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Decreased energetics in murine hearts bearing the R92Q mutation in cardiac troponin T

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The thin filament protein cardiac troponin T (cTnT) is an important regulator of myofilament activation. Here we report a significant change in cardiac energetics in transgenic mice bearing the missense mutation R92Q within the tropomyosin-binding domain of cTnT, a mutation associated with a clinically severe form of familial hypertrophic cardiomyopathy. This functional domain of cTnT has recently been shown to be a crucial modulator of contractile function despite the fact that it does not directly interact with the ATP hydrolysis site in the myosin head. Simultaneous measurements of cardiac energetics using ^{31}P NMR spectroscopy and contractile performance of the intact beating heart revealed both a decrease in the free energy of ATP hydrolysis available to support contractile work and a marked inability to increase contractile performance upon acute inotropic challenge in hearts from R92Q mice. These results show that alterations in thin filament protein structure and function can lead to significant defects in myocardial energetics and contractile reserve.

Introduction

Hypertrophic cardiomyopathy is a disease characterized by the presence of a nondilated, usually hypertrophied left ventricle (LV) and greater susceptibility to arrhythmias and sudden death. A dominant feature of most patients with this disease is abnormal diastolic function due to impaired relaxation and reduced LV compliance in spite of preserved or even hyperdynamic LV systolic function (1). In about 60% of these patients, the disease is inherited as an autosomal-dominant familial hypertrophic cardiomyopathy (FHC) (2). Most of the FHC mutations affect the proteins in the sarcomere. One of the most clinically malignant FHC mutations is a missense mutation in the gene for the thin-filament protein cardiac troponin T (cTnT) that results in an amino acid exchange of glutamine (an uncharged amino acid) for

arginine (a positively charged amino acid) at residue 92 (R92Q). Individuals with this mutation represent a unique clinical subset of FHC characterized by early sudden death in the absence of overt ventricular hypertrophy; approximately 50% of affected individuals die by the third decade of life.

Troponin T plays a crucial role in the regulation of the contractile cycle because it binds to tropomyosin (TM) where the two TM polypeptide chains overlap (3–5). Recent biophysical data (6) have shown that when the TnT tail binds to this region, changes in the structure of the C-terminal domain of TM occur that affect the flexibility of the entire TM filament. This in turn determines the affinity of the TM-TnT complex for actin. Two recent reports, one using cTnT (3) and the other skeletal muscle TnT (4), have found that in the absence of the other regulatory thin filament proteins, the tail portion of TnT exhibits a multifunctional role in that it inhibits myosin subfragment-1 ATPase activity, slows in vitro motility of actin-TM filaments on heavy meromyosin, and increases the cooperativity of myosin subfragment-1 binding to thin filaments. Thus, in addition to a structural role, TnT plays an important functional role in thin filament activation and crossbridge cycling in striated muscles. Several authors have suggested that the differing structures of the tail domain of TnT's explain the differences between skeletal and cardiac thin filament properties (4, 7), differences in the properties of TnT isoforms (8), and differences in properties of the sarcomeres in hypertrophic and dilated cardiomyopathic hearts (9). The R92Q mutation studied here is located

in the elongated tail domain of cTnT at the critical site of overlap of the TM monomers, and missense mutations at this site in the cTnT tail have been shown to alter thin filament function (6, 10).

The conformational changes that occur in normal thick and thin filament proteins during the contractile cycle are energetically unfavorable and must be coupled to the hydrolysis of ATP in order to proceed. The chemical driving force available to drive ATPase reactions is quantitatively expressed as the free energy of ATP hydrolysis, ΔG_{-ATP}. The free energy of ATP hydrolysis is the sum of the constant value for ATP hydrolysis under standard conditions corrected for the actual concentrations of ATP, ADP, and inorganic phosphate (Pi) in the cytosol. The relationship between ΔG_{-ATP} and contractile performance in the normal myocardium shows that decreasing the chemical driving force for ATPase reactions limits contractile performance (11). Diastolic performance in normal (12) and hypertrophied (13) myocardium is impaired by the accumulation of the hydrolysis products of ATP, namely ADP and Pi, and thus leads to a lower chemical driving force.

While it is now clear that the concentration of ATP (as well as the primary high-energy reserve compound phosphocreatine [PCr]) is lower in the severely failing human heart than for normal heart (14), little information is available defining the energetics of hearts of either FHC patients or animal models of FHC. One study using ^{31}P NMR spectroscopy of hearts of asymptomatic patients with hypertrophic cardiomyopathy showed the ratio of PCr to ATP concentrations was decreased (15). A ^{31}P NMR spectroscopy study of hearts isolated from a mouse bearing a missense mutation at the 403 position of myosin heavy chain made the unexpected observation that this mutation in the actin-binding domain of myosin led to decreased PCr concentrations and less favorable ΔG_{-ATP} (16). These hearts demonstrated diastolic dysfunction and reduced contractile reserve with inotropic challenge.

