Morphological and Functional Alterations in Ventricular Myocytes From Male Transgenic Mice With Hypertrophic Cardiomyopathy

M. Charlotte Olsson, Bradley M. Palmer, Brian L. Stauffer, Leslie A. Leinwand, Russell L. Moore

Abstract—Familial hypertrophic cardiomyopathy (FHC) is a human genetic disorder caused by mutations in sarcomeric proteins. It is generally characterized by cardiac hypertrophy, fibrosis, and myocyte disarray. A transgenic mouse model of FHC with mutations in the actin-binding domain of the α-myosin heavy chain (MyHC) gene displays many phenotypes similar to human FHC. At 4 months, male transgenic (TG) mice present with concentric cardiac hypertrophy that progresses to dilation with age. Accompanying this latter morphological change is systolic and diastolic dysfunction. Left ventricular (LV) myocytes from male TG and wild-type (WT) littermates at 5 and 12 months of age were isolated and used for morphological and functional studies. Myocytes from 5- and 12-month-old TG animals had shorter sarcomere lengths compared with WT. This sarcomere length difference was abolished in the presence of 2,3-butanedione monoxime, suggesting that the basal level of contractile element activation was increased in TG myocytes. Myocytes from 12-month-old TG mice were significantly longer than those from age-matched WT controls, and TG myocytes exhibited Z-band disorganization. When cells were paced at 0.5 Hz, TG myocyte relengthening and relaxation were slowed when compared with cells from age-matched WT controls. Moreover, an increased amount of β-myosin heavy chain protein was found in hearts from TG compared with WT. Thus, myocytes from the α-MyHC TG mouse model display many morphological and functional abnormalities that may help explain the LV dysfunction seen in this TG mouse model of FHC. (Circ Res. 2004;94:201-207.)

Key Words: 2,3-butanedione monoxime ■ calcium ■ relaxation ■ heart failure

Familial hypertrophic cardiomyopathy is a clinically and genetically heterogeneous disease resulting from mutations in sarcomeric proteins of the heart. The first gene implicated in FHC was the α-myosin heavy chain (MyHC) gene displays many phenotypes similar to human FHC. At 4 months, male transgenic (TG) mice present with concentric cardiac hypertrophy that progresses to dilation with age. Accompanying this latter morphological change is systolic and diastolic dysfunction. Left ventricular (LV) myocytes from male TG and wild-type (WT) littermates at 5 and 12 months of age were isolated and used for morphological and functional studies. Myocytes from 5- and 12-month-old TG animals had shorter sarcomere lengths compared with WT. This sarcomere length difference was abolished in the presence of 2,3-butanedione monoxime, suggesting that the basal level of contractile element activation was increased in TG myocytes. Myocytes from 12-month-old TG mice were significantly longer than those from age-matched WT controls, and TG myocytes exhibited Z-band disorganization. When cells were paced at 0.5 Hz, TG myocyte relengthening and relaxation were slowed when compared with cells from age-matched WT controls. Moreover, an increased amount of β-myosin heavy chain protein was found in hearts from TG compared with WT. Thus, myocytes from the α-MyHC TG mouse model display many morphological and functional abnormalities that may help explain the LV dysfunction seen in this TG mouse model of FHC.
single cell level and compared it to whole heart tissue both in 5- and 12-month-old male mice. We found that many of the TG-induced alterations in LV morphology and contractile function in the intact heart are readily reconcilable with the types of cellular abnormalities reported herein.

Materials and Methods

Animals
Male transgenic mice with a missense allele, R403Q, and an actin-binding deletion in the cardiac α-MyHC were used in this study. Specifically, the transgene coding region consists of a rat α-MyHC cDNA containing a 61445A point mutation, resulting in R403Q and a deletion of amino acids 468 to 527 bridged by the addition of nine nonmyosin amino acids (SerSerLeuProHisLeuLys-Leu). Four groups of mice were used in this study: 5-month-old transgenic (5TG) and their wild-type littermates (5WT), and 12-month-old transgenic (12TG) and wild-type (12WT).

