F110I and R278C Troponin T Mutations That Cause Familial Hypertrophic Cardiomyopathy Affect Muscle Contraction in Transgenic Mice and Reconstituted Human Cardiac Fibers*

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We have studied the physiological effects of the troponin T (TnT) F110I and R278C mutations associated with familial hypertrophic cardiomyopathy (FHC) in humans. Three to four-month-old transgenic (Tg) mice expressing F110I-TnT and R278C-TnT did not develop significant hypertrophy or ventricular fibrosis even after chronic exercise challenge. The F110I mutation impaired acute exercise tolerance, whereas R278C did not. Skinned papillary muscle fibers from transgenic mice expressing F110I-TnT demonstrated increased Ca2+ sensitivity of force and ATPase activity, and likewise an increased Ca2+ sensitivity of force was observed in F110I-TnT-reconstituted human cardiac muscle preparations. In contrast, no changes in force or the ATPase-pCa dependencies were observed in transgenic R278C fibers or in human fibers reconstituted with the R278C-TnT mutant. The maximal level of force development was dramatically decreased in both transgenic mice. However, the maximal ATPase was not different (R278C-TnT) or only slightly less (F110I-TnT) than that of non-Tg and WT-Tg littermates. Consequently, their ratios of ATPase/force (energy cost) at all Ca2+ concentrations were dramatically higher compared with non-Tg and WT-Tg fibers. This increase in energy cost most likely results from a decrease in force per myosin cross-bridge, because forcing all cross-bridges into the force generating state by substitution of MgADP for MgATP in maximum contracting solutions resulted in the same increase in maximal force (15%) in all transgenic and non-transgenic preparations. The combination of increased Ca2+ sensitivity and energy cost in the F110I hearts may be responsible for the greater severity of this phenotype compared with the R278C mutation.

Tropin T (TnT) plays many important roles in striated muscle contraction, providing structural stability to the troponin complex and actively participating in the Ca2+-dependent regulation of contraction

11, 13). Thus, the clinical significance of an isolated report of a sudden death in a carrier of the R278C mutations may be questionable.

In this report, we present studies on the physiological consequences of the TnT F110I and R278C mutations in skinned papillary muscle fibers obtained from transgenic mice expressing these human cardiac TnT mutations. We also characterize human cardiac-skinned fibers reconstituted with these and other TnT mutants and evaluate them versus previously described reconstituted porcine cardiac muscle fibers (14). The F110I mutation occurs in a conserved region of TnT that interacts with actin and tropomyosin (Tm), and the R278C mutation is located in a region of positively charged residues reported to interact with Tm, troponin I (TnI), and troponin C (TnC) (15–18). In vitro studies from our laboratory, as well as others, indicated functional defects in the contractile apparatus involving these TnT mutations and suggested some mechanisms that might be involved in the F110I- and R278C-dependent pathophysiology of FHC (14, 19, 20). Utilizing skinned porcine cardiac fibers reconstituted with recombinant human cardiac F110I- or R278C-TnT mutants, we have previously shown an increase in Ca2+ sensitivity of force development and F110I-dependent alterations in the level of maximal ATPase activity in reconstituted thin filaments (14). In addition, we have suggested a decrease in binding
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affinity of F110I-TnT and its binding protein partners in these reconstituted skinned fibers (14). In order to have an in vivo model of these two TnT mutations and to possibly clarify some of the reconstituted skinned fiber data, we have produced several lines of transgenic mice that express the human cardiac F110I-TnT or R278C-TnT mutant proteins (F110I-Tg, R278C-Tg). Fibers from these mice were examined for Ca\(^{2+}\) sensitivity of force development and ATPase activity measured simultaneously under isometric conditions and for the maximal levels of force and ATPase activity. We also present physiological studies utilizing human cardiac muscle preparations reconstituted with the F110I-, R278C-, and, for comparison, I79N-TnT mutants. Both muscle systems, the transgenic animal models and the reconstituted human cardiac muscle fibers, demonstrate increased Ca\(^{2+}\) sensitivity of force/ATPase for F110I and I79N mutants whereas no change in the Ca\(^{2+}\) sensitivity of force/ATPase was observed for the R278C-TnT mutant.

Our transgenic data showed that whereas the maximal level of ATPase was not very different in all experimental groups, the maximal force development was dramatically decreased in F110I- and R278C-TnT cardiac mouse fibers. Therefore, the ratio of ATPase/force (energy cost), which was nearly the same for both transgenic TnT mutants, was much higher than that observed in the cardiac fibers of non-Tg and WT-Tg littermates. In summary, our results suggest that this decrease in force and increase in energy cost observed in both F110I- and R278C-TnT mutant mice might be a key factor in triggering cardiac pathophysiology like hypertrophy as seen in human patients harboring these mutations. The combination of increased Ca\(^{2+}\) sensitivity and energy cost in the F110I-mutated myocardium may be responsible for the greater severity of the F110I phenotype compared with the R278C mutation.

