Hypertrophy, Fibrosis, and Sudden Cardiac Death in Response to Pathological Stimuli in Mice With Mutations in Cardiac Troponin T

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Background—Transgenic mouse models expressing a missense mutation (R92Q) or a splice donor site mutation (trunc) in the cardiac troponin T (cTnT) model familial hypertrophic cardiomyopathy (FHC) in humans. Although males from these strains share the unusual property of having significantly smaller ventricles and cardiac myocytes, they differ with regard to systolic function, fibrosis, and gene expression. Little is known about how these phenotypes affect the responses to additional pathological stimuli.

Methods and Results—We tested the ability of hearts of both sexes of wild-type and mutant mice to respond to defined pathological, pharmacological, hypertrophic stimuli in vivo. Hearts of mutant cTnT models of both sexes were able to undergo hypertrophy in response to at least one stimulus, but the extent differed between the 2 mutants and was sex specific. Interestingly, the trunc-mutant mouse heart was resistant to the development of fibrosis in response to pharmacological stimuli. Stimulation with 2 adrenergic agonists led to sudden cardiac death of all male but not female mutant animals, which suggests altered adrenergic responsiveness in these 2 models of FHC.

Conclusions—Hypertrophic signaling is differentially affected by distinct mutations in cTnT and is sex modified. Hearts can respond with either an augmented hypertrophic and fibrotic response or a diminished hypertrophy and resistance to fibrosis. Sudden cardiac death is related to adrenergic stress and is independent of the development of fibrosis but occurred only in male mice. These results suggest that patients with certain TnT mutations may respond to certain pathological situations with a worsened phenotype. (Circulation. 2004;110:2102-2109.)

Key Words: cardiomyopathy — catecholamines — death, sudden — hypertrophy — remodeling

Familial hypertrophic cardiomyopathy (FHC) is a heterogeneous disease caused by autosomal-dominant mutations in contractile proteins.1 Mutations in the cardiac troponin T (cTnT) gene are a common cause of FHC, accounting for 7% to 15% of cases.2,3 Mutations in cTnT are often associated with relatively mild hypertrophy but a high incidence of sudden death.4 We have created transgenic mouse lines expressing mutant murine cTnT that mimic human mutations.5,6 One mutant is a truncated cTnT protein (TnT-trunc) missing the last coding exon. Mouse lines expressing 4% and 6% of their TnT protein as the truncated isoform exhibit smaller left ventricles, severe diastolic and milder systolic dysfunction, and myocardial disarray but no fibrosis and no induction of hypertrophic markers.5 The decrease in cardiac mass is due to a reduced number of smaller cardiac myocytes. As a control line, mice were created expressing Myc-tagged wild-type murine cTnT (TnT-WT-Myc) with no phenotype.5

A missense allele (R92Q) was analyzed in transgenic mouse lines (TnT-R92Q). Lines were established expressing 30%, 67%, and 92% of their total cTnT protein as the missense allele.6 These mice also have smaller left ventricles, but in contrast to the mice with truncated TnT, they have significant fibrosis and induction of the hypertrophic markers atrial natriuretic factor (ANF) and β-myosin heavy chain (MyHC). The smaller, isolated cardiac myocytes show sarcomeric activation, impaired relaxation, and shorter sarcomere length.6 Isolated, working heart preparations demonstrate hypercontractility but severe diastolic dysfunction.

The signaling pathways affected by mutant sarcomeric proteins that lead to FHC are poorly understood. Overexpression of the β-adrenergic receptor leads to rapid progression of disease in a mouse model of FHC harboring an MyHC mutation.7 Genetic crosses between the mutant MyHC model and models with inhibition of the β-adrenergic kinase pathway or the phospholamban-null suggested that both of these
signaling pathways contribute to the FHC phenotype. More recently, a pressure-overload stimulus on a different MyHC mutant led to the conclusion that similar pathways contribute to both FHC and pressure overload.

