Cardiac troponin I Pro82Ser variant induces diastolic dysfunction, blunts β-adrenergic response, and impairs myofilament cooperativity

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FAMILIAL HYPERTROPHIC CARDIOMYOPATHY (HCM) is the most common inherited cardiovascular disease. In general, it is associated with autosomal dominant inheritance, and a number of animal models have replicated the human phenotype. Sarcomere mutations lead to the development of HCM and predispose to sudden cardiac death. Up to ~5–6% of familial HCM patients may have either double (≥1 mutation in same gene) or compound (≥1 mutated gene) genotypes (17).

Cardiac troponin I (cTnI) is a key sarcomere protein that regulates myocardial contraction and relaxation by acting as a molecular switch (28). Mutations in cTnI may result in HCM, restrictive (RCM), or dilated cardiomyopathy (DCM) phenotypes (12, 21, 46). However, the role of cTnIP82S mutations in elderly patients with late-onset hypertrophy remains uncertain. Initial studies showed that ~20% of late-onset hypertrophy cases have an identifiable genetic cause and concluded that cTnIP82S was a disease-causing mutation (36). However, a study on a larger cohort of HCM families concluded that, because cTnIP82S was present in 3% of Afro-Caribbean controls, it was likely to be a non-disease-causing polymorphic variant (32). Unfortunately, conducting a phenotype-genotype segregation analysis has been prohibitive due to the small size of cTnIP82S families studied to date.

We found that a heterozygous proline to serine mutation at cTnI (cTnIP82S) mutation is present in 3% of apparently normal African Americans subjects (11) (from a normal population control panel, Coriell Institute for Medical Research Biorepository); however, in a small cohort of hypertensive young African American men, cTnIP82S significantly correlated with increased left ventricle (LV) mass (4). In addition, cTnIP82S variant coexistence with MHY7 R453S mutation was associated with a severe phenotype in an African American female patient (11). Thus it is plausible that cTnIP82S could promote or exacerbate cardiac dysfunction, particularly under conditions of hemodynamic stress, such as that present during chronic hypertension, where afterload is persistently elevated. Yet, despite TnIP82S association with late-onset HCM, a causal relationship between this mutation and the appearance of cardiac dysfunction, especially under conditions of acute or chronic hemodynamic stress, remains to be firmly established.

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This study was designed to address the functional impact of TnIP82S. Using a transgenic (Tg) mouse model of cTnIP82S, here we assessed the functional effects of the cTnIP82S sequence variant on whole heart and in isolated muscle function. We hypothesized that cTnIP82S variant causes cardiac dysfunction during aging and with imposition of chronic stress. Using isolated intact cardiac trabeculae, tissue Doppler imaging (TDI) and pressure-volume relationships, we document a late-onset of cardiac dysfunction. Furthermore, young male Tg-cTnIP82S showed a blunted response to β-adrenergic stimulation and an exacerbated hypertrophic response when pressure overload was imposed by transverse aortic constriction (TAC).

METHODS

Tg model. The cDNA for rat cTnI was subjected to site-directed mutagenesis (Stratagene, San Diego, CA) to mutate proline 82 to serine (cTnIP82S), confirmed by sequencing and then ligated into the Tg expression vector downstream of the mouse α-myosin heavy chain (MHC) promoter (Fig. 1A) (kind gift of Dr. J. Robbins). To generate multiple lines of Tg-cTnIP82S, a linearized vector was used to inject pronuclear embryos (C57BL6 X A/J), as previously described (34), and crossed for at least six generations to C57BL6. Genotyping was performed by PCR (Fig. 1A), with forward G05 5’-ATG-GCG-GAT-GAG-AGC-GAT-G-3’ and reverse AG05 5’-CAA-TGT-CCT-CCT-TCT-TCA-CCT-GCT-TG-3’ primers. A total of three mouse lines were established: two were used for the majority of experiment (270 and 273), although a third establish line (265) was also included in the echocardiographic analysis of aged mice. All protocols were performed in accordance with the “Guide for the Use and Care of Laboratory Animals” published by the National Institutes of Health and approval of the Institutional Animal Care and Use Committee.

