Cardiac Dysfunction in Hypertrophic Cardiomyopathy Mutant Tropomyosin Mice Is Transgene-Dependent, Hypertrophy-Independent, and Improved by β-Blockade

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Abstract—Familial hypertrophic cardiomyopathy (FHC) has been linked to mutations in the contractile proteins of the cardiac sarcomere, including α-tropomyosin (Tm). Mice expressing αTm in the heart were developed to determine the effects of FHC mutant Tm on cardiac structure and function from single cardiac myocytes to whole organ function in vivo. Expression of E180G mutant Tm did not produce cardiac hypertrophy or detectable changes in cardiac muscle morphology. However, E180G mutant Tm expression increased the Ca\(^{2+}\) sensitivity of force production in single cardiac myocytes in a transgene expression–dependent manner. Contractile dysfunction in single myocytes manifested organ level dysfunction, as conductance-micromanometry showed E180G Tm mice had significantly slowed relaxation (diastolic dysfunction) under physiological conditions. The diastolic dysfunction in E180G Tm mice was no longer evident during β-blockade because propranolol eliminated the effect of E180G Tm to slow myocardial relaxation. Cellular and organ level dysfunction were evident in E180G Tm mice in the absence of significant cardiac structural abnormalities normally associated with FHC. These findings therefore suggest that diastolic dysfunction in FHC may be a direct consequence of FHC mutant protein expression. In addition, because diastolic dysfunction in E180G Tm mice is dependent on inotropic status, cardiovascular stress may play an important role in FHC pathogenesis. (Circ Res. 2002; 91:255-262.)

Key Words: tropomyosin ■ hypertrophic cardiomyopathy ■ calcium ■ heart ■ mice

Familial hypertrophy cardiomyopathy (FHC) has been linked to mutations in the contractile proteins of the cardiac sarcomere, including the β-myosin heavy chain (MyHC) and associated light chains (MLC), and the cardiac isoforms of myosin binding protein C, troponin T (TnT), troponin I (TnI), actin, and α-tropomyosin (Tm).\(^{1}\) Although FHC is generally characterized by unexplained cardiac hypertrophy, diastolic dysfunction, myocardial disarray, and increased risk of sudden death, FHC is clinically and genetically divergent with variable disease penetrance.\(^{1}\) While patients with the A63V, K70T, and E180G Tm mutations show mild to moderate cardiac hypertrophy and family histories of sudden cardiac death, patients with the D175N Tm mutation have a consistent, benign clinical outcome.\(^{2}\) It is still unclear, and of interest to understand, how specific mutations affect contractile protein structure and function, cardiac contractility, and contribute to the differential clinical features of FHC.

A combination of in vitro biochemical and in vivo gene-targeted and transgenic mouse studies has begun to establish the dominant-negative effects of FHC mutations on contractile protein structure and function and heart function.\(^{3,4}\) Recently, characterization of mice with cardiac-specific expression of murine D175N mutant Tm was reported.\(^{5}\) Mice with high levels of mutant Tm expression (>60% mutant Tm) had a mild cardiac phenotype of impaired contraction and relaxation with sparse myocyte disarray and no significant cardiac hypertrophy. However, adenoviral-mediated gene transfer to adult cardiac myocytes in vitro has recently shown that the FHC mutant Tm proteins display an allele-specific hierarchy (A63V>K70T>E180G>D175N= wild-type) causing hypersensitivity of the Ca\(^{2+}\) activation of cardiac muscle contraction.\(^{6}\) Therefore, we hypothesized that mice expressing an E180G FHC mutant Tm might reveal a more marked FHC phenotype and help establish the role of different Tm mutations in the differential pathogenesis of FHC.

In this report, we show the functional effects of cardiac-specific expression of human E180G mutant Tm in transgenic mice. We determined the transgene dose-dependent effect of E180G Tm expression on Ca\(^{2+}\) activation of cardiac muscle force generation in single cardiac myocytes. In addition, using conductance-micromanometry to measure pressure-volume relationships in the heart,\(^{7}\) we examined the impact of...
Expression Analysis

Total RNA was isolated from mouse hearts using RNeasy (Qiagen). Northern blots used a random-prime–labeled (Amersham) human Tm cDNA or murine GAPDH as probes and were quantified by phosphorimaging. Total protein expression was analyzed by Western blotting as described. Myofilament-bound protein was analyzed from myocytes permeabilized with 0.1% Triton X-100 in relaxing solution as described. Western blots were probed with the monoclonal Tm antibody Tm311 (Sigma, 1:10,000), TnI antibody MAB 1691 (Chemicon), or TnT antibody IF-2 (Research Diagnostics). The migration of human E180G Tm was confirmed in extracts from HEK-293 cells treated with AdvTmE180G.

