

## **Ca<sup>2+</sup>-Independent Alterations in Diastolic Sarcomere Length and Relaxation Kinetics in a Mouse Model of Lipotoxic Diabetic Cardiomyopathy**

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# Ca<sup>2+</sup>-Independent Alterations in Diastolic Sarcomere Length and Relaxation Kinetics in a Mouse Model of Lipotoxic Diabetic Cardiomyopathy

Thomas P. Flagg, Olivier Cazorla, Maria S. Remedi, Todd E. Haim, Michael A. Tones, Anthony Bahinski, Randal E. Numann, Attila Kovacs, Jean E. Schaffer, Colin G. Nichols, Jeanne M. Nerbonne

**Abstract**—Previous studies demonstrated increased fatty acid uptake and metabolism in MHC-FATP transgenic mice that overexpress fatty acid transport protein (FATP)1 in the heart under the control of the  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter. Doppler tissue imaging and hemodynamic measurements revealed diastolic dysfunction, in the absence of changes in systolic function. The experiments here directly test the hypothesis that the diastolic dysfunction in MHC-FATP mice reflects impaired ventricular myocyte contractile function. In vitro imaging of isolated adult MHC-FATP ventricular myocytes revealed that mean diastolic sarcomere length is significantly ( $P<0.01$ ) shorter than in wild-type (WT) cells ( $1.79\pm 0.01$  versus  $1.84\pm 0.01$   $\mu\text{m}$ ). In addition, the relaxation rate ( $dL/dt$ ) is significantly ( $P<0.05$ ) slower in MHC-FATP than WT myocytes ( $1.58\pm 0.09$  versus  $1.92\pm 0.13$   $\mu\text{m/s}$ ), whereas both fractional shortening and contraction rates are not different. Application of 40 mmol/L 2,3-butadionemoxime (a nonspecific ATPase inhibitor that relaxes actin–myosin interactions) increased diastolic sarcomere length in both WT and MHC-FATP myocytes to the same length, suggesting that MHC-FATP myocytes are partially activated at rest. Direct measurements of intracellular Ca<sup>2+</sup> revealed that diastolic [Ca<sup>2+</sup>]<sub>i</sub> is unchanged in MHC-FATP myocytes and the rate of calcium removal is unexpectedly faster in MHC-FATP than WT myocytes. Moreover, diastolic sarcomere length in MHC-FATP and WT myocytes was unaffected by removal of extracellular Ca<sup>2+</sup> or by buffering of intracellular Ca<sup>2+</sup> with the Ca<sup>2+</sup> chelator BAPTA (100  $\mu\text{mol/L}$ ), indicating that elevated intracellular Ca<sup>2+</sup> does not underlie impaired diastolic function in MHC-FATP ventricular myocytes. Functional assessment of skinned myocytes, however, revealed that myofilament Ca<sup>2+</sup> sensitivity is markedly increased in MHC-FATP, compared with WT, ventricular cells. In addition, biochemical experiments demonstrated increased expression of the  $\beta$ -MHC isoform in MHC-FATP, compared with WT ventricles, which likely contributes to the slower relaxation rate observed in MHC-FATP myocytes. Collectively, these data demonstrate that derangements in lipid metabolism in MHC-FATP ventricles, which are similar to those observed in the diabetic heart, result in impaired diastolic function that primarily reflects changes in myofilament function, rather than altered Ca<sup>2+</sup> cycling.

**Key Words:** metabolism ■ diabetes ■ myofilaments ■ remodeling

Mounting evidence indicates that cardiac metabolism and disease are intimately related. In this regard, altered energy metabolism is a prominent feature of and, in some instances, may cause heart failure.<sup>1,2</sup> It is also now well recognized that patients with diabetes mellitus have an increased risk of cardiac disease that is independent of the presence of secondary risk factors such as coronary artery disease.<sup>2</sup> These observations suggest that derangements of cardiac metabolism have a direct consequence on cardiac function. The molecular mechanisms potentially linking al-

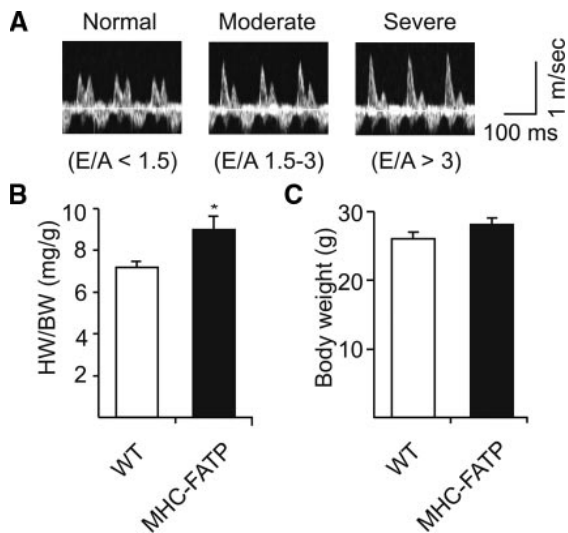
terations in metabolism with cardiac pathology are numerous,<sup>2</sup> although poorly understood.

