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Functional Effects of a Restrictive Cardiomyopathy linked Cardiac Troponin I mutation (R145W) in Transgenic Mice

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Abstract

The human cardiac troponin I (hcTnI) mutation, R145W, has been associated with restrictive cardiomyopathy. In this study, simultaneous measurements of ATPase activity and force in transgenic skinned papillary fibers from hcTnI R145W transgenic mice (Tg-R145W) were explored. The Tg-R145W fibers showed a ~13 to ~16% increase in the maximal Ca²⁺ activated force and ATPase activity compared to hcTnI wild type transgenic mice (Tg-WT). The force generating cross-bridge turnover rate (g) and energy cost (ATPase/force) was the same in all groups of fibers. Also, the Tg-R145W fibers showed a large increase in the Ca²⁺ sensitivity of both force development and ATPase. In intact fibers, the mutation caused prolonged force and intracellular [Ca²⁺] transients and increased time to peak force. Analysis of force and Ca²⁺ transients showed that there was a 40% increase in peak force in Tg-R145W muscles which was likely due to the increased Ca²⁺ transient duration. The above cited results suggest that: 1) there would be an increase in resistance to ventricular filling during diastole resulting from the prolonged force and Ca²⁺ transients that would result in a decrease in ventricular filling (diastolic dysfunction); and 2) a large (approximately 53%) increase in force during systole which may help to compensate, in part, for the diastolic dysfunction. These functional results help to explain the mechanisms by which these mutations give rise to the restrictive phenotype.

Keywords

RCM; R145W; Troponin; Cross-bridge; Ca²⁺ and Force Transients; Energy Cost

Introduction

Cardiac muscle diseases (cardiomyopathies) are classified as either extrinsic or intrinsic cardiomyopathies. Primary cardiomyopathies (intrinsic) can be classified into three major

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types: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and restrictive cardiomyopathy (RCM) based upon a range of morphological and functional criteria. Unlike HCM and DCM which are described by changes in the heart morphology, RCM is characterized by impaired physiological functions: restrictive ventricular filling, reduced diastolic volume and increased end diastolic pressure in the presence of normal systolic function. Morphology in RCM hearts show normal or near normal myocardial wall thickness and cavity size.¹ Among these three cardiomyopathies, RCM is associated with the greatest morbidity and mortality.² RCM can be idiopathic or secondary to some systemic disorder, such as metabolic disorders (Fabry's disease) and infiltrative disorders (amyloidosis).^{3,4}

Six mutations in the highly conserved region of human cardiac troponin I (hcTnI) (L144Q, R145W, A171T, K178E, D190G and R192H) were found to correlate with RCM.⁴ Cardiac troponin I (cTnI) inhibits actomyosin interactions in the absence of Ca^{2+} .⁵ A number of researchers have studied the effects of these six RCM-linked cTnI mutations on actomyosin interactions and contractility. They showed that these RCM-related cTnI mutations increase the Ca^{2+} sensitivity of force development and basal force levels in the absence of Ca^{2+} in porcine⁶ and rabbit⁷ skinned fibers reconstituted with mutant TnI. Additionally, Gomes et al.⁶ showed a decrease in the ability of the RCM cTnI mutants to inhibit actomyosin ATPase activity in the actin-Tm-activated myosin-ATPase assay in the presence of 1.0 mM EGTA. In rat cardiac myocytes transduced with R193H, Davis et al.⁸ found an increased Ca^{2+} sensitivity of force and a reduced basal sarcomere length in resting myocytes.

Interestingly, two different amino acid replacements at residue 145 in cTnI are associated with different cardiomyopathies.^{4,9} Since previous *in vitro* studies showed similar results in both cTnI mutations at this residue associated with HCM and RCM, there was no clear cut answer to this dilemma.^{6,10} In this study, using the transgenic mouse model, we address the question of how a single amino acid mutation at the same position in cTnI causes either HCM or RCM? We have already published our results on the cTnI R145G mutation that results in HCM.¹¹ Here we present our cTnI R145W experimental results and possible interpretation of how R145W causes RCM.