Morphological and Histological Analysis

Hearts were weighed and then fixed in 4% paraformaldehyde in PBS and paraffin embedded or frozen and sectioned. Paraffin sections were stained with hematoxylin and eosin (Sigma) or Masson’s Trichrome (Sigma). Frozen sections were immunolabelled with monoclonal sarcomeric Tm antibody CH-1 (Sigma) as described.

Echocardiography

Mice were anesthetized with 1.5% isoflurane. Two-dimensionally guided M-mode recordings were obtained from the short axis view at the level of the papillary muscles using an Acuson Sequoia system and a 15-MHz linear array transducer (Acuson). Left ventricular end-systolic (ESD) and end-diastolic (EDD) dimensions, as well as systolic and diastolic wall thickness, were measured from the M-mode tracing using the leading-edge convention of the American Society of Echocardiography. Left ventricular shortening fraction (SF) was calculated using the following formula: SF = (EDD − ESD)/EDD.

Single Adult Cardiac Myocyte Isometric Force Measurements

The Ca2+-activated force production in single permeabilized cardiac myocytes from 4-month-old transgenic mice was measured as previously described.

In Vivo Cardiac Hemodynamics

Conductance micromanometry was modified from methods previously described. Mice aged 10 to 11 months were anesthetized by inhalation of 5% to 2% isoflurane and body temperature was maintained at 37°C with a heating pad. The exposed trachea was cannulated with a 20-gauge tubing adapter, and the mouse was ventilated with 98% O2, 1.5% to 2% isoflurane at 120 breaths per minute using a pressure-controlled rodent ventilator (Kent Scientific). The jugular vein was cannulated with a 30-gauge needle. Mice were infused with NaCl, 10% human serum albumin to maintain resting blood pressure. A midline thoracotomy was performed under a dissecting microscope using cautery to minimize bleeding. A 1.4 French miniaturized pressure-conductance catheter (SPR-719, Millar Instruments) was inserted into the left ventricle via an apical stab made with a 25-gauge needle. Pressure-volume loops were collected online at 1000 Hz. Dobutamine (20 ng·min⁻¹·kg⁻¹) was infused over 3 minutes and propranolol (75 mg per kg IP) was used to examine cardiovascular function under increased and decreased β-adrenergic stimulation, respectively. The conductance attributed to parallel conductance was estimated by injection of a 10-μL bolus of 20% saline into the jugular vein as previously described. Individual blood conductance was calibrated using fresh heparinized blood in 4 cylindrical chambers drilled in a lucite block with diameters of 2 to 5 mm as described. In preliminary experiments, the volume calibration method was validated using a Doppler flow probe (2R, Transonic Systems, Inc) attached to the descending aorta to estimate cardiac output. Two-second data sweeps
(10 to 20 loops) were analyzed with PVAN 2.7 software (Millar Instruments Inc).

**Back-Phosphorylation Assay**

Back-phosphorylation experiments were performed as described, and methods are included in the online data supplement.

**Statistics**

Data presented are mean±SEM. Statistical differences were determined by ANOVA and a Student-Neuman-Keuls post hoc test for statistical significance (*P*<0.05).

An expanded Materials and Methods section can be found in the online data supplement at http://www.circresaha.org.

**Results**

**Transgenic Mice Expressing Human αTm and Human αTm E180G**

The E180G mutant Tm protein displays unique migration on SDS-PAGE allowing for direct assessment of αTmE180G protein expression by Western blotting (Figure 1B). Human αTm and mouse αTm differ by one conserved amino acid substitution and are indistinguishable on SDS-PAGE. However, transgene-derived transcripts were detected by Northern analysis using a human αTm probe (Figure 1C). Line 169 WT Tm showed levels of transgene RNA transcripts similar to that of line160 E180G. Line 169 WT Tm was used as a control for mRNA overexpression of normal human αTm. Line 135 E180G (50% E180G Tm) and line 160 (60% E180G Tm) were both analyzed to address possible transgene insertion effects. In addition, expression of mutant Tm in these lines is close to that hypothesized to be present in FHC patients heterozygous for the E180G Tm mutation. Previously we have shown that E180G Tm incorporates normally into sarcomeres when expressed in isolated cardiac myocytes.