Mouse Ventricular Myocyte Isolation
Ca²⁺-tolerant cardiac myocytes were obtained from the left ventricular free wall and septum (henceforth referred to as LV) using a protocol described previously with a few modifications. The most notable modification was that these cells were not isolated in the presence of 2,3-butanedione monoxime (BDM). Isolated cells in suspension were divided into one large (80%) and one small (20%) aliquot that were plated onto laminin-coated glass coverslips and incubated for 2 to 8 hours at 37°C with 5% CO₂/20% O₂.

Cardiocyte Morphology and Sarcomere Measurements
Video images of individual cardiocytes (between 75 to 105 in each group) with or without BDM were analyzed for maximal length, width, and area as previously described. Sarcomere lengths were assessed from white-light microscopy video images (between 100 myocytes in each group) using IonOptix analysis software (IonOptix Corp).

Electron Microscopy
Myocytes (no BDM) and whole heart tissue were fixed and sectioned for electron microscopic inspection. The coherence of Z-band alignment was assessed by regression analysis of groups of 3 Z-bands in series across at least 9 sarcomeres in each group. The residual variance of regression lines through 3 serial Z-lines provided a metric of the error in estimating Y from X; large residual variance of regression lines through 3 serial Z-lines was used. Simple effects were examined to determine differences between the WT and TG subgroups for both 5- and 12-month-old mice. To reduce the possibility of committing a Type II interpretive error, ie, a false-negative, significance was reported at both the P<0.05 and P<0.10 levels.

Measurement of Intracellular [Ca²⁺] and Shortening Dynamics
Myocyte shortening dynamics were determined using a commercially available video edge detection system (Crescent Electronics). Simultaneously, ratiometric fura-2 fluorescence measurements were made using a system (IonOptix Corp) fitted with optical excitation filters of 400 and 360 nm. This choice of filters yields a linear relationship between [Ca²⁺], and the fluorescence ratio and, therefore, more reliable estimates of the temporal characteristics of the [Ca²⁺] transient. Electrically paced (0.5 Hz via field stimulation) cardiocytes were superfused with a normal Tyrode’s solution containing (in mM/L) NaCl 140, KCl 6, MgCl₂ 1, CaCl₂ 10, glucose 2, pyruvate, and 5 HEPES, at pH 7.4 and 25°C. Cardiocyte fluorescence background was taken as the fluorescence recording remaining after cell lysis with a nominal Ca²⁺ superfusate containing 1 μmol/L digitonin.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Whole heart tissue was homogenized and loaded onto a 6% SDS-PAGE gel, run at 16 mA/gel for 4.5 hours at 8°C according to methods by Warren and Greaser. Gels were subsequently silver stained following previously published methods, and the percent α- and β-MyHC were determined using densitometry.

Results

Myocyte Morphology and Sarcomere Lengths
In the absence of BDM, sarcomere lengths in myocytes isolated from 5- and 12-month-old TG animals were significantly shorter (5% and 6%, respectively) than sarcomeres measured in myocytes from age-matched WT animals (*P<0.05, different from same group without BDM). In the presence of BDM, the 12-month-old groups had significantly longer sarcomeres than the 5-month-old groups (‡P<0.05, different from 5-month-old groups with BDM).

Figure 1. Cardiocyte sarcomere lengths in the presence and absence of 10 mmol/L BDM. Myocytes from 5- and 12-month-old transgenic (TG) groups had significantly shorter sarcomeres than wild-type (WT) littermates and wild-type littermates with BDM (*P<0.05, different from age-matched WT without BDM). BDM-treated myocytes had longer sarcomeres compared with the same group without BDM (*P<0.05, different from same group without BDM). In the presence of BDM, the 12-month-old groups had significantly longer sarcomeres than the 5-month-old groups (‡P<0.05, different from 5-month-old groups with BDM).
and slightly narrower, and surface area stayed the same. Similar to sarcomere length, the greatest change in myocyte length due to BDM treatment was found in 5 and 12 TG myocytes (5WT 5%; 5TG 7%; 12WT 2%; and 12TG 9% longer myocytes with BDM). BDM-treated myocytes from 12TG were significantly longer than age- and BDM-matched WT myocytes, as would be expected from a dilated heart (Figure 2A). An example of the differential BDM-mediated myocyte elongation in a 12 WT and a 12 TG myocyte is provided in Figure 3. Increased cardiocyte length to width ratio has been found in myocytes from human dilated hearts.\textsuperscript{14,15} We found a significant TG versus WT increase in length/width ratio in myocytes from 12-month-old hearts (Figure 2D).