Q-trap nano-liquid chromatography tandem mass spectrometry and multiple reaction monitoring analysis. Multiple reaction monitoring (MRM) analysis was performed to determine the content of cTnIP82S in Tg mice. Myofilaments were isolated, as previously described (38). The mixture of peptides from the in gel digestion of cTnI proteins was reconstituted with 20 μL of HPLC water containing 0.1% formic acid. MRM analyses were performed on a 4000 QTRAP hybrid triple quadrupole/linear IT mass spectrometer (AB SCIEX), as previously described (50), operating with Analyst 1.4.2 software scheduled experiments in positive ion mode. Peak detection and quantification of peak area were determined with Multiquant software (SI Heidelberg), as previously described (38). Force was expressed in mili-Newton per millimeter squared (mN/mm²) of cross-sectional area. Muscles size average was as follows, width = 0.17 ± 0.03 mm, thickness = 0.28 ± 0.04 mm, length = 1 ± 0.12 mm, and cross-sectional area = 0.04 ± 0.01 mm². The muscles underwent isometric contractions superfused with no 2,3-butanedione monoximine K-H solution at a rate of 10–12 ml/min, with a voltage stimulus of 3.5–5 V, pacing frequency of 0.5 Hz, at room temperature (21–22°C), and external Ca²⁺ concentration of 2.0 mM. The resting length was set such that resting force was 10–15% of total force development (optimal muscle length). This resting muscle length, corresponding to a resting sarcomere length of 2.2–2.3 μm, as determined previously by laser diffraction, was maintained throughout the experiment. Muscles were allowed to stabilize for 30–45 min, before diffusional loaded with 50 μg fura 2-AM (Invitrogen), as previously described (23). Calcium transients were calculated by the ratio of fluorescent signals excited at 340 and 380 nm, and emitted at 510 nm, using a photomultiplier tube PMT (R1527, Hamamatsu, Japan). The ratio of calcium-sensitive ratiometric indicator was subtracted after subtracting back-ground fluorescence at 340 and 380 nm and expressed as arbitrary units. For skinned fiber studies, similar muscles were exposed for 5–10 min to 1% Triton X-100 in relaxing solution. Skinned steady-state force-extracellular Ca²⁺ concentration relations were obtained as previously described (13), fit to the Hill equation to yield Fmax, or maximal Ca²⁺-activated force; ECa50, the extracellular Ca²⁺ concentration required for 50% of maximal activation; and the Hill coefficient (n Hill).

LV function pressure-volume studies. Pressure-volume relationships were assessed in young mice at baseline and after isoproterenol infusion, and at 10 wk post-TAC, as previously described (2, 6).

Protein analysis. Myofilaments were isolated from snap-frozen ventricles, as previously described (38), and separated by 4–12% bis-its Novex SDS-PAGE (Invitrogen) or by Mn-Phos-tag (Phos-tag Consortium, Japan) acrylamide gels, as previously described (22, 26). Western blots were performed using antibodies against cTnI 1:5,000 (1–87, Spectral Diagnostics), phospho-tropinin I (TnI) (Ser23/Ser24) 1:2,000 (Cell Signaling, Danvers, MA), and myosin-binding protein C (MyBP-C) 1:10,000 (kind gift of Dr. S. Sudayappan).

Molecular modeling cTnIP82S mutation. The molecular models of WT and TnIP82Ser were rendered using the Kortemme Lab server (http://kortemma.gene.com) (43). The resulting molecular model of hTnIP82Ser and WT human cardiac

Chronic pressure overload model. TAC was performed on anesthetized and mechanically-ventilated mice, as previously described (6). Serial echocardiography, including the estimation of LV mass, and physiological studies were performed on these mice.

Histology. Mice were euthanized with a pentobarbital overdose (75 mg/kg) intraperitoneal injection. Hearts were harvested, quickly rinsed in PBS (pH 8.0), then fixed in 4% formaldehyde in PBS (pH 8.0), paraffin-embedded, sectioned (8–10 μm thick), and stained with hematoxylin and eosin and Masson’s trichrome, as previously described (34).