MATERIALS AND METHODS

Clone Construction

The clone for the human cardiac WT-TnT has been previously described (21). The F110I- and R278C-human cardiac TnT (HCTnT) mutant cDNAs were obtained by overlapping sequential PCR (22) using the WT-HCTnT clone (14) and a set of primers specific for each mutation. The WT and mutant cDNAs were constructed to have an NcoI site at the N-terminal ATG and a BamHI site following the stop codon to facilitate cloning into pET-3d (Novagen), which was used for bacterial expression of the proteins.

Protein Expression and Purification

All HCTnT proteins were expressed and purified as previously described (14). The correct sequence for both F110I- and R278C-HCTnT clones were verified before expressing mutant proteins.

Transgene Construct

The 876-base pair cDNAs of the F110I- and R278C-HCTnT were cloned into the unique Sall site of a plasmid containing a 5\'-myosin heavy chain promoter and a downstream 630-base pair region of the 3\'-untranslated region (3\'-UTR) of the human growth hormone (hGH) (clone 26, a generous gift from Dr. Jeffrey Robbins).

Generation of Transgenic Mice

All animal studies were conducted in accordance with institutional guidelines. The transgene construct described above was purified on a cesium chloride gradient and digested with NotI to release a 7-kilobase fragment that was used for microinjection. This fragment was purified by agarose gel electrophoresis, followed by electroelution (23) and resuspension in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 5 \(\mu\)g/ml. Mouse pronuclei were injected and the surviving embryos were implanted using standard methods (24). Founder mice were identified by preparing tail clip DNA and analyzing its hybridization to a probe corresponding to the 3\'-untranslated region of the human growth hormone (a 630-base pair HindIII/EcoRI fragment from the transgenic construct). Founder transgenic mice were bred to B6/SJL mice to establish stable transgenic lines.

RNA Analysis of Transgenic Mice

RT-PCR of Heart Tissue from HCTnT Transgenic and Non-transgenic Animals—Standard RT-PCR with 2 \(\mu\)g of RNA extract from the hearts of transgenic and non-transgenic animals was performed. A 5\'-primer specific for the 5\'-UTR of the transgenic transcript or a 3\'-primer specific for the 5\'-UTR of the endogenous mouse mRNA, and a 3\'-antisense primer that anneals to both the human and the mouse CTnT cDNA was used to analyze the RNA (Fig. 1, A and B).

Northern Blots—Northern blots were performed on RNA extracted from tissues from Non-Tg and Tg mice. Autoradiographs of membranes with 10 \(\mu\)g of total RNA isolated from transgenic and non-transgenic mouse hearts were incubated with a 32P-radiolabeled human growth hormone probe. Only lanes containing the transgenic transcripts have a band of the expected transcript size of 1550 nucleotides (Fig. 1C). This probe did not hybridize with RNA from non-transgenic hearts or transgenic liver (not shown). A probe to endogenous mouse GADPH was used as the internal loading control.

Protein Analysis of Transgenic Mice

The amount of human cardiac TnT protein in the transgenic mice was determined by Western blot. Experimental mouse and control human heart samples were homogenized in 20 mM Tris-HCl, pH 7.4, 1% SDS, 1% \(\beta\)-mercaptoethanol, 10% glycerol on ice, and the total protein concentrations were determined using the Bio-Rad protein assay. Two identical SDS-PAGE gels were simultaneously run with a total of 2 \(\mu\)g of protein for each lane. Protein was then transferred to nitrocellulose membranes and immunostained with: 1) a 1:3000 dilution of an anti-CTnT polyclonal antibody (produced in this laboratory) to recognize both human and mouse CTnT or 2) a 1:2000 dilution of an anti-HCTnT monoclonal antibody (clone 7G7 antibody, Research Diagnostics, Inc., Flanders, NJ), which recognizes only the human CTnT. A standard curve was produced with different ratios of mouse and human protein totaling 2 \(\mu\)g of protein in each lane. The density of the bands stained with monoclonal antibody was divided by the density of the corresponding bands stained with the polyclonal antibody to normalize total protein loaded for each sample. Immunoreactivity was detected using goat anti-mouse IgG labeled with horseradish peroxidase or rabbit anti-goat IgG labeled with horseradish peroxidase (both used at 1:3000 dilution; Sigma), and developed using diaminobenzidine/H\(_2\)O\(_2\). The optical density of immunostained protein was determined by scanning the membrane and analysis was carried out on a Dell Pentium Computer using Scion Image software.
Mice Exercise Protocol

A swimming protocol described by Geisterfer-Lowrance et al. (25) was utilized with minor modification. Male and female mice from the wild type, F110L, and from two of the R278C lines of 2-month-old animals were exercised by swimming. Mice were adapted to the swimming program by beginning with 10-min sessions two days a day separated by 4 h. Swimming time was incremented by 10 min a day until reaching 90 min a session. The mice were exercised 7 days a week for a total of 56 days. An equivalent number of age- and sex-matched mice from each line were kept sedentary. All animals were weighed weekly. During each session, animals were monitored for the inability to sustain the exercise and/or sudden death. At the conclusion of the exercise program, the exercised mice and the sedentary counterparts were sacrificed and the hearts to body weight ratios were determined. For accuracy, excised hearts were trimmed and gently pressed to remove any remaining blood before determining mass. The hearts were then immediately prepared for pathology evaluation and for fiber studies.

