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Effect of exercise on passive myocardial stiffness in mice with diastolic dysfunction

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ABSTRACT

Heart failure with preserved ejection fraction (HFpEF) is a complex syndrome, characterized by increased diastolic stiffness and a preserved ejection fraction, with no effective treatment options. Here we studied the therapeutic potential of exercise for improving diastolic function in a mouse model with HFpEF-like symptoms, the TtnΔIAjxn mouse model. TtnΔIAjxn mice have increased diastolic stiffness and reduced exercise tolerance, mimicking aspects of HFpEF observed in patients. We investigated the effect of free-wheel running exercise on diastolic function. Mechanical studies on cardiac muscle strips from the LV free wall revealed that both TtnΔIAjxn and wildtype (WT) exercised mice had a reduction in passive stiffness, relative to sedentary controls. In both genotypes, this reduction is due to an increase in the compliance of titin whereas ECM-based stiffness was unaffected. Phosphorylation of titin's PEVK and N2B spring elements were assayed with phospho-site specific antibodies. Exercised mice had decreased PEVK phosphorylation and increased N2B phosphorylation both of which are predicted to contribute to the increased compliance of titin. Since exercise lowers the heart rate we examined whether reduction in heart rate per se can improve passive stiffness by administering the heart-rate-lowering drug ivabradine. Ivabradine lowered heart rate in our study but it did not affect passive tension, in neither WT nor TtnΔIAjxn mice. We conclude that exercise is beneficial for decreasing passive stiffness and that it involves beneficial alterations in titin phosphorylation.

1. Introduction

Heart Failure with preserved Ejection Fraction (HFpEF) is a complex syndrome with diastolic stiffening that leads to higher diastolic pressures and insufficient filling, the culmination of which eventually leads to decreased cardiac reserve, and pulmonary edema [1,2]. The prevalence of HFpEF is increasing and is predicted to exceed 8% of persons older than 65 by the year 2020 [3]. Unlike heart failure with reduced ejection fraction (HFrEF), pharmacological approaches to treating HFpEF do not currently exist and clinical trials focused on HFpEF have had neutral results [4,5].

Physical training has been reported to have beneficial effects on diastolic function under a range of conditions and earlier studies [6,7] suggest that exercise could be a potential therapy for HFpEF through decreasing diastolic stiffness. The Exercise Training in Diastolic Heart Failure trial (Ex-DHF) studied HFpEF patients and concluded that exercise training improved exercise capacity and diastolic function [8]. However, the mechanistic basis of how exercise affects passive stiffness in HFpEF is not well established. Recent studies have indicated that rodents have decreased phosphorylation at titin's PEVK element following exercise and that this hypo-phosphorylation might play a role in lowering titin-based stiffness [6,9,10]. Although this data suggests that exercise is a promising therapy for lowering diastolic stiffness, mechanistic studies and passive stiffness measurements are required.

In the present study, we investigated the effects of exercise on the passive stiffness of LV wall muscle strips and evaluated both extracellular matrix (ECM) and titin-based stiffness. Wildtype (WT) mice were studied as were TtnΔIAjxn mice that have the I-A junction of titin removed resulting in increased diastolic stiffness and reduced exercise tolerance, mimicking aspects of HFpEF observed in patients [11]. A beneficial effect of exercise on passive stiffness was found in both genotypes (WT and TtnΔIAjxn) and extraction studies showed that the effect was derived from an increase in titin compliance. Additionally, we performed phosphorylation studies and focused on the phosphorylation status of the PEVK element, in which an increase in phosphorylation has been shown to increase stiffness [12], and the N2B element, in which an increase in phosphorylation has been shown to decrease stiffness [13]. Because it is known that exercise reduces heart rate [14] we

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also studied the effect of the heart-rate lowering drug ivabradine. Ivabradine is a selective inhibitor of the cardiac pacemaker If-current [15], effectively lowering heart rate [16] with recent work that suggests that ivabradine improves diastolic function [17,18]. Hence we included in our work a study of the effects of ivabradine on passive stiffness.

2. Materials and methods

2.1. Mice

Male 4-month-old mice on a C57BL/6 J background were used for all experiments. TtnΔIAjxn mice had the I-A junction region of titin removed, for details see Granzier et al. [11]. All experiments were approved by the University of Arizona Institutional Animal Care and Use Committee and followed the U.S. National Institutes of Health *Using Animals in Intramural Research* guidelines for animal use.

2.2. Exercise

For the exercise protocol, a free rotating running wheel exercise protocol was used as previously described [19]. Mice were housed individually in a cage that contained an 11.5 cm-diameter wheel with a 5.0 cm wide running surface (6208; PetSmart; Phoenix, AZ) equipped with a digital magnetic counter (BC600, Sigma Sport, Olney Il) activated by wheel rotation. All animals were given water and standard rodent feed ad libitum. Daily exercise values for time and distance run were recorded and the average speed was calculated from these recorded values. Sedentary animals were housed individually in cages without running wheels. 28 days was selected in order to allow an initial acclimatization period of ~1 week followed by enough time to reach steady-state exercise levels and ensuing adaptations in protein expression.