The diverse phenotypes of mice with 2 different mutations in the same gene (cTnT) led us to test the response of hearts of the TnT-mutant mice to defined pathological stimuli in vivo. We chose isoproterenol (ISO) and/or phenylephrine (PE) as β- and α-adrenergic agonists, respectively, because of the possible involvement of these signals in the progression of FHC. We also tested the response of these hearts to angiotensin II (Ang II), which is one of the most potent stimuli of fibrosis and hypertrophy in the myocardium.

**Methods**

**Mice**

Twelve-week-old C57BL6/J mice bearing Myc-tagged murine cTnT with the R92Q mutation (TnT-R92Q, 67%), the truncated allele (TnT-trunc, 4%), or wild-type cTnT (TnT-WT-Myc) were generated as previously described. Sibling mice were used as nontransgenic controls. Animals were maintained in accordance with NIH guidelines, and the protocol was approved by the Animal Care Committee of the University of Colorado and followed current NIH and American Physiological Society guidelines.

**Long-Term Growth Factor Infusion**

Osmotic minipumps (Alzet, Durect Corp) containing ISO (30 mg · kg⁻¹ · d⁻¹), PE (30 mg · kg⁻¹ · d⁻¹), Ang II (200 ng · kg⁻¹ · min⁻¹), or vehicle (VEH, saline containing 50 mmol/L ascorbic acid) were implanted subcutaneously after midscapular incision. They were left in place for 7 days (ISO, PE) or 14 days (Ang II). For Ang II, this dose has been shown to induce hypertrophy without producing a significant increase in blood pressure, and it has therefore been found to be a nonpressor dose. At the end of this period, mice were humanely killed by Metofane inhalation followed by cervical dislocation; hearts were excised and weighed; and ventricles were either frozen in LN₂ or fixed in 10% neutral-buffered formalin. Body weights without the pumps were recorded at the beginning and the end of the study.

**ECG Monitoring**

Mice were anesthetized with tribromoethanol (Avertin, 250 mg/kg). ECGs were recorded at baseline for 5 minutes at a sampling rate of 5 kHz. The electrodes were placed to emulate a lead I equivalent. The baseline recording was followed by injection of 1 mg/kg epinephrine IP and recording for 15 minutes.

**Northern Blotting and Ribonuclease Protection Assays**

RNA isolation and Northern blotting for glyceraldehyde 3-phosphate dehydrogenase, ANF, and sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) 2a mRNAs were performed as previously described. For analysis of MyHC mRNAs, 10 μg total RNA was hybridized overnight with a molar excess of radiolabeled RNA corresponding to 300 bases of the β-MyHC mRNA with a 170-base overlap to α-MyHC. These complexes were digested with RNase T1, and protected fragments were analyzed on a 6% urea/polyacrylamide gel. The gel was dried and exposed on a PhosphorImager (Molecular Dynamics, Amersham).

**Histological Analysis**

Hearts fixed in neutral-buffered formalin were desiccated by sequential incubations in 70% ethanol and 100% ethanol, embedded in paraffin, and sectioned. Sections were stained with Masson’s trichrome as previously described and analyzed by light microscopy.

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**Figure 1.** A, Cardiac hypertrophy in vivo in male mutant troponin T–transgenic mice. Increase in heart weights in response to hypertrophic stimuli compared with vehicle (saline containing vitamin C). Ang II was given at 200 ng · kg⁻¹ · min⁻¹ for 14 days; PE at 30 mg · kg⁻¹ · d⁻¹ for 7 days; and ISO at 30 mg · kg⁻¹ · d⁻¹ for 7 days. n=5 or 6 for all groups. Abbreviations are as defined in text. B, Cardiac hypertrophy in vivo in female mutant troponin T–transgenic mice.

**Figure 2.** Survival curves in male mice after long-term infusion of adrenergic substances PE (30 mg · kg⁻¹ · d⁻¹ for 7 days) and ISO (30 mg · kg⁻¹ · d⁻¹ for 7 days). VT-Myc mice, n=10; R92Q mice, n=5; Trunc mice, n=5. Female mice, n=5 or 6 per group. Abbreviations are as defined in text.
Statistical Analysis

Differences between groups of WT and mutant mice were assessed by unpaired Student’s t tests and the nonparametric Mann-Whitney test. To detect differences between all groups, ANOVA analysis was performed. Data are expressed as mean±SEM. *P<0.05 was considered significant.