Histopathology of Mouse Cardiac Tissue

Hearts from each animal were immediately fixed in 10% buffered formalin or prepared for fiber studies at the end of the exercise session. The sedentary counterpart served as control. Hematoxylin & eosin and trichrome-stained ventricular sections of formalin-fixed hearts were obtained as previously described (26). A board-certified pathologist examined cross-sections of representative hearts from each animal line (sedentary and exercised) for cardiac pathologies. Cellular hypertrophy, myocyte disarray, and fibrosis were scored on a 4-point scale in a blinded fashion as previously described (21).

Cardiac Fiber Experiments

Simultaneous Force and ATPase Measurements in Fresh (Not Glycinated) Skinned Fibers—Small preparations (~1 mm long and 50–90-μm in diameter) of mouse papillary muscles were dissected free in relaxing solution and then treated in relaxing solution containing 1% Triton X-100 for 30 min. Relaxing solutions are test solutions containing no added calcium. The skinned fibers test solutions contain 85 mM Tris for 30 min. Relaxing solutions are test solutions containing 1% Triton X-100 for 24 h at 4 °C. Then the fibers were transferred to the same solution without Triton X-100 and stored at −20 °C for about 2 weeks. Reconstitution of these papillary muscle fibers with recombiant human cardiac WT-TnT and I79N-, F110L-, or R278C-TnT mutants was performed as previously described for porcine fibers (14). These reconstituted fibers were examined for Ca2+ sensitivity of force development at pH 7 and at pH 6.5 (21).

Graded Treadmill Exercise Tolerance Test

For experimental trials, mice were randomly assigned to groups of five and placed individually into special chambers of the motorized rodent treadmill (Exer-6M, Columbus Instruments, Columbus, OH). The electrical shock grid located behind the belt of the treadmill was activated with every experimental session. The slope of the treadmill was kept constant at 15° inclination at a starting speed 16 m/min with incremental increases in treadmill belt speed by 2 m/min every 2 min until mouse exhibited signs of exhaustion. Exhaustion was defined as the mouse spending >50% of the time or >15 s consecutively on the shock grid during any 60-s period (30). The total running distance was recorded and used as indicator of maximum exercise tolerance. To avoid complication from psychological stress, animals were trained to run on the treadmill for 2 days prior to experimental trials two times a day for 20 min at a fixed speed of 16 m/min for each training session was separated by at least 3 h. These training sessions reduced the amount of electrical stimulation necessary to maintain running behavior during the experimental trial.

RESULTS

Generation and Characterization of Transgenic Mice

The wild-type HCTnT transgenic mice (WT-Tg) as well as I79N-Tg mice have previously been described (21). This report describes one transgenic mouse line for the F110L mutation (F110L-Tg line 1) and four lines for the R278C mutation (R278C-Tg lines 1, 3, 5, and 7). RT-PCR of heart tissue from representatives of each HCTnT transgenic line and non-transgenic littermates was performed. Two typical PCR reactions for each sample using 3 μg of cDNA and primers specific for the transgenic and non-transgenic C1Tn transcript are shown on Fig. 1. A and B. A PCR product of the expected size (150 bp) was only present in the PCR reaction of transgenic animals when the specific primers against the sequence of human cardiac TnT were used (Fig. 1A). When primers

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specific for the mouse endogenous cardiac TnT (MCTnT) were used, the PCR reaction for all transgenic and non-transgenic littermates produced a PCR product of 120 bp, indicating the presence of endogenous MCTnT transcript (Fig. 1B). Northern blot analysis using 10 μg of total RNA isolated from transgenic and non-transgenic mouse hearts hybridized with a 32P-radiolabeled probe specific for the 3′-untranslated region of the hGH in the transgene are shown in Fig. 1C. Only RNA from transgenic animals hybridized with the transgene-specific probe. When the same membrane was stripped and rehybridized with a 32P-radiolabeled probe specific for mouse GADPH, both transgenic and non-transgenic RNA showed expected bands of 1500 bp in an autoradiograph of Northern blot membrane with 10 μg of total RNA isolated from transgenic and non-transgenic mouse hearts hybridized with 32P-radiolabeled 3′-UTR of hGH probe (C) and GAPDH probe (D).

We first determined the effect of the HCTnT mutations on heart weight. None of the 5 mutant lines (F110I, R278C-1, and R278C-3) had evidence of cardiac hypertrophy when comparing average heart to body weight ratios to ratios of Non-Tg or WT-Tg mice (Fig. 3), although there was a non-significant trend (p = 0.07) toward increased heart to body weight ratios in the F110I line. The variability of heart weights was also significantly larger in this line compared with all other transgenic lines (p < 0.001 by Levene’s Test for Equal Variance). Surprisingly, the R278C line that expressed the lowest amount of mutant TnT (line 1, 35%) had significantly lower average heart to body weight ratios. The significance of these two findings is not clear and will have to be confirmed in more long term studies.