Electron Microscopy

During the sarcomere length measurements (previous section) at the light microscope level, we observed that myocyte images from 12-month TG cells (and to a lesser extent 5-month TG cells) had less clear cross striations than the age-matched WT myocytes. We wanted to determine if this observation reflected an intrinsic cell morphology difference resulting from expression of the transgene, or if it was an artifact from our isolation technique (ie, our isolation procedure was more damaging to the TG cells compared with the WT cells). Therefore, both freshly isolated cells and whole heart tissue were fixed and mounted for later viewing with an electron microscope. Figure 4 shows sections from fixed

![Figure 2](image1)

![Figure 3](image2)

![Figure 4](image3)
isolated cardiac myocytes. The TG myocyte images, both from 5- and 12-month-old mice, had abnormal dispersion and disorganization of the Z-bands (Figures 4B and 4D), whereas WT myocytes (Figures 4A and 4C) showed the typical registry of Z-bands. Similarly, in sections from fixed whole heart tissue misaligned Z-bands were more prevalent in the TG heart (Figures 4F) compared with the WT heart (Figure 4E). The images taken from fixed isolated cardiocytes were almost indistinguishable from intact heart tissue images, indicating that disorganized cross-striation is a result of the α-MyHC mutation, not an artifact from our cell isolation methods.

In order to more objectively determine the degree of misalignment of the Z-bands between TG and WT, we calculated the residual variance from a regression analysis of Z-bands in series (see Materials and Methods). Analyses were performed on isolated myocytes in primary culture and on sections of LV taken from whole hearts from WT and TG mice at 5 and 12 months. A higher residual variance value indicated an increased occurrence of misaligned Z-bands. Results of a 2×2 ANOVA are indicated by mutation main effect; AGE, age main effect; and X, MUT by AGE interaction. Simple effect differences are indicated, *P<0.05, difference from age-matched WT; †P<0.05, difference from age main effect, P<0.001. Mutation main effect, P<0.001

Values reported are mean±SEM. Table contains residual variance values derived from regression analysis of Z-bands in series (see Materials and Methods). Analyses were performed on isolated myocytes in primary culture and on sections of LV taken from whole hearts from WT and TG mice at 5 and 12 months. A higher residual variance value indicated an increased occurrence of misaligned Z-bands. Results of a 2×2 ANOVA are indicated by mutation main effect. Simple effect differences are indicated, *P<0.05, difference from age-matched WT.

| Transgene and Age Effects on Cardiomyocyte Shortening/Relaxation and [Ca2+]i Dynamics |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Cardiac myocyte shortening, relaxation, and Ca2+ parameters from transgenic and wild-type mice at 5 and 12 months are given in Table 2. Representative fura-2 fluorescence ratio and cardiomyocyte shortening data are presented in Figure 5. Myocyte resting length and max shortening rate constant (∂dS/∂t/∂S) were not affected by transgene or age (Table 2). Myocytes from the 12-month-old mice shortened slightly more (in percent) than myocytes from the 5-month-old mice (age main effect, P<0.05). Moreover, with age, percent shortening did not change in the TG myocytes, whereas the myocytes from the WT groups shortened a little more at 12 months compared with 5 months (age×mutation interaction, P<0.08). Relaxation was attenuated in myocytes from the TG hearts compared with WT (Table 2). The maximal