Isolated cardiac muscle studies. Male or female mice (3–9 mo old) were anesthetized with pentobarbital (50 mg/kg) and heparinized (100 U) intraperitoneally. Hearts were excised and perfused retrogradely (~15 ml/min) with dissecting Krebs-Henseleit (K-H) solution equilibrated with 95% O₂ and 5% CO₂. The dissecting K-H solution is composed of the following (in mM): 120 NaCl, 20 NaHCO₃, 5 KCl, 1.2 MgCl₂, 10 glucose, 0.5 CaCl₂, and 2.3-butanedione monoxime, pH 7.35–7.45, at room temperature (21–22°C). Trabeculae or small papillary muscles were quickly dissected from hearts and mounted between a force transducer and a micromanipulator. Force was measured by a custom-made basket attached to a force transducer (SI Heidelberg), as previously described (38). Force was expressed in mili-Newton per millimeter squared (mN/mm²) of cross-sectional area. Muscles size average was as follows, width = 0.17 ± 0.03 mm, thickness = 0.28 ± 0.04 mm, length = 1 ± 0.12 mm, and cross-sectional area = 0.04 ± 0.01 mm². The muscles underwent isometric contractions superfused with no 2,3-butanedione monoximine K-H solution at a rate of 10–12 ml/min, with a voltage stimulus of 3.5–5 V, pacing frequency of 0.5 Hz, at room temperature (21–22°C), and external Ca²⁺ concentration of 2.0 mM. The resting length was set such that resting force was 10–15% of total force development (optimal muscle length). This resting muscle length, corresponding to a resting sarcomere length of 2.2–2.3 μm, as determined previously by laser diffraction, was maintained throughout the experiment. Muscles were allowed to stabilize for 30–45 min, before diffusional loaded with 50 μg fura 2-AM (Invitrogen), as previously described (23). Calcium transients were calculated by the ratio of fluorescent signals excited at 340 and 380 nm, and emitted at 510 nm, using a photomultiplier tube PMT (R1527, Hamamatsu, Japan). The ratio of calcium-sensitive ratiometric indicator was subtracted after subtracting back-ground fluorescence at 340 and 380 nm and expressed as arbitrary units. For skinned fiber studies, similar muscles were exposed for 5–10 min to 1% Triton X-100 in relaxing solution. Skinned steady-state force-extracellular Ca²⁺ concentration relations were obtained as previously described (13), fit to the Hill equation to yield Fmax, or maximal Ca²⁺-activated force; ECa50, the extracellular Ca²⁺ concentration required for 50% of maximal activation; and the Hill coefficient (n Hill).

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The TgcTnP82S mouse model displays normal histology. A: a fragment of cardiac troponin I (cTnI) cDNA where the mutation was introduced is shown in a gray shaded box. This mutant was cloned into a SalI site downstream murine α-myosin heavy chain (MHC) promoter. Right: DNA 1% agarose gel showing two PCR products using primers specific for transgenic (Tg) mice illustrates two founders, from left to right, 270 and 273, positive genotype. B: top: a representative total ion chromatogram from a multiple reaction monitoring (MRM) assay quantifying the relative content of wild-type (WT) cTnI and cTnI P82S peptides with respect to total cTnI. Bottom: a representative calibration curve for mutant cTnIP82S peptide. A serial dilutions of mutant peptide were used to produce a 7-point calibration curve at 10, 20, 30, 40, 50, 80, and 100 pmol/μl for mutant P82S peptide [C(CAM)QSLELDGLGFEELQDLC(CAM)R]. C: TgcTnP82S normal histology in representative hematoxylin and eosin (H&E), and lack of fibrosis in Masson’s staining (×40 top row, and ×100 bottom row). Ntg, nontransgenic.

cTnI PDB files was downloaded, visualized, and aligned in PyMOL version 1.5 for iPad. Statistics. Statistical analysis of the data was performed using Student’s t-test and two-way ANOVA with repeated measurements (twitching muscles relaxation, Ca²⁺ transients kinetics, and echocardiography baseline and serial post-TAC analysis). A value of P < 0.05 was used to indicate significant differences between control and Tg mice. Pooled data were expressed as means ± SE.

RESULTS

TgcTnP82S protein expression by MRM. To detect a mutant protein that has no tags, we developed a MRM assay using radiolabeled synthetic peptides as internal standards (50). We isolated myofilaments from three snap-frozen hearts for non-Tg (Ntg) and each Tg line (270 and 273). These samples...
were processed in parallel to quantify the expression of cTnIP82S by mass spectrometry. The MRM assay was analyzed by a 4000 QTRAP hybrid triple quadrupole/linear IT mass spectrometer (AB SCIEX). Our results revealed that the stoichiometry of cTnIP82S Tg replacement was 7.85% (7.57% ± 0.29 for line 270 and 8.13% ± 0.06 for line 273) (Fig. 1B).