Results

Hypertrophic Response In Vivo

The response of TnT-transgenic mice to pathological cardiac hypertrophic stimuli was tested by long-term infusion of ISO, PE, or Ang II in a nonvasopressor dose. Surprisingly, despite the markedly smaller cardiac mass in male mutant TnT mice,
hearts of female TnT-R92Q mice at baseline had a size similar to that of TnT-WT-Myc hearts, whereas TnT-trunc mice of both sexes had smaller hearts. In general, heart weight–body weight ratios were significantly higher in female mice in all groups. The 3 agents induced an increase in cardiac mass in relation to body weight in male and female TnT-WT-Myc mice (Table 1). In TnT-R92Q mice, the hypertrophic response to adrenergic stimulation was significantly augmented (Figure 1). The response to Ang II showed a clear sex difference in TnT-R92Q mice, with an augmented response in male mice and a blunted response in females compared with the response in TnT-WT-Myc mice (Table 1 and Figure 1).

TnT-trunc mice showed a hypertrophic response to ISO. However, in male mice, PE and Ang II each led to a decrease in body weight, and therefore, heart weight–body weight ratios were higher (Table 1), but the absolute heart mass did not change (Figure 1A). In female TnT-trunc mice, this reduction of body weight did not occur, and a significant hypertrophic response could be detected in response to Ang II but not for PE (Table 2 and Figure 1B).

Although no deaths occurred with individual agonists, both adrenergic agonists led to sudden cardiac death in 100% of male TnT-R92Q (n = 5) and TnT-trunc mice (n = 5), but only 1 death was observed in 10 mice of the TnT-WT-Myc group (Figure 2). Female mice of all lines (n = 5 or 6 each) survived the treatment with no apparent phenotype (Figure 2). The hypertrophic response was stronger than for either ISO or PE alone and was augmented in TnT-R92Q mice (Table 2 and Figure 1B).

Fibrotic Response In Vivo

The degree of fibrosis induced by hypertrophic stimulation in the transgenic TnT mice was assessed by light microscopy and by measurement of collagen 1A1 (COL1A1) mRNA. Treatment with ISO or Ang II led to significant fibrosis in TnT-WT-Myc mice (Figure 3A), paralleling the induction of COL1A1 mRNA that was observed with all 3 agonists (Figure 4). TnT-R92Q mice had fibrosis at baseline that was increased by treatment with ISO or Ang II (Figures 3A and 4). All 3 agonists increased COL1A1 mRNA even further in TnT-R92Q mice (Figure 4). Surprisingly, TnT-trunc mice were resistant to the development of fibrosis as assessed by both histology and quantification of COL1A1 mRNA when treated with ISO or Ang II (Figures 3A and 4). TnT-trunc mouse hearts had numerous areas of fibroblasts with no collagen deposition, whereas these areas were “hot spots” of collagen accumulation in TnT-R92Q mice (Figure 3B).

Molecular Responses In Vivo

Pathological hypertrophy is typically associated with changes in gene expression that include induction of the “fetal gene program.” We also wished to examine genes central to Ca\(^{2+}\) handling. Levels of mRNAs encoding ANF, \(\beta\)- and \(\alpha\)-MyHC, SERCA, and phospholamban (PLB) were measured. In male and female TnT-WT-Myc mice, all 3 pharmacological agents increased ANF mRNA (Figure 4). However, \(\beta\)-MyHC was induced only by ISO (Figure 4). The magnitude of the induction of \(\beta\)-MyHC mRNA by ISO was different between the sexes, with higher induction in female TnT-WT-Myc mouse hearts. Despite a basal increase in the expression of ANF and \(\beta\)-MyHC in R92Q mice, both mRNAs were further induced by ISO, with varying degrees of induction by PE and Ang II. There was a pronounced sex difference between the basal levels of ANF mRNA in the TnT-R92Q mice, with a 22-fold increase in males versus a 4.5-fold increase in females. TnT-trunc mice showed very mild changes in gene expression at the basal state, and in the stimulated state, these varied somewhat with sex and stimulus (Figure 4). The ratio of SERCA-PLB mRNA was unchanged in all animals by either stimulus (data not shown).
Electrophysiological Response In Vivo