We next examined if the mutations caused abnormalities of myocyte or tissue morphology. Microscopic examination of hematoxylin & eosin- or trichrome-stained ventricular cross-sections revealed no significant increase in focal myocyte hypertrophy, disarray, or myocardial fibrosis in any of the transgenic lines. As illustrated in TABLE ONE, even non-Tg or WT-Tg mice had focal areas of abnormalities that were rated mild on a 4 point scale (normal, mild, moderate, severe). On average, mild myocyte hypertrophy or disarray was observed with similar frequency in all groups (TABLE ONE). No excess mortality was observed in any of the HCTnT transgenic lines during the first 5 months of life. Long term survival studies were not conducted.

**Effect of Chronic Swimming Exercise on HCTnT Transgenic Mice**

Chronic swimming exercise has been previously shown to exacerbate the pathologic phenotype of mice expressing FHC-linked mutations (25). Thus, we subjected groups from all transgenic lines to a 56-day swimming exercise program. During the exercise program, two male F110I-Tg mice died during the first week of swimming. The hearts of these animals, immediately prepared for microscopic evaluation, revealed no myocyte disarray, myocyte hypertrophy, nor myocardial fibrosis (TABLE ONE). All other animals survived the scheduled protocol. Chronic exercise significantly increased the heart to body weight ratio in all groups (p < 0.01 by two-way analysis of variance (ANOVA)). However, neither the F110I nor the R278C lines demonstrated a significantly larger hypertrophic response to exercise compared with non-Tg or WT-Tg mice, as evidenced by the lack of a statistical interaction between genotype and exercise (p = 0.08 by two-way ANOVA). Accordingly, no apparent trends in cardiac histopathology were
observed (TABLE ONE). Myocyte hypertrophy ranged from normal to a few hypertrophic cells in the cardiac tissue of all lines (TABLE ONE). Myocyte disarray was typically normal to mild, occurring mostly in the free and post left ventricle area and in the septum, with no identifiable pattern between sedentary and exercised animals (TABLE ONE). No myocardial fibrosis was identified in any of the animals expressing HCTnT transgenes.

### Maximum Exercise Tolerance of HCTnT Transgenic Mice

Because the transgenic lines appeared to tolerate the chronic swimming exercise without difficulties, we next examined whether TnT mutants have a differential effect on acute maximum exercise tolerance. Maximum exercise tolerance provides a measure of overall cardiopulmonary function. Thus, we subjected all groups of mice to a graded

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TABLE ONE

Cardiac pathology of representative transgenic mice

Myocyte hypertrophy was identified as normal, few, several, or many. For the degree of myocyte disarray and myocardial fibrosis a scale of normal, mild, moderate, or severe was used.

| Transgene | Sex | Condition | Myocyte hypertrophy | Myocyte disarray | Myocardial fibrosis |
|-----------|-----|-----------|----------------------|-------------------|---------------------|
|           |     |           |                      | Degree | Site |                      |                      |
| Non-Tg    | M   | Sedentary | Few                  | Mild   | Free LV<sup>b</sup> | Normal              |
|           | M   | Sedentary | Normal               | Mild   | Free LV | Moderate             |
|           | F   | Sedentary | Few                  | Moderate | Free LV | Normal              |
|           | F   | Swimming  | Normal               | Normal | —        | Normal              |
|           | M   | Swimming  | Normal               | Normal | Septum  | Normal              |
| WT        | M   | Sedentary | Normal               | Normal | —        | Normal              |
|           | M   | Sedentary | Few                  | Mild   | Post LV | Normal              |
|           | F   | Sedentary | Few                  | Mild   | Post LV, septum | Normal              |
|           | F   | Swimming  | Normal               | Mild   | Septum  | Normal              |
|           | M   | Swimming  | Few                  | Normal | —        | Normal              |
|           | F   | Swimming  | Normal               | Normal | —        | Normal              |
| F110I     | M   | Sedentary | Few                  | Normal | —        | Normal              |
|           | M   | Sedentary | Normal               | Normal | —        | Normal              |
|           | M   | Sedentary | Normal               | Mild   | Post LV | Normal              |
|           | M   | Sedentary | Few                  | Mild   | Post LV, septum | Normal              |
|           | M<sup>e</sup> | Swimming | Normal               | Normal | —        | Normal              |
|           | F<sup>e</sup> | Swimming | Normal               | Normal | —        | Normal              |
|           | F   | Sedentary | Few                  | Normal | —        | Normal              |
| R278C-1   | M   | Sedentary | Few                  | Mild   | Free LV | Normal              |
|           | F   | Sedentary | Few                  | Normal | —        | Normal              |
| R278C-3   | M   | Sedentary | Normal               | Normal | —        | Normal              |
|           | M   | Sedentary | Few                  | Mild   | Free LV, post LV | Normal              |
|           | M   | Swimming  | Normal               | Moderate | Post LV | Normal              |
| R278C-5   | M   | Sedentary | Normal               | Normal | —        | Normal              |
| R278C-7   | M   | Sedentary | Few                  | Mild   | Free LV | Normal              |

<sup>a</sup> M, male.

<sup>b</sup> LV, left ventricle.

<sup>c</sup> F, female.

<sup>d</sup> Not specified.

<sup>e</sup> Died during swimming protocol.

