in mouse hearts of occult diastolic failure but not in wild type or transgenic mouse hearts with mildly lowered systolic function or with increased diastolic function. The chronic or TAC-induced increase of desmin showed no proportional increase in phosphorylation, implicating an up-regulated expression rather than a decreased protein turnover. The data demonstrate a novel early response specifically to diastolic heart failure, indicating a function of the Z-disk in the challenging clinical condition of heart failure with preserved ejection fraction (HFpEF).

Keywords

α -Actinin; Cardiac muscle sarcomere; Desmin; Diastolic heart failure; Thin filament regulatory proteins; Z-disk

1. Introduction

Diastolic heart failure (DHF), or heart failure with preserved ejection fraction (HFpEF), contributes to approximately 50% of all heart failure cases and is a major cause of morbidity and mortality in cardiovascular diseases [1]. The clinical syndrome of DHF is characterized by decreased heart function with preserved ejection fraction (EF) due to diastolic dysfunctions [1–3], *i.e.*, decreased relaxation velocity, — dP/dt, increased isovolumic

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Disclosures
None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2015.10.035>.

relaxation time, increased chamber stiffness, and slow and incomplete filling of the ventricles [4].

It has become apparent that DHF and systolic heart failure (SHF) are caused by different pathophysiological mechanisms [4]. In contrast to the current knowledge of SHF, the development and pathophysiology of DHF are much less understood. Patients with DHF generally demonstrate a concentric pattern of ventricular hypertrophy with a normal or near-normal end-diastolic volume, while those with SHF demonstrate a pattern of eccentric remodeling with increased end-diastolic volume [5]. The shape, size and molecular composition of cardiomyocytes also differ in these two distinct types of heart failure [5].

DHF could result from myocardial and/or extra myocardial abnormalities [2]. The myocardial abnormalities include changes in calcium homeostasis and energetics, or mutations in myofilament and cytoskeletal proteins. It was reported that in comparison to SHF patients, DHF patients had ~20% higher myofilament density and shifts of titin splice forms [6]. Cardiomyocytes from DHF patients showed higher resting tension [7], indicating abnormality in sarcomeric relaxation.

Desmin is a cytoskeleton intermediate filament protein [8,9]. The desmin network, which connects the Z-disks in adjacent myofibrils and the myofibrils to nuclear envelope and sarcolemma [8], is critical for the structural integrity of cardiomyocytes. Changes in desmin filaments have been reported in hypertrophic and failing hearts. Whereas increased levels of desmin or disorganization of desmin filaments have been found in cardiac hypertrophy and heart failure [10–13], other studies observed no change in desmin filaments in cardiomyocytes from hearts with pressure overload-induced hypertrophy [14,15].

α -Actinin is a member of the spectrin superfamily. Four isoforms of α -actinin have been identified, of which α -actinin-2 is the major isoform in cardiac muscle [16]. α -Actinin is known as an actin-cross linker in the Z-disks in striated muscle myofibrils [17]. Protein binding assays have shown that α -actinin-2 interacts with multiple proteins and plays essential functions in the structure and organization of sarcomeres [18]. As a major component of the Z-disk [19], α -actinin may play a role in force bearing and transmission in cardiomyocytes.

Increasing evidence has demonstrated that the sarcomeric Z-disk (Z- band) is a sensor and responder of mechanical tension and stretch during cardiomyocyte adaptation to pressure and strain alterations due to hemodynamic demands [20,21]. The Z-disks respond to changes of ventricular strain and diastolic volume [22] and function as a mediator and sensor in cellular signaling cascades [20]. Multiple proteins in the Z-disk form a structural network to maintain the integrity of the sarcomeres and myofibrils, critical to the conduction of systolic force and diastolic tension [23,24]. Mutations in Z-disk proteins are known to cause cardiomyopathies and heart failure [25,26]. In the present study, we investigated desmin and α -actinin regulations in representative mouse models of hearts failure. The results showed that desmin and α -actinin significantly increased in the cardiac myofibrils of young mouse hearts with diastolic dysfunction but not the hearts with systolic dysfunction. This early

indication of diastolic myocardial dysfunction suggests a Z-disk adaptation specifically in diastolic heart failure.

2. Materials and methods

2.1. Genetically modified mouse models of heart failure

Seven transgenic or gene-targeted mouse lines were studied as representative models of diastolic or systolic heart failure. These previously characterized mouse lines are: a) transgenic mice with α -myosin heavy chain (MHC) promoter-driven postnatal over-expression of a hypertrophic cardiomyopathy mutation, E180G, in α -tropomyosin, causing impaired myocardial relaxation [27]; b) mice with a hypertrophic cardiomyopathy mutation, A8V, knocked-in the gene encoding cardiac troponin C (cTnC), also causing impaired myocardial relaxation [28]; c) mice with α -MHC promoter-driven over-expression of a mutation, K118C, in cardiac troponin I (cTnI), causing impaired myocardial relaxation [29]; d) mice with MCK-cre driven conditional knockout of the Gs subunit α (Gsa-KO) in cardiac muscle, causing systolic heart failure [30]; e) mice with α -MHC promoter-driven over-expression of cardiac troponin T (cTnT) with a deletion of the exon 7-encoded segment, causing dilated cardiomyopathy due to systolic heart failure [31]; f) mice with α -MHC promoter-driven over-expression of N-terminal truncated cardiac TnT (cTnT-ND), causing moderately slower myocardial contractile velocity [32]; and g) mice with α -MHC promoter-driven over-expression of N-terminal truncated cardiac TnI (cTnI-ND), resulting in increased myocardial relaxation velocity [33].

To focus on early primary adaptations, 2–3 months old young adult mice of both sexes without clinical signs of heart failure were used in our study. Since the mouse lines are in full C57BL/6 background, either littermate or purchased wild type C57BL/6 mice were used as controls. Mice were maintained on a 12 h:12 h light-dark cycle (6:00 AM/ 6:00 PM) and fed a standard pellet diet. The animal studies were approved by the Institutional Animal Care and Use Committee.