The ATP generated in the myocardium that supports cell and organ function, including contraction, is derived largely from 2 metabolic pathways: fatty acid oxidation and glycolysis. Under normal conditions,  $\approx 60\%$  of the ATP is produced by fatty acid oxidation, with the remainder resulting from the glycolytic metabolism of glucose. Dramatic shifts in this distribution can occur during disease. For example, up to 90% to 100% of the ATP produced in the diabetic myocardium is

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**Figure 1.** MHC-FATP transgenic hearts are hypertrophic and exhibit a range of diastolic dysfunction. The ratio of early to late filling velocities (E/A) was used as an indicator of the degree of diastolic dysfunction. A, Typical transmitral echocardiographic findings obtained in MHC-FATP transgenic animals. All animals used for cellular studies in the present study exhibited moderate or severe diastolic dysfunction. Compared to WT controls, the HW/BW ratio (B) was significantly ( $P < 0.05$ ,  $t$  test) elevated in MHC-FATP animals, consistent with cardiac hypertrophy ( $n = 32$  WT and 24 MHC-FATP animals), whereas body weight (C) did not differ significantly between the 2 groups ( $P > 0.05$ , Student  $t$  test).

generated from fatty acid oxidation.<sup>3,4</sup> In the case of the diabetic heart, the increased reliance on fatty acid oxidation arises from the interplay of depressed insulin signaling and increased circulating free fatty acids, leading to a suppression of glucose uptake and glycolytic flux by modulating both the production and activity of glycolytic enzymes. Similar changes in glycolytic flux can be observed in rodents fed a high-fat diet, suggesting that the relative increase in free fatty acid uptake, storage, and usage is likely to be an important component of the cardiac pathophysiology.

An early hallmark of diabetic cardiomyopathy is diastolic dysfunction,<sup>5</sup> defined as increased diastolic pressure in the absence of a decrease in fractional shortening (FS). In vivo, this can be observed in Doppler echocardiograms as restrictive ventricular filling (early/late atrial [E/A] wave ratio,  $> 1$ ) resulting from increased diastolic pressure.<sup>6</sup> Similarly, diastolic dysfunction can be inferred when significant atrial hypertrophy, to compensate for increased diastolic pressure, is observed.<sup>7</sup>

Several previous studies have specifically examined cardiac dysfunction in rodent models of diabetes mellitus.<sup>8–11</sup> Interestingly, however, considering these studies as a whole, there is no consistent factor that explains the pathophysiological phenotype of the diabetic myocardium. In particular, some models exhibit alterations of  $Ca^{2+}$  handling that causes a depression of myocardial contractility, whereas in others, no changes in  $Ca^{2+}$  regulation are evident. Other studies suggest that insulin insensitivity is linked to electric remodeling in the diabetic cardiomyocytes<sup>12</sup> and may have consequences for myocardial function.

We have recently generated and characterized transgenic animals (MHC-FATP) overexpressing fatty acid transport protein (FATP)1 under the transcriptional control of the  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter to drive expression specifically in cardiac tissue.<sup>13</sup> MHC-FATP hearts exhibit a significant increase in fatty acid uptake, accumulation, and usage.<sup>13</sup> In addition, in the absence of systemic metabolic disturbances, the alterations in cardiomyocyte lipid homeostasis in these MHC-FATP animals results in diastolic dysfunction similar to what occurs in diabetic cardiomyopathy in its earliest stages. The present study was undertaken to examine directly the intracellular  $Ca^{2+}$  transients and cellular contractile phenotype in isolated ventricular myocytes to test whether alterations of normal intracellular  $[Ca^{2+}]$  homeostatic control mechanisms underlie the impaired diastolic function evident in MHC-FATP hearts.

## Materials and Methods

All procedures complied with the standards for the care and use of animal subjects as stated in the *Guide or the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996), and all protocols were approved by the Animal Studies Committee at Washington University School of Medicine. The MHC-FATP transgenic line has been described previously.<sup>13</sup> An expanded Materials and Methods section is provided in the online data supplement at <http://circres.ahajournals.org>.

### Echocardiographic Measurements

Longitudinal noninvasive transthoracic echocardiograms were obtained as described previously.<sup>13</sup> Diastolic dysfunction was defined based on the ratio of early (E) to late (atrial [A]) transmitral flow velocity (Figure 1). All cells used for in vitro experiments were isolated from MHC-FATP mice exhibiting moderate or severe diastolic dysfunction.