In the present study using the intact heart, we test the hypothesis that the structural changes caused by the R92Q missense mutation in the TM-binding domain of cTnT lead to increased ATP utilization and impair the ability of the heart to recruit its contractile reserve upon acute inotropic challenge. If this is the case, it would be the first time that a mutation in a thin filament protein is shown to result in altered energetics in the intact heart. We used a well-characterized mouse model that expresses a *c-myc*–tagged full-length murine cTnT bearing the R92Q mutation (67% replacement of native cTnT) specifically in the heart (17). This model was chosen because it recapitulates many of the aspects of the human disease including myocyte disarray and diastolic dysfunction in the absence of ventricular hypertrophy. We used the non-invasive tool of ^{31}P NMR spectroscopy to measure the energetic state of the heart simultaneously with measurements of systolic and diastolic contractile performance (18). We found that the presence of the single amino acid missense mutation R92Q in cTnT, known to occur within the cTnT-TM binding site that determines thin filament structure and function, leads to increased ATP utilization and impaired ability of the heart to recruit its contractile reserve.

Methods

Mice. Sixteen-week-old mice bearing *c-myc*–tagged murine cTnT with the R92Q mutation (R92Q) or WT cTnT with the *c-myc* tag (WT-Tg) were generated as previously described (17); sibling mice were used as non-transgenic (NTG) controls. Each animal was genotyped by PCR-amplified tail DNA and restriction enzyme digestion to confirm the presence or absence of the mutation. Animals were maintained in accordance with NIH guidelines for the care and use of laboratory animals. The experimental protocol was approved by the Standing Committee on Animals of Harvard Medical Area and followed current NIH and American Physiologic Society guidelines.

Isolated perfused heart preparation. Hearts with cardiac-specific R92Q mutation in cTnT (R92Q) (*n* = 15) and their NTG littermate controls (*n* = 12), and hearts with normal cTnT bearing the *c-myc* tag (WT-Tg) (*n* = 4) and their littermate controls (*n* = 4) were isolated and perfused in the Langendorff mode as described (19). Hearts were initially perfused with a phosphate-free Krebs-Henseleit buffer containing 118 mM NaCl, 5.3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 0.5 mM EDTA, 25 mM NaHCO₃, and 11 mM glucose equilibrated with 95% O₂ and 5% CO₂, pH 7.4. A water-filled balloon custom-made of polyvinylchloride film was inserted through the mitral valve into the LV via an incision in the left atrium. The balloon was connected to a pressure transducer (Statham P23Db; Gould Statham Inc., Medical Provision Department, Oxnard, California, USA) for continuous recording of LV pressures and heart rate. The size of the balloon matched the size of the ventricular cavity. The balloon was inflated to set LV end diastolic pressure to approximately 10 mmHg and the balloon volume was then held constant. Isovolumic contractile performance data were collected online at a sampling rate of 200 Hz using a commercially available data acquisition system (MacLab; ADInstruments Pty., Milford, Massachusetts, USA). LV developed pressure (DevP), i.e., the difference between systolic pressure and end diastolic pressure, and the minimum and maximum values within a beat of the first derivative of left ventricular pressure (+dP/dt and –dP/dt) were calculated offline. All hearts were paced at 7 Hz using monophasic square-wave pulses delivered from a stimulator (model S88; Grass Instrument Co., Quincy, Massachusetts, USA) through salt-bridge pacing wires consisting of PE-90 tubing filled with 4 M KCl in 2% agarose.

Experimental protocols. In one protocol, ^{31}P NMR spectra of the whole heart (eight sibling NTG and nine R92Q hearts and four each sibling NTG and WT-Tg hearts) were obtained during baseline perfusion with

buffer containing only glucose. The buffer was then switched to one containing both glucose and pyruvate (0.5 mM) and another ^{31}P NMR spectrum was obtained. Adding pyruvate to the buffer maximized the ratio of PCr to ATP. Finally hearts were studied at high workload achieved by increasing the total [Ca^{2+}] to 4.0 mM in a perfusate buffer containing both glucose and pyruvate. Data defining isovolumic contractile performance were collected continuously throughout the three sequential 16-minute periods required for these NMR measurements. In the second protocol, myocardial oxygen consumption was measured in four NTG and five R92Q isolated perfused hearts as described (20). At the end of the experiments, each heart was either rapidly weighed or freeze-clamped, and then stored at –80°C for subsequent biochemical assays.