RESULTS

Generation and characterization of Tg-WT and Tg-R145W

The expression of human cTnI (hcTnI) WT and R145W in mouse hearts were driven by the α -MHC promoter in the mouse heart (Figure 1a). The WT and mutant protein expression level which incorporate into the thin filament were assessed on the basis of gel densitometry (Figure 1b). hcTnI WT and R145W protein expression was normalized to total cTnI content (murine cTnI (mcTnI) + hcTnI). The hcTnI replaced the mcTnI in the heart, so the mcTnI was reduced. Two lines (L) of Tg-WT generated ~10% (L1) and ~65% (L2) of hcTnI compared to the whole cTnI amount in the mouse heart. A total of 4 lines carrying the hcTnI R145W mutation were analyzed. Two of these lines could not be studied in details because of early death and paucity of transgenic offspring. In fact, one of the founders died prematurely and the other one died soon after labor. Both of the remaining two lines expressed ~10% mutant protein (Figure 1b and 1c). L2 of Tg-WT and L1 of Tg-R145W consistently produced the expected transgene product levels and were chosen for further functional studies. In both skinned and intact fiber experiments, 3–7 months old mice were used and matched by gender. Measurements were also performed with 12.0±1.0 months old mice. No differences in the functional studies were observed between these different age-groups.

Functional studies

Skinned papillary muscle fibers—Simultaneous ATPase-pCa and force-pCa measurements were performed in freshly skinned papillary muscle fibers from all groups of transgenic versus control mice under isometric conditions. An increased Ca^{2+} sensitivity in Tg-R145W fibers compared to Tg-WT fibers was seen in the steady-state force development measurements with $\text{pCa}_{50} = 5.34 \pm 0.02$ ($n = 15$) and $\text{pCa}_{50} = 5.16 \pm 0.02$ ($n = 13$) for Tg-R145W and Tg-WT, respectively. The $\Delta\text{pCa}_{50} = 0.18$ was statistically significant ($P < 0.05$). There was no change in the Hill coefficient of force in Tg-R145W fibers ($n_H = 2.47 \pm 0.14$) compared to Tg-WT fibers ($n_H = 2.52 \pm 0.12$) (Figure 2a). Figure 2b showed that the Ca^{2+} sensitivity measured by ATPase activity was also increased in Tg-R145W fibers compared to Tg-WT fibers. The pCa_{50} was 5.64 ± 0.02 ($n = 14$) and 5.38 ± 0.02 ($n = 13$) for Tg-R145W and Tg-WT fibers, respectively. The $\Delta\text{pCa}_{50} = 0.26$ between Tg-R145W and Tg-WT fibers was also statistically significant ($P < 0.05$). No significant difference in pCa_{50} and Hill coefficient were observed between the Tg-WT and Non transgenic mice (NTg) fibers as shown in Figure 2c and 2d. In Figure 3, maximal force values (10^5 N/m^2) and maximal ATPase rates (s^{-1} per myosin S1 head) were presented for NTg, Tg-WT and Tg-R145W skinned fibers. As shown, there was ~13% increase in maximal Ca^{2+} activated force between Tg-R145W fibers (0.78 ± 0.03) and Tg-WT fibers (0.69 ± 0.02) (Figure 3a). Also, there was ~16% increase in maximum Ca^{2+} activated ATPase in Tg-R145W fibers (6.17 ± 0.19) compared to Tg-WT fibers (5.33 ± 0.24) (Figure 3b). No significant difference was observed comparing the maximal Ca^{2+} activated force and maximal ATPase rates between NTg and WT mice fibers (Figure 3a and 3b). There is no change in the fractional cross-bridge attachment at maximal Ca^{2+} activation in all groups of fibers (data not shown). As illustrated in Figure 4, the rate of cross-bridge turnover (g) as a function of activation state (fraction of maximum activated force) was the same in all groups of fibers. These results show that the hcTnI R145W RCM mutation does not statistically affect the turnover rate of the cross bridge at Ca^{2+} activating levels. Also, there was no statistically difference in energy cost (ATPase/Force) as a function of activation state in all groups of fibers (Figure 5).