<sup>f</sup> RV, right ventricle.
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FIGURE 4. Maximum exercise tolerance of HCTnT transgenic lines. Transgenic mice expressing either the I79N-TnT or F110I-TnT mutant demonstrate significantly reduced maximum exercise tolerance compared with mice expressing the R278C-TnT mutant (R278C, line 1), human WT TnT (WT) or non-transgenic littersmates (Non-Tg). Total running distance of a graded exercise tolerance test was used to compare non-Tg (n = 13), WT-Tg (n = 13), I79N-Tg line 8 (n = 8), R278C-Tg line 1 (n = 11), R278C-Tg line 7 (n = 8), and F110I-Tg (n = 8) mice. Data are the average ± S.E.; *, p < 0.05 versus non-Tg, WT, R278C-1, and R278C-7; #, p < 0.05 versus non-Tg, WT, R278C-1.

exercise protocol using a motorized treadmill. Mice expressing the I79N-TnT mutation, which have a fiber phenotype of increased Ca\(^{2+}\) sensitivity and decreased maximum force similar to that of F110I-Tg mice (see below and Ref. 21), were also included for comparison. On average, the maximum exercise tolerance of R278C-Tg mice from two different transgenic lines, 1 (35%) and 7 (52%) was not statistically different from that of WT-Tg or non-Tg mice (Fig. 4). In contrast, I79N-Tg and F110I-Tg mice demonstrated a significant reduction of maximal exercise tolerance (Fig. 4) compared with non-Tg, WT-Tg, and R278C-Tg mice.

Fiber Studies

Transgenic Mice—We next performed multiple experiments to examine the Ca\(^{2+}\) sensitivity of force development and ATPase activity on freshly skinned papillary muscle fibers from WT-Tg, F110I-Tg, and R278C-Tg mice. The control fibers were derived from non-Tg littermates. Prior to measurement, the fibers (diameter of ~70 ± 20 μm) were skinned with Triton X-100. As demonstrated in Fig. 5A, the Ca\(^{2+}\) sensitivity of the ATPase activity for WT-Tg was pc50 = 5.199 ± 0.052 (n = 39) while that of F110I was pc50 = 5.421 ± 0.026 (n = 20) and the difference between them, Δpc50 = 0.22. There was a difference of Δpc50 ~0.1 between the WT-Tg and non-Tg fibers most likely because of the human TnT expressed in mouse hearts. A similar effect of the F110I mutation was seen on the steady state force development and is demonstrated in Fig. 5B. The midpoint of the force-pCa dependence of WT-Tg was pc50 = 5.068 ± 0.019 (n = 39) whereas that of F110I was pc50 = 5.224 ± 0.026 (n = 20), with Δpc50 = 0.156. The large increase in Ca\(^{2+}\) sensitivity of force and ATPase of F110I-Tg compared with WT fibers was not observed for R278C transgenic fibers. As demonstrated in Fig. 6, there was again no difference in either the Ca\(^{2+}\) sensitivity of ATPase (Fig. 6A) or force (Fig. 6B) between WT-Tg and R278C-Tg fibers. The respective Δpc50 values between the WT-Tg and R278C-Tg were ~0.033 (ATPase, n = 36) and ~0.044 (force, n = 36). In Figs. 7 and 8, maximal ATPase rates (s\(^{-1}\)) and maximal force (10\(^{14}\) N/mol) are presented for Non-Tg, WT-Tg, F110I-Tg, and R278C-Tg skinned fibers. Maximal ATPase activity was decreased in F110I-Tg fibers compared with WT-Tg (p = 0.0127). The ATPase rates were equal 6.014 ± 0.345 (n = 39) and 4.743 ± 0.139 (n = 20) for WT-Tg and F110I-Tg, respectively. In contrast, no significant changes in maximal ATPase rates were observed between WT-Tg and R278C-Tg fibers (Fig. 7). However, there was a large decrease in maximal force observed for F110I-Tg and R278C-Tg fibers (Fig. 8). The maximal force for WT-Tg fibers was 4.002 ± 0.316 (n = 39) whereas those for Tg F110I and R278C were 1.828 ± 0.086 (n = 20, p = 9.86e-6) and 2.284 ± 0.092 (n = 36, p = 3.33e-6), respectively. Fig. 9 illustrates the energy cost or rate of cross-bridge dissociation (ATPase/force). It shows that the maximum energy cost or rate of dissociation of force generating myosin cross-bridges is higher in both F110I-Tg fibers (Fig. 9A) and R278C-Tg fibers (Fig. 9B) when compared with WT-Tg fibers. This means that a higher amount of energy is going to be used per unit isometric force in both transgenic TnT mice (F110I and R278C) compared with WT-Tg mouse fibers. There were no differences in the ATPase and force measurements between the sedentary and exercised groups of mice.