^{31}P NMR spectroscopy. ^{31}P NMR spectra were obtained at 161.94 MHz using a GE-400 wide-bore spectrometer (Omega, General Electric, Fremont, California, USA). Hearts were placed in a 10-mm glass NMR tube and inserted into a custom-made ^{1}H/^{31}P double-tuned probe situated in a superconducting magnet (89-mm bore, 9.4 T). Spectra were collected without proton decoupling at a pulse width of 16 μs, pulse angle of 60°C, recycling time of 2.14 seconds, and sweep width of 6,000 Hz. Spectra for individual hearts were obtained by signal averaging 416 free induction decays; the spectra shown in Figure 1 are the sum of six such spectra. Spectra were analyzed using 20-Hz exponential multiplication and zero and first-order phase corrections. The resonance areas corresponding to ATP, PCr, and Pi were fitted to Lorentzian functions calculated using the commercially available program NMR1 (NMR1, Syracuse, New York, USA) (21) and corrected for saturation [ATP (1.0), PCr (1.2), and Pi (1.15)].

Biochemical measurements. Five to 10 mg of LV tissue from NTG and R92Q hearts was homogenized for 10 seconds at 4°C in potassium phosphate buffer containing 1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.4 (final concentration, 5 mg tissue/ml). Aliquots were removed for assays of protein (22) using BSA as the standard and for total creatine content (23). Triton X-100 was then added to the homogenate at a final concentration of 0.1%. Tissue activities of creatine kinase; the glycolytic enzymes phosphofructokinase, GAPDH, and lactate dehydrogenase; the mitochondrial enzyme citrate synthase and the isoenzyme distributions of creatine kinase and lactate dehydrogenase were measured as described (24–27). All reagents came from Sigma Chemical Co. (St. Louis, Missouri, USA) and were at least analytical grade.

Metabolite concentrations. To determine the cytosolic concentration of ATP, the absolute resonance areas corresponding to ATP (average of β-phosphate and γ-phosphate resonance areas) in the ^{31}P NMR spectra during baseline perfusion were normalized by heart weight. Mean ATP area units/mg wet weight for NTG and R92Q hearts were 0.63 ± 0.04 and 0.58 ± 0.08 during glucose perfusion and 0.55 ± 0.03 and 0.50 ± 0.07 for perfusion

with glucose and pyruvate, respectively. We made the assumption that the ratio of intracellular volume to cardiac mass of 0.48 μl/mg wet weight was the same for all hearts (28). Thus, regardless of the buffer used, the NTG and R92Q hearts have indistinguishable [ATP].

Cytosolic [ADP] was calculated using the equilibrium expression for the creatine kinase reaction and values for ATP, PCr, creatine, and H$^+$ concentrations obtained by NMR spectroscopy and biochemical assays: [ADP] = ([ATP][free creatine])/([PCr][H$^+$]K_{eq}); where K_{eq} is 1.66×10^9 M^{-1} for [Mg^{2+}] of 1.0 mM (29, 30).

The free energy released by ATP hydrolysis (ΔG_{-ATP}) drives the ATPase reactions in the cell. Because ΔG_{-ATP} is a negative number, we describe changes in ΔG_{-ATP} in absolute values, denoted as $|\Delta G_{-ATP}|$. ΔG_{-ATP} is calculated as $|\Delta G_{-ATP}|$ (kJ/mol) = $|\Delta G° + RT \ln ([ADP][Pi]/[ATP])|$ where $\Delta G°$ (–30.5 kJ/mol) is the value of ΔG_{-ATP} under standard conditions of molarity, temperature, pH, and [Mg^{2+}] (28), R is the gas constant (8.3 J/mol K), and T is the temperature in degrees Kelvin.

Statistical analysis. All results are expressed as mean ± SEM. The paired (for repeated measures performed on the same heart) or unpaired Student t test or factorial ANOVA was used to compare the NTG and transgenic hearts as appropriate. All statistical analyses were performed with STATVIEW (Brain Power Inc., Calabasas, California, USA), and a value of $P < 0.05$ was considered significant.