Intact papillary muscle fibers—In another series of experiments, simultaneous force and $[\text{Ca}^{2+}]$ transient measurements were performed in electrically stimulated intact papillary muscles (Figure 6 and Table 1). Intact papillary muscles from Tg-R145W mice, Tg-WT mice and NTg mice were examined for both force and $[\text{Ca}^{2+}]$ transients. The muscle was stimulated at 1.0 Hz and force as well as fluorescence signals were recorded simultaneously (see Materials and Methods for details). It is not possible to measure force per cross-sectional area in the intact mouse papillary muscles because of their irregular shapes and cross-section along the length of the fibers. Therefore, we looked for an indirect method for determining the relative transient force differences during a twitch for the Tg-WT and mutant papillary muscle. Unique to this study, from the skinned fiber experiments, we know that the cross-bridge turnover rate (g) was the same for Tg-WT and Tg-R145W fibers for a given level of activation (Figure 4). Additionally from our measurements, the fractional force generating cross-bridge attachment is the same for both types of fibers. Based on the Huxley 1957 model,¹² the fractional force generating cross-bridge attachment equals to $f/(f+g)$. Since g and fractional cross-bridge attachments are the same in Tg-WT and Tg-R145W, we can conclude that the rate parameters f and g should be the same for Tg-WT and Tg-R145W papillary muscles at all levels of activation. So by matching the normalized rate of rise in Tg-R145W force transient (Figure 6a gray solid trace) to Tg-WT force transient (Figure 6a black solid trace), we can conclude that the relative peak force in Tg-R145W force transient (Figure 6a, gray dash trace) is approximately 40% higher than the Tg-WT. This type of analysis only works when the initial phase of the Ca^{2+} transient is identical in the two muscle types, which is actually the case in Tg-R145W. As shown in Figure 6b, the intracellular Ca^{2+} transients are the same for both Tg-R145W and Tg-WT papillary muscles up to the peak. It is only during the falling phase that

the Ca^{2+} transients diverge, and the Tg-R145W calcium transient decays more slowly than the Tg-WT. So the increased time to peak force and higher relative peak force in Tg-R145W papillary muscle results from the fact that the Ca^{2+} transient is longer, causing the papillary muscle to contract longer before the force starts to decay.

Table 1 summarizes the t_{50} and t_{10} values in milliseconds from the peak to 50% and 10%, of force and $[\text{Ca}^{2+}]$ in relaxation, respectively. The t_{50} and t_{10} value of force transients were both ~1.5-fold longer in Tg-R145W papillary muscles compared to those of Tg-WT. At the same time, the t_{50} and t_{10} value of transients in Tg-R145W papillary muscles were ~13% and ~20% longer than in Tg-WT papillary muscles respectively. There were no significant differences in force and $[\text{Ca}^{2+}]$ transients (Figure 6c and 6d) between Tg-WT and NTg mouse fibers.

DISCUSSION

In this study, the physiological changes caused by the hcTnI R145W mutation in transgenic mouse fibers are as follows: 1) a large increase in Ca^{2+} sensitivity of force development and ATPase activity (Figure 2), this increase is even larger than what we observed in Tg-R145G; 2) no significant change in the Hill coefficient in the force-pCa relationship in contrast to a decreased Hill coefficient in the Tg-R145G (Figure 2); 3) a significant ~13%–16% increase in both maximal Ca^{2+} activated force and maximal Ca^{2+} activated ATPase activity (Figure 3) in contrast to a decrease in the maximal force in Tg-R145G; 4) no significant change in fractional cross-bridge attachment at maximal Ca^{2+} activation (data not shown); 5) no statistically significant change in the rate of cross-bridge turnover as a function of activation state (fraction of maximum activated force) (Figure 4); 6) no significant difference in the energy cost (ATPase/force) in contrast to an increase in the Tg-R145G (Figure 5); 7) both force and intracellular Ca^{2+} transients were prolonged (Figure 6); 8) time to peak force increased in contrast to no change for Tg-R145G (Figure 6); and 9) matching normalized rate of rise of Tg-R145W force transient to Tg-WT showed there was approximately a 40% higher peak force and longer time to peak in the Tg-R145W force transient compared to Tg-WT in contrast to no change for the Tg-R145G (Figure 6a, gray dashed line).

As can be seen in Figure 2, there was a large increase in Ca^{2+} sensitivity with no change in the slope of the force-pCa curve. No significant change in the Hill coefficient indicates that the hcTnI R145W mutation does not interfere with regulatory units interaction in the thin filament during activation.¹³ The large increase in Ca^{2+} sensitivity of force development demonstrated in the skinned papillary fibers from Tg-R145W is similar to what was previously shown in skinned porcine fibers reconstituted with mutant R145W hcTnI when compared to wild type hcTnI. These results lead us to the following question: what is responsible for the increase in Ca^{2+} sensitivity? There are two major possibilities: 1) a decrease in the cross-bridge turnover rate¹³ and 2) a decrease in the off-rate of Ca^{2+} from cardiac troponin C.^{14,15} It is well known that the cross-bridge turnover rate can affect the Ca^{2+} sensitivity of skinned fibers.^{16,17,18} Since in this study simultaneous ATPase and force measurements were made in the same fibers, it was possible to determine the reason for the increase in Ca^{2+} sensitivity of force and ATPase. Figure 4 shows that the rate of cross-bridge turnover g is not affected by the mutation, so changes in g as a reason for the increase in Ca^{2+} sensitivity of force and ATPase activity was eliminated. The most likely explanation for this increase is that the hcTnI R145W mutation produces a conformational change in the Tn complex resulting in a decrease in the off-rate of Ca^{2+} from TnC. This conclusion is also in accordance with previous fluorescence data showing that this specific mutation leads to an increase in the Ca^{2+} affinity for cTnC in reconstituted thin filament.¹⁹