cTnIP82S expression impairs diastolic function of aged-mice. To delineate whether cTnIP82S murine heart expression replicates the signs of hypertrophy and/or cardiac fibrosis, cardiac function was evaluated in aged Tg and Ntg mice (14.5 ± 1.8 mo old). For the initial characterization of this Tg model, we used M-mode echocardiography and TDI. Interestingly, TDI of aged Tg mice consistently displayed an ~19% increase in isovolumetric relaxation time (IVRT) (Ntg 24.06 ± 0.12 ms vs. Tg 28.64 ± 0.18 ms, P = 0.022), increased Tei index (Ntg 1.25 ± 0.03 vs. Tg 1.46 ± 0.04, P = 0.025), and normal ejection time and isovolumetric contraction time (Table 1). In addition, the dynamics of tissue Doppler waves velocities were analyzed [as early diastolic myocardial velocity (Ea) and late diastolic myocardial velocity (Aa) cm/s] and their respective Ea-to-Aa ratios. In agreement with Tei index, Ea was significantly slower, and Ea-to-Aa ratios significantly larger in Tg (Table 1), also indicative of diastolic dysfunction. In contrast, there were no echocardiographic differences between Tg and Ntg littermates at younger age (4.2 ± 0.02 mo old) (Table 2). Interestingly, no signs of overt hypertrophy or fibrosis were evident in old or young Tg mice, as determined by heart-to-body weight ratios (Table 1) and by light microscopy analysis of hematoxylin and eosin and Masson’s stained slides (Fig. 1C). Thus the selective impairment of diastolic function in aged mice, in the absence of cellular hypertrophy or fibrosis, suggests that cTnIP82S expression might alter myofilament properties and diastolic function in an age-dependent manner without findings suggestive of a typical HCM phenotype.

Table 1. Aged-mice contractility and LV chamber dimensions by M-mode, tissue Doppler echocardiography, and gross pathology

<table>
<thead>
<tr>
<th></th>
<th>Ntg</th>
<th>Tg/cTnIP82S</th>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Age, mo</td>
<td>13.8 ± 1.2</td>
<td>15.2 ± 2.1</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>677 ± 33</td>
<td>682 ± 34</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.86 ± 0.03</td>
<td>2.88 ± 0.04</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.21 ± 0.03</td>
<td>1.24 ± 0.04</td>
</tr>
<tr>
<td>FS, %</td>
<td>57.85 ± 0.15</td>
<td>57.16 ± 0.16</td>
</tr>
<tr>
<td>IVCT, ms</td>
<td>16.73 ± 0.09</td>
<td>17.00 ± 0.01</td>
</tr>
<tr>
<td>ET, ms</td>
<td>33.00 ± 0.12</td>
<td>31.64 ± 0.13</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>24.06 ± 0.12</td>
<td>28.64 ± 0.18*</td>
</tr>
<tr>
<td>Tei index</td>
<td>1.25 ± 0.03</td>
<td>1.46 ± 0.04*</td>
</tr>
<tr>
<td>Ea, cm/s</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Aa, cm/s</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Ea/Aa ratio</td>
<td>1.35 ± 0.05</td>
<td>0.78 ± 0.05*</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>152 ± 15</td>
<td>147 ± 7</td>
</tr>
<tr>
<td>Heart weight/body weight ratio, mg/g</td>
<td>4.68 ± 2.41</td>
<td>4.81 ± 2.4</td>
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</table>

Values are averages ± SE; n, no. of mice. Ntg, nontransgenic; HR, heart rate; LVEDD, left ventricle end-diastolic dimension; LVESD, left ventricle end-systolic dimension; FS, fractional shortening; IVCT, isovolumetric contraction time; ET, ejection time; IVRT, isovolumetric relaxation time; Tei index = (IVCT + IVRT)/ET; Ea, tissue Doppler imaging early diastolic myocardial velocity; Aa, tissue Doppler imaging late diastolic myocardial velocity. *P value ≤ 0.05.

Table 2. Young mice (17 wk) contractility and LV chamber dimensions by M-mode and tissue Doppler echocardiography

<table>
<thead>
<tr>
<th></th>
<th>Ntg</th>
<th>Tg/cTnIP82S</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>711 ± 12</td>
<td>688 ± 5</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.94 ± 0.05</td>
<td>2.93 ± 0.04</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.14 ± 0.03</td>
<td>1.12 ± 0.03</td>
</tr>
<tr>
<td>FS, %</td>
<td>61.3 ± 0.15</td>
<td>69.9 ± 0.55</td>
</tr>
<tr>
<td>IVCT, ms</td>
<td>16.43 ± 0.15</td>
<td>17.67 ± 0.13</td>
</tr>
<tr>
<td>ET, ms</td>
<td>43.71 ± 0.25</td>
<td>43.33 ± 0.2</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>19.29 ± 0.18</td>
<td>20.33 ± 0.15</td>
</tr>
<tr>
<td>Tei index</td>
<td>0.82 ± 0.04</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td>Ea, cm/s</td>
<td>0.06 ± 0.003</td>
<td>0.09 ± 0.015</td>
</tr>
<tr>
<td>Aa, cm/s</td>
<td>0.05 ± 0.004</td>
<td>0.06 ± 0.011</td>
</tr>
<tr>
<td>Ea/Aa ratio</td>
<td>1.45 ± 0.1</td>
<td>1.36 ± 0.1</td>
</tr>
<tr>
<td>LV mass</td>
<td>91.5 ± 3.27</td>
<td>95.99 ± 4.68</td>
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</table>

