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Original article

Diastolic dysfunction and thin filament dysregulation resulting from excitation–contraction uncoupling in a mouse model of restrictive cardiomyopathy

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ABSTRACT

Restrictive cardiomyopathy (RCM) has been linked to mutations in the thin filament regulatory protein cardiac troponin I (cTnI). As the pathogenesis of RCM from genotype to clinical phenotype is not fully understood, transgenic (Tg) mice were generated with cardiac specific expression of an RCM-linked missense mutation (R193H) in cTnI. R193H Tg mouse hearts with 15% stoichiometric replacement had smaller hearts and significantly elevated end diastolic pressures (EDP) *in vivo*. Using a unique carbon microfiber-based assay, membrane intact R193H adult cardiac myocytes generated higher passive tensions across a range of physiologic sarcomere lengths resulting in significant Ca^{2+} independent cellular diastolic tone that was manifest *in vivo* as elevated organ-level EDP. Sarcomere relaxation and Ca^{2+} decay was uncoupled in isolated R193H Tg adult myocytes due to the increase in myofilament Ca^{2+} sensitivity of tension, decreased passive compliance of the sarcomere, and adaptive *in vivo* changes in which phospholamban (PLN) content was decreased. Further evidence of Ca^{2+} and mechanical uncoupling in R193H Tg myocytes was demonstrated by the biphasic response of relaxation to increased pacing frequency versus the negative staircase seen with Ca^{2+} decay. In comparison, non-transgenic myocyte relaxation closely paralleled the accelerated Ca^{2+} decay. Ca^{2+} transient amplitude was also significantly blunted in R193H Tg myocytes despite normal mechanical shortening resulting in myocyte hypercontractility when compared to non-transgenics. These results identify for the first time that a single point mutation in cTnI, R193H, directly causes elevated EDP due to a myocyte intrinsic loss of compliance independent of Ca^{2+} cycling or altered cardiac morphology. The compound influence of impaired relaxation and elevated EDP represents a clinically severe form of diastolic dysfunction similar to the hemodynamic state documented in RCM patients.

1. Introduction

Inherited restrictive cardiomyopathy (RCM) is a malignant heart muscle disease that can transition to heart failure and early morbidity [1,2]. RCM is relatively rare, evident in roughly 2% of diagnosed inherited cardiomyopathy cases [1,2]. The clinical hallmark of RCM is near normal systolic function accompanied by marked diastolic dysfunction resulting from impaired ventricular filling and an extremely stiff heart [1,2]. Ventricular wall thickness in RCM hearts is normal or near normal with variable fibrosis that can range from non-existent to severe [1]. RCM was thought to be idiopathic until the recent identification of six mutations in the gene that encodes cardiac troponin I (cTnI), *TNNI3*, cosegregated with RCM in human patients [3].

Cardiac TnI is a key component of the thin filament protein complex responsible for regulating cardiac muscle contraction. It functions as a molecular switch that inhibits actin–myosin interaction during diastole. During systole, cTnI moves from its inhibitory position on actin to one in contact with troponin C (cTnC) in response to intracellular elevation in the activating ligand Ca^{2+} [4]. This TnI switch further facilitates the binding of Ca^{2+} to cTnC [4]. The cTnI locus is a hotspot for cardiomyopathy mutations, as over twenty different mutations have been linked to either hypertrophic cardiomyopathy (HCM) [5] or those associated with RCM [3]. HCM is clinically heterogeneous disease, distinct from RCM, with an estimated prevalence of 0.2% of the population [2]. In contrast to RCM, the clinical hallmarks of HCM include ventricular hypertrophy with myocyte disarray and fibrosis, normal or hyperdynamic systolic function, and diastolic dysfunction [2,6–9]. HCM is the most common cause of sudden cardiac death in athletes and adolescents [9]. Approximately 75% of the identified cardiomyopathy mutations in cTnI cluster in the carboxy-terminal domain (C-terminal, amino acids 164–210). TnI's C-terminus contains a secondary actin–tropomyosin binding domain that is necessary for fully inhibiting cross-bridge cycling during

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diastole [10–13]. Delineating the mechanistic basis of how mutations in the same functional domain of cTnI can result in the distinct clinical presentations of RCM or HCM has remained elusive.