### Cell Contractility and Calcium Transient Measurements

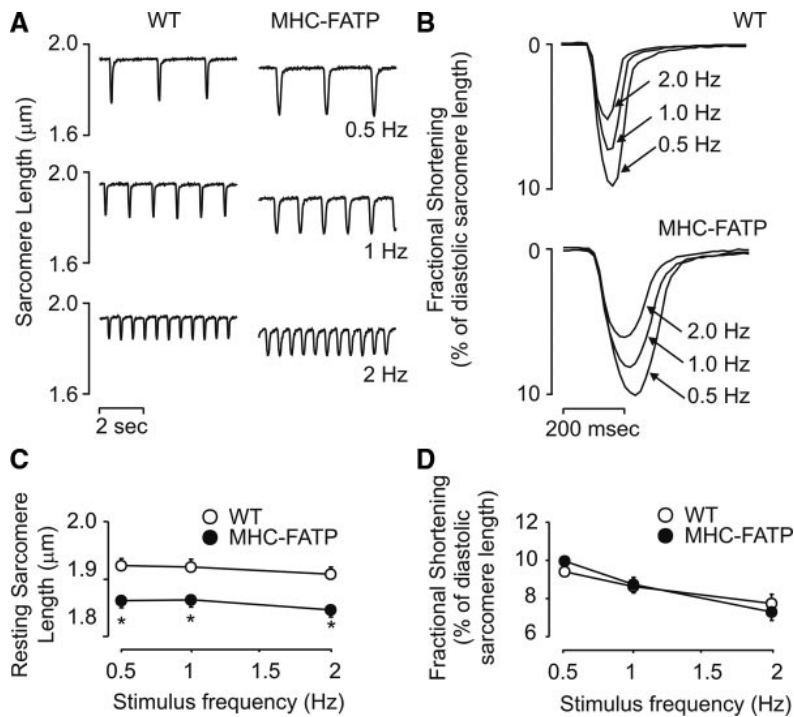
Unloaded cell shortening and calcium transients were measured in freshly isolated ventricular myocytes, prepared as described previously.<sup>14</sup> Cells were field stimulated to contract at 0.5, 1 or 2 Hz as noted in the text. Where applicable, cells were loaded with BAPTA-AM (100  $\mu$ mol/L) or treated with 2,3-butadionemonoxime (BDM) (40 mmol/L). To measure intracellular  $Ca^{2+}$ , isolated cells were loaded with fluo-4-AM (4  $\mu$ mol/L). All experiments were performed at room temperature.

### Force Measurements in Permeabilized Cardiomyocytes

Isometric force was measured in single permeabilized cardiomyocytes at different  $Ca^{2+}$  concentrations at sarcomere lengths of 1.9 and 2.3  $\mu$ m as described previously.<sup>15</sup> Slack sarcomere lengths were measured before attachment of the cells, and mean ( $\pm$ SEM) values were not significantly different in wild-type (WT) ( $1.87 \pm 0.01$   $\mu$ m) and FATP-MHC ( $1.90 \pm 0.01$   $\mu$ m) myocytes. Force was normalized to the cross-sectional area, measured from the imaged cross-section, and force-pCa relations were fitted to a Hill equation.

### Western Blots

Proteins were isolated from frozen ventricular tissues, separated on gradient SDS-PAGE (8% to 16%) gels, and transferred to nitrocellulose membranes for immunoblotting as previously described.<sup>15</sup>



**Figure 2.** Impaired diastolic, but not systolic, function in isolated MHC-FATP ventricular myocytes. Shown in A are typical records of sarcomere shortening obtained from isolated WT and MHC-FATP ventricular myocytes. Myocytes were field-stimulated to contract at 0.5, 1, or 2 Hz as indicated. Averaged recordings of fractional shortening (B) were determined from experiments shown in A. Single averaged contractions are overlaid for each frequency tested. C, Summary data from all experiments (n=65). There was a significant shortening of the resting sarcomere length in FATP transgenic myocytes, reflecting impaired diastolic function (C). D, Consistent with intact systolic function, fractional sarcomere shortening in WT and MHC-FATP cardiomyocytes was not significantly different.

## Results

### MHC-FATP Animals Exhibit Impaired Diastolic Heart Function

Overexpression of FATP1 in the myocardium (MHC-FATP) recapitulates the metabolic and contractile profile of the early stages of diabetic cardiomyopathy.<sup>13</sup> As expected, MHC-FATP hearts exhibit a 4-fold increase in fatty acid storage and use more fatty acid and less glucose than WT hearts, whereas systemic metabolism is not altered. Moreover, transgenic animals exhibited a restrictive filling pattern assessed with transthoracic echocardiography ( $E/A > 1.5$ ) consistent with moderate to severe diastolic dysfunction (Figure 1A). In addition, MHC-FATP animals had a larger mean ( $\pm$ SEM) heart weight-to-body weight ratio (Figure 1B) with no difference in the mean body weights (Figure 1C), consistent with cardiac hypertrophy. In the experiments here, diastolic function/dysfunction in MHC-FATP transgenic and in WT animals was assessed in all animals. Ventricular myocytes were isolated from MHC-FATP transgenic animals displaying moderate to severe diastolic dysfunction to determine directly if the observed restrictive filling of the heart *in vivo* reflects impaired diastolic function at the cellular level. Parallel experiments were completed on cells from WT littermates.