### TABLE 1. Assessment of Z-Band Registry in Wild-Type (WT) and Transgenic (TG) Myocardium

<table>
<thead>
<tr>
<th>Isolated Myocytes</th>
<th>Whole Heart Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>5WT 0.66±0.18</td>
<td>1.62±0.36</td>
</tr>
<tr>
<td>5TG 7.33±3.06*</td>
<td>25.87±5.67*</td>
</tr>
<tr>
<td>12WT 1.87±0.84</td>
<td>3.47±0.78</td>
</tr>
<tr>
<td>12TG 11.28±2.19*</td>
<td>22.68±7.16*</td>
</tr>
</tbody>
</table>

ANOVA Mutation main effect, P<0.001. Mutation main effect, P<0.001

| Table 2. Shortening, Relaxation, and [Ca2+]i Dynamics From Paced WT and TG Cardiomyocytes at 5 and 12 Months |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Shortening                                      | Shortening                                      | Shortening                                      | Shortening                                      | Shortening                                      |
| Resting length, μm                             | 113±4                                          | 110±3                                          | 107±4                                          | 108±5                                          |
| Percent shortening                             | 1.62±0.14                                      | 1.97±0.25                                      | 2.53±0.29                                      | 2.02±0.24                                      |
| ∂dS/∂t/∂S, s⁻¹                                | −14.39±0.57                                    | −14.81±0.71                                    | −13.96±0.48                                    | −13.61±0.72                                    |
| +dS/∂t/∂S, s⁻¹                                | 3.06±0.18                                      | 2.89±0.16                                      | 3.63±0.19                                      | 2.82±0.17*                                     |
| Time to 90% relaxation, sec                    | 1.19±0.03                                      | 1.29±0.04†                                     | 1.09±0.05                                      | 1.29±0.05*                                     |
| No. of cells                                   | 36                                             | 36                                             | 38                                             | 29                                             |
| [Ca2+]i                                        | R⁰ = 0.121±0.010                               | 0.082±0.007*                                   | 0.244±0.023                                    | 0.261±0.033                                    |
|                                               | R∞ = 0.021±0.003                               | 0.013±0.003                                    | 0.046±0.004                                    | 0.057±0.009                                    |
|                                               | k₉₀ = 5.21±0.21                                | 4.94±0.26                                      | 5.81±0.27                                      | 4.75±0.31*                                     |
| No. of cells                                   | 35                                             | 27                                             | 37                                             | 21                                             |

Values reported are mean±SE. Results of a 2×2 ANOVA are indicated at *P<0.05 or †P<0.10. MUT indicates mutation main effect; AGE, age main effect; and X, MUT by AGE interaction. Simple effect differences are indicated, *P<0.05, difference from age-matched WT; †P<0.10, difference from age-matched WT. NS indicates nonsignificant.
relaxation rate constant, $+dS/dt/\Delta S$, was slower in the TG hearts (mutation main effect, $P<0.05$). There was a differential $+dS/dt/\Delta S$ response by the WT and TG cells to age. The TG cardiocytes were not affected by age, whereas WT myocytes tended to increase $+dS/dt/\Delta S$ from 5 to 12 months (age X mutation interaction, $P=0.08$). In both 5TG and 12TG, myocytes took longer to reach 90% relaxation compared with age-matched WT cardiocytes (mutation main effect, $P<0.05$).

An age-dependent (5 versus 12 months) difference in $R_{rest}$, suggestive of an increase in resting $[Ca^{2+}]_c$, was found (age main effect, $P<0.05$). A statistically significant decrease in $R_{rest}$ was found in the 5TG myocytes compared with 5WT (simple effect, $P<0.05$). Similar to $R_{rest}$, the 12-month-old myocytes had higher $R_{rel}$ relative to 5-month-old, but no TG versus WT changes were found in the $R_{aut}$ transient magnitude (Table 2). The monoeponential constant $k_{rel}$, which describes the rate of $[Ca^{2+}]_c$ decline, was smaller in myocytes from the TG groups (mutation main effect, $P<0.05$) relative to the WT groups, suggestive of a decreased ability of the TG myocytes to reduce cytosolic $[Ca^{2+}]_c$ during relaxation.