The primary effects of the highly malignant RCM C-terminal mutant cTnI, R193H, on cardiac myocyte function have been elucidated using acute adenoviral gene transfer to adult cardiac myocytes [14]. In that study, the R193H mutant cTnI caused a novel Ca^{2+} independent cellular diastolic tone and hypersensitized the myofilaments to Ca^{2+}, which slowed myocyte relaxation and Ca^{2+} transient decay. Biochemical reconstitution preparations also found that R193H cTnI increased myofilament Ca^{2+} sensitivity in ATPase and tension assays [15–17]. The R193H mutation has been engineered into the mouse cTnI sequence and expressed in a published mouse model in which a 25% replacement elicited a small increase in myofilament Ca^{2+} sensitivity [18] that manifest itself *in vivo* as diastolic dysfunction beginning around 6 months of age and becoming more prominent with a loss of myocardial compliance at 12 months of age [19,20]. From these studies it was concluded that myocardial diastolic dysfunction in R193H mice can be ascribed to the following properties measured in isolated myocytes: short end diastolic sarcomere lengths, slow relaxation, and slow Ca^{2+} transient decay that occurs in the absence of altered SR-load or Ca^{2+} handling proteins [18]. These results are similar to those obtained with acute adenoviral gene transfer of rodent R193H cTnI to isolated cardiac myocytes.

Despite these published results several key questions still remain unanswered. (1) What is the physiologic basis for diastolic dysfunction in R193H Tg mice and is cardiac performance altered with physiologic stressors? (2) Given that R193H mutant myocytes have a high Ca^{2+}-independent diastolic tone and altered cellular morphology, do R193H cTnI Tg mice exhibit altered left ventricular end diastolic pressures? (3) If R193H mice have reduced myocardial compliance is it due to cell intrinsic or extrinsic properties? (4) Given the current paradigm that Ca^{2+} sensitizing cardiomyopathic sarcomeric proteins increase the buffering capacity of the myofilaments, does Ca^{2+} handling adapt to accommodate an R193H cTnI that elicits a greater change in myofilament Ca^{2+} sensitivity when expressed *in vivo*?

To address these important questions we generated independently a cTnI R193H Tg mouse model that was engineered with rodent cTnI and found to elicit a 3-fold increase in myofilament Ca^{2+} sensitivity despite a low level of replacement. Using real-time hemodynamic measurements we uncovered the new finding that R193H cTnI Tg mice have significantly elevated LV end diastolic pressure (EDP) that becomes more severe with increased expression of R193H cTnI. Notably this elevated organ-level EDP could be directly attributed to the significantly reduced compliance in R193H Tg myocytes as demonstrated by their resistance to passive stretch. This poor cellular distensibility, in combination with the high Ca^{2+}-independent diastolic tone described in R193H myocytes [14], suggests that the resistance to passive stretch is due to overactivation of the R193H myofilaments during diastole. Furthermore functional measurements in isolated myocytes directly demonstrate that diastolic dysfunction, elevated LV EDP, and poor compliance are Ca^{2+}-independent as the Ca^{2+} cycling was uncoupled from myofilament function in isolated R193H Tg myocytes.

Taken together, the results of altered myocardial passive-elastic properties (altered pressure–volume loops), loss of cellular compliance and slowed relaxation provide a basis for the *in vivo* diastolic dysfunction and poor running performance of R193H Tg mice. Unlike previous reports, the expression of Ca^{2+} handling proteins were altered in this R193H model which is likely due to homeostatic preservation of Ca^{2+} handling in the context of highly Ca^{2+} sensitized and "stiff" myofilaments. Despite the corrected Ca^{2+} transient decay myocyte relaxation still remained impaired suggesting that the R193H mutant cTnI directly influences cardiac performance. Overall R193H transgenic mice have a unique hybrid cardiomyopathic phenotype characterized by elevated diastolic pressures, diastolic dysfunction, and hypercontractility.