Results

Characteristics of mice. NTG and R92Q transgenic littermates had similar body weights (26.2 ± 0.6 vs. 27.2 ± 0.7 g, respectively). The atria of R92Q hearts were larger (NTG, 7.2 ± 0.6 vs. R92Q, 10.2 ± 0.6 mg), while both the right ventricular (NTG, 20.1 ± 1.3 vs. R92Q, 17.3 ± 10.8 mg) and LV (NTG, 91.7 ± 4.2 vs. R92Q, 73.6 ± 35 mg) weights of R92Q hearts were smaller than those of NTG hearts. Because the overall heart weight of the R92Q hearts was about 15% less than that of NTG hearts (NTG, 119.1 ± 5.4 vs. R92Q, 100.9 ± 3.9 mg), the

Figure 1
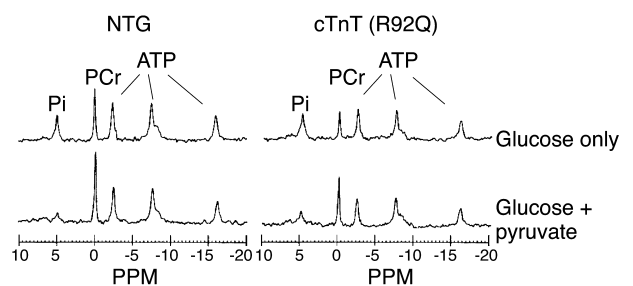Sum of ^{31}P NMR spectra from six 16-week-old NTG (left) and R92Q (right) hearts obtained during baseline perfusion with buffer containing either 11 mM glucose (top spectra) or 11 mM glucose with 0.5 mM pyruvate (bottom spectra). Resonance areas from left to right correspond to Pi, PCr, and γ-, α-, and β-phosphates of ATP. R29Q hearts had lower PCr and higher Pi resonance areas than did NTG hearts. PPM, parts per million.

Table 1

Indices of isovolumic contractile performance of NTG and cTnT (R92Q) hearts at baseline and in response to inotropic stimulation

	NTG		R92Q	
	$[Ca^{2+}] = 2.5$ mM	$[Ca^{2+}] = 4.0$ mM	$[Ca^{2+}] = 2.5$ mM	$[Ca^{2+}] = 4.0$ mM
n	8	7	9	9
SP	94.4 ± 5.9	141.5 ± 10[A]	83.9 ± 9.2[B]	86.3 ± 12.6[B]
EDP	9.9 ± 0.6	9.4 ± 1.1	9.7 ± 0.3	11.1 ± 0.8
+dP/dt	3,500 ± 220	5,500 ± 500[A]	3,400 ± 470[B]	3,900 ± 700 [B]
−dP/dt	2,700 ± 250	4,900 ± 360[A]	2,000 ± 300[A,B]	2,300 ± 440 [B]
DevP	84.4 ± 6.0	132.1 ± 9.7[A]	74.3 ± 9.3[B]	75.2 ± 12.5[B]
RPP × 10⁻³	39.1 ± 2.7	59.2 ± 2.8[A]	34.7 ± 4.3[B]	33.6 ± 5.3 [B]

NTG and cTnT (R92Q) hearts were perfused with buffer containing glucose and pyruvate, and contractile performance was measured at baseline (2.5 mM calcium) and in response to inotropic stimulation with 4.0 mM calcium. [A]$P < 0.05$ compared with NTG baseline. [B]$P < 0.05$ vs. NTG high work. Heart rate equals 460 beats per minute. EDP, end diastolic pressure. SP (systolic pressure), EDP, and DevP are given in mmHg; dP/dt is in mmHg/s; RPP is in mmHg/min.

heart weight to body weight ratio of the R92Q mice was about 20% smaller (NTG, 4.6 ± 0.2 vs. R92Q, 3.7 ± 0.1). Thus, in agreement with previous reports (17), the transgenic hearts bearing the R92Q mutation in cTnT were smaller than sibling NTG hearts. Hearts bearing the *c-myc* tag on WT cTnT (WT-Tg) were not different in weight from sibling NTG hearts (129 ± 8.1 vs. 113 ± 5.9 mg for WT-Tg and NTG mice, both weighing 25.6 g, respectively).

Contractile performance. When perfused in the Langendorff mode (Table 1), murine hearts bearing the R92Q mutation in cTnT exhibited similar indices of systolic contractile performance (assessed as systolic pressure, peak DevP, +dP/dt, and rate pressure product [RPP]) as NTG hearts. Diastolic function (assessed as −dP/dt) was impaired in R92Q hearts.