The next question is how a decrease in the off-rate of Ca^{2+} from TnC would translate into a physiological defect that would result in RCM? If the off-rate of Ca^{2+} is decreased *in vivo* the

expected results would be prolonged Ca^{2+} and force transients. This is because cTnC is one of the major Ca^{2+} buffers in the cardiac muscle myoplasma and thereby influences Ca^{2+} uptake by the sarcoplasmic reticulum during the relaxation phase of muscle contraction.^{14,20} Figure 6 shows that in intact papillary muscles both the Ca^{2+} and force transients are lengthened in Tg-R145W compared to Tg-WT in addition to time to peak force. Thus, diastolic $[\text{Ca}^{2+}]$ would be increased, especially at high heart rates, resulting in a residual active force during diastole. This would be expected to cause an increase in the resistance to ventricular filling during relaxation (RCM) and also cause diastolic dysfunction by decreasing the filling of the ventricle with blood. This would result in a decrease in stroke volume in severe circumstances, such as under stress. Compensatory hypertrophy (HCM) would be expected in order to maintain stroke volume. The problem is that in RCM there is no hypertrophy associated with it by definition.

Therefore, the hcTnI R145W mutation must somehow improve the ability of the heart to eject blood in order to maintain stroke volume despite the decrease in preload associated with the increased resistance to filling. From previous analysis it is predicted that there would be a 40% increase in peak force during a twitch because of the prolongation of the Ca^{2+} transient (Figure 6a gray dash line). In addition, Figure 3 shows that there is a 13% increase in force per cross-section as well as a similar increase in ATPase activity. In addition to these, the large increase in Ca^{2+} sensitivity of the Tg-R145W fibers, as shown in Figure 2, would also cause an increase in total cross-bridges attached and increase the maximal force.

In conclusion, the increase in contractility for the hcTnI R145W mutation during a twitch comes from two sources: 1) The largest increase comes from the increase in the duration of the Ca^{2+} transient (Figure 6a); 2) Skinned fiber data analysis shows that there would be an additional 13% increase in force due to the extra cross-bridge attachment. This 13% increase in cross-bridge attachment in the Tg-R145W muscles makes the diastolic dysfunction even more severe. These two functional changes add up to a predicted 53% increase in the Tg-R145W cardiac muscle twitch force compared to Tg-WT as shown in Figure 7. This large increase in contractility for the Tg-R145W cardiac muscle is the most important difference between the Tg-R145G and Tg-R145W muscles containing these mutations.

What is responsible for the 13% increase in force per cross-section in the skinned Tg-R145W fibers? There are at least three reasons. First, there could be an increase in the force per cross-bridge. This however cannot be the case since there is no significant change in cross-bridge turnover rate and energy cost. Energy cost equals to ATPase rate/force and is proportional to g/f_{avg} (cross-bridge turnover rate (g)/average force per cross-bridge (f_{avg})). Since both g and energy cost are not changed, f_{avg} cannot change. The second reason would be a decrease in the rate of dissociation of force generating cross-bridges, which is not the case as shown in Figure 4. The third reason is that there was an increase in the number of cross-bridges that can interact with actin. This is the most likely reason since the first two cited reasons are ruled out. The argument for an increase in the number of cross-bridges that can form is that both maximum force and ATPase activity statistically increased proportionately (Figure 3). Since cTnI is part of the thin filament regulatory complex, it may be that in the presence of Ca^{2+} the hcTnI R145W mutation changes the equilibrium between dynamically interconverting open (active) and primed-closed (partially active) conformers of TnC-TnI²¹ such that more myosin binding sites on actin are exposed to interact with myosin.