2.2. Characterization of systolic and diastolic functions of ex vivo mouse working hearts

Mouse working heart studies were performed in a genotype-blinded manner as described previously [29,31]. Briefly, mice were heparinized and anesthetized with pentobarbital (100 mg/kg body weight i.p.). Hearts were rapidly isolated and cannulated through the aorta to start Langendorff retrograde perfusion within 3 min after opening the chest. The left atrium was then cannulated through a pulmonary vein for perfusion in the working mode. Base-line heart function was measured at 10 mmHg preload and 55 mmHg afterload by recording $\pm dP/dt_{\max}$, $LVP_{\max}$, $LVP_{\min}$, stroke volume (SV) ($\mu\text{L}/\text{mg}$ heart weight), and pressure-volume (P–V) loop using a 1.2F P–V catheter (Transonic, FTS-1212B-3518). The catheter was calibrated for volume measurement using manufacturer-provided standard wells (10 to 50 μL) filled with the perfusion buffer at 37 °C. Due to the small size of young mouse heart, a slight change of the position of the catheter tip would affect the accuracy of volume measurement. Therefore, we also directly measure the stroke volume as the sum of actual aortic and coronary flows to correct the volume measured using the P–V catheter. All hearts

studied were confirmed for their genotypes by polymerase chain reaction (PCR) and Western blot analysis of the genetic and myocardial protein modifications.

2.3. SDS-PAGE and Western blot

Muscle tissue was rapidly removed from the left ventricular free wall, immediately frozen in liquid nitrogen, and stored at -80°C until the analysis. Total protein was extracted by high-speed homogenization in SDS-PAGE sample buffer containing 50 mmol/L Tris-HCl, pH 8.8, 10% glycerol, 0.1% bromophenol blue, 2% SDS, and 1% β -mercaptoethanol. The protein extract was heated at 80°C for 5 min and then centrifuged at 14,000 rpm in a microcentrifuge (Beckman Coulter Microfuge 18) for 5 min to remove insoluble materials. Samples were resolved on 14% Laemmli gel with an acrylamide-to-bisacrylamide ratio of 180:1. The protein bands were stained with Coomassie Blue R250 or electrically transferred to blot nitrocellulose membrane using a BioRad mini-gel tank at 300 mA for 2 h. The membranes were blocked with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and individually incubated with mouse anti-desmin monoclonal antibody (mAb) D1033 (Sigma, clone DE-U-10), mouse anti- α -actinin mAb A7811 (Sigma, clone EA-53), mouse anti-cardiac TnT mAb CT3 [34] or mouse anti-TnI mAb TnI-1 [35] at 4°C overnight. The subsequent washes, incubation with alkaline phosphatase-labeled anti-mouse IgG secondary antibody (Santa Cruz Biotechnology), and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium substrate reaction were carried out as previously described [36].

2.4. Pro-Q diamond phosphoprotein staining

Pro-Q diamond phosphoprotein staining of SDS-gels was performed according to the manufacture's instruction (Invitrogen) with minor modifications. Briefly, the gels were fixed in 50% methanol, 10% acetic acid overnight. After washing 3 times in deionized water for 10 min each, the gels were stained with Pro-QDiamond in a dark box at room temperature for 90 min. After destaining four times in 20% acetonitrile, 50 mmol/L sodium acetate, pH 4.0, for 30 min each, the gels were washed twice with deionized water for 5 min each and then scanned on a Typhoon 9410 fluorescence imager (GE Healthcare) using fluorescence mode (450 V, high sensitivity, green laser excitation at 532 nm and recording emission at 560 nm long pass).

2.5. Isolation of adult mouse cardiomyocytes

Cardiomyocytes were enzymatically isolated from adult wild type and transgenic mouse hearts as previously described [37]. Mice were heparinized and anesthetized as above. The heart was rapidly excised together with the large vessels and immediately immersed in Ca^{2+} -free Joklik medium containing 10 mmol/L NaHCO_3 , 10 mmol/L butanedione monoxime, 0.1% BSA, pH 7.2, at 37°C . The heart was cannulated through the aorta and perfused at 37°C on a Langendorff apparatus at a constant flow of 3 mL/min with non-circulating plain Joklik medium and then with circulating Joklik medium containing 300 U/ml collagenase II (Worthington) for 15–20 min until the tissue color was turning pale. The heart was further perfused with non-circulating plain Joklik medium for 3 min. The ventricle was dissected, cut into small pieces, and gently agitated in Ca^{2+} free Joklik medium containing 10% FBS to disperse cardiomyocytes. The cell suspension was filtered through a 100-mesh screen to

remove tissue debris. The isolated cardiomyocytes were seeded on laminin-coated coverslips. After 15 min adhesion, the cardiomyocytes were fixed with 75% ethanol and 25% acetone for 15 min and stored in -20°C for immunofluorescence studies.

2.6. Preparation of cardiac myofibrils

Adult mouse cardiac myofibrils were isolated from left ventricular muscle as described previously [38]. After euthanasia of the mice, hearts were rapidly isolated and cannulated through the aorta and perfused with ice-cold Krebs buffer containing 50 mmol/L KCl for 3 min to relax the cardiac muscle. The left ventricle was cut on ice into approximately 1 mm^3 pieces. 10 mL of washing buffer (0.1 mol/L KCl, 2 mmol/L MgCl_2 , 2 mmol/L EGTA, 10 mmol/L Tris-HCl, pH 6.8, 2 mmol/L NaF, 0.5 mmol/L DTT, 0.1 mmol/L PMSF) was added for each left ventricle for gentle homogenization using a Polytron-type of mechanical homogenizer. Triton X-100 was added to 0.5% from 5% stock to wash the tissue homogenate with rotation at 4°C for 30 min. The homogenate was passed through two layers of cheesecloth and centrifuged at 2000 g for 15 min. After two washes with the washing buffer without Triton X-100, the pellet was stored at -20°C in the washing buffer plus 50% glycerol for later microscopic studies.