### MHC-FATP Cardiomyocytes Display Altered Contractile Properties

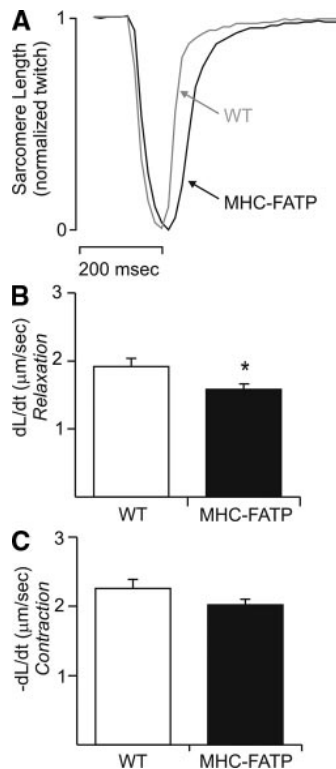
To test whether impaired MHC-FATP heart function originates from altered myocyte contractility, unloaded cell shortening was assessed in freshly isolated ventricular myocytes field stimulated at 0.5, 1, and 2 Hz. As shown in Figure 2, FS was not different in WT and MHC-FATP myocytes at any stimulus frequency examined, demonstrating that systolic function remains intact in transgenic cells. In contrast, dia-

stolic function was significantly compromised in the MHC-FATP myocytes. The baseline (diastolic) sarcomere length was significantly ( $P < 0.01$ ) reduced in MHC-FATP, compared to WT, cells, suggesting that transgenic myocytes are more contracted than WT cells in the resting state. Similarly, in cells stimulated at 1 Hz, the peak relaxation rate ( $dL/dt$ ) was significantly ( $P < 0.05$ ) reduced in MHC-FATP myocytes (Figure 3), consistent with impaired diastolic function. Collectively, these data mirror previous results using a working heart model,<sup>13</sup> in which the relaxation rate was significantly slower in MHC-FATP, compared to WT, hearts. Moreover, the results here suggest that the diastolic dysfunction of the intact MHC-FATP heart originates from a defect at the cellular level.

### Decrease in Diastolic Sarcomere Length Does Not Depend on Increased $[Ca^{2+}]_i$

The observation that diastolic sarcomere length is shorter in MHC-FATP transgenic myocytes suggests that these cells are more contracted and that diastolic tension is greater in these cells at rest than in WT cells. Addition of the chemical phosphatase BDM (40 mmol/L), which inhibits actin-myosin interactions,<sup>16</sup> causes a significant ( $P < 0.05$ ) lengthening of diastolic sarcomere length in both MHC-FATP and WT myocytes (Figure 4A). Importantly, both MHC-FATP and WT cells reach the same final relaxed sarcomere length, indicating that the difference in starting sarcomere length under normal conditions reflects increased resting contraction in the transgenic myocytes.

To test the possibility that the decrease in sarcomere length was the direct result of an increase in the baseline  $[Ca^{2+}]_i$ , the diastolic sarcomere length in myocytes loaded with BAPTA-AM (100  $\mu$ mol/L) for 1 hour was compared with unloaded myocytes. As shown in Figure 4B, BAPTA-AM had no significant effect on diastolic sarcomere length in

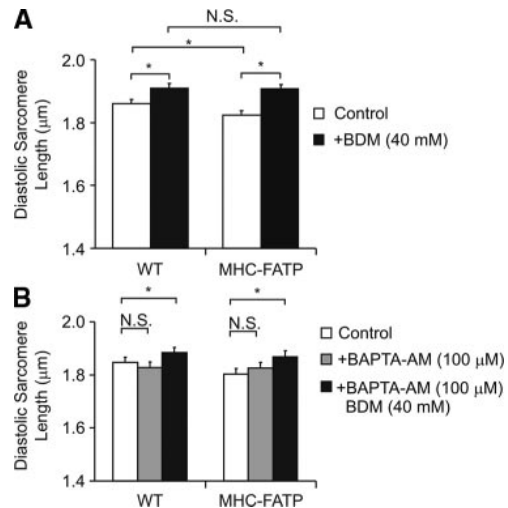


**Figure 3.** Relaxation rate is reduced in isolated MHC-FATP ventricular myocytes. A, Exemplar normalized contraction records illustrating the difference in relaxation kinetics in WT and MHC-FATP ventricular myocytes stimulated at 1 Hz. B and C, Peak relaxation rate (dL/dt) (B) was significantly ( $P < 0.05$ ,  $t$  test) reduced in MHC-FATP ( $n = 106$ ) compared with WT cells ( $n = 108$ ), whereas peak contraction rates ( $-dL/dt$ ) (C) did not differ ( $P > 0.1$ ) between the 2 groups.

either WT or MHC-FATP myocytes, suggesting that increased resting  $[Ca^{2+}]_i$  does not underlie the observed shortening of the diastolic sarcomere length in MHC-FATP myocytes. In addition, similar to the results obtained in non-BAPTA-loaded cells (Figure 4A), exposure of BAPTA-loaded WT and MHC-FATP cells to BDM (40 mmol/L) resulted in significant ( $P < 0.05$ ) lengthening of the sarcomere length (Figure 4B), consistent with a direct effect of BDM on the myofilaments. Taken together, these data indicate that the observed decrease in diastolic sarcomere length in isolated MHC-FATP myocytes does not result from an increase in resting  $[Ca^{2+}]_i$ .