Differences between our results in transgenic fibers and previous reported results in reconstituted skinned fibers are that they reported a decrease in maximum force in contrast to our increase, and a decrease in the ability of the fibers to relax in the absence of Ca^{2+} , different from our findings of no effect. The most likely reason for the above differences is because of the differences in the reconstituted *in vitro* experiments and skinned fibers from transgenic mice. Another contributing factor to the differences is likely due to the amount of mutated

versus WT protein incorporated (near 100% in the reconstituted fibers and only 10% in the transgenic fibers) into the thin filament of these two systems. However others have shown that different ratios of protein expression did not have large effects on the phenotypes.²²

Why RCM has such a high mortality rate can only be speculated. Even though under normal conditions the RCM heart is able to maintain normal stroke volume, they always start out with a decrease in preload (decreased end diastolic volume) compared to a normal heart. This will decrease cardiac reserve and the maximum volume that can be ejected will also be decreased. Because an increase in cardiac output is required during exercise, cardiac reserve will be further decreased and limited by this fact. Once this limit is approached, increases in the heart rate normally used to increase cardiac output will only cause summation of end diastolic Ca^{2+} resulting in a further decrease in ventricular filling and cardiac reserve. This defect in Ca^{2+} homeostasis could also result in disruption of cell to cell conduction through the gap junctions^{23,24} caused by high diastolic intracellular Ca^{2+} resulting in defective pathways of depolarization resulting in fibrillation and sudden death in humans where the length of the pathways of depolarization are much greater than they are in the mouse. Additionally, lack of cardiac reserve in conditions of hormonal stress caused by fear or excitement could result in a loss of blood pressure as a result of a decrease in peripheral resistance and a complete loss of cardiac reserve finally resulting in heart failure and sudden death. However we did not observe any mortality or histological difference between Tg-R145W and Tg-WT (data not shown).

In conclusion, the main difference between Tg-R145G and Tg-R145W is a decrease in maximum force per cross-sectional area in Tg-R145G and an increase in Tg-R145W. The decrease in maximum force and the physiological evidence of diastolic dysfunction in the R145G mutation can only be compensated for by hypertrophy (HCM). In contrast, the R145W mutation causes an increase in force per cross-section, which will offset the necessity for hypertrophy by ejecting more blood in spite of our physiological evidence for diastolic dysfunction.

Materials and Methods

Generation and characterization of transgenic mice

Transgene construction and transgenic mice generation—The wild type (WT) cDNA for human cardiac TnI (hcTnI) was obtained by RT-PCR using total RNA isolated from human cardiac ventricle and cloned into the pET-3d vector (Novagen) at NCO 1/Bam H1 sites. The hcTnI R145W mutation was made by using overlapping sequential PCR²⁵ and cloned into the same vector as hcTnI WT. The WT and R145W cDNAs were released from pET-3d and subcloned into the Sall site of the plasmid, α -myosin heavy chain promoter (a gift from Dr. J. Robbins, Cincinnati Children's Hospital Medical Center). The resulting constructs contained about 5.5 kb of the mouse α -myosin heavy chain promoter, including the first two exons and part of the third, followed by WT/R145W-cDNA and a downstream 630-base pair region of the 3'-untranslated region (3'-UTR) of the human growth hormone (hGH). The transgene construct described above was 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 and resuspension in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at a final concentration of 20 $\mu\text{g}/\text{ml}$. Mouse pronuclei were injected and the surviving embryos were implanted using standard methods.²⁶ Founder mice were identified by preparing tail clip DNA and analyzing their PCR products corresponding to the α -myosin heavy chain promoter and R192 residue in the cTnI. Stable transgenic lines were generated by breeding founders to non-transgenic (NTg) B6SJL mice. The hcTnI transgenes were confirmed by standard PCR using a pair of primers specifically amplifying a 600-bp fragment of the hcTnI of the transgenic construct. Each PCR reaction also included a set of primers specific for the mouse-actin gene to confirm successful genomic amplification (eliminating false negative animals and producing

a 200-bp PCR product). Two lines of Tg-WT and Tg-R145W (Figure 1b) were produced and the lines with higher protein expression were used for skinned and intact fiber studies.