**Effect of $\alpha$-MyHC Mutation on Cardiac Myosin Heavy Chain Isoform Expression**

Left ventricular homogenates from TG animals contained a significantly increased amount of $\beta$-MyHC protein compared with age-matched WT controls (Figure 6C). At 4 months, the amount of $\beta$-MyHC protein was $\approx 7\%$ of total MyHC in the TG group compared with $\approx 2\%$ in the WT group. Similarly, at 9 months, TG hearts contained $\approx 13\%$ $\beta$-MyHC protein compared with $\approx 3\%$ in the WT hearts. Representative gels on which 6 dilutions of each sample are loaded are shown in Figures 6A and 6B.

**Discussion**

This study demonstrated that the expression of the mutant $\alpha$-MyHC transgene in murine hearts affected myosin heavy chain isoform expression, basal myofilament interaction, myocyte dimension, and sarcomeric organization. Moreover, cardiocytes from TG animals exhibited slowed relaxation and a slowed fall of intracellular $[Ca^{2+}]_c$ relative to WT controls. We demonstrated that sarcomeric abnormalities seen in the whole heart were preserved in isolated cells and represented true transgene-associated sarcomere disorganization rather than being an artifact of cell isolation techniques.

Myocyte and sarcomeric resting lengths are determined by both $Ca^{2+}$-dependent and $Ca^{2+}$-independent myofilament mechanisms, and perturbations known to affect myofilament interaction such as the addition of BDM, would be expected to increase resting cell length.16,17 Our observation that sarcomere lengths increased to a greater extent in BDM-treated TG myocytes relative to WT cells strongly suggests that basal levels of contractile element activation were elevated in TG myocytes (Figure 1). To our knowledge, published estimates of sarcomere lengths in hypertrophic cardiomyopathy hearts have to date been assessed quantitatively only in a transgenic cardiac troponin T mouse model (TnT-TG).9 Similar to our results, Tardiff et al9 found shorter sarcomeres in the TnT-TG myocytes compared with myocytes from WT littermates. In an $\alpha$-MyHC mouse model of FHC, Kim et al18 reported no difference in sarcomere lengths between the transgenic and wild-type littermates; however, no quantitative measurements were provided.

We have previously demonstrated that male TG mice present with mild hypertrophy at 3 to 4 months, but by 8 to 10 months of age, they display ventricular wall thinning and mild dilation.6–8 Cardiac hypertrophy and remodeling are
often reflected at the cellular level by changes in myocyte size and dimension. In human hypertrophic cardiomyopathy where significant myocardial hypertrophy is present, myocyte diameter is greater when compared with nonhypertrophied control tissue. In our study, we found no TG versus WT changes in myocyte dimensions in the 5-month-old mice. This is not too surprising in view of the fact that the extent of myocardial hypertrophy is quite modest (5% to 7%) in male TG mice at this early age. However, in humans with dilated cardiomyopathy and heart failure, myocytes appeared elongated and the ratio of myocyte length to myocyte width was increased when compared with cells isolated from healthy human hearts. Consistent with these findings in humans, we found that in the presence of BDM, myocyte length and length:width ratios were increased in cells isolated from 12TG mice relative to cells isolated from 12WT controls.

Sarcomeric disorganization and contractile protein abnormalities are commonly found in cardiac tissue from humans with hypertrophic cardiomyopathy, and dilated cardiomyopathy. Using electron microscopy, we demonstrated structural changes in isolated LV myocytes and whole heart tissue from the TG animals. Misaligned Z-bands and disorganized sarcomeres were much more prevalent in TG myocytes compared with WT (Figure 4, Table 1). It is interesting to note that Z-band register and alignment were significantly more organized in thin sections taken from single myocytes when compared with those derived from whole heart preparations. We speculate that this may have been due to a greater degree of variability in the local conditions under which tissue from whole hearts was fixed, and subsequently sectioned.