To determine whether R92Q hearts were able to increase contractile performance in response to inotropic challenge, we measured contractile performance in sibling NTG and R92Q hearts perfused with normal and high $[Ca^{2+}]$ (Table 1). In the NTG hearts, increasing $[Ca^{2+}]$ from 2.5 to 4 mM resulted in a greater than 50% increase in all indices of contractile performance: systolic pressure, peak DevP, and +dP/dt. The rate of relaxation also increased. In contrast, the R92Q hearts failed to increase contractile performance in response to increases in $[Ca^{2+}]$; none of the indices of either systolic or diastolic performance changed. Thus, transgenic hearts bearing the R92Q mutation in cTnT exhibit diastolic dysfunction under baseline conditions and fail to increase contractile performance in response to inotropic challenge.

Energetics. We used ³¹P NMR spectroscopy to measure ATP, PCr, and Pi concentrations of sibling NTG and R92Q hearts at baseline and at high $[Ca^{2+}]$ to determine whether the R92Q mutation in cTnT causes a change in energetics at baseline and to test whether the decreases in contractile reserve are due to decreases in energy reserve (12). We also made comparable measurements in sibling NTG and WT-Tg hearts to test whether the presence of the *c-myc* tag alone affected whole-heart energetics.

Figure 1 shows ³¹P NMR spectra acquired during baseline perfusion from NTG and R92Q hearts per-

fused with either glucose only (top) or glucose plus pyruvate (bottom). The ATP resonance areas/mg wet weight, and thus the amount of ATP/mg tissue, were similar for the NTG and R92Q hearts, but the PCr resonance areas were smaller and Pi resonance areas higher in the R92Q heart, regardless of the exogenous oxidizable substrate supplied to the heart.

Table 2 and Table 3 show the mean values of [ATP], [ADP], [PCr], and [Pi] determined at baseline with glucose-containing perfusate and at baseline and high workload conditions when both glucose and pyruvate were the exogenous substrates supplied to support ATP synthesis for NTG and R92Q hearts. Regardless of exogenous substrate supplied to support ATP synthesis, NTG and R92Q hearts had similar [ATP] but R92Q hearts had lower [PCr] (by ~20%) and higher [Pi] (2.2- to 3.7-fold). The decrease in [PCr] in R92Q hearts was not due to a decrease in total creatine concentration, which was not different between NTG and R92Q hearts (Table 4). Instead, the decrease in [PCr] is due to increased ATP utilization. R92Q hearts had higher cytosolic [ADP] and higher [Pi] than NTG hearts. Intracellular pH was similar in NTG and R92Q hearts and averaged 7.14.

Changes in [ATP], [ADP], and [Pi] can be expressed as a single number, the free energy of ATP hydrolysis, ΔG_{-ATP}. This value represents the driving force for the ATPase reactions in the cell and describes the energy state of the cell. Regardless of which substrate was sup-

Table 2

Energetics of NTG and cTnT (R92Q) hearts perfused with buffer containing glucose and 2.5 mM Ca^{2+}

	NTG	R92Q
n	8	10
ATP (mM)	9.9 ± 0.01	10.0 ± 0.02
PCr (mM)	10.7 ± 0.7	8.7 ± 1.0
Pi (mM)	7.9 ± 1.1	17.2 ± 2.4[A]
pH	7.14 ± 0.01	7.15 ± 0.02
ADP (μM)	136 ± 18	233 ± 50[A]
$|\Delta G_{-ATP}|$ (kJ/mol)	54.3 ± 0.6	51.3 ± 0.5[A]

[A]$P < 0.05$ compared with NTG.

Table 3
Energetics of NTG and cTnT (R92Q) hearts perfused with glucose and pyruvate at baseline and in response to inotropic stimulus

| | NTG | | R92Q | |
	$[Ca^{2+}]$ = 2.5 mM	$[Ca^{2+}]$ = 4.0 mM	$[Ca^{2+}]$ = 2.5 mM	$[Ca^{2+}]$ = 4.0 mM
n	7	6	9	8
ATP (mM)	8.8 ± 0.3	8.8 ± 0.3	9.4 ± 0.6	8.1 ± 0.6[C]
PCr (mM)	14.7 ± 0.6	11.7 ± 0.6[A]	12.3 ± 0.3[A]	10.0 ± 0.9[A,C]
Pi (mM)	2.7 ± 0.5	4.9 ± 0.5	10.1 ± 1.8[A,B]	10.8 ± 0.9[A,B]
pH	7.14 ± 0.01	7.14 ± 0.02	7.11 ± 0.06	7.11 ± 0.02
ADP (μM)	64 ± 8	113 ± 25	101 ± 19	129 ± 31
$|\Delta G_{-ATP}|$ (kJ/mol)	58.9 ± 0.8	55.9 ± 0.4[A]	54.8 ± 0.7[A]	53.4 ± 0.7[A,B,C]