Analysis of protein expression—The expression of hcTnI protein in the transgenic mice was determined by Western blot analysis. The experimental mouse heart and control human heart tissues were minced in a solution of 1% (v/v) β -mercaptoethanol, 1% (w/v) SDS, 1 mM PMSF, 1 mM EDTA, and Protease Inhibitor Cocktail (Sigma). These samples were homogenized in 20 mM Tris-HCl, pH 7.4, 1% SDS, 1% β -mercaptoethanol, 10% glycerol on ice, and the total protein concentration of each cleared homogenate was determined by Bio-Rad Coomassie Plus assay. SDS-PAGE gels (15%) were run with a total of 2 μ g of protein for each lane. Protein was then transferred to nitrocellulose membranes (Bio-Rad, Hercules, GA, USA). The membrane was blocked in Rockland blocking buffer for 30 mins at room temperature. A monoclonal antibody 6F9 (Fitzgerald Industries international Inc.) was used at 1:4,000 dilution for an hour to detect both human and mouse cTnI. The gel mobility of hcTnI is faster than that of mouse TnI due to its lower molecular mass (24 kDa versus 25 kDa, respectively). Immunoreactivity was detected using goat anti-mouse IgG antibody labeled with CY5.5 fluorescent dye at 1:3000 dilution for an hour in room temperature and the reaction signal blot was scanned with Odyssey Infrared Imager (LICOR) and analysis was carried out on a Dell Pentium Computer using Scion Image software. The percentage of transgenic protein expression was calculated as: X100

Functional studies in skinned papillary muscle fibers

The rationale for using simultaneous measurements of force and ATPase activity is as follows: Force provides a measure of the number of force generating cross-bridges attached. ATPase provides a measure of the rate that ATP is being hydrolyzed. By combining this information it is possible to determine the rate of cross-bridge turnover, energy cost, and changes in force per cross-bridge which is not possible to determine by either force or ATPase measurements alone.

Simultaneous force and ATPase measurements—Mouse papillary muscles, ~1mm long and 60–90 μ m in diameter, were dissected from excised mouse hearts in relaxing solution and then skinned in relaxing solution containing 1% (v/v) Triton X-100 for 30 minutes at room temperature. Standard solution contains 85 mM K^+ , 2 mM $MgATP^{2-}$, 1 mM Mg^{2+} , 7 mM EGTA, 10^{-9} M or $10^{-3.4}$ M Ca^{2+} , and propionate as the major anion. Ionic strength is adjusted to 0.15 and pH is maintained at 7.00 ± 0.02 with imidazole propionate. Relaxing solutions are solutions with no Ca^{2+} added. Maximal contracting solutions are solutions that give maximal tension. For ATPase measurements, solutions contain, in addition to the constituents described above, 5 mM phosphoenol pyruvate (PEP), 0.4 mM NADH, 100 units/ml pyruvate kinase (PK), and 140 units/ml L-lactic dehydrogenase (LDH). The concentrations of the various ionic species in the relaxing and maximal contracting solutions are determined by a computer program using binding constants from the literature.²⁷ The Ca^{2+} concentration in the cuvette perfusing the skinned preparation was varied by use of a gradient maker (Scientific Instruments GmbH, Heidelberg) to mix two solutions (relaxing and maximal contracting) of known Ca^{2+} and ionic composition together. A complete description of the method is given in Allen *et al.*²⁸ The Ca^{2+} concentration in the cuvette perfusing the skinned preparation was varied continuously from pCa 9 to pCa 3.4. The fluorescent Ca^{2+} indicator, Calcium Green-2 (Molecular Probes), was used to calibrate and calculate $[Ca^{2+}]$ during measurements. Calcium Green-2 changes its fluorescence over the range of $[Ca^{2+}]$ required for activation of contraction and ATPase activity. The K_d of Calcium Green-2 used to calculate pCa was $10^{-5.53}$ M. The concentration of Calcium Green-2 in the gradient solution was 1.0 μ M. The Calcium Green-2 fluorescence was excited at 480nm and the fluorescence measured with a cut-off filter at 515 nm.

The skinned fibers were placed in a quartz cuvette and mounted in the Guth Muscle Research System (skinned fibers are held in stainless steel clips attached to a force transducer at one end and a linear motor on the other end),²⁹ which allowed for simultaneous measurements of force and ATPase.^{14,30,31} The length of the fibers was set by removing the slack from the fiber and stretching the fiber 10%. The solution in the cuvette was changed every 20s using a peristaltic pump triggered by a computer. The hydrolysis of ATP was measured by the NADH fluorescence method in which ATP is regenerated from ADP and PEP by the enzyme PK.^{29,32} This reaction is coupled to the oxidation of NADH (fluorescent) to NAD (nonfluorescent) and the reduction of pyruvate to lactate by L-lactic dehydrogenase (LDH).^{14,29,32} In this reaction 1mol of PEP and NADH are used to produce 1mol of ATP and NAD. The solution surrounding the fiber in the quartz cuvette was excited at 340 nm and the decrease in NADH concentration detected by a decrease in the fluorescence signal $\lambda = 450$ nm. The solution in the cuvette was changed every 20s, and the fluorescence change taking place between each solution change was converted to the rate of ATP hydrolysis by comparison to NADH standards. The force and ATPase activity were plotted as a function of pCa. Each curve is the averaged data, as shown in Figure 2.