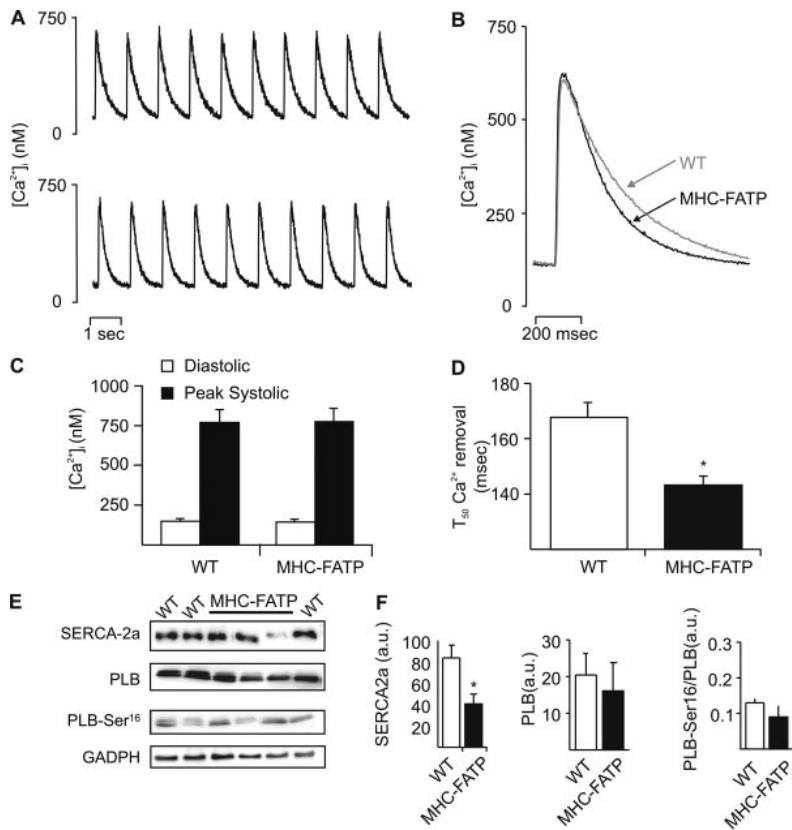
### Altered $Ca^{2+}$ Cycling Cannot Explain Impaired Relaxation of MHC-FATP

The results above suggest that the observed decrease in diastolic sarcomere length in MHC-FATP myocytes does not reflect a change in  $[Ca^{2+}]_i$ . It remains possible that slowed  $Ca^{2+}$  removal mechanisms explain the reduced relaxation rate observed in MHC-FATP myocytes. To determine whether slowed  $Ca^{2+}$  removal underlies the reduced relaxation rate in MHC-FATP myocytes,  $[Ca^{2+}]_i$  levels were measured during contraction in WT and MHC-FATP cells loaded with the fluorescent  $Ca^{2+}$  indicator, fluo-4-AM. The results of these experiments are summarized in Figure 5A through 5D.



**Figure 4.** BDM, but not BAPTA-AM, relaxes resting MHC-FATP and WT ventricular myocytes. A, Mean diastolic sarcomere length increased significantly ( $P < 0.05$ ;  $t$  test) in both WT and MHC-FATP ventricular myocytes after application of 40 mmol/L BDM. Importantly, there was no difference in diastolic sarcomere length between the 2 groups in the presence of BDM, suggesting that MHC-FATP myocytes relax to the same extent as WT cells. B, Treatment of isolated myocytes with BAPTA-AM (100  $\mu$ mol/L) for 1 hour did not significantly affect diastolic sarcomere length in either MHC-FATP or WT cells, indicating that lower resting sarcomere length in MHC-FATP myocytes does not result from increased resting  $[Ca^{2+}]_i$ . Similar to the results illustrated in A, application of BDM to BAPTA-loaded cells also resulted in relaxation. In addition, in the presence of BDM, MHC-FATP and WT myocytes relaxed to the same resting sarcomere length. As expected, BAPTA-AM loading abolished contraction in both WT and MHC-FATP ventricular myocytes.