Values are averages ± SE; n, no. of mice. LV mass = 1.055 × [(LVESD + LVEDD + LVPWDTED)\(^3\)] − (LVEDD\(^3\)), where IVSD is interventricular septal thickness at end-diastole, and LVPWDTED is LV posterior wall thickness at end diastole. *P value ≤ 0.05.
The diastolic response to β-adrenergic stimulation was “negative”, meaning that Tg mice rates of relaxation become slower rather than faster, delta for minimum rate constant of pressure rise \( (dP/dt_{\text{min}}) \) (Ntg \(-1.231 \pm 450 \text{ mmHg/s} \) vs. Tg \(+2.266.7 \pm 1.430 \text{ mmHg/s}, n = 6 \) vs. \( n = 5 \), \( P < 0.05 \) (Fig. 3E) and delta for isovolumetric relaxation constant \( (\tau) \) (Ntg \(-0.47 \pm 0.21 \text{ vs. Tg} \:+0.26 \pm 0.21, n = 6 \) vs. \( n = 5 \), \( P < 0.05 \) (Fig. 3F). These negative effects on relaxation rates did not seem to be mediated by altered myofilament phosphorylation in response to β-adrenergic stimulation. Myofilament phosphorylation pattern was estimated by SDS-PAGE followed by phosphor-TnI Ser23/24 WB (Fig. 3G) or by ProQ Diamond and Sypro Ruby staining (Fig. 3H). These results suggest that cTnI P82S blunts augmentation of Ca\(^{2+}\) transient amplitude and whole organ contractility in response to acute β-adrenergic stimulation.
cTnI proline 82 serine exacerbates pressure overload-induced maladaptive hypertrophy. Because cTnIP82S has been potentially associated with elderly onset HCM in humans, we tested if cTnIP82S exacerbated hypertrophy when the Tg mice were subjected to chronic pressure overload by TAC. To this end, cardiac function was assessed by echocardiography at baseline (pre-TAC) and followed at 1, 2, 3, 5, 7, and 10 wk post-TAC. Interestingly, our data revealed that, although 4-mo-old Tg mice appear normal, at 1 wk post-TAC, they displayed a significant increase in IVRT; see representative M-mode and Fig. 3.

**Fig. 3.** In vivo β-adrenergic dose-response is blunted in TgcTnIP82S. A: representative Ntg (n = 6) and Tg (n = 5) pressure-volume (PV) loops at baseline (solid line) and at highest point of Iso dose-response (10, 20, 40, and 80 ng·kg⁻¹·min⁻¹) (dashed line). Notice on left, a leftward and upward shift of Ntg PV loop, typical of adequate contractile adrenergic response, whereas the right shows the failure of TgcTnIP82S to respond to β-adrenergic stimulation. LVP, left ventricular pressure; LVV, left ventricular volume; ESPVR, end-systolic PV relationship. B: mean results for Iso dose-response of maximal rate constant of pressure rise (dP/dt max). TgcTnIP82S mice show a blunted dose-dependent augmentation of systolic function vs. Ntg. *P < 0.001 is for group interaction terms analysis by two-way ANOVA repeated measures (RM). C: change (Δ) in dP/dt max. D: maximal rate of pressure rise normalized to instantaneous pressure (dP/dt/Tmax). E: 100% peak negative dP/dt (dP/dt min). F: isovolumetric relaxation constant (τ), which was calculated by logistic regression. G: myofilament from Ntg and TgcTnIP82S that were subject to Iso dose-response showed no difference in TnI phosphorylation of Ser23/24. H: myofilament preparations from Ntg and TgcTnIP82S that were subject to Iso dose-response were determined by Pro-Q staining (left) and normalized to total protein content, determined by Sypro Ruby (middle). Comparison of normalized phosphorylation levels expressed as ratio of ProQ/Sypro Ruby signals. Phosphorylation pattern is not significantly different (right). MyBP-C, myosin-binding protein C; MLC, myosin light chain. Values are means ± SE.
TDI echocardiography (Fig. 4A) (NTg 28.7 ± 0.39 ms vs. Tg 35.57 ± 0.44 ms, n = 8 vs. n = 7, P < 0.05), which is suggestive of diastolic dysfunction (Fig. 4B). In addition, 7 wk post-TAC Tg mice showed, by means of echocardiography, that genotype exacerbated hypertrophic response (Fig. 4C), where LV mass (NTg 158.35 ± 1.3 mg vs. Tg 196.79 ± 1.48 mg, n = 8 vs. n = 7, P < 0.05 two-way ANOVA) and LV chamber dilatation were significantly increased (Fig. 4D). Furthermore, terminal LV pressure-volume studies (see representative traces in Fig. 4E) revealed that cTnIP82S mice had impaired overall systolic, as judged by dP/dt max normalized to instantaneous pressure (Fig. 4F) or by dP/dt max (Fig. 4G), and abnormal diastolic function, as evidenced by reduced dP/dt min (Fig. 4H) and prolonged τ (Fig. 4I), compared with NTg after chronic pressure overload (See Table 3). These results suggest that cTnIP82S amplifies hypertrophic response to TAC, worsens adverse negative chamber remodeling, and exacerbates TAC-induced heart failure.