2. Methods

2.1. Engineering transgenic mice carrying R193H mutant cTnI construct

The R193H Tg construct utilized a cardiac specific α-myosin heavy chain (αMHC) promoter (kindly provided by Dr. Jeffrey Robbins, Children's Hospital, Cincinnati, OH) to drive expression of murine R193H mutant cTnI (Fig. 1A). This construct also contained a 3′ flag epitope tag, which has been shown previously not to affect isolated myocyte Ca^{2+} sensitivity of tension, pH sensitivity, shortening, or Ca^{2+} cycling when expressed [14,21,22]. In previous reports transgenic animals expressing cTnI with a 5′ flag tag have also been shown to function normally [23–25]. The R193H construct was enzymatically removed from a pDC315 vector (Admax, Microbix) [14] by *EcoRI* digestion and subcloned into pBluescript 2SK plasmid (Stratagene). A second subcloning step was performed using restriction enzymes *XhoI* and *NotI* to transfer the R193H cTnI construct into the plasmid containing the αMHC promoter and SV40 polyadenylation sequence (Fig. 1A). The transgenic construct was verified by DNA sequencing and given to the transgenic animal core (University of Michigan) for DNA purification and injection into C57BL/6×SJL F_2 fertilized eggs and surgically transplanted into pseudopregnant females. Ninety-five potentially transgenic mice were PCR screened for the

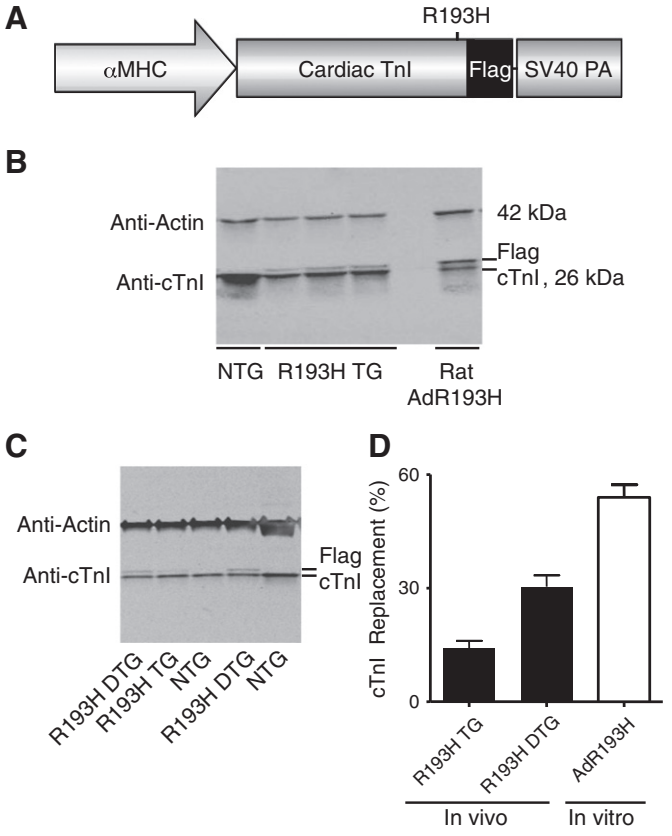

Fig. 1. The expression and replacement of mutant R193H cTnI in transgenic mouse hearts. (A) Transgenic construct containing the cardiac specific α-myosin heavy chain (αMHC) promoter driving expression of a C-terminal flag epitope-tagged mutant R193H cTnI with a simian virus 40 (SV40) polyadenylation signal. (B) Representative Western blot of whole heart homogenates from R193H Tg line 594, non-transgenic (NTG), and rat myocytes adenovirally transduced for 2.5 days with R193H cTnI. (C) Representative Western blot of whole heart homogenates from R193H double Tg mice derived from inbreeding line 594 (R193H DTG) and single Tg mice from line 594 (R193H TG). Blots were probed with anti-cTnI and anti-sarcomeric actin specific primary antibodies. Anti-cTnI antibody displays the relative replacement of the native cTnI with the flag epitope-tagged R193H cTnI. There were no detectable changes in other thin filament proteins. (D) A summary of the percent replacement of native cTnI for the R193H mutant cTnI. Values mean ± SEM, n = 6.