2.7. Immunofluorescence microscopy

As described previously [39], cardiac muscle thin frozen sections, cardiomyocytes and myofibrils on cover slips were incubated with anti-desmin mAb at 4°C overnight. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20, the samples were incubated with FITC-conjugated anti-mouse IgG second antibody (Sigma, F1010) and TRITC-conjugated phalloidin (Sigma, P1951) at room temperature for 1 h and washed again before mounting on glass slides for examination using a Zeiss Axiovert 100 H phase contrast-epifluorescence microscope.

2.8. Transverse aortic constriction

A minimally invasive transverse aortic constriction (TAC) method [40] was used in our study. Anesthetized with isoflurane inhalation, the mouse was placed supine on a heating pad to maintain body temperature at 37°C . After removing fur, the neck and upper chest area was sterilized with 70% alcohol. A 5 mm horizontal skin incision was made at the level of the suprasternal notch and the thyroid was retracted to expose the trachea. The soft tissue under the sternum was bluntly dissected with a small cotton ball to avoid extensive bleeding when cutting the sternum. A 5 mm longitudinal cut was made in the sternum, and the thymus was retracted to allow visualization of the aortic arch. A curved 23-gauge needle was used to place a 6–0 silk suture under the aorta arch between the origin of the right innominate and left common carotid arteries. A bent 27-gauge needle was placed next to the aortic arch, the suture was securely tied around the needle and aorta, and the needle was then immediately removed. The skin was closed and the mouse was allowed to recover in a clean cage placed on a heating pad. The TAC-treated mice were euthanized after 2 weeks for the study of early myocardial adaptations.

2.9. Echocardiography

Echocardiography studies were performed using a Vevo 2100 highresolution *in vivo* imaging system (VisualSonics, Toronto, ON, Canada). To exclude experimental bias, all measurements were done by an examiner blinded to the genotypes. The mice were anesthetized with 1.2% isoflurane and placed on a heating pad to maintain body temperature at 37 °C. Hair on the precordial region was removed and the region was covered with ultrasound transmission gel. B-mode images were taken to measure the ventricular and aortic structure and dimensions. Data and images were saved and analyzed to evaluate the effects of short term TAC treatment.

2.10. Data analysis

Densitometry analysis of Western blots was performed on images scanned at 600 dpi using NIH ImageJ 1.42 software. Quantitative data are shown as means $\pm$ SE. Statistical significance of differences between the mean values was analyzed by two-tail Student's *t*-test or one-way ANOVA. *P* < 0.05 was considered to be significantly different.

3. Results

3.1. Characteristics of the representative heart failure mouse models

At the young adult age examined in the present study, all of the mouse models had no signs of clinical heart failure, indicating a compensated state of cardiac function that is suitable for the investigation of primary pathophysiology and adaptation of the cardiac muscle.

To characterize the alterations of intrinsic cardiac function without the influences from vascular and neurohumoral feedbacks, *ex vivo* working heart studies revealed distinct phenotypes of the mouse models with specific molecular modifications in the cardiac muscle (Fig. 1). Reflecting diastolic dysfunctions, cTnC-A8V, α -tropomyosin-E180G and cTnI-K118C hearts showed decreased relaxation velocity ($-dP/dt$) and increased end diastolic pressure (LVP_{min}) with preserved systolic function ($+dP/dt$). Reflecting systolic dysfunctions, Gs α -KO and exon 7-deleted cardiac TnT (cTnT-E7) hearts showed decreased contractile velocity ($+dP/dt$). These features validate these genetically modified mouse models for use as representative experimental systems to investigate cardiac muscle adaptations specific to diastolic or systolic dysfunction.

3.2. Increased levels of desmin in the hearts of mouse models of cardiac diastolic dysfunction

To validate the effective extraction of myofibril proteins and the linear range of Western blot densitometry for reliable quantifications, serial dilutions of SDS-gel samples extracted using sample buffer containing 2% SDS or with the addition of 8 M urea and 2 M thiourea were examined. The results in Supplement Fig. S1 showed excellent positive linear correlations with wide range of detections, justifying the use of Western blot densitometry to quantitatively detect desmin (and α -actinin) in our present study. The reliability of normalization to actin band in Coomassie Blue stained SDS-gels was confirmed by trial normalizations to Western blots of actin (Supplement Fig. S1).

We first demonstrated increases of desmin in the three unrelated diastolic heart failure models.

The A8V mutation knocked-in the cardiac/slow TnC gene results in higher Ca^{2+} sensitivity of cardiac myofilaments [28], consistent with the slower relaxation velocity with preserved systolic function and stroke volume of *ex vivo* working hearts (Fig. 1). Western blots showed that the total protein extracts from cardiac muscle of young adult cardiac TnC-A8V mice had significantly more desmin ($31.9 \pm 3.6\%$ higher) than that of wild type hearts (Fig. 2).

The hearts of transgenic mice over-expressing the E180G mutant of α -tropomyosin exhibited significantly slower relaxation velocity in previous studies [27] and in our *ex vivo* working heart experiments (Fig. 1) than wild type controls. Similar to that seen in the cTnC-A8V mouse hearts, Western blots showed that the total protein extracts from cardiac muscle of α -tropomyosin-E180G mice had significantly more desmin ($44.7 \pm 10.3\%$ higher) than that of wild type hearts (Fig. 2).

The hearts of transgenic mice over-expressing cardiac TnI with an amino acid substitution, K118C, in the helix interfacing with TnT exhibits moderate but statistically significant decreases in diastolic function [29]. Western blot analysis showed that the total protein extracts from cardiac muscle of cTnI-K118C mice also had a significant increase in the level of desmin ($31.8 \pm 4.4\%$ higher) than that of wild type hearts (Fig. 2).