Altered Ca dynamics and slower relaxation properties have been found previously in tissue samples and cardiocytes taken from humans in end-stage heart failure, and in myocytes from an α-MyHC animal model of FHC. In our transgenic mouse model of FHC, we found similar results where TG myocytes displayed a slowed decline of [Ca2+]i, occurring in conjunction with a slower mechanical relaxation when compared with WT myocytes. The TG myocytes in our study displayed increased basal myofilament activation where shorter sarcomeric lengths might have increased the resistance to shortening, and accelerated the relaxation properties, which would be strongly suggestive of true relaxation abnormalities in the TG myocytes. We have previously shown impaired LV relaxation that worsened with age in hearts from both young and mature adult male TG mice. In the present study, we found that myocytes from TG male mice displayed slower \( k_{\text{off}} \), decreased \( + \Delta S/\Delta t \), and prolonged time to 90% return to baseline, and these indications of relaxation abnormalities were more pronounced in the 12TG myocytes. Virtually any study involving the use of fura-2 in situ is dependent on a variety of assumptions, which include uniformity in fura-2 loading and dissociation constant, \( K_d \) values across experimental groups, and age. However, our estimates of the temporal characteristics of the [Ca2+]i transients are little affected by the potential errors inherent in qualitative R measurements. The reason for this is that we used an excitation wavelength pairing that takes advantage of the linear relationship between [Ca2+]i, and the fluorescence ratio. As a result, the slower \( k_{\text{off}} \) found in our study represent valid measures of intracellular relaxation abnormalities. In our quantitative R measures we found an increase in \( R_{\text{max}} \) and \( R_{\text{off}} \) with age. This could be a true difference in [Ca2+]i, between the two groups, or result from different fura-2 loading or compartmentalization in myocytes from 5- and 12-month-old mice. However, an increased \( R_{\text{max}} \) with age occurred concomitantly with an age-dependent increase in percent shortening in myocytes from 12-month-old mice. Because mechanical shortening is related to intracellular Ca2+ movements, it is tempting to speculate a linkage between these two observations.

As mentioned earlier, we found age-dependent improvements in some of our mechanical and [Ca2+]i, parameters. Percent shortening and \( R_{\text{off}} \) were increased with age, which would suggest improved contractile performance in hearts from 12-month-old mice compared with 5 months. It should be noted however, that our changes with age occurred between young to mature adulthood, and not senescence. Our data are consistent with the results of rat studies where no change or slight augmentation in cardiac performance between young and mature rats was observed.

The change in MyHC isoforms composition could have significant functional consequences for contractile function. It has long been appreciated that contractile velocity and myosin isoforms composition are correlated. It has previously been shown that expression of small amounts of either α- or β-MyHC in rodent hearts or cardiac myocytes can have significant effects on myofibrillar ATPase or contractile function. Therefore, the significant differences in MyHC composition in TG versus WT cells may contribute to contractile alterations in this model of FHC.

In summary, myocytes from the α-MyHC TG model described herein displayed the types of aberrant sarcomeric organization, altered morphology, and mechanical dysfunction that is typically observed in human hypertrophic cardiomyopathies. In addition, the age- and mutation-dependent changes in LV myocyte dimension observed in this study are consistent with our previous observations that TG hearts (male) demonstrate an age-dependent progression from a mild concentric LV hypertrophy to a dilated eccentric hypertrophy. In view of the striking cell and organ similarities that exist across our murine FHC model and human hypertrophic cardiomyopathy, the R403Q mutant could prove to be of considerable utility in the elucidation of the molecular and cellular mechanisms that are ultimately responsible for human hypertrophic cardiomyopathy and the failing heart phenotype.

Acknowledgments

This work was supported in part by grants from the NIH (HL40306 to R.L.M., and HL50560 to L.A.L.), the American College of Sports Medicine (Foundation Research Grant and Graduate Student Scholarship for Women to M.C.O.), and American Heart Association, Northland Affiliate (to M.C.O.). We wish to acknowledge valuable gel electrophoresis consultation of Dr Richard Moss, as well as the support provided by his laboratory (PHS Grant HL63167). Special thanks to Katherine Esquivel for technical assistance.
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Circ Res. 2004;94:201-207; originally published online December 11, 2003;
doi: 10.1161/01.RES.0000111521.40760.18

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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