cTnIP82S selectively impairs myofilament cooperativity. To gain further insights into the impact of cTnIP82S variant on myofilament function, we evaluated force-calcium relationships in skinned fibers (Fig. 5A). Surprisingly, Tg mice exhibited a marked depression in myofilament cooperativity, as estimated by the n Hill coefficient (Tg 1.72 ± 0.17 vs. NTg 3.09 ± 0.44, n = 7 vs. n = 5, P < 0.05) (Fig. 5D), while no significant effect was observed on myofilament calcium sensitivity.
Fig. 5. Steady-state force-Ca$^{2+}$ relationships in skinned fibers. A: pool raw force data averaged and fitted to modified Hill equation: Ntg ($n = 5$), Tg ($n = 6$). $B$: averaged maximal Ca$^{2+}$ activated force ($F_{\text{max}}$). Note the trend of Tg to reduced $F_{\text{max}}$ vs. Nonsignificant. $C$: Ca$^{2+}$ sensitivity of Tg is modestly increased. $EC_{50}$, extracellular Ca$^{2+}$ concentration required for 50% of maximal activation $D$: myofilament cooperativity (n Hill, Hill coefficient) is markedly reduced in Tg muscles. * $P < 0.05$. $E$, left: TnI phosphorylation on Ser23/24 is not affected. PhosTag-SDS-PAGE show that phosphorylation stoichiometry is unchanged for TnI (middle) and MyBP-C (right). F: phosphorylation of myofilament preparations from Ntg and TgcTnIP82S were determined by Pro-Q staining (left) and normalized to total protein content, determined by Sypro Ruby (middle). Molecular weight markers are labeled 1 (Precision-C, Bio-Rad), 2 (Peppermint Stick, Inv), and 3 (MagicMark XP, Inv) with myofilament protein migration highlighted by a line. Right: comparison of normalized phosphorylation levels expressed as ratio of ProQ/Sypro Ruby signals. Phosphorylation pattern is not significantly different. Values are means ± SE.

**DISCUSSION**

Here we report three major novel findings. First, cTnIP82S induces a late-onset diastolic dysfunction, without the presence of hypertrophy, tissue disarray, or fibrosis. The second major observation is that, in younger Tg mice, this mutation blunts β-adrenergic response and exacerbates maladaptive hypertrophy as well as pump failure in pressure-overloaded hearts. Finally, at the myofilament level, cTnIP82S selectively impairs cooperativity and, consequently, overall cardiac contractility.

cTnIP82S leads to age-dependent diastolic dysfunction. Since its first identification, cTnIP82S has been considered a potential disease-causing variant (36). However, later studies suggested that it was a benign polymorphic variant (31).
Previously, our group reported a patient with MHY7 R453S and TNNI3 cTnIP82S compound mutations, had severe disease (11). Based on this association as well as the confirmation that this is a variant present in ~3% of the African American population, we hypothesized that cTnIP82S could be a relatively common disease-causing mutation or a disease modifier. In this study, we provide strong evidence that, despite low levels of protein expression, cTnIP82S has adverse effects on cardiac function, and that these effects seem to be amplified by hemodynamic stress and by advanced age. Echocardiography confirmed altered diastolic dysfunction in aging mice. However, notably, cardiac dysfunction occurred in the absence of evidence for adverse hypertrophy or cardiac fibrosis. Intriguingly, present data confirmed previous observations showing the occurrence of diastolic dysfunction (as indexed by TDI) before the onset of hypertrophy or tissue abnormalities. This sequence of events was documented in several animal models of HCM (3, 10, 15, 42), RCM (8), and even in patients with β-MHC (16), MyBP-C (5, 29, 35), Tnl (30), and troponin T (TnT) (35) mutations. Given the consistency of this observation in different experimental and human cardiomyopathy contexts, diastolic dysfunction detected by TDI has been proposed as an early diagnosis (16, 35) or prognosis tool (24) in genotype (+) but hypertrophy-negative individuals. While diastolic dysfunction appears in aged cTnIP82S mice, it is also notable the profound effect of increased afterload in the cTnIP82S mice, which have more severe systolic and diastolic dysfunction when TAC is performed in younger mice. In the latter, recapitulating the full adverse features of the cTnIP82S mutation required the presence of sustained hemodynamic stress. Overall, these data suggest that TnIP82S could predispose a small but significant percent of the African American population to cardiac diastolic dysfunction late in life and to more profound effects if exposed to additional stressors earlier in adulthood. Given the compelling evidence of increased risk of hypertension in African Americans (9), this may be quite important on a population-wide basis.