In addition to Western blot densitometry normalized to Coomassie Blue stained actin band, the increase of desmin in the three diastolic heart failure models was further confirmed using densitometry analysis normalized to the Western blots of actin (Supplement Fig. S2).

3.3. No change in the level of desmin in mouse hearts with systolic failure

The hearts of mice with MCK-cre-induced conditional knockout of the Gsa subunit showed hypertrophy (Fig. 1) with no change in left ventricular end diastolic volume [30]. The functional parameters measured on *ex vivo* working hearts of Gs α -KO mice demonstrated decreased contractile velocity (Fig. 1), indicating systolic dysfunction. Western blot analysis of total protein extracts showed no change in the level of desmin in the young Gs α -KO mouse hearts as compared with wild type control (Fig. 3).

The hearts of transgenic mice over-expressing cTnT- E7 exhibits decreased pumping function and cardiomyopathy phenotypes [31]. *Ex vivo* working heart studies demonstrated decreased systolic function (Fig. 1). Similar to that in Gs α -KO mouse hearts, Western blot analysis showed no change in the level of desmin in the total protein extracts from cTnT-E7 hearts as compared with wild type control (Fig. 3).

3.4. Parallel increases of desmin and α -actinin in mouse hearts of diastolic failure

Densitometry quantification of Western blots showed that the levels of α -actinin significantly increased in total cardiac muscle protein extracts of cTnC-A8V, α -tropomyosin E180G and cTnI-K118C mice but did not change in Gs α -KO and exon 7-deleted cTnT hearts as compared with wild type controls (Fig. 4A). Linear regression analysis showed a

significantly positive correlation between the levels of α -actinin and desmin in the mouse models studied (Fig. 4B).

3.5. Increased desmin and α -actinin in the cardiac myofibrils of mouse hearts with diastolic failure

To demonstrate the subcellular locations of the increased desmin in a representative of diastolic heart failure model, cardiac muscle of cTnC-A8V mice showed apparently normal distribution of desmin in immunofluorescence microscopic images, displaying a striated pattern and localizations at the Z-disks and intercalated disks (Fig. 5). The results suggest that the increased amount of desmin in diastolic heart failure did not alter its cellular distribution. The relative intensity of desmin fluorescence in cTnC-A8V cardiac muscle sections was increased by $52.6 \pm 11.9\%$ as compared with that in wild type control (Fig. 5), proportional to the Western blot quantification of total cardiac muscle protein extracts (Fig. 2).

In another representative model of diastolic heart failure, isolated cardiomyocytes and myofibrils from α -tropomyosin-E180G mouse hearts further showed increased levels of desmin and α -actinin with a normal pattern of sarcomeric distribution and localization at the Z- disks (Fig. 6). Western blot quantification determined that the levels of desmin and α -actinin in α -tropomyosin-E180G cardiac myofibrils were increased by $32.5 \pm 1.4\%$ and $67.3 \pm 18.1\%$, respectively, as compared with that in wild type myofibrils (Fig. 6), proportional to the increase in whole muscle homogenate (Fig. 2). These results demonstrate that the increased desmin and α -actinin in diastolic heart failure was primarily in the myofibrils.

3.6. Two weeks of TAC induced increases of desmin and α -actinin in cardiac muscle of a mouse model of occult diastolic heart failure

Short term TAC was applied to test the effect of hemodynamic stress on inducing desmin and α -actinin in representative mouse heart failure models. Echocardiography was performed in wild type mice to confirm the aorta arch constriction in mice treated with 2 weeks of TAC (Fig. 7A and B). Histology analysis further showed that the thickness of ascending aorta wall was significantly increased after 2 weeks of TAC in all of the mice studied (Fig. 8A and B). Consistent with a previous report that 2 weeks of TAC induced remodeling of the ascending aorta [41], the data indicate the effectiveness of TAC with increased aortic pressure and, thus increased left ventricular systolic load. (3-Myosin heavy chain was re-expressed in the mouse hearts after 2 weeks of TAC, further indicating the effective induction of myocardial adaptation (data not shown).

Western blots and densitometry quantification showed that the short term pressure overload with 2 weeks of TAC did not induce significant change in the levels of desmin and α -actinin in wild type mouse hearts (Fig. 8C and D), or in the hearts of transgenic mice over-expressing cTnT-ND, which exhibit a moderately decreased systolic function [32] (Fig. 8C and D).

The hearts of cTnI-K118C mice showed rather mild diastolic dysfunction in contrast to that of cTnC-A8V and α -tropomyosin-E180G hearts (Fig. 1), providing an experimental system to test the role of the primary type of heart failure in the up-regulation of desmin and α -

actinin. Compared with the untreated group, 2 weeks of TAC induced further increases of desmin ($29.9 \pm 5.7\%$) and α -actinin ($32.7 \pm 4.5\%$) in cTnI-K118C mouse hearts (Fig. 8C and D). In contrast, 2 weeks of TAC did not increase the level of desmin or α -actinin in transgenic mouse hearts over-expressing cTnI-ND (Fig. 8C and D), a model of increased diastolic function [30]. These results further support the notion that the up-regulation of desmin and α -actinin is a response specific to diastolic heart failure.

Ex-vivo working heart studies further showed that the 2 weeks of TAC treatment of wild type mice effectively induced hypertrophy and reduced ventricular relaxation with preserved systolic function but decreased stroke volume, a typical HFpEF phenotype (Fig. 7C). Although the specific responses of the genetically modified mouse models to 2 weeks of TAC remain to be assessed in future studies, this baseline validation confirms our reproducibility of the short term TAC model for producing and augmenting cardiac diastolic dysfunctions.