Further, skeletal muscle from HCM patients with a Tm mutation show equivalent amounts of normal and mutant Tm. Tm stoichiometry was determined by measuring the total Tm expression per milligram total protein or the ratio of total Tm to troponin T expression (Figure 1D). Despite marked expression of E180G αTm in lines 135 and 160, there was no significant change in the total amount of Tm protein expression (Figure 1D). This maintenance of contractile protein stoichiometry is consistent with previous studies using transgenesis to express Tm and cardiac contractile proteins in the heart.

**FHC Mutant Tm Expression and Heart Morphology**

Histological analysis of hearts from 12-month-old mice expressing WT Tm or E180G Tm revealed no detectable changes in gross chamber morphology, interstitial fibrosis, or myocyte disarray when compared with NTG littermates (Figure 2). However, the HW/BW ratio was slightly decreased in line 135 E180G mutant Tm mice (4.01±0.12, n=10; *P*<0.05 versus NTG and WT Tm) and line 160 E180G mutant mice (4.35±0.16, n=10; *P*<0.05 versus WT Tm) compared with NTG (4.67±0.08, n=32) and line 169 WT Tm mice (4.85±0.10, n=10). This difference was not seen at 3 to 4 months of age (data not shown). Given the age dependence and the lack of any fibrosis or necrosis in the E180G Tm mice, cardiac myocyte–expressing E180G Tm mice likely fail to hypertrophy normally with age as has been shown FHC mutant TnT and mutant MLC mice.

Measurements of wall thickness and EDD by M-mode echocardiography were unchanged in 10- to 12-month-old mice expressing E180G Tm (line 160) indicating the lack of significant hypertrophy or dilatation (Table 1). However, a significant increase in shortening fraction was apparent in mice expressing E180G Tm compared with NTG mice and mice expressing WT Tm. Expression of WT Tm in transgenic mice had no significant effect on any of the measurements.

**Table 1. Heart Morphology in 11- to 12-Month-Old Transgenic Mice by Echocardiography**

<table>
<thead>
<tr>
<th>Variable</th>
<th>NTG</th>
<th>WT Tm, line 169</th>
<th>E180G Tm, line 160</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDD, cm</td>
<td>0.366±0.013</td>
<td>0.334±0.012</td>
<td>0.336±0.012</td>
<td>NS</td>
</tr>
<tr>
<td>ESD, cm</td>
<td>0.225±0.012</td>
<td>0.190±0.012</td>
<td>0.164±0.016</td>
<td>0.05*</td>
</tr>
<tr>
<td>IVS, cm</td>
<td>0.073±0.002</td>
<td>0.081±0.004</td>
<td>0.080±0.003</td>
<td>NS</td>
</tr>
<tr>
<td>LVPW, cm</td>
<td>0.084±0.006</td>
<td>0.098±0.006</td>
<td>0.077±0.006</td>
<td>NS</td>
</tr>
<tr>
<td>SF, %</td>
<td>38.9±1.8</td>
<td>43.0±2.1</td>
<td>52.1±3.3</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

Data are mean±SEM. ANOVA with a Student-Neuman-Keuls test was used for statistics. IVS indicates interventricular septum; LVPW, LV posterior wall.

*NTG vs E180G Tm line 160; †WT Tm line 169 vs E180G Tm line 160.
made by M-mode echocardiography compared with NTG littermates.

When compared with NTG littermate mice, the pattern of sarcomeric Tm immunofluorescence was unchanged in mice expressing WT Tm or E180G Tm (Figure 3). The lack of increased diffuse staining of Tm in mice overexpressing WT Tm or E180G Tm suggests that the expressed Tm is incorporating appropriately into the cardiac sarcomere and not accumulating in the myoplasm. Electron micrographs showed no detectable change in sarcomeric ultrastructure in mice expressing E180G Tm (not shown).