Human Cardiac Fiber Studies—Previous studies examining porcine fibers reconstituted with recombinant human cardiac WT and some of the FHC-TnT mutants including I79N-, F110I-, or R278C-TnT demonstrated an increase in Ca\(^{2+}\) sensitivity of force development generated by these mutants (14). We have repeated these reconstitution experiments utilizing human cardiac muscle preparations. In agreement with our previous study (14), an increase in the Ca\(^{2+}\) sensitivity of force was observed for the I79N- and F110I-TnT mutants (TABLE TWO). However, fibers reconstituted with R278C-TnT did not change the Ca\(^{2+}\) sensitivity of force and behaved similar to the R278C-transgenic fibers reported above in Fig. 6B. As shown in TABLE TWO, the pc50 of the force-pCa relationship determined at pH 7 was 5.452 ± 0.012 for WT (n = 4), 5.657 ± 0.017 for I79N (n = 4), 5.571 ± 0.007 for F110I (n = 4), and 5.438 ± 0.021 for R278C (n = 4). The largest increase in the Ca\(^{2+}\) sensitivity of force at pH 7.0 was observed between the WT and the I79N mutant with Δpc50 = 0.205 and the second largest difference was observed between WT and F110I (Δpc50 = 0.119). To evaluate the effects of the TnT mutations at lowered pH, simulating the condition the muscle experiences during strenuous exercise and/or ischemic injury, the Ca\(^{2+}\) sensitivity of force development in these reconstituted human papillary muscle fibers was determined at pH 6.5 (TABLE TWO). Similar to WT fibers, all mutant-reconstituted fibers showed decreased Ca\(^{2+}\) sensitivity at pH 6.5 compared with pH 7.0. The greatest difference in the Δpc50 (pH 7.0 to pH 6.5) was observed for WT-reconstituted fibers (Δpc50 = 0.507 ± 0.016). As reported earlier (21), the I79N mutant showed the greatest acidic pH resistance, Δpc50 = 0.368 ± 0.028 (TABLE TWO). However, the F110I- and R278C-reconstituted fibers also demonstrated acidic pH resistance, with Δpc50 (pH 7.0 to 6.5) equal to 0.409 ± 0.011 and 0.439 ± 0.029, respectively (TABLE TWO). All differences between WT and mutant-reconstituted fibers were statistically significant (p < 0.01; TABLE TWO). These results suggest a mutant-related deficiency in this putative adaptive mechanism of cardiac muscle to lower pH.

DISCUSSION

This study is the first to describe transgenic animal models for the FHC human cardiac TnT F110I and R278C mutations that have been generated under the control of the murine α-myosin heavy chain promoter. All transgenic lines demonstrated similar levels of protein expression, no cardiac hypertrophy and no ventricular histopathology. A large difference in the Ca\(^{2+}\) sensitivity of force and ATPase activity, determined in skinned papillary muscle fibers, was observed between the F110I transgenic mice and the other lines tested including WT, R278C, or non-transgenic animals. A shift in the force-pCa dependence toward lower Ca\(^{2+}\) concentrations in F110I-Tg mouse fibers was also observed in reconstituted porcine (14) and human cardiac muscle fibers reconstituted with recombinant F110I-TnT protein. In contrast to
FIGURE 5. Simultaneous ATPase (A) and force (B) measurements in skinned papillary muscle fibers of F110I-TnT transgenic mice. Small preparations (~1-mm long and 50–90-μm in diameter) of mouse papillary muscles were dissected free in relaxing solution, treated with 1% Triton X-100 and mounted in the Guth Muscle Research System (27), which allows for simultaneous force and ATPase measurements (see “Materials and Methods”). About 20 transgenic mouse fibers expressing F110I mutations were subjected to these measurements. The midpoints (pCa50) of ATPase-pCa dependence for F110I-Tg, WT-Tg, and Non-Tg (NTg) fibers were: 5.301 ± 0.021 (n = 20), 5.199 ± 0.052 (n = 39), 5.301 ± 0.021 (n = 25). The pCa50 values of force-pCa relationship for dependence for F110I-Tg, WT-Tg, and Non-Tg fibers were: 5.090 ± 0.020 (n = 20), 5.068 ± 0.019 (n = 39), 5.090 ± 0.020 (n = 25).
FIGURE 6. Simultaneous ATPase (A) and force (B) measurements in skinned papillary muscle fibers of R278C-TnT transgenic mice. Simultaneous force and ATPase measurements were performed as depicted in Fig. 5. The midpoints (pCa_{50}) of ATPase-pCa dependence for R278C-Tg was 5.218 ± 0.013 (n = 36) whereas that of force-pCa, 5.024 ± 0.013 (n = 36). The pCa_{50} values for WT-Tg and Non-Tg fibers are shown in Fig. 5.
F110I-Tg mouse fibers, no increase in the Ca$^{2+}$ sensitivity of force and ATPase was seen for R278C-Tg mouse fibers and no change in force was observed for human fibers reconstituted with R278C-TnT protein. Our previous studies with reconstituted porcine fibers showed an increase in the Ca$^{2+}$ sensitivity of force for all three TnT mutations (14). Interestingly, cardiac fibers of both F110I and R278C transgenic mice displayed dramatic decreases in maximal force, 54% decrease by F110I and 43% decrease by R278C, compared with those of WT-Tg mice. Similar decreases in maximal force were previously observed in our I79N-Tg mouse study (21).