3.7. Desmin phosphorylation showed no change in the transgenic mouse hearts of diastolic dysfunction, but decreased in cTnI-K118C hearts after 2 weeks of TAC

Phosphorylation is a known regulatory post-translational modification of desmin [42]. Pro-Qdiamond phosphoprotein staining and densitometry quantification showed that in comparison with the wild type control, the level of desmin phosphorylation relative to total desmin protein was not significantly changed in the diastolic or systolic heart failure models studied (Fig. 9A and B).

Two weeks of TAC had no effect on the level of desmin phosphorylation in wild type, cTnT-ND and cTnI-ND transgenic hearts (Fig. 9C and D), which had no change in total desmin protein (Fig. 8C and D). Compared with that in untreated cTnI-K118C hearts, 2 weeks of TAC resulted in a lower proportion of phosphorylated desmin (Fig. 9C and D) while the level of total desmin was increased (Fig. 8C and D). Microarray study found that the hearts from young adult wild type and cTnI-K118C mice had no difference in the levels of desmin and α -actinin mRNA (unpublished). Phosphorylation was correlated to disassembly of the desmin filaments in cardiomyocytes [58]. Therefore, the decreasing proportion of phosphorylated desmin upon TAC induction of diastolic heart failure and the unchanged mRNA levels suggest that the increased levels of total desmin protein in cTnI-K118C hearts may be due to more stable desmin filaments and thus a slower protein turnover. Further investigation is required to establish the mechanisms.

4. Discussion

Desmin is a major intermediate filament protein in higher eukaryotic cells and a key component in vertebrate striated muscle cells [8]. While increases in desmin are seen in heart failures [12,13], desmin deficiency also causes cardiomyopathy with myocyte destructions [43,44]. Our present study compared several previously characterized mouse models bearing definitive molecular modifications to produce phenotypes representative of diastolic or systolic heart failure. To focus on early and primary pathophysiological adaptations, young adult mice were studied to seek mechanistic insights.

Our design of this study first examined three diastolic (cTnC-A8V, TM-180E, cTnI-118C) versus two systolic (cTnT exon 8 deletion, Gs α -KO) heart failure models to establish the responses of desmin and α -actinin specific to diastolic dysfunction or failure. Two additional models were then employed in short term TAC studies to further establish the causal relationship between reduced ventricular relaxation and up-regulation of desmin and α -actinin: cTnT-ND hearts were examined as a model of mildly decreased systolic function and cTnI-ND hearts as a model of increased diastolic function together with the cTnI-118C hearts with mild diastolic dysfunction.

4.1. Increases of desmin and α -actinin in diastolic but not systolic heart failure

Increased desmin has been found in explanted failing human hearts [11,45], hypertrophic cardiac muscle [12] and cardiomyopathy [13]. However, the findings from different studies have been controversial [10–12,14,15]. Up-regulation and down-regulation of desmin were both observed in cardiomyopathy patients [13]. To link desmin adaptation to the primary alterations of cardiac function in heart failure, our present study carried out a direct comparison of several representative mouse models of diastolic or systolic cardiac dysfunction. The finding that desmin was up-regulated at early stage in diastolic but not systolic heart failure gives a novel lead to understanding this potentially adaptive up-regulation and its functional significance.

Previous studies reported a disarrangement of sarcomeric α -actinin in cardiomyopathy [46] but no increase of α -actinin in chronic pressure overload-induced cardiac hypertrophy [47]. However, decreased α -actinin was seen in heart failure patients after treatment with left ventricular-assist device [48]. Our finding of the concurrent increases of α -actinin and desmin in hearts with diastolic dysfunction due to mutations in different myofilament proteins (Fig. 4) provides new evidence suggesting that desmin and α -actinin up-regulations may be coordinated in a specific adaptation of cardiomyocytes to reduced ventricular relaxation with various primary causes.

A previous immunohistochemistry study [13] suggested four types of desmin distribution in cardiomyopathy: Type I is that in normal cardiac muscle; type IIA is increased level of desmin with normal distribution at Z-disks; type IIB is increased level of desmin with protein aggregates; and type III is decreased level or lack of desmin. While type III changes indicated poor prognosis [49] and desmin null cardiomyocytes displayed severely disorganized and damaged myofibrils as well as loss of cell adhesions [44], type IIA and type IIB increases of desmin are proposed to be adaptive and maladaptive, respectively [13]. In our present study, we found that desmin and α -actinin increased with normal locations at Z-disks and intercalated disks in diastolic heart failure models (Figs. 5 and 6), which is consistent with the adaptive type IIA change. Therefore, the increased desmin and α -actinin in diastolic heart failure may represent a novel mechanism of compensatory adaptation.

Titin is a major determinant of passive mechanical properties in cardiomyocytes with two splice forms that produce different passive tensions [50]. We examined the titin splice forms in wild type and cTnI-K118C mouse before and after 2 weeks of TAC and found no significant difference (Supplement Fig. S3).

4.2. Potential function of increased desmin and α -actinin at Z-disks of cardiac myofibrils in diastolic heart failure

Cardiomyocytes function in a dynamic equilibrium, balancing extrinsic stressors with intrinsically developed force at the Z-disks that link the neighboring sarcomeres. In pathophysiological conditions, disruption of this balance initiates adaptations in the myocytes to remain homeostasis [20]. α -Actinin anchors the thin filament to Z-disks and desmin filament mechanically links the adjacent Z-disks and links the Z-disks to the costameres [23]. The parallel increases of desmin and α -actinin at the Z-disks in diastolic heart failure indicate a potentially adaptive compensation for a specific dysfunction in cardiac muscle contractility. This adaptation may contribute to a preserved systolic function and prevent progressive dilation of end diastolic volume in diastolic heart failures [4]. This hypothesis is supported by the observation that moderate overexpression of desmin (~3-fold) was not detrimental but increased the shortening fraction of isolated cardiomyocytes and sustained diastolic function, whereas loss-of-function mutation in desmin impaired the systolic as well as diastolic functions [51].