**Effects of E180G Tm Expression of Ca$^{2+}$-Activated Force Production**

Ca$^{2+}$-activated force production was measured in single permeabilized adult cardiac myocytes isolated from NTG littermates, WT Tm, and multiple independent lines of E180G Tm transgenic mice (Figure 4A). There was no significant effect ($P>0.05$) of expressing WT human Tm in the murine heart on the Ca$^{2+}$-activated force production of the adult cardiac myocytes (Figure 4B). However, expression of E180G Tm resulted in a marked increase in the force produced at submaximal Ca$^{2+}$ concentrations, signifying increased Ca$^{2+}$ sensitivity within single cardiac myocytes.

Often a broad range of transgene expression is obtained based on variable transgene copy number and efficiency of transgene expression. The Ca$^{2+}$-activated force properties from myocytes isolated from multiple transgenic lines expressing E180G Tm were characterized to establish a dose-dependent effect of E180G mutant Tm expression on contractile dysfunction. After each experiment, E180G Tm expression was determined in permeabilized cardiac myocytes by Western blot and the Ca$^{2+}$ sensitivity of force production (pCa$_{50}$) was plotted versus the percentage of E180G Tm expression (Figure 4B). Compared with NTG...
Effects of E180G Tm on Murine Hemodynamic Function In Vivo

Murine hemodynamic function was examined in 10- to 12-month-old NTG, WT Tm, and E180G Tm mice. Figures 5A and 5B show representative pressure-volume data measured in NTG, line 169 WT Tm, line 135 (50% E180G Tm), and 160 (60% E180G Tm) transgenic mice. The shapes of the pressure-volume loops in mice expressing E180G Tm are remarkably similar to NTG littermate mice and mice expressing WT Tm. However, the most significant and consistent finding in mice expressing E180G mutant Tm was a slowing of rate of myocardial relaxation (Figure 5C).

Table 2 summarizes the baseline hemodynamic parameters measured in mice, which are highly comparable to those reported by Georgakopoulos et al. Overexpression of WT Tm in the murine heart did not produce significant changes in hemodynamic function. Indices of cardiac contractility were largely unchanged in both lines of E180G Tm mice [cardiac output (CO), stroke work (SW), maximum rate of pressure development (dP/dt max), and maximum developed pressure (P max)]. Consistent with echocardiography, ejection fraction (Table 2) was increased in both E180G Tm–expressing lines (although the difference in line160 E180G did not reach statistical significance compared with NTG mice). Previous work has suggested that in situ measurements of +dP/dt are confounded by potential effects of cardiac preload and afterload. Therefore, the slope of the end-systolic pressure-volume relationship after brief IVC occlusion (E es) was compared (Table 2). In agreement with the +dP/dt measurements, there was no significant change in E es in E180G Tm–expressing mice compared with NTG littermates or 169 WT Tm mice. In contrast, relaxation performance was significantly impaired in both lines of E180G Tm–expressing mice with a significant decrease in the maximal rate of relaxation (dP/dt min) and a significant increase in the time constant (τ) for cardiac muscle relaxation (versus WT and NTG mice). Because line 160 E180G Tm mice showed slightly lower left ventricle (LV) volumes (Tables 1 and 2) compared with WT mice, it is possible that the hearts in this line are operating under slightly decreased afterload, which could in turn slow myocardial relaxation. However, analysis of the −dP/dt versus V max relationship under basal conditions in a large number of NTG mice indicates a slope of −73.0 mm Hg/s per microliter (r²=0.441). This suggests that a >25 μL change in V max would be required to produce the effect on diastolic function seen in the 160E180G Tm line. In addition, the primary effect of E180G Tm on diastolic function is supported in the line 135 E180G Tm mice. Line 135 E180G Tm mice operate at similar pressures and volumes as NTG mice yet have decreased diastolic function similar to line 160. We speculate that the lower ventricular volumes in the line160 E180G Tm mice may be a secondary consequence of decreased filling under these baseline physiological conditions.