We performed baseline ECG monitoring in male transgenic mice. Baseline heart rates were not significantly different in all models (550 to 600 bpm, data not shown). On stimulation with epinephrine, which stimulates both α- and β-adrenergic receptors, we observed multiple premature ventricular beats and occasional short ventricular runs (Figure 5) in addition to sinus arrhythmias and runs of atrial fibrillation (data not shown). Shown in Figure 5 is an example of sinus bradycardia before (318 bpm) and after (345 bpm) an episode of a ventricular run (542 bpm).

Discussion

We chose several well-characterized hypertrophic stimuli to probe signaling pathways in distinct models of FHC. A recent
report tested the response of a different model of FHC to pressure overload and found no significant difference between WT and MyHC-mutant animals. Short-term stimulation with ISO in a mutant line (I79N) in cTnT led to cardiac dysfunction and sudden cardiac death in the absence of hypertrophy or fibrosis.

We show in this study that TnT-transgenic mice can respond to some but not all pathological stimuli in vivo with an increase in cardiac mass but show significant differences in the extent, dependent on the allele, stimulus, and sex (summarized in Table 2). TnT-trunc mice had blunted responses to many of the stimuli. Only ISO was able to elicit a uniform hypertrophic response independent of allele or sex. One unexpected finding was the augmented hypertrophic response of TnT-R92Q mice to stimuli, modified by sex. All stimuli led to an augmented response in male TnT-R92Q mice. Female TnT-R92Q mice had a diminished response to Ang II infusion compared with males but preserved an augmented response to adrenergic stimuli (ISO and PE). Female TnT-trunc mice, however, developed hypertrophy on stimulation with Ang II, in contrast to their male counterparts. These findings lend strength to the hypothesis of sex-specific modification of phenotypes of primary cardiac disease, which has been supported by several reports during the past few years (see Leinwand). Many recent clinical reports suggest sex differences in pressure-overload hypertrophy and FHC. In addition, the penetrance of FHC has been shown to be significantly lower in women. A recent report describes that women diagnosed with FHC are older (postmenopausal) and present with a more severe form of the disease with higher mortality. Similarly, we have observed sex-specific differences in our MyHC-mutation mouse model of FHC. In a recent report, the α-adrenergic receptor has been identified as a mandatory component of normal postnatal heart growth and physiological hypertrophy, but only in male mice, whereas female mice null for the α-adrenergic receptor had a normal phenotype. This effect was independent of estrogen, because ovariectomized females still had normal heart growth.

The changes in gene expression that we observed add another dimension to the complexity of the hypertrophic response in these mice and likely, to FHC in general (see Table 2 for a summary of gene expression data). ANF is dramatically induced in male TnT-R92Q mice and to a lesser degree in female mice. ANF expression is further increased with hypertrophic stimuli, suggesting that the pathological stimulus of the missense mutation has not maximally stimulated the pathway leading to ANF activation. Alternatively, multiple parallel pathways may lead to ANF activation. In TnT-trunc mice, there is some basal activation but a mild increase with hypertrophic stimuli. It is now widely believed that ANF is indeed a protective factor, and its upregulation in disease is not merely a marker of pathology. It has been shown that ANF inhibits fibroblast growth and production of extracellular matrix proteins as well as cardiac hypertrophy. It has also been shown that the effects of ANF in pressure-overload hypertrophy differ in male versus female mice, making it one of the possible targets to explain sex differences in the development of hypertrophy. These properties of ANF, however, cannot explain the lack of fibrosis and hypertrophy in TnT-trunc mice, because there was no elevation of this factor. Along the same line, the lower activation in TnT-R92Q females also argues against a protective role in this model.