The stiffness of cardiomyocytes from diastolic heart failure was increased [7]. Consequently, the mechanical stretch in cardiomyocytes with reduced ventricular relaxation is increased [1]. As a stretch sensor [26], Z-disks are in the frontline to respond to the increased strain, which may induce the early increases of desmin and α -actinin in diastolic dysfunction.

The Z-disk also serves as a nodal point for myocyte signaling, particularly in mechanosensation and mechanotransduction [23]. The role of Z-disks in cell signaling has become pivotal in unraveling short- and long-term regulations of cardiac function [20]. Numerous signaling proteins including kinases, phosphatases, and Ca^{2+} -binding proteins have been identified in the Z-disks of cardiac muscle. The Z-disk adaptation in early stage of diastolic heart failure as indicated by the increases in desmin and α -actinin may contribute to various signaling cascades, and is worth further investigation.

4.3. Pressure overload selectively induces up-regulation of desmin and α -actinin in mouse hearts with occult diastolic failure

Previously studies demonstrated increases of desmin in human failing hearts [11], hypertrophic mouse hearts, and after 4 weeks to 6 months of TAC in guinea pigs [47]. While systolic function was preserved, reduced ventricular relaxation was seen 4 weeks after TAC [52]. It was shown that left ventricular systolic performance declined immediately after TAC in mice and recovered to baseline in 10–20 days [53]. Two weeks of TAC would result in preserved diastolic function and systolic function in wild type mouse [40]. Therefore, we applied two-week TAC in selected mouse models to investigate the adaptive responses of desmin and α -actinin expression in cardiac muscle. Echocardiography showed that our TAC procedure narrowed the aorta arch by ~67% and resulted in increased thickness of the proximate aortic wall (Fig. 8) and detectable myocardial hypertrophy (Fig. 7). Supporting our hypothesis, *ex-vivo* working heart function of wild type mice showed that the diastolic velocity was significantly decreased after 2 weeks of TAC, whereas the systolic function was largely preserved (Fig. 7).

The finding that 2 weeks of TAC did not increase desmin and α -actinin in wild type mouse hearts but did effectively in cTnI-K118C hearts that had a mild impairment of diastolic function [29] (Fig. 8) suggests that occult diastolic dysfunction is a prerequisite for stress conditions to induce this adaptation. This observation is further supported by the results that 2 weeks of TAC did not induce up-regulation of desmin and α -actinin in cTnT-ND mouse hearts that had moderately decreased systolic function or in cTnI-ND mouse hearts that have been chosen as a model of enhanced diastolic function (Fig. 8). These data conclude that reduced ventricular relaxation promotes the stress-induced up-regulation of desmin and α -actinin in cardiomyocytes as a specific and potentially compensatory adaptation.

4.4. The increases of desmin in diastolic heart failure is likely due to decreased protein turnover

Our microarray results (unpublished) found no changes in the levels of desmin and α -actinin mRNA in cTnI-K118C mouse hearts, precluding transcriptional up-regulation. Previous studies showed that phosphorylated and non-phosphorylated desmin represent the non-filamentous and filamentous forms, respectively [42]. We found no change in desmin phosphorylation while the total desmin protein was increased in diastolic failure models of cTnC-A8V and TM-E180G mouse hearts, and the baseline condition of cTnI-K118C mouse hearts (Fig. 9A and B). However, the finding that the proportion of phosphorylated desmin was decreased in cTnI-K118C hearts after the treatment of 2 weeks of TAC to produce higher level of total desmin protein (Fig. 9C and D) indicates that a) the increased desmin was in filamentous form; b) desmin up-regulation is a rapid adaptation in the preexistence of diastolic dysfunction before the myocyte and Z-disk remodeling reaching an equilibrium; and c) the absence of hyper-phosphorylation precludes changes such as aggregation-related accumulation, such as that seen in desmin-related myopathy or in type 1 skeletal muscle fibers of cytoplasmic body myopathy [54]. Therefore, the increases of total desmin in diastolic failing hearts may be from decreased protein turnover due to more non-phosphorylated desmin in filamentous forms [42].

There is limited information on α -actinin phosphorylation and its synthesis/degradation in cardiomyocytes, and we did not see significant change in the phosphorylation of α -actinin in our study. Therefore, further investigation is needed to explore the possible mechanism for the increases of α -actinin in diastolic heart failure.

4.5. Role of thin filament regulatory proteins in myocardial diastolic dysfunction and adaptation

Diastolic dysfunction is a common clinical condition that contributes nearly 50% of all heart failure cases. Mutations in multiple sarcomeric proteins have been identified to cause cardiomyopathy and heart failure [55]. Except for the myosin-based thick filament that does not directly connect with the Z-disk, three of the four myofilaments in the sarcomere, actin, titin and nebulin/nebulette, are anchored at the Z-disk. In the present study, we found adaptive increases of desmin and α -actinin in three genetically modified mouse models bearing mutations in the cardiac thin filament regulatory proteins, TnC, TnI and tropomyosin, which produce diastolic heart failure phenotypes (Fig. 1). This finding suggests that thin filament regulation may be a critical factor in the diastolic function and adaptation of cardiac muscle.

Cardiac TnC is the Ca^{2+} receptor subunit of the troponin complex [56], cTnI is the inhibitory subunit of cardiac troponin and tropomyosin relays the Ca^{2+} signal to regulate the interaction between actin and myosin filaments [57]. The three models of diastolic heart failure have different impairments in abnormally high Ca^{2+} sensitivity or decreased relaxation velocity of the cardiac muscle. However, these different causes of reduced ventricular relaxation in the young transgenic mice in the absence of significant remodeling of myocardial structure and chamber morphology had an early common adaptation of increased levels of desmin and α -actinin.