To address how the expression of E180G Tm affects cardiac function under conditions where the demand for cardiac performance is altered, hemodynamic function was assessed under both β-adrenergic stimulation and blockade. After assessment of baseline cardiovascular function, 20 ng/g per minute dobutamine, or a 1 mg/kg bolus propranolol injection were used in each mouse to maximally stimulate or block, respectively, β-adrenergic stimulation of the heart. Infusion with dobutamine produced increases in cardiac output (Figure 6) and contractility (dP/dt max, mm Hg/sec, 13 286±425, 13 251±652, 12 456±671, and 11 965±425 in NTG, 169 WT, 160 E180G, and 135 E180G mice, respectively) to levels similar (P>0.05) among all experimental groups. The effect of dobutamine to increase heart rate (HR), CO, +dP/dt reached statistical significance by paired t test.
within all experimental groups (P<0.05). Despite this marked change in cardiac contractility, parameters of diastolic function in all lines of mice were unaffected by dobutamine infusion (P>0.05 paired t test within basal versus dobutamine all experimental groups). Notably, the significant effect of E180G Tm to slow myocardial relaxation was preserved during dobutamine infusion in both transgenic lines (Figure 6). Propranolol treatment markedly diminished myocardial cardiac output in all experimental groups to similar levels (Figure 6) by reducing myocardial contractility (dP/dt max, not shown). Interestingly, the slowing of relaxation induced by E180G Tm expression that was observed under basal and dobutamine infusion conditions was no longer evident in the presence of propranolol treatment. Diastolic function in both E180G Tm lines during β-adrenergic blockade was not significantly different (P>0.05) than NTG and WT Tm mice (Figure 6). The effects of propranolol to reverse the effects of E180G Tm on cardiovascular function suggested that basal myofilament phosphorylation may be altered in E180G Tm–expressing mice. However, back-phosphorylation assays of E180G Tm–expressing mice indicated that basal troponin I phosphorylation was unaltered in both E180G Tm–expressing lines (Figure 6E). Although we did not assess all cardiac phosphoproteins, the effect of propranolol to reverse diastolic function in E180G Tm mice is likely due to a secondary effect of β-blockade on cardiac function, independent of myofilament phosphorylation.

Discussion

Tropomyosin is a key component of the thin filament of the cardiac sarcomere that is involved in the regulation of contraction by intracellular Ca2+. In the present study, we show that adult single cardiac myocytes isolated from mice expressing a FHC mutant Tm display a linear, transgene expression–dependent increase in the Ca2+ sensitivity of isometric force production. Hemodynamic studies under physiological conditions in vivo revealed that contractile dysfunction at the cellular level manifests itself primarily as diastolic dysfunction at the organ level, all in the absence of alterations in muscle structure or chamber morphology. The relative impairment of murine diastolic function caused by E180G Tm expression is alleviated when cardiovascular performance is depressed by β-adrenergic blockade.

Given the absence of cardiac hypertrophy and fibrosis in E180G Tm mice, we conclude that the diastolic abnormalities in E180G Tm mice are directly due to the increased Ca2+ sensitivity of the contractile apparatus. The increase in the Ca2+ sensitivity of force production seen in single cardiac myocytes isolated from multiple independent lines of E180G Tm mice is transgene expression dependent. The establishment of a dose-dependent effect on contractile function in multiple lines is important because high levels of overexpression of reporter genes and normal proteins in some individual transgenic mouse lines can induce cardiomyopathy.17,18 Our results suggest that gene- or protein-based therapies may not need to completely eliminate mutant Tm expression to provide a therapeutic effect. The linearity of the change in Ca2+ sensitivity with increasing E180G Tm expression suggests that mutant tropomyosin proteins, even in heterodimers with normal proteins, exert equally potent dominant-negative effects on contractile function.

Diastolic dysfunction is a hallmark of the FHC phenotype in humans but has been largely attributed to the increases in wall stiffness associated with cardiac hypertrophy and myocardial fibrosis. The presence of diastolic dysfunction without cardiac hypertrophy or myocardial fibrosis in E180G Tm–expressing mice suggests that diastolic dysfunction may be a direct consequence of the E180G mutant Tm to increase the