β-Adrenergic stimulation and increased afterload precipitate cardiac dysfunction in Tg cTnIP82S mice. Diastolic dysfunction is a silent early feature, common to HCM, RCM, and DCM, that can often be uncovered by exercise and dobutamine echo test (40, 49). Accordingly, baseline echocardiography in young adult (17–19 wk) Tg cTnIP82S did not reveal any abnormalities. However, under β-adrenergic stimulation, LV pressure-volume analysis uncovered an underlying cardiac dysfunction. Interestingly, this was true even at maximal concentrations of isoproterenol. Our findings are in agreement with others; since Tg TnT(I79N) mice also displayed minimal hypertrophy with no fibrosis under basal conditions, normal whole heart contractility in the presence of abnormal myofilament properties, such as increased myofilament Ca$^{2+}$ sensitivity and Mg-ATPase activity (9). However, when challenged with β-adrenergic stimulation, Tg TnT(I79N) showed no significant systolic and diastolic response. Chronic β-adrenergic stimulation resulted in overall cardiac dysfunction and sudden cardiac death by ventricular arrhythmias (25). Likewise, TgTnIP82S excitation-contraction coupling analysis uncovered that β-adrenergic-dependent increase of Ca$^{2+}$ transient amplitude was significantly lessened in Tg muscles. In addition, when TAC was induced to increase afterload, there was a prolongation of IVRT at 1 wk after the procedure, which suggests a deficient compensatory hemodynamic response; however, by 7 wk post-TAC, a progressive chamber remodeling and exacerbated LV hypertrophy was evident. Our findings are in contrast with those of HCM model α-MHC (R403Q) Tg mouse. Although Tg α-MHC R403Q mice also displayed abnormal excitation contraction coupling and reduced myofilament cooperativity (14), when subjected to TAC the mice did not develop more LV hypertrophy than Ntg mice (39). While these differences can be attributed to diverse strain genetic backgrounds, cTnIP82S may confer a particular susceptibility to LV hypertrophy with coexisting pressure overload, such as uncontrolled systemic hypertension.

Our data suggests that cTnIP82S might recapitulate some of the phenotypic features, both at the myofilament and whole heart levels, of previously reported HCM mouse models where TnT or α-MHC function is compromised.