As is evident in Figure 5, diastolic  $[Ca^{2+}]_i$  is similar in WT and transgenic myocytes, consistent with results above indicating that the decrease in MHC-FATP diastolic sarcomere length does not reflect an increase in resting  $[Ca^{2+}]_i$ . In addition, the peak stimulated  $[Ca^{2+}]_i$  was similar in WT and MHC-FATP cells, consistent with the observation that systolic function is unaltered in MHC-FATP myocytes. The kinetics of  $[Ca^{2+}]_i$  clearance were also examined to determine whether reduced  $[Ca^{2+}]_i$  extrusion rates underlie the reduced relaxation rates (Figure 3) observed in MHC-FATP cells. Unexpectedly, the rate of  $[Ca^{2+}]_i$  removal was significantly faster in MHC-FATP, compared with WT, ventricular myocytes. To examine whether increased sarcoplasmic reticular  $Ca^{2+}$  ATPase (SERCA) expression might underlie the increased rate of  $Ca^{2+}$  removal in MHC-FATP myocytes, SERCA and phospholamban (PLB) levels were assessed by Western blot (Figure 5E and 5F). Surprisingly, these experiments revealed that SERCA-2a expression is actually decreased, whereas PLB expression and phosphorylation (PLB-Ser16) are unaffected in MHC-FATP ventricles. These results suggest that increased SERCA expression or activity does not contribute to the increased rate of  $[Ca^{2+}]_i$  extrusion observed in MHC-FATP cells. Moreover, the data clearly suggest that the observed impairment of myocardial relaxation in MHC-FATP cells is not caused by altered  $Ca^{2+}$  cycling.



**Figure 5.** Altered  $[Ca^{2+}]_i$  cycling cannot explain impaired relaxation kinetics of MHC-FATP ventricular myocytes. A and B, Representative raw recordings of  $[Ca^{2+}]_i$  transients obtained in WT and MHC-FATP transgenic ventricular myocytes stimulated at 1 Hz (A) and averaged  $Ca^{2+}$  transient obtained from more than 10 events (B). C, Mean ( $\pm$ SEM) diastolic and peak systolic  $[Ca^{2+}]_i$  values were not significantly ( $P > 0.1$ ; *t* test) different in WT ( $n=47$ ) and MHC-FATP ( $n=38$ ) myocytes. D, Whereas there was no difference in the diastolic or peak  $[Ca^{2+}]_i$ , the rate of  $[Ca^{2+}]_i$  removal ( $T_{50}$ =time to 50% decline in peak  $[Ca^{2+}]_i$ ) was significantly ( $P < 0.05$ ) faster in MHC-FATP compared with WT cells. E and F, Representative Western blots (E) and cumulative protein expression data (F) for SERCA-2a and PLB. As is evident, SERCA expression was decreased, whereas PLB expression was unaffected in MHC-FATP, compared with WT, ventricles.

### Increased Myofilament $Ca^{2+}$ Sensitivity in MHC-FATP Myocytes

Contrary to the initial hypothesis, alterations in  $[Ca^{2+}]_i$  cycling do not link the altered lipid uptake and metabolism in MHC-FATP transgenic ventricles to the impaired relaxation observed in MHC-FATP transgenic animals. These results suggest that the origin of myocardial contractile dysfunction lies downstream of  $Ca^{2+}$  signaling, namely at the level of the myofilaments. To examine directly myofilament function, the  $Ca^{2+}$  sensitivity of steady-state isometric force development was measured in skinned WT and MHC-FATP cardiomyocytes. Whereas there was no significant difference in the maximal active tension developed, myofilament  $Ca^{2+}$  sensitivity, indexed by  $pCa_{50}$ , was significantly ( $P < 0.01$ ) greater in MHC-FATP, than in WT, myocytes at sarcomere lengths of 1.9  $\mu$ m and 2.3  $\mu$ m (Figure 6). The shift in myofilament  $Ca^{2+}$  sensitivity between short and long sarcomere lengths, an index of length-dependent activation, however, was similar in WT ( $\Delta pCa_{50} = 0.22 \pm 0.01$ ) and MHC-FATP ( $\Delta pCa_{50} = 0.25 \pm 0.01$ ) myocytes. Passive tension, measured by stretching the cell from slack length to a sarcomere length of 2.3  $\mu$ m in relaxing solution, was significantly higher in MHC-FATP, than in WT, myocytes. In addition, the slope of the tension– $pCa$  relation ( $n_H$ ), an index of cooperative myofilament activation, was significantly reduced at short sarcomere length and increased at long sarcomere length in MHC-FATP, as compared with WT, myocytes. Taken together, these results demonstrate that myofilament function in MHC-FATP myocytes is perturbed and suggest a primary role for myofilament dysfunction in determining the altered relaxation evident in MHC-FATP ventricular myocytes.