It is worth noting that one of the two systolic heart failure mouse models showing no adaptive changes in desmin and α -actinin was produced by Gs α -KO with a defect in (β -adrenergic signaling pathway that results in diminished PKA phosphorylation of cardiac TnI and exhibits cardiac phenotypes of decreased systolic and diastolic function [30]. However, the levels of desmin and α -actinin did not change in the hearts of Gs α -KO mice, suggesting that preserved or increased systolic force may be necessary for various diastolic dysfunctions to induce this specific adaptation. The hypothesis that active force generation plays a role in myocardial adaptation to primary diastolic dysfunctions is worth investigating, toward better understanding of the mechanisms for pathophysiological remodeling of cardiac muscle in heart failure, especially HFpEF.

The up-regulations of desmin and α -actinin in the thin filament regulatory protein mutation-generated models of diastolic heart failure also indicate that the Z-disks may be a direct sensor of thin filament force and stretch and a responder with structural remodeling to maintain the homeostasis in myofibril function. The adaptive change of desmin and α -actinin levels also suggests that the stoichiometry of Z-disk proteins may be a regulatory mechanism in myocardial adaptation. The three diastolic heart failure models used in the present study provide valuable experimental systems to further investigate the role of Z-disk in myofibril structure and function.

In conclusion, the coordinated up-regulations of desmin and α -actinin specifically in the early stage of diastolic heart failure mouse models indicate a novel myocardial response. It is important to further understand the adaptive or maladaptive significance of the changes in desmin and α -actinin in diastolic heart failure and the mechanistic link to cardiac function and remodeling. While our present study focuses on desmin and α -actinin, changes in other proteins may also present in the heart failure models studied. More research is needed to understand the early adaptation in diastolic heart failure as well as the role of increased desmin and α -actinin in cardiac muscle structure and function. The specific increases of desmin and α -actinin and the role of Z-disk adaptation in diastolic heart failure offer an attractive leads to explore the pathophysiology of diastolic heart failure and a potential target for the development of new treatment. Although our present study was limited to mouse models, the new findings laid a foundation for future investigations on human heart failure and follow-up studies in large animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BSA	bovine serum albumin
CON	control group
cTnC	cardiac troponin C
cTnI	cardiac troponin I
cTnT	cardiac troponin T
cTnI-ND	N-terminal truncated cardiac troponin I
cTnT- E7	exon 7-deleted cardiac troponin T
cTnT-ND	N-terminal truncated cardiac troponin T
DHF	diastolic heart failure
$\pm$ dP/dt	contractile and relaxation velocities
EF	ejection fraction
FBS	fetal bovine serum
HFpEF	heart failure with preserved ejection fraction
LVP_{max}	left ventricular maximum pressure
LVP_{min}	left ventricular minimum pressure
mAb	monoclonal antibody
MBP-C	myosin-binding protein C
MHC	myosin heavy chain
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
P-V loop	pressure-volume loop
RLC	regulatory light chain

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHF	systolic heart failure
SV	stroke volume
TAC	transverse aortic constriction
TBS	Tris-buffered saline

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Fig. 1.

Characteristics of mouse hearts with diastolic or systolic dysfunction. Normalized to wild type controls, the functional parameters measured in *ex vivo* working hearts of the five mouse models demonstrated features representative of diastolic or systolic failure. To reflect overall cardiac function, left ventricular stroke volume (SV) was normalized to heart weight (B). It is worth noting that the heart weight to body weight ratio of cTnC-A8V and α -tropomyosin (TM)-E180G mice was significantly decreased whereas that of Gs α -KO mice significantly increased as compared to wild type control (A). LVP_{max}, left ventricular

maximum pressure; LVP_{min} , left ventricular minimum pressure; $+dP/dt$, contractile velocity; $-dP/dt$, relaxation velocity. $n = 3-8$ in each wild type group, $n = 5$ in cTnC-A8V group, $n = 4$ in TM- E180G group, $n = 6$ in cTnI-K118C group, $n = 7$ in Gs α -KO group, and $n = 5$ in exon 7-deleted cTnT (cTnT- E7) group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. wild type. Statistic analysis was performed using two-tail Student's t -test.

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Fig. 2.

Increases of desmin in mouse hearts with diastolic failure. SDS-PAGE gels and Western blots (A) and densitometry analysis normalized to the level of actin (B) showed increases of desmin in cTnC-A8V (31.9%), α -tropomyosin (TM)-E180G (44.7%), and cTnI-K118C (31.8%) mouse hearts compared with that in wild type hearts. Data are presented as means $\pm$ SE; $n = 4$ mice each in wild type groups, $n = 5$ mice in cTnC-A8V group, $n = 10$ mice in TM-E180G group, $n = 5$ mice in cTnI-K118C group. * $P < 0.05$ vs. wild type. Statistic analysis was performed using two-tail Student's t -test. Whereas the cTnC-A8V knock-in

mutation was confirmed using PCR (data not shown), the expression of TM-E180G mutation was confirmed with mAb CH1 Western blot and the expression of cTnI-K118C was confirmed with mAb TnI-1 Western blot.

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Fig. 3.

No change in the level of desmin in mouse hearts of systolic failure. The SDS-PAGE gel and Western blots (A) and densitometry analysis normalized to the level of actin (B) showed that the levels of desmin in Gα-KO and exon 7-deleted cTnT (cTnT- E7) mouse hearts were both similar to that in wild type hearts. Data are presented as means $\pm$ SE; $n = 4$ mice each in Gα-KO and control wild type groups, $P = 0.476$ vs. wild type; $n = 5$ mice each in cTnT- E7 and control wild type groups, $P = 0.934$ vs. wild type. Statistic analysis was performed using two-tail Student's t -test. The genotype of MCK-cre, Gα-flox genotype was confirmed

using PCR (data not shown) and the over-expression of cTnT- E7 was confirmed with mAb CT3 Western blot.

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Fig. 4.