[A]$P < 0.05$ compared with baseline. [B]$P < 0.05$ compared with NTG (4.0mM calcium). [C]$P < 0.05$ compared with cTnT (R92Q) (2.5 mM calcium).

plied to the heart to support ATP synthesis, the values of $|\Delta G_{-ATP}|$ are approximately 3–4 kJ/mol lower in R92Q hearts. Thus, hearts bearing the R92Q cTnT missense mutation have a lower driving force for ATPase reactions. The presence of the *c-myc* tag did not change the driving force for the ATPase reactions regardless of exogenous substrate supplied to the hearts: values for $|\Delta G_{-ATP}|$ for WT-Tg and sibling NTG hearts perfused with glucose and pyruvate were 60.7 and 60.8 kJ/mol, respectively.

Contractile performance and energetics. The relationships between physiologic performance of the heart assessed as RPP and $|\Delta G_{-ATP}|$ for NTG, WT-Tg, and R92Q hearts perfused using buffers containing 2.5 and 4.0 mM $[Ca^{2+}]$ are shown in Figure 2. Both NTG and WT-Tg hearts demonstrated the expected decrease in $|\Delta G_{-ATP}|$ as RPP increased in response to inotropic challenge (12, 16), and the quantitative relationships between RPP and $|\Delta G_{-ATP}|$ were indistinguishable for NTG and for WT-Tg hearts. In contrast, R92Q hearts not only had lower $|\Delta G_{-ATP}|$ at baseline conditions but failed to support an increase in RPP with the equivalent inotropic challenge. Upon inotropic challenge, the free energy of ATP hydrolysis for R92Q hearts fell by about 1.4 kJ/mol and RPP did not increase. Thus the R92Q hearts were not able to transduce the small amount of free energy released from ATP hydrolysis into mechanical work.

To rule out that the inability of R92Q hearts to increase contractile performance was due to decreased capacity for ATP synthesis, we also measured myocardial oxygen consumption (MVO_2) at different RPPs, which were varied by changing perfusate $[Ca^{2+}]$ (Figure 3). The data for four NTG and five R92Q hearts were fit well by the same linear relationship: $MVO_2 = 1.6 \times 10^{-5}$ RPP + 4.7. The capacity for ATP synthesis via oxidative phosphorylation assessed by citrate synthase activity (a good marker of mitochondrial mass), via glycolysis (measured as activities of phosphofructokinase, GAPDH, and lactate dehydrogenase), and via the creatine kinase reaction was the same in R92Q and NTG hearts (Table 4). The isoenzyme distributions for lactate dehydrogenase and creatine kinase were unchanged (data not shown). In addition, the capacity for ATP synthesis was unchanged in WT-Tg hearts

(data not shown). Thus, neither the capacity for ATP synthesis nor the coupling of MVO_2 and RPP differed between NTG and R92Q hearts.

Discussion
There are two major findings in the studies reported here. First, hearts bearing the R92Q mutation in cTnT have decreased [PCr] and increased [Pi] and [ADP], leading to a large decrease (~4 kJ/mol) in the free energy of ATP hydrolysis, $|\Delta G_{-ATP}|$. This result shows that this single amino acid replacement in the TM-binding domain of cTnT at a site known to cause changes in thin filament structure and function is sufficient to increase ATP utilization in the intact beating heart. Second, upon acute inotropic challenge, the small amount of free energy expended in the cTnT mutant hearts (~1.4 kJ/mol) failed to support an increase in contractile performance. The observation that R92Q hearts fail to recruit their contractile reserve suggests that the R92Q mutation in the TM-binding domain of cTnT disrupts the normal transmission of the Ca^{2+} signaling cascade from troponin C (cTnC) to TM. Mutant cTnT hearts also exhibit work-induced diastolic dysfunction. Importantly, since hearts (and myocytes) bearing the cardiac-specific R92Q mutation are smaller than littermate NTG hearts, these energetic and contractile

Table 4
Biochemical characteristics of NTG and cTnT (R92Q) mouse hearts

	NTG	R92Q
n	8	8
Protein content (mg/mg wet weight)	0.173 ± 0.008	0.174 ± 0.004
[Cr] (mM)	21.56 ± 1.0	21.2 ± 1.99
CK (IU/mg protein)	8.0 ± 0.2	8.7 ± 0.4
LDH (IU/mg protein)	0.93 ± 0.03	1.0 ± 0.06
GAPDH (mIU/mg protein)	940 ± 78	919 ± 59
PFK (mIU/mg protein)	315 ± 23	316 ± 23
CS (mIU/mg protein)	805 ± 37	799 ± 45