### Table 2. Baseline Hemodynamic Function In Vivo

<table>
<thead>
<tr>
<th></th>
<th>NTG (n=25)</th>
<th>WT Tm, line 169 (n=8)</th>
<th>E180G Tm, line 135 (n=10)</th>
<th>E180G Tm, line 160 (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>547±7</td>
<td>558±12</td>
<td>511±14</td>
<td>551±12</td>
<td>0.05†</td>
</tr>
<tr>
<td>V max, μL</td>
<td>35.7±1.2</td>
<td>38.5±2.3</td>
<td>36.4±1.1</td>
<td>30.7±1.6</td>
<td>0.05§</td>
</tr>
<tr>
<td>V min, μL</td>
<td>15.8±1.0</td>
<td>18.4±1.7</td>
<td>13.9±1.0</td>
<td>11.6±0.9</td>
<td>0.0§§</td>
</tr>
<tr>
<td>P max, mm Hg</td>
<td>94.9±1.1</td>
<td>94.0±1.8</td>
<td>92.9±1.9</td>
<td>89.6±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>P min, mm Hg</td>
<td>1.9±0.3</td>
<td>1.8±0.5</td>
<td>3.2±0.5</td>
<td>3.4±0.5</td>
<td>0.05‡</td>
</tr>
<tr>
<td>Ees, %</td>
<td>90.1±1.2</td>
<td>90.8±2.1</td>
<td>87.0±2.4</td>
<td>83.4±1.9</td>
<td>0.05†</td>
</tr>
<tr>
<td>Pes, mm Hg</td>
<td>6.4±0.4</td>
<td>7.5±0.8</td>
<td>8.7±0.6</td>
<td>8.7±0.4</td>
<td>0.05‡</td>
</tr>
<tr>
<td>SV, μL</td>
<td>19.9±0.7</td>
<td>20.0±0.8</td>
<td>22.5±0.6</td>
<td>19.2±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>EF, %</td>
<td>56.4±1.6</td>
<td>52.7±1.9</td>
<td>62.0±2.0</td>
<td>62.4±1.7</td>
<td>0.05‡§</td>
</tr>
<tr>
<td>CO, μL/min</td>
<td>10 892±391</td>
<td>11 196±570</td>
<td>11 472±444</td>
<td>10 581±585</td>
<td>NS</td>
</tr>
<tr>
<td>SW, mm Hg · μL</td>
<td>1594±56</td>
<td>1551±67</td>
<td>1727±45</td>
<td>1423±98</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/sec</td>
<td>9796±193</td>
<td>9466±395</td>
<td>9736±207</td>
<td>1015±375</td>
<td>NS</td>
</tr>
<tr>
<td>Fα</td>
<td>4.27±0.34</td>
<td>3.83±0.52</td>
<td>4.72±0.52</td>
<td>4.90±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt min, mm Hg/sec</td>
<td>-9361±144</td>
<td>-9173±259</td>
<td>-7775±226</td>
<td>-7535±285</td>
<td>0.001†‡§</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>5.42±0.10</td>
<td>5.66±0.23</td>
<td>6.78±0.22</td>
<td>6.76±0.19</td>
<td>0.001†‡§</td>
</tr>
</tbody>
</table>

Data collected using murine conductance micromanometry. Data are mean±SEM. ANOVA with a Student-Neuman-Keuls post hoc test was used for statistics. P max indicates end systolic pressure; P min, end diastolic pressure; EF, ejection fraction; and Ees, end-systolic pressure-volume relationship.

*NTG vs 135 E180G; †109 WT vs 135 E180G; ‡NTG vs 160 E180G Tm; §169 WT Tm vs 160 E180G Tm.
**Ca^{2+}** sensitivity of force production. The single myocyte dose-response results suggest that even small modulations of steady-state **Ca^{2+}** sensitivity (Figure 5) can translate to large effects on murine myocardial relaxation under physiological conditions in vivo. The direct effect of E180G Tm on diastolic function in the absence of cardiac hypertrophy suggests diastolic dysfunction may be an important early target in the prevention of progression of FHC.