β-Adrenergic response, TnI interactions, and myofilament cooperativity. Site-specific phosphorylation of cTnI, in concert with MyBP-C, is one of the major regulators of systolic and diastolic kinetic properties, both at rest and during β-adrenergic stimulation (18, 19, 45). β-Adrenergic stimulation increases Ca$^{2+}$ transients through a rapid phosphorylation and functional activation of key calcium handling proteins (27). Although Tg cTnIP82S did increase their calcium transient amplitude in response to isoproterenol, the steepness of their β-adrenergic dose response was significantly blunted. Steady-state force-Ca$^{2+}$ studies in skinned fibers showed a clear isolated defect in myofilament cooperativity (n Hill) in Tg cTnIP82S, whereas myofilament Ca$^{2+}$ sensitivity (ECa_{50}) and F_max were minimally lower, but did not reach statistical significance. Mechanistically, the myofilament cooperativity defect could not be attributed to abnormalities in myofilament phosphorylation patterns. In fact, there were no changes in phosphorylation of cTnI and MyBP-C (See Fig. 5E). For example, phos-Tag acrylamide SDS-PAGE followed by immunoblots demonstrated no significant differences in phosphorylation stoichiometry. Similarly, phosphorylation at cTnI Ser23/Ser24 was unchanged between Ntg and Tg, at baseline or during isoproterenol stimulation. Previously, it has been suggested that cardiac muscle relaxation rates are dictated by myofilament intrinsic properties (20). Thus, because the Ca$^{2+}$ transient’s amplitude was overall lower but produced similar force, it is possible that myofilaments might be sensitized to Ca$^{2+}$ in the concentration range of dynamic twitching muscles, without showing evident changes in ECa_{50} estimated by skinned muscles during steady-state force-Ca$^{2+}$ relationships (38). Such increase in myofilaments Ca$^{2+}$ sensitivity could contribute significantly to the impairment of diastolic function found in the intact Tg cTnIP82S heart. A TnI mutant (TnIR193H) increases myofilament ECa_{50} in skinned muscles during steady-state force-Ca$^{2+}$ relationships, and it is associated with RCM and severe diastolic dysfunction. However, a recent Tg TnIR193H mouse model study demonstrated impaired myofilament cooperativity with normal myofilament Ca$^{2+}$ sensitivity in skinned muscles steady-state force-Ca$^{2+}$ relationships. Their steady-state findings were in sharp contrast with the phenotype observed in dynamic twitching muscles with clear blunted Ca$^{2+}$ transients amplitude and slower kinetics of decay, which ultimately explain the in vivo elevated diastolic pressure and diastolic dysfunction (8).
The cTnPro82Ser missense mutation occurs in an evolutionarily conserved proline residue that is localized in the hinge of IT arm and TnI-TnT interactions in H2 region (see Fig. 6) and thus likely to affect function. Our molecular modeling of Pro82Ser mutant show that this amino acid residue change may alter the hinge in the IT arm and set H2 TnI region further apart from TnT (See TnIWT and TnIP82Ser overimposed structures, Fig. 6A). Similarly, the model of TnIP82Ser of Ramachandran and colleagues (37) pointed out that serine substitution might result in a hydrogen bond interaction with the butyl ammonium nitrogen of Lys234 in TnT and perturb TnI-TnT interactions. Thus TnIP82Ser appear to have similar structural and phenotypic effects as the dominant negative mutation in TnI Cys111Arg, which also decrease the binding affinity of TnI for TnT. Remarkably similar to the TnIP82Ser mutation, cTnI Cys111Arg also impairs diastolic function and blunts the adrenergic response of cardiac muscle (1, 47, 48). Thus TnIPro82Ser appear to have similar structural and phenotypic effects as the dominant negative mutation in TnI Cys111Arg, which also decrease the binding affinity of TnI for TnT. Remarkably similar to the TnIP82Ser mutation, cTnI Cys111Arg also impairs diastolic function and blunts the adrenergic response of cardiac muscle (1, 47, 48).

Translational implications. Our novel findings are of special clinical interest if we take into consideration that cTnIP82S variant is moderately prevalent (~3%) in certain ethnic groups, such as African Americans (11) and/or Afro-Caribbean (32), and that familial HCM patients (~5–6%) may have either double (>1 mutation in same gene) or compound (>1 mutated gene) genotypes (17). In particular, the Tg cTnIP82S mouse model ads mechanistic weight to our previous clinical observation that led us to speculate that a cTnIP82S + β-MHC R453S genotype was associated with a more severe phenotype (11). A phenotype-genotype cosegregation analysis would greatly help to understand the role of cTnIP82S in families with asymptomatic heterozygous carriers. This study holds potential in helping to clarify the clinical significance of cTnIP82S mutation when found in young asymptomatic, or in older, patients displaying LV hypertrophy or diastolic dysfunction.

Limitations. This Tg cTnIP82S mouse model achieved modest TnI replacement in vivo, an MRM assay estimated an ~7–8% proportion of mutant protein. The latter is a possible explanation for the cTnIP82S late-onset phenotype and cardiac dysfunction only under hemodynamic stress. The absolute level of expression of the mutant protein is generally unknown in human HCM; however, it has been demonstrated in murine models even low levels of mutant myofilament protein may lead to significant physiological effects (23, 33, 44). It is likely that several lines with increasing percentages of TnI replacement would be needed to assess the impact of higher levels of TnIP82S protein, although it is striking that even low expression leads to a late onset and stress-related phenotype, likely analogous to the “elderly onset” human HCM phenotype originally ascribed to this mutation.

Conclusions. Our study reveals that sustained myocardial expression of cTnIP82S in mice is sufficient to cause late-onset cardiac diastolic dysfunction. Furthermore, cTnIP82S mutant blunts the heart’s ability to respond during adrenergic stimulation and exacerbates maladaptive hypertrophy, as well as pump failure in response to pressure overload stress. The precise mechanism(s) underlying the development of late-onset HCM in the presence of a cTnIP82S mutation in humans is still unknown. Our data indicate that cTnIP82S is a significant myofilament mutation that may justify life-long monitoring of diastolic function in heterozygous carriers, as well as aggressive treatment of comorbid conditions, such as hypertension or coronary artery disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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