Perhaps, all three mutations that lower force per cross-sectional area may in the long-term result in systolic dysfunction and the hearts of patients harboring these mutations will be prone to using more energy to maintain blood pressure and blood flow at normal levels. In support of this idea the ratios of ATPase/force (energy cost) at all Ca$^{2+}$ concentrations for both transgenic F110I- and R278C-TnT mutants were dramatically higher than for Non-Tg and/or WT-Tg animals. A higher energy cost can be the result of at least two obvious reasons. Normally, it would be assumed to be a result of an increase in the rate of dissociation of force generating myosin cross-bridges during muscle contraction. This is because it is generally assumed that the average force per cross-bridge does not change. However, a decrease in the average force per cross-bridge could be just as effective in increasing energy cost. Because the maximum ATPase (also a measure of myosin cross-bridge turnover rate) for both mutants does not change or changes very little, one is left with the strong possibility that it is the maximum force per

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**FIGURE 7.** Maximal ATPase rates in skinned papillary muscle fibers of F110I-TnT and R278C-TnT transgenic mice. Maximal ATPase rates ($s^{-1}$) for F110I-Tg, R278C-Tg, WT-Tg, and Non-Tg fibers were: $4.743 \pm 0.139 (n = 20)$, $6.014 \pm 0.345 (n = 36)$, $6.014 \pm 0.345 (n = 39)$, and $5.254 \pm 0.530 (n = 25)$. The difference between WT-Tg and F110I-Tg was statistically significant, $p = 0.0127$.

**FIGURE 8.** Maximal force in skinned papillary muscle fibers of F110I-TnT and R278C-TnT transgenic mice. Maximal force ($10^{14} \text{N per mol myosin}$) for F110I-Tg, R278C, WT-Tg, and Non-Tg fibers were: $1.828 \pm 0.086 (n = 20)$, $2.284 \pm 0.092 (n = 36)$, $4.002 \pm 0.316 (n = 39)$, and $3.529 \pm 0.278 (n = 25)$. The differences between WT-Tg and F110I-Tg as well as between WT-Tg and R278C-Tg were statistically significant with respective $p = 9.84e^{-6}$ and $3.33e^{-6}$. 
cross-bridge that was decreased by the TnT mutations. This possibility is supported by the fact that substituting MgADP for MgATP in the maximal contracting solutions, in order to force all cross-bridges into the force-generating state, produced only a 15% increase in force regardless of whether the skinned preparations were from the WT-TnT, R278C-TnT, F110I-TnT, or non-transgenic mice. How this could happen can only be speculated, but perhaps these TnT mutations can affect the flexibility of tropomyosin in such a way as to prevent the myosin head from rotating properly during the power stroke or the mutation alters the structure of troponin (15, 16), which in turn alters the structure of actin and this changes the actin-cross-bridge interaction.

FIGURE 9. Maximum energy cost in transgenic mouse fibers expressing F110I-TnT (A) and R278C-TnT (B) proteins. Maximum Energy Cost was plotted as ratio of ATPase/Force measured simultaneously under isometric conditions. It expresses the rate of cross-bridge dissociation ($y_{ave}$).

One intriguing result of our study is that mice expressing the R278C mutation, which significantly reduces maximum force of skinned fibers, had a normal maximal exercise tolerance, (Fig. 4). This suggests that, at least in the acute setting, mice are able to compensate for a decreased maximum force and an increased energy cost of contraction imparted by the R278C mutation. This result may not be as surprising as it might appear, since it is well recognized that in the intact ventricular myocardium, peak intracellular Ca$^{2+}$ concentration never reaches values that are high enough to produce maximum contractile force, even under conditions of maximum inotropic stimulation (31). For example, in intact rabbit and rat myocytes, a maximum myoplasmic free [Ca$^{2+}$] of
pCa \sim 5.40 was reached during the optimum twitch. This was much lower than the free [Ca\(^{2+}\)] \_ necessary for the full activation of the myofilaments (pCa \sim 4.90) (31). These results suggest that the cardiac muscle operates on the exponential range of the pCa-force relationship and probably never reaches free [Ca\(^{2+}\)] values beyond the pCa\(_{50}\) of the myofilaments (compare also pCa\(_{50}\) values listed in Fig. 5). Furthermore, since steady-state conditions are not present in a beating cardiac muscle, effects of the mutation on dynamic properties of muscle contraction may be more important than their effects on maximum steady-state force. Consistent with this idea, mice expressing the Ca\(^{2+}\)-sensitizing TnT mutations (I79N, F110I) demonstrated significantly impaired exercise capacity. This suggests that in vivo the impaired relaxation conveyed by the Ca\(^{2+}\)-sensitizing effect of TnT mutants (26) is hemodynamically more important than any changes of maximal developed force, which was depressed to a similar extent by all three mutations (compare Fig. 8 and Ref. 21).

Another surprising finding was that mutant transgenic lines lacked the histopathological findings (TABLE ONE) that are considered hallmarks of FHC in humans. Perhaps the expression levels of mutant protein may have been too low, since other investigators have observed an apparent “threshold” for histopathological changes around 50% of mutant protein load (32). However, since the TnT mutations cause an autosomal dominant disorder and most patients are heterozygous for the mutation, it can be predicted that expression levels of mutant TnT protein is less than 50%, although exact values are presently unknown. This would favor an alternative explanation, namely that the animals under study were simply too young to demonstrate significant histopathology. Consistent with this idea, we have recently reported preliminary data suggesting that a histopathological phenotype developed in HCTnT I79N transgenic mice aged 15–20 months (33). Studies are in progress to test whether similar changes also occur in HCTnT R278C and F110I transgenic mice.