Mechanistically, several important factors contribute to the regulation of murine and human myocardial relaxation. These factors include phospholamban (PLB) regulation of the sarcoplasmic reticulum **Ca^{2+}** ATPase and thin filament troponintropomysin deactivation of the contractile apparatus, both of which are targets for phosphorylation by protein kinase A. It has been proposed that myocardial relaxation in mice is primarily regulated by PLB regulation of **Ca^{2+}** sequestration, because PLB knockout mice display enhanced cardiac relaxation and reduced effects of β-adrenergic stimulation in isolated heart preparations.\(^5\) We show here that in mice in vivo, with physiological HR and contractility, that mutation of a contractile protein can markedly slow myocardial relaxation. In addition, stimulation of mouse hearts under these conditions with dobutamine enhances contractility without enhancing myocardial relaxation in NTG, WT Tm, and E180G Tm mice. Indeed, TnI is also nearly maximally phosphorylated under physiological conditions and normal physiological conditions this suggests that under normal physiological conditions in mice, cardiac reserve is low and additional β-adrenergic stimulation cannot markedly enhance myocardial relaxation by phosphorylating phospholamban. Because E180G mutation in Tm causes slow phosphorylation of TnI in cardiac myofilaments from lines 7, 160 E180G; and 135 E180G, \(^*\) Line 160 E180G significantly different than both NTG and line 169 WT Tm mice (P<0.05). *Line 160 E180G significantly different than both NTG and line 169 WT Tm mice (P<0.05). N.S. indicates no significant differences among groups.

The foregoing discussion may help explain why diastolic dysfunction is no longer evident in E180G Tm mice in the presence of β-adrenergic blockade. Our findings suggest that when murine myocardial contractility is reduced by β-blockade, a new rate-limiting step in the regulation of relaxation is revealed. Perhaps **Ca^{2+}** sequestration or crossbridge detachment becomes rate limiting in myocardial relaxation, thereby obscuring the effects of E180G mutant Tm on relaxation. Indeed, the effect of propranolol to slow heart rate may be important for eliminating the effect of E180G Tm on diastolic function. By slowing heart rates, propranolol may also improve ventricular filling in E180G Tm mice, which could improve relaxation by a preload dependent mechanism. Alternatively, it is possible that E180G Tm behaves differently depending on the phosphorylation status of the cardiac TnI. However, in isolated permeabilized cardiac myocytes, where the phosphorylation status of the thin filament is relatively low, E180G Tm still enhances the **Ca^{2+}** sensitivity of contraction (Figure 4),\(^6\) and basal phosphorylation of TnI is unaltered in E180G Tm–expressing mice (Figure 6).

Because diastolic dysfunction caused by E180G Tm is more pronounced under conditions where demand for cardiac output is greater and enhanced ventricular filling is required (basal and isoproterenol versus β-blockade), cardiovascular

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**Figure 6.** Effects of manipulating adrenergic signaling on cardiovascular function in NTG (▲), 169 WT Tm (●), 160 E180G Tm (▼), and 135 E180G Tm (▲) transgenic mice. CO (A), HR (B), dP/dt\(_{\text{max}}\) (C), and the time constant for relaxation, tau (D), are shown. Data are mean±SEM with n=25, NTG; n=7 to 8 169 WT Tm; n=7, 160 E180G; and n=10, 135 E180G Tm. \(^*\) Line 135 E180G significantly different than both NTG and line 169 WT mice (P<0.05). *Line 160 E180G significantly different than both NTG and line 169 WT Tm mice (P<0.05). N.S. indicates no significant differences among groups.

E. Representative back-phosphorylation assay of cTnI in cardiac myofilaments from lines 135 and 160 of E180G Tm transgenic (Tg) mice and nontransgenic (NTG) littersmates. Treatment groups are Basal, Propranolol (Inderal, 1 mg/kg IP), and Dobutamine (20 ng/g IP) and show the dynamic range of the assay. Hearts were extracted 6 minutes after IP injection. Phosphorylation of TnI in the absence of PKA was 3.75±0.6% (n=8) of phosphorylation detected in the presence of PKA (not shown). The relative amount of TnI phosphorylation present in Tg and NTG mouse hearts was not significantly different based on densitometric analysis and normalization for protein loading (NTG, n=3; E180G Tg, n=5; P<0.05).
stress may be an important environmental factor for the phenotype. Interestingly, many FHC patients display abnormal pressure responses during exercise and the exercise capacity is, in part, determined by the degree of diastolic dysfunction. In addition, many, but not all FHC patients respond quite favorably to β-blockers or Ca²⁺ antagonists that improve diastolic function. However, the mechanistic differences in the regulation of Ca²⁺ handling between human myocardial cells and those of other mammalian species has only recently been appreciated. Understanding the differential Ca²⁺ handling and basal myofilament phosphorylation between mammalian species will be important for comparing the mechanisms of contractile dysfunction in murine model systems and in human cardiac muscle.

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