Results for NTG and R92Q hearts are indistinguishable. Results for *c-myc*–tagged WT and sibling NTG hearts are also indistinguishable (data not shown). Cr, creatine; PFK, phosphofructokinase; LDH, lactate dehydrogenase; CS, citrate synthase. Data are mean ± SEM.
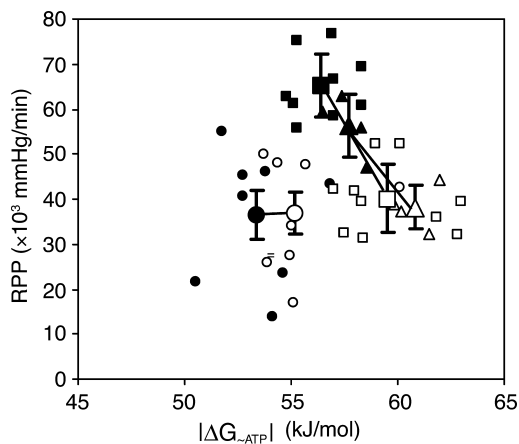
Figure 2

Plot of the relationship between RPP and $|\Delta G_{\sim ATP}|$ for NTG (squares, $n = 9$), WT-Tg (triangles, $n = 4$), and R92Q (circles, $n = 8$) hearts perfused with buffers containing glucose and pyruvate with 2.5 mM (open symbols) and 4.0 mM (filled symbols) Ca^{2+}. The means of the NTG hearts (large squares), WT-Tg hearts (large triangles), and R92Q hearts (large circles) for each workload are connected. The relationships for NTG and WT-Tg mice are indistinguishable; the relationship for R92Q hearts differs from that of both NTG and WT-Tg hearts.

defects are not a consequence of cardiac hypertrophy or LV dilatation but are the result of the mutation in the thin filament protein cTnT.

The known molecular mechanisms that can lead to decreased PCr with normal total creatine and increased Pi and ADP concentrations leading to lower $|\Delta G_{\sim ATP}|$ include: increased use of myofibrillar ATP (by far the major ATPase in the myocyte); a very large increase in ATP utilization by the sarcoplasmic reticular Ca^{2+}-ATPase (SERCA); and decreased ATP synthesis, singly or in any combination (31). The possibility that the R92Q mutation in cTnT leads to a change in the conformation of cTnC that in turn leads to increased ATP utilization by SERCA can be ruled out. As noted by Tobacman and colleagues (3), cTnC is physically distant from the cTnT tail. Instead, the physical interaction that occurs is between the cTnT tail and the flexible region of the TM filament, thereby altering the TM-actin interaction, which in turn alters crossbridge cycling. The physical constraints of this highly ordered system must lead to increased ATP utilization by the myofibril, not by SERCA. Additional evidence ruling out increased ATP utilization by SERCA includes the observation that myocytes bearing the R92Q mutation in cTnT have indistinguishable levels of SERCA mRNA (17) and protein (J.C. Tardiff, unpublished results). Finally, using the same protocol described here, hearts in which SERCA levels varied by as much as 50% have a normal relationship between RPP and $|\Delta G_{\sim ATP}|$ (I. Pinz, R. Tian, W. Dillman, and J.S. Ingwall, unpublished results).

Decreased free energy of ATP hydrolysis and the inability of the R92Q heart to increase contractile performance upon demand are not due to decreased ATP

synthesis since none of the following differed in R92Q and NTG hearts: ATP and total creatine concentrations; the capacity for ATP synthesis by oxidative phosphorylation, glycolysis, or via the creatine kinase reaction; and the MVO_2/RPP relationship. In addition, the changes in $|\Delta G_{\sim ATP}|$ were independent of the exogenous substrate supplied.

Thus, the results presented here show that the R92Q mutation in the TM-binding domain of cTnT changes the cTnT-TM interaction in such a way that whole-heart ATP utilization rates are chronically higher than those of NTG hearts, as evidenced by a decrease in PCr and increases in Pi and ADP concentrations. The magnitude of the difference between cTnT and NTG hearts in the driving force for the ATPase reactions is large (~4 kJ/mol) and is particularly surprising in view of the relatively modest effects of the R92Q mutation on the flexibility of the TM filament and affinity for actin compared with the R92L and R92W mutations observed in careful biophysical measurements using isolated polypeptides (6). This illustrates the importance of making these measurements in the intact heart, where the impact of apparently small perturbations at the polypeptide level can be summed over a large number of myofibrils. The magnitude of the difference in the driving forces for the ATPase reaction between R92Q and NTG hearts is comparable to the change in energy reserve that led to decreased contractile reserve in normal hearts (11), and is sufficient to explain the decrease in contractile reserve observed for the R92Q hearts. As reported previously for normal and hypertrophied hearts (12, 13), the relatively high concentrations of Pi and ADP observed for the R92Q hearts are also sufficient to cause the slowed rate of relaxation.