Fractional cross-bridge attachment at maximal Ca^{2+} activation—At the end of each experiment, the substitution of 10 mM MgADP for 2 mM ATP was made to the maximal Ca^{2+} activating solution in the cuvette perfusing the fiber. This substitution forces all cross bridges into the force generating state producing the maximal force that the fiber can develop. The maximal Ca^{2+} activated force is then divided by the maximum force the muscle can develop in the presence of 10 mM MgADP. This ratio is the fractional cross-bridge attachment at maximal Ca^{2+} activation.

Measurements of the rate of cross-bridge detachment (g)—The force that a muscle develops can be characterized by the formation of force generating states rate (f) and the dissociation of force generating rate (g) according to the two state model of Huxley (1957), as shown in the scheme below¹²

Based on this kinetic scheme, g can be calculated by the following equation:

$$g = \text{ATPase} / \text{total crossbridges attached}$$

Total cross-bridges attached = [myosin subfragment 1 head] x fractional cross-bridge attachment at any Ca^{2+} concentration. The fractional cross-bridge attachment at any Ca^{2+} concentration equals to the fractional cross-bridge attachment at maximal Ca^{2+} activation times the normalized force. The total intracellular myosin subfragment 1 head concentration in muscle is approximately 154 μM .³³ In this study, g was calculated based on the above equation. Figure 4 illustrates g as a function of normalized force.

Functional studies in intact papillary muscle fibers

Measurements of $[\text{Ca}^{2+}]$ and force transients—Following CO_2 euthanasia of the mice, hearts were removed quickly and soaked in ice-cold saline (0.9% NaCl). Intact papillary muscle was dissected quickly from right ventricles in oxygenated Krebs–Henseleit solution (119 mM

NaCl, 4.6 mM KCl, 11 mM glucose, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2mM MgSO₄ 1.8mM CaCl₂) containing 30mM 2,3-butanedione monoxime and mounted in the Guth Muscle Research System.¹⁴ Fibers were loaded in oxygenated Krebs-Henseleit solution without 2,3-butanedione monoxime for an hour at room temperature to allow the muscle to adapt to the extracellular environment. The fibers were then loaded with 5 μ M Fura-2 AM (AM, acetoxymethyl ester form) for 1 h at room temperature in oxygenated Krebs-Henseleit solution containing 0.5% (v/v) of the non-cytotoxic detergent Cremophor, which increases the solubility of Fura-2 AM. The muscle was stimulated at 1.0 Hz through the two tweezers attached to the ends of the preparation. Once the preparation was mounted in the apparatus, muscle length was adjusted until maximum active twitch force was obtained. Force and fluorescence corresponding to either 340 nm or 380 nm excitation were recorded by the computer. The fluorescence 340 nm/380 nm ratio was calculated and plotted along with force. Force and calcium transients were normalized and then averaged and plotted as a function of time, as shown in figure 6.

Statistical analysis

Data are expressed as the average of *n* experiments $\pm$ S.E. (standard error). Statistically significant differences were determined using an unpaired Student's *t*-test (Sigma Plot 8.0), with significance defined as * *P*<0.05 and ** *P*<0.01.

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Figure 1.

Transgene construct and Western Blot (a) hcTnI transgene construct: A schematic diagram of the human cardiac TnI transgene to generate hcTnI R145W mutant overexpressing mice. Sequences corresponding to the α MHC promoter/gene enhancer sites are shown as open boxes. Sequences corresponding to the hcTnI cDNA are represented as a gray box. The point mutation introduced in the hcTnI cDNA, relevant restriction sites and transcription start site are indicated. (b) Western blots of heart extracts from Tg-WT lines (Tg-WT1 indicates Tg-WT line1 and Tg-WT2 indicates Tg-WT line 2) and Tg-R145W lines (Tg-R145W1 indicates Tg-R145W line1 and Tg-R145W2 indicates Tg-R145W line 2) versus NTg and Human Heart extraction. The protein expression levels in transgenic mice were assessed on the basis of densitometry. Western blots labeled with monoclonal troponin I 6F9 antibody and followed by a goat anti-mouse secondary antibody. (c) Summary of all generated lines for Tg-WT and Tg-R145W. Two lines of Tg-WT express $9.84\% \pm 2.37$ and $66.4\% \pm 2.26$; Two lines of Tg-

R145W express $11.20\% \pm 1.44$ and $9.57\% \pm 1.5$. The results are the mean $\pm$ S.E. of 5 measurements.