Concurrent increases of desmin and α -actinin in mouse hearts with diastolic failure. A. Western blots showed that α -actinin was increased in cTnC-A8V, α -tropomyosin (TM)-E180G and cTnI-K118C mouse hearts, but not in Gs α -KO and cTnT- E7 hearts. The protein inputs are as that in the SDS-gel shown in Figs. 2 and 3 and the effective extraction of α - actinin was confirmed in Supplement Fig. S1. B. Linear regression analysis demonstrated a significantly positive correlation between the levels of desmin and α -actinin in the transgenic and wild type mouse hearts studied with increases in the hearts of diastolic failure. Among the three diastolic failure models, TM-E180G hearts showed the highest levels of both desmin and α -actinin. $n = 4$ mice for wild type, $n = 10$ mice for TM-E180G, $n = 5$ mice for cTnC-A8V, $n = 5$ mice for cTnI-K118C, $n = 4$ mice for Gs α -KO, and $n = 5$ mice for cTnT- E7 groups.

Fig. 5.

Normal distribution of increased desmin in mouse hearts with diastolic failure. A. Immunofluorescence microscopic images showed that desmin in cTnC-A8V mouse heart sections was located at the Z-disks and intercalated disks as well as co-localized with F-actin in the sarcomeres similarly to that in wild type hearts. B. Relative intensity of desmin quantified using Image-J software showed a significantly higher level in cTnC-A8V hearts than that in wild type hearts, consistent with the Western blot results in Fig. 2. $n = 4$ mice in each group. Statistical analysis was performed using two-tail Student's t -test. * $P < 0.05$.

Fig. 6.

Increased desmin and α -actinin in cardiac myofibrils of mouse hearts with diastolic failure. A. Immunofluorescence microscopic images of cardiomyocytes isolated from α -tropomyosin-E180G mouse hearts showed locations of desmin and α -actinin at the Z-disks. B. Immunofluorescence staining of isolated myofibrils confirmed the locations of desmin and α -actinin at the Z-disks. C. Western blots and densitometry quantification confirmed the increases of desmin and α -actinin in the myofibrils. * $P < 0.05$ vs. wild type, $n = 3$ mice in each group.

Fig. 7. Echocardiography and *ex-vivo* working heart function of wild type mouse hearts treated with 2 weeks of TAC. A. Representative echocardiographs clearly showed the aortic constriction between innominate and left carotid arteries (pointed by the arrow). B. Measurements based on the echocardiographs showed that the diameter of aorta was 1.44 ± 0.03 mm in control group (CON) and 0.84 ± 0.01 mm in the TAC group. $n = 7$ mice for CON and $n = 4$ mice for TAC groups. Further confirming the result of TAC, the luminal area of the ligation site was 33.58% of CON. *** $P < 0.001$ vs. wild type. C. Post mortem studies

showed that the heart weight/body weight ratio of wild type mice was significantly increased after 2 weeks of TAC. *Ex-vivo* working heart study showed that 2 weeks of TAC did not change left ventricular systolic and diastolic pressures. However, the stroke volume normalized to heart weight showed a trend of decreasing after 2 weeks of TAC together with significantly decreased diastolic but not systolic velocity, indicating an effective induction of HFpEF. $*P < 0.05$ vs. wild type. $n = 4$ mice for CON and $n = 4$ mice for TAC groups.

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Fig. 8.

Two weeks of TAC increased the level of desmin in cTnI-K118C but not cTnT-ND or cTnI-ND mouse hearts. H&E stained sections (A) and wall thickness measurements (B) of cross-sections of mouse ascending aorta (upstream of the ligation site) from 2-week TAC-treated mice showed significantly increased wall thickness in comparison with that of the same region of untreated mice ($*P < 0.05$), verifying the effectiveness of TAC in the transgenic mouse models studied. SDS-PAGE and Western blots (C) and densitometry quantification (D) showed that 2 weeks of TAC treatment increased the expression of desmin ($29.9 \pm 5.7\%$) and α -actinin ($32.7 \pm 4.5\%$) in cTnI-K118C mouse hearts but not in wild type, cTnT-ND or cTnI-ND hearts as compared with the untreated control. $n = 3$ mice for wild type CON, $n =$

4 mice for wild type TAC, n = 3 mice for cTnI-K118C CON, n = 3 mice for cTnI-K118C TAC, n = 4 mice for cTnT-ND CON, n = 5 mice for cTnT-ND TAC, n = 3 mice for cTnI-ND CON and n = 5 mice for cTnI-ND TAC groups. * $P < 0.05$ vs. control. Statistic analysis was performed using two-tail Student's t -test.

Fig. 9.

The level of desmin phosphorylation relative to total desmin protein decreased after 2 weeks of TAC. Coomassie Blue and Pro-QDiamond phosphoprotein stained SDS-PAGE gels of total protein extracts from wild type and transgenic mouse hearts (A) and densitometry quantification (B) showed that normalized to the level of total desmin protein, no significant change of desmin phosphorylation was detected in the diastolic or systolic failing mouse hearts. $n = 4$ mice for wild type, $n = 10$ mice for TM-E180G, $n = 5$ mice for cTnC-A8V, $n = 5$ mice for cTnI-K118C, $n = 4$ mice for Gsα-KO and $n = 5$ mice for cTnT- E7 groups.

Coomassie Blue and Pro-QDiamond phosphoprotein stained SDS-PAGE gels (C) and densitometry quantification (D) further showed that 2 weeks of TAC treatment resulted in decreased proportion of phosphorylated desmin relative to the level of total desmin protein in cTnI-K118C hearts but not cTnT-ND or cTnI-ND hearts (* $P < 0.05$). $n = 3$ mice for wild type CON, $n = 4$ mice for wild type TAC, $n = 3$ mice for cTnI-K118C CON, $n = 3$ mice for cTnI-K118C TAC, $n = 4$ mice for cTnT-ND CON, $n = 5$ mice for cTnT-ND TAC, $n = 3$ mice for cTnI-ND CON and $n = 5$ mice for cTnI-ND TAC groups. Statistic analysis was performed using two-tail Student's t -test. MBP-C, myosin-binding protein C; RLC, myosin regulatory light chain.