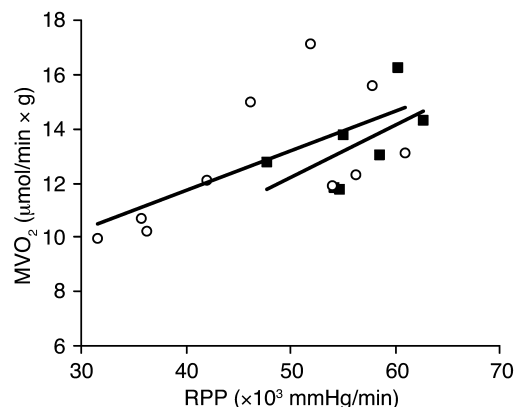The only other mouse model of FHC in which energetics and contractile performance have been report-

Figure 3

Relationship between MVO_2 and RPP in NTG (filled squares) and R92Q (open circles) hearts. Hearts were perfused with the same buffers described in Figure 2 legend. The equations for the linear regressions are similar in both groups: NTG ($n = 4$, seven data points), $MVO_2 = 1.9x + 2.65$; R92Q ($n = 5$, ten data points), $MVO_2 = 1.4x + 5.94$; however, the R92Q hearts fall on the lower part of the relationship.

ed in an intact heart is the R403Q missense mutation (50% replacement) in the murine α-myosin heavy chain gene (αMyHC) (16). The comparison with the mouse hearts bearing the R92Q mutation in the *cTnT* gene is instructive. The most striking similarity between the hearts bearing the R403Q mutation in the *αMyHC* gene and the R92Q mutation in the *cTnT* gene is the unexpected defects in energetics; both hearts have decreased PCr and increased Pi concentrations, leading to a decrease in the free energy of ATP hydrolysis. It is important to emphasize that, although neither amino acid residue is physically near the ATP-hydrolysis site in the myosin head, a common result of both the R403Q mutation in the actin-binding domain of the myosin head and the R92Q mutation in the TM-binding domain of cTnT is greater ATP utilization. Taken together, these results show that altering the actin-myosin interaction either by a charge change in the actin-binding domain of myosin (R403Q) or by altering TM flexibility and affinity for actin via a charge change in cTnT (R92Q) leads to structural changes in the thin filament that cost energy at the level of the cardiac sarcomere. As elegantly shown by others for both cardiac and skeletal muscle TnT's, the tail portion of TnT influences the size of the cooperative unit and the transition from weak to strong binding states, two ways that mutated filaments can alter energy utilization. Of note, in 1998, Sweeney and colleagues suggested that cTnT-related mutations in the N-terminal domain could increase the energy cost of force production; the data presented here have shown for the first time that this is indeed the case (32).

An important difference between the two FHC models is that the αMyHC mutant mouse heart was able to use the free energy available from ATP hydrolysis to support an increase in contractile performance elicited by the same inotropic challenge used in this study for the cTnT mutant mouse. The change in $|\Delta G_{-ATP}|$ for the R403Q αMyHC mouse heart was over 4 kJ/mol, the same change observed for their (and the present) littermate controls. For the cTnT mouse hearts bearing the R92Q mutation, there was only a small change in $|\Delta G_{-ATP}|$ upon challenge with high Ca^{2+} perfusion (~1.4 kJ/mol), which was unable to support an increase in contractile performance. Although speculative, it seems likely that the differences in energetic and contractile phenotypes observed for these two thick and thin filament FHC-associated mutations in these murine models may have a clinical correlate and contribute to the strikingly different clinical courses seen in patients with mutations in different sarcomeric proteins. A recent report using a murine mutant cTnT model (I79N) with characteristics similar to the R92Q mutation studied here demonstrated that while the I79N mice were able to tolerate a training exercise regimen, mice subjected to intraperitoneal isoproterenol injection exhibited a high frequency of sudden death (33). The findings reported here are consistent with a

potential pathogenic mechanism for FHC whereby acute adrenergic challenge of hearts with chronic low driving force for ATPase reactions leads to cardiac decompensation. If this is the case, the results reported here suggest that lethality associated with FHC need not be due solely to ventricular hypertrophy, but may also result from discrete alterations in the regulation of myofilament activation at the level of the cardiac sarcomere. These changes may well contribute to the broad range of clinical phenotypes that have been observed in patients with cTnT-related FHC.

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