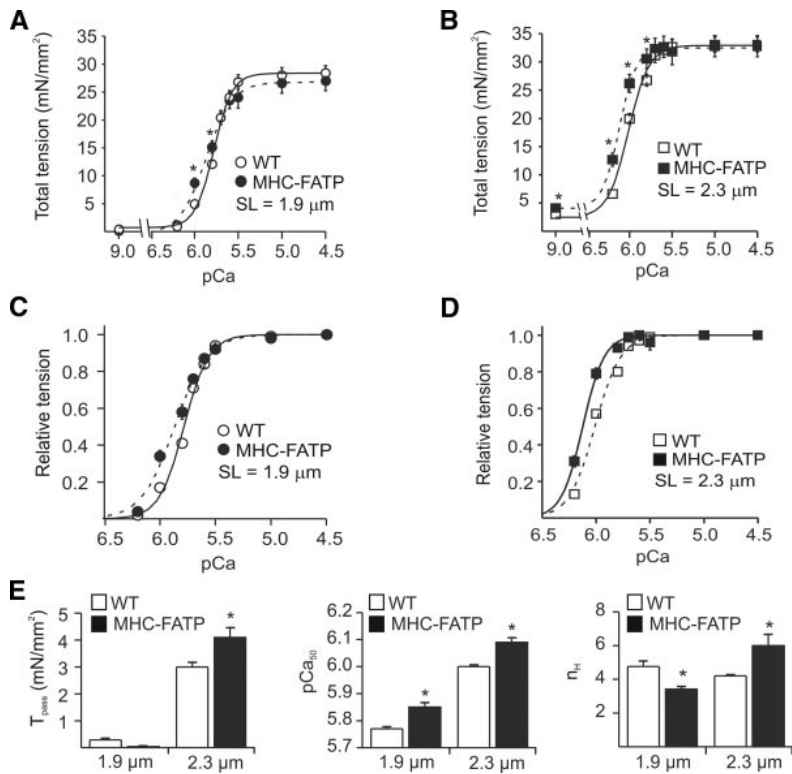
### $\beta$ -MHC Expression Is Increased in MHC-FATP Ventricles

To explore potential mechanisms underlying the observed deceleration of relaxation in MHC-FATP myocytes, the expression levels of 2 myofilament regulatory proteins, myosin binding protein (MyBP)-C<sup>17</sup> and troponin I (TnI),<sup>18</sup> that have been linked to increased  $Ca^{2+}$  sensitivity and slowed cardiac relaxation, were examined. Western blot experiments revealed that total MyBP-C and TnI expression, as well as the expression levels of phosphorylated MyBP-C<sup>Ser282</sup> and PKA-phosphorylated TnI, do not differ in WT and transgenic myofilaments (Figure 7), suggesting that altered regulation of these proteins does not appear to be involved in the impaired relaxation of MHC-FATP myocytes. However, further experiments revealed a significant increase in  $\beta$ -MHC expression in transgenic ventricles (Figure 7). Whereas increased expression of this isoform alone cannot account for increased myofilament  $Ca^{2+}$  sensitivity, the slower ATP hydrolysis and cross-bridge detachment rates associated with the  $\beta$ -MHC isoform<sup>19</sup> likely contribute to the reduced peak rate of relaxation in MHC-FATP myocytes (Figure 2).

## Discussion

### Cellular Basis of Diastolic Dysfunction

Overexpression of the FATP1 in the heart recapitulates the increase in the uptake, storage, and usage of fatty acid seen in the diabetic myocardium.<sup>13</sup> Moreover, MHC-FATP animals exhibit a restrictive filling pattern in echocardiograms consistent with diastolic dysfunction, which is a hallmark phenotype of diabetic cardiomyopathy at the earliest stages.<sup>5</sup> The



**Figure 6.** The  $\text{Ca}^{2+}$  sensitivity of myofilaments is increased in MHC-FATP ventricular myocytes. Mean ( $\pm$ SEM) steady-state total (A and B) and normalized (C and D) isometric tension plotted as a function of  $[\text{Ca}^{2+}]_i$  obtained in skinned WT ( $n=39$ ) and MHC-FATP ( $n=24$ ) ventricular myofibers, determined at sarcomere lengths of 1.9 and 2.3  $\mu\text{m}$ , respectively. E, At both sarcomere lengths,  $\text{Ca}^{2+}$  sensitivity ( $\text{pCa}_{50}$ ) was significantly increased ( $P < 0.05$ ,  $t$  test) in MHC-FATP, compared with WT, myocytes. Passive tension ( $T_{\text{pass}}$ ) and Hill coefficient ( $n_H$ ) were also increased in MHC-FATP, compared with WT, myocytes at the 2.3- $\mu\text{m}$  sarcomere length. A reduction in  $n_H$  was also observed in MHC-FATP, compared with WT, myocytes at the shorter (1.9- $\mu\text{m}$ ) sarcomere length.

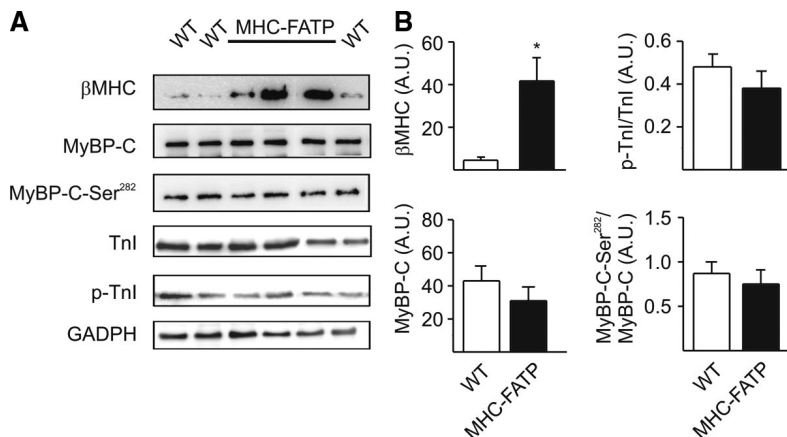
results presented here demonstrate that increased fatty acid transport, storage, and usage correlate with altered ventricular myocyte relaxation both in the resting state and during the relaxation phase of the cardiac cycle.