2.3. Ivabradine treatment

Ivabradine (Waterstone Technology) was added to the drinking water for twenty-eight days. In order to get a correct dosage, mice where weighed and water intake tracked and ivabradine was dissolved in water to achieve a dose of 20 mg/kg BW/day. (The addition of the drug did not affect water intake.) Ivabradine is water soluble so controls received water alone. Heart rate was measured using the tail-cuff measurement system (MC4000 MultiChannel, Hatteras Instruments, Cary, NC). Briefly, mice are placed on a warmed holder and the tail is thread through a balloon cuff and placed under a LED. Pulse is measured as a LED passes light through the tail and a photodiode detects changes in absorption due to blood flow with each beat. Mice were allowed to acclimate to the system for ~10 min before measurements were taken each day. In order to account for stress on the mice due to the system, measurements were then taken for three consecutive days each week with the last day taken as the measurement.

2.4. Echocardiography

Echocardiography was performed as previously described [20–22] on ivabradine treated WT and TtnΔIAjxn mice as well as untreated controls of both genotypes. Mice were anesthetized with 1–3% isoflurane (USP, Phoenix), then placed in dorsal recumbence on a heated platform for echocardiography. Transthoracic echo images were obtained with a Vevo 2100 High-Resolution Imaging System (Visual-Sonics, Toronto, Canada) using the model MS550D scan head designed for murine cardiac imaging. Care was taken to avoid animal contact and excessive pressure which could induce bradycardia. Body temperature was maintained at 37 °C. Imaging was performed at a depth setting of 1 cm. Images were collected and stored as a digital cine loop for off-line calculations. Doppler and functional calculations were obtained according to American Society of Echocardiography guidelines. Passive LV filling peak velocity, E (cm/s), and atrial contraction flow peak velocity, A (cm/s), were acquired from the images of mitral valve Doppler flow from tilted parasternal long axis views. A sweep speed of 100 mm/s was used for studies. The heart rate of animals during the echocardiographic study was maintained in the range of 500–550 beats/min. Measures of diastolic function, specifically the relationship between early and late filling (E/A ratio) and how quickly the flow velocity declines in early diastole (E-wave deceleration time), are reported.

2.5. Muscle mechanics

Mice were placed under isoflurane anesthesia, cervically dislocated, and the ribcage was rapidly opened to access the heart. One ml of HEPES pH 7.4(in mM: NaCL, 133.5; KCl, 5; NaH2PO4, 1.2; MgSO4,1.2; HEPES, 10) solution containing and additional 30 mM KCl and 3 mM BDM was injected into the LV through the apex, after which time the heart noticeably relaxed and ceased pumping. The heart was removed and the LV was isolated from the other chambers. The apex and base were removed from the LV leaving a cross-sectional slice approximately 2 mm thick. The septum and RV attachment regions were discarded and the LV free wall tissue was placed in relaxing solution (in mM; 20 BES, 10 EGTA, 6.56 MgCl2, 5.88 NaATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate, pH 7.0). Endocardial fibers (apical to base orientation) were dissected and discarded. Once visualized, mid-myocardial fibers (circumferential orientation) were carefully removed and skinned in fresh relaxing solution pH 7.0 with 1% Triton-X-100 (Pierce, IL, USA) overnight at 4 °C and protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; leupeptin, 0.04 mM; E64, 0.01 mM), then washed for one hour with relaxing solution. Phosphatase inhibitor cocktail 2 (P5726, Sigma-Aldrich) 1 ml was added into 100 ml of relaxing solution. In experiments shown in Supplementary Fig. 3 papillary muscles were also carefully dissected immediately after placing the LV in relaxing solution. Following a wash with relaxing solution, 150–250 μm (diameter) strips were dissected and aluminum clips were placed on both ends of the preparation. The aluminum clips were attached to a strain gauge force transducer and high-speed length motor and the preparation was submersed in relaxing solution. A laser diffraction system was used to measure the sarcomere length by taking advantage of Bragg's law ($d\sin\theta = m\lambda$) to calculate the lattice spacing (d) based on the wavelength (λ), peak order (m), and angle (θ). The length and cross-sectional area (CSA) of the fiber then measured in order to normalize measured forces to CSA. Once the fiber was mounted and measured, a stretch-hold-release protocol was used in which a skinned fiber dissected from the circumferentially aligned free wall was stretched to a given sarcomere length (SL) within the physiological range (2.0 to 2.3 μm) at 100%/s, held for 90 s, and subjected to a sinusoidal frequency sweep from 0.1 Hz to 100 Hz at ±2%. The sinusoidal frequency sweep was used to quantify the viscous and elastic modulus. Viscous and elastic moduli are defined as $(\sigma/\varepsilon)\sin(\theta)$ and $(\sigma/\varepsilon)\cos(\theta)$ respectively where σ is stress, ε is strain, and θ is the phase shift. In order to quantify the relative contributions of titin and ECM, skinned muscle strips were extracted using first 0.6 M KCl in relaxing solution followed by 1.0 M KI in relaxing solution to depolymerize the thick and thin filaments respectively, removing the anchoring points of titin but keeping ECM intact [23,24].

In this study, both skinned papillary muscles as well as muscle strips from the LV free wall at the mid-level myocardium where fibers are circumferentially aligned were used. Papillary muscles are a popular specimen for mechanical studies as they are generally easy to dissect and thought to reflect the properties of the heart wall. However, this assumption might not be valid as ECM expression differs throughout the heart [25,26]. LV free wall strips, while more difficult to dissect, are thus thought to be more representative of the surrounding myocardium. In order to test if there was indeed a difference between papillary