An important hallmark of pathological cardiac hypertrophy is the isoform switch from the fast α- to the slower β-isoform of MyHC. Normal mouse hearts express exclusively α-MyHC. In this study, ISO but not the other agonists induced β-MyHC in WT animals. This might be explained by the lesser hypertrophy with these agents compared with ISO. It is possible that higher doses of Ang II or PE might be able to increase β-MyHC, as has been documented in Wistar rats. TnT-R92Q mice already express significant amounts of β-MyHC at baseline, and it is further increased with ISO.
In TnT-trunc mice, β-MyHC induction is blocked even in the presence of hypertrophy after ISO stimulation.

An intriguing finding was the resistance of hearts of TnT-trunc mice to the development of fibrosis when treated with Ang II. Cardiac fibroblasts normally respond to Ang II in vitro with proliferation and increased synthesis of extracellular matrix proteins. The number of fibroblasts in the TnT-trunc hearts was higher than in normal hearts but without accumulation of collagen. Furthermore, although the hearts of these animals responded to ISO with an increase in cardiac mass, they did not respond to ISO with a significant increase in ANF or β-MyHC. We hypothesize that truncated TnT leads to a block in growth factor production within the cardiomyocytes that signals for fibrosis and may be involved in induction of hypertrophic markers. Further experiments need to clarify this phenomenon.

We chose 3 defined pharmacological stimuli to ask how common disorders such as arterial hypertension or diabetes affect the phenotype of FHC. In an MyHC model, no aggravated hypertrophy or fibrosis was reported in response to aortic constriction. The authors concluded that the pathways leading to FHC and induced pressure overload do not interact and are independent. In contrast to this study, we found aggravated fibrosis and/or hypertrophy, dependent on the stimulus, and that sudden cardiac death occurred with adrenergic stimulation. However, it may be that mutations in different molecules result in distinct responses to hypertrophic signaling.

The sudden cardiac death that occurred on stimulation with both adrenergic receptors highlights the importance of avoiding extreme adrenergic stress in individuals with FHC. This is the second report showing a high incidence of sudden cardiac death in mouse models of FHC without overt heart failure. The proposed mechanism is the development of ventricular tachycardias, which we also observed on adrenergic stimulation. In contrast to the study by Knollmann et al., we observed death in mice only on stimulation of both adrenergic pathways. Stimulation with ISO alone at a dose similar to the one used for the short-term challenge in a previous study was insufficient to induce sudden cardiac death in our distinct TnT-mutant models. Interestingly, both TnT models exhibited sudden cardiac death despite different histological phenotypes. Therefore, in these models, sudden cardiac death is not a consequence of fibrosis but either is a consequence of myocellular disarray, a feature common to both alleles, or is dependent on proarrhythmic properties within the cardiac myocytes themselves. Because sudden cardiac death does not occur in female mice, it is likely that arrhythmic signaling is independent of histological structure. This is supported by the study in the TnT-I79N-mutant model because it lacks both fibrosis and disarray. Further support comes from a recent study showing decreased cardiac energetics in our R92Q mutant model that were exacerbated during increased workload, such as the one provided by adrenergic stress. Studying factors that prevent arrhythmogenesis in female TnT mice might be useful for identifying therapeutic options for arrhythmias in this disease.

We believe that this study adds a new dimension to understanding the pathogenesis of FHC as well as to the biology of the cardiovascular system. First, it highlights once more that sex is a modifying factor for the phenotype of cardiovascular disease. There were sex effects in response to the transgene, agonist stimulation, gene expression, and sudden cardiac death. Most striking, however, was that there was only one phenotype shared by both mutations and both sexes: the hypertrophic response to ISO. Second, our results emphasize that sudden cardiac death in FHC is not merely dependent on fibrosis. Adrenergic stress is clearly a major contributor to the sudden cardiac death reported here. Third, the resistance of TnT-trunc mice to fibrosis in response to Ang II provides an excellent model for studying cardiac fibrosis.

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