Figure 2.

The effects of the hcTnI R145W mutation on the Ca^{2+} sensitivity of steady-state force and ATPase activity were measured simultaneously in isometric muscle fibers. (a), (c) The force-pCa relationship in Tg-R145W ($n = 15$) versus Tg-WT ($n = 13$) and NTg ($n = 11$) versus Tg-WT ($n = 13$) mouse fibers, respectively. (b), (d) The ATPase-pCa relationship between Tg-R145W ($n = 14$) versus Tg-WT ($n = 13$) and NTg ($n = 11$) versus Tg-WT ($n = 13$) mouse fibers, respectively. The pCa values and Hill coefficients for force curve and pCa values for ATPase curve are shown in the graphs.

Figure 3.

The effect of the hcTnI R145W mutation on maximal force (10^5 N/m²) and maximal ATPase activity (s^{-1} per myosin head) in transgenic papillary muscle fiber. (a) The maximal Ca^{2+} activated force is ~13% higher in Tg-R145W fibers than in Tg-WT fibers. (b) The maximal Ca^{2+} activated ATPase is ~16% higher in Tg-R145W fibers than in Tg-WT fibers. No statistically significant difference in both maximal Force and ATPase between NTg and Tg-WT fibers ($P > 0.05$). * represent statistically significant difference ($P < 0.05$). Data are expressed as mean of n experiments $\pm$ S.E.

Figure 4.

The rate of cross-bridge turn over (g) as a function of activation state (fraction of maximum activated force) in transgenic skinned papillary muscle fibers. As shown, the dissociation of cross bridge rate (g) was the same in Tg-R145W (n = 15), Tg-WT (n = 13) and NTg (n = 11) fibers.

Figure 5.

The energy cost (ATPase/force) as a function of activation state (fraction of maximum activated force) in all groups of skinned papillary fibers. There was no statistically significant difference in energy cost in Tg-R145W (n = 15), Tg-WT (n = 13) and NTg (n = 11) fibers.

Figure 6.

Force and Ca^{2+} transients in intact transgenic papillary muscle fibers. (a) Force and (b) $[\text{Ca}^{2+}]$ transients in Tg-R145W *versus* Tg-WT mouse intact papillary fibers. In figure a, gray line indicates normalized Tg-R145W force transient and gray dash line indicates matching the normalized rate of rise in Tg-R145W to Tg-WT. (c) Force and (d) $[\text{Ca}^{2+}]$ transients in NTg *versus* Tg-WT mouse intact papillary fibers. Intact papillary muscle fibers were stimulated at 1.0 HZ. Fluorescence produced by Fura-2 is recorded at the same time. As shown, Force and $[\text{Ca}^{2+}]$ transients are both prolonged in Tg-R145W fibers compared to Tg-WT fibers. As predicted, force and $[\text{Ca}^{2+}]$ transients do not have significant difference in Tg-WT fibers compared to NTg fibers. Data are expressed as mean of n (5 ~ 9) experiments $\pm$ SE.

Figure 7.

The predicted twitch force in Tg-R145W compared to Tg-WT. The higher predicted peak force in the Tg-R145W is the sum of the extra force due to a longer calcium transient (40%) and more cross-bridges able to attach (13%).

Table 1

Force and [Ca²⁺] transients in intact papillary muscles from Non-Tg, Tg-WT and Tg-R145W transgenic mice

mouse	Force Transients		Ca ²⁺ Transients		n
	t ₅₀ (Duration)(ms)	t ₁₀ (Duration)(ms)	t ₅₀ (Duration)(ms)	t ₁₀ (Duration)(ms)	
NTg	79.0±4.4	164.0±12.4	165.8±12.4	432.6±9.3	5
Tg-WT	85.3±4.3	180.4±12.2	167.7±6.8	489.7±15.3	9
Tg-R145W	121.7±9.0 ^a	264.2±10.8 ^a	189.7±10.2 ^a	540.2±14.5 ^a	9

t₅₀ (Duration) is the time from peak to 50% of force or Ca²⁺ in relaxation. t₁₀ is the time from peak to 10% of force or Ca²⁺ in relaxation. Data are the mean of the n experiments ±SE.

^a significantly different compared with Tg-WT (P<0.05). There are no significant differences between Tg-WT and NTg.