There are studies from other laboratories examining transgenic animal models for other TnT mutations than those described in our previous (21) or current report, namely truncated TnT (34), or the R92Q-TnT mutation (32, 35). Transgenic animals expressing the truncated form of TnT demonstrated myocellular disarray and a reduced number of cardiac myocytes that were also smaller in size (34). The ventricles of mice expressing the R92Q mutation were smaller than those of WT-Tg (32). In contrast to the truncated TnT mice, the R92Q-TnT hearts demonstrated significant induction of atrial natriuretic factor and \(\beta\)-myosin heavy chain transcripts, interstitial fibrosis, and mitochondrial pathology (32). These studies suggest that different cellular mechanisms lead to TnT-linked FHC, which is generally characterized by mild hypertrophy, but also, sudden cardiac death (8).

We have also examined the effect of lowering pH, from pH 7.0 to 6.5, on the Ca\(^{2+}\) sensitivity of force in human cardiac muscle fibers reconstituted with the F110I, R278C mutations as well as with the previously described TnT I79N and WT proteins (21). We have shown that all of the TnT-reconstituted fibers decreased the Ca\(^{2+}\) sensitivity of force development as well as maximal force when the pH was lowered from 7.0 to 6.5. This desensitization to Ca\(^{2+}\) (rightward shift), expressed as a difference in \(\Delta pCa_{50}\) units between force monitored at pH 7 versus that at pH 6.5, was the highest for the fibers reconstituted with WT-TnT and then R278C-> F110I > I79N mutants. Large shifts in the Ca\(^{2+}\) sensitivity of force upon lowering the pH during cardiac muscle contraction is thought to protect the muscle when the heart undergoes potential ischemic processes. Therefore, the smallest change in \(\Delta pCa_{50}\) as observed for I79N-reconstituted fibers, implies FHC-linked alterations in the pH-dependent adaptation of the I79N-TnT muscle to acidosis. A similar effect was observed in our previous I79N-TnT transgenic mouse fibers (21). However, impairment in the pH-dependent shift was also seen in the F110I- and R278C-reconstituted fibers compared with WT-TnT-reconstituted human cardiac muscle preparations. These differences between the WT and mutant-reconstituted fibers were statistically significant (p < 0.01; TABLE TWO). Both mutations decreased the protective effect to changes in pH on the Ca\(^{2+}\) sensitivity that are observed in cardiac muscle similar to what we have reported for other FHC mutations (36).

In summary, our results show that the TnT mutations F110I and R278C, associated with FHC in humans, are characterized in skinned fibers by a decrease in force at all Ca\(^{2+}\) concentrations, but little or no significant effect on fiber ATPase. This phenomenon resulted in a dramatically increased energy cost for both F110I and R278C transgenic fibers at all Ca\(^{2+}\) concentrations. The common thread in these TnT mutation studies is that force is decreased resulting in an increased energy cost. This would suggest that the myocardium of an animal with normal blood pressure and cardiac output might have greater energy consumption than a normal animal. Perhaps these processes initiate and/or contribute to the development of FHC and cardiac failure. The mechanism for the increased energy consumption appears to result from a decrease in force per cross-bridge as judged by the fact that forcing all cross-bridges into the force generating state caused the same increase in force in wild-type, non-Tg, and mutant fibers. The decrease in force and the increase in energy cost observed in both the F110I- and the R278C-TnT mutant mice might be a key factor in triggering cardiac hypertrophy as seen in human patients harboring these mutations. However, an increase in the Ca\(^{2+}\) sensitivity of force as well as in energy cost that was seen with the F110I mutation compared with the R278C mutation, which did not affect Ca\(^{2+}\) sensitivity of force/ATPase, may be a key factor in understanding the greater severity of the F110I-mutated myocardium in humans.

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### TABLE TWO

| Recombinant TnT protein* | \(pCa_{50}\) (pH 7.0) | \(pCa_{50}\) (pH 6.5) | \(\Delta pCa_{50}\) (pH 7.0-pH 6.5) |
|-------------------------|----------------------|----------------------|----------------------------------|
| WT                      | 5.452 ± 0.012         | 4.945 ± 0.019\(^b\)  | 0.507 ± 0.016\(^{cd,e}\)          |
| I79N                    | 5.657 ± 0.017         | 5.289 ± 0.039         | 0.368 ± 0.028\(^c\)              |
| F110I                   | 5.571 ± 0.007         | 5.162 ± 0.014         | 0.409 ± 0.011\(^d\)              |
| R278C                   | 5.438 ± 0.021         | 4.999 ± 0.036         | 0.439 ± 0.029\(^a\)              |

* At least four fibers were subjected to measurements.
\(^a\) Errors are expressed as S.D.
\(^b\) The differences between WT and I79N were statistically significant (p < 0.01).
\(^c\) The differences between WT and F110I were statistically significant (p < 0.01).
\(^d\) The differences between WT and R278C were statistically significant (p < 0.01).
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