At rest, there was a significant decrease in sarcomere length, indicating that MHC-FATP ventricular myocytes are more contracted than their WT counterparts during diastole. Moreover, BDM causes relaxation of both WT and MHC-FATP myocytes to the same resting sarcomere length. This observation indicates that actin-myosin cross-bridges are important contributors to myocardial diastolic function and, in addition, suggests that diastolic tension is greater in MHC-FATP, compared with WT, cardiomyocytes. In the context of the whole heart, this would be expected to cause increased ventricular pressure and to restrict ventricular filling during diastole. In addition, myocyte relaxation was significantly slowed in MHC-FATP myocytes. This observa-

tion agrees with previous hemodynamic studies demonstrating that MHC-FATP hearts exhibited slower relaxation ( $-\text{dP}/\text{dt}$ ) than WT hearts.<sup>13</sup> The present findings obtained in isolated myocytes, therefore, demonstrate a cellular correlate of the intact MHC-FATP heart.

### Altered $[\text{Ca}^{2+}]$ Cycling Cannot Explain Impaired Diastolic Function in MHC-FATP Myocytes

Contrary to our expectation, the observed diastolic dysfunction in MHC-FATP myocytes cannot be explained by alterations in  $[\text{Ca}^{2+}]_i$  cycling. Direct measurement of  $[\text{Ca}^{2+}]$  transients revealed no significant differences between MHC-FATP and WT ventricular myocytes in either diastolic or peak stimulated  $[\text{Ca}^{2+}]_i$ . Taken together with the observation that chelation of intracellular  $[\text{Ca}^{2+}]$  with BAPTA-AM did not increase the diastolic sarcomere length in MHC-FATP cells, these data suggest that the observed shortening of



**Figure 7.**  $\beta\text{-MHC}$  expression is increased in MHC-FATP ventricles. A and B, Representative Western blots (A) and cumulative expression data (B) for several proteins involved in myofilament relaxation, MyBP-C, Tnl, and  $\beta\text{-MHC}$ . As is evident,  $\beta\text{-MHC}$  was increased approximately 9-fold in MHC-FATP, compared with WT, ventricles, whereas the expression levels of phosphorylated and nonphosphorylated MyBP-C (MyBP-C-Ser<sup>282</sup>) and Tnl (PKA p-Tnl) were found to be similar in MHC-FATP and WT ventricles.

diastolic sarcomere length is independent of  $[Ca^{2+}]_i$ . Similarly, the clearance rate of  $Ca^{2+}$  is significantly faster in MHC-FATP, compared with WT, myocytes and, thus, cannot explain the slower relaxation kinetics observed in MHC-FATP cells. Interestingly, these observations are consistent with the conclusion of a recent report demonstrating that myocyte relaxation is dissociated from the decline of  $[Ca^{2+}]_i$ .<sup>20</sup>

Although the faster rate of  $[Ca^{2+}]_i$  removal in MHC-FATP myocytes cannot explain the observed diastolic dysfunction, these observations do reveal that  $[Ca^{2+}]$  cycling is perturbed in transgenic myocytes. The increased rate of  $Ca^{2+}$  removal in MHC-FATP ventricular myocytes suggests either that (1) additional  $Ca^{2+}$  clearance mechanism(s) and/or buffer(s) are activated or (2) an existing  $Ca^{2+}$  clearance pathway is upregulated in MHC-FATP transgenic cells. In this regard, increased  $Ca^{2+}$  sensitivity of the myofilaments might explain an increase in the cytoplasmic  $Ca^{2+}$  buffering capacity. Alternatively, it is possible that, as a consequence of increased fatty acid storage in the MHC-FATP heart, alterations of the sarcolemmal phospholipids change the  $Ca^{2+}$  binding properties of the membrane affecting the kinetics of the  $Ca^{2+}$  transients.<sup>21</sup> Although the experiments here revealed that SERCA protein expression is decreased in MHC-FATP myocytes, it remains possible that upregulation of SERCA activity contributes to the increased rate of  $Ca^{2+}$  removal. PLB-dependent regulation of SERCA appears not to play a role, however, because total expression of PLB as well as phosphorylated PLB is not different in MHC-FATP and WT cells. It should be noted, however, that changes in the expression or the functioning of PLB, phosphorylated on Thr17, or in the activity of the sodium–calcium exchanger could play a role in regulating the rate of  $Ca^{2+}$  transient decay, because these have not been investigated in the present study. Interestingly, overexpression of SERCA has been used to normalize systolic function in streptozotocin-injected mice and rats that display many features of type I diabetes.<sup>22,23</sup> It is possible that the faster clearance of  $[Ca^{2+}]_i$  observed in the MHC-FATP cardiomyocytes confers a resistance to the onset of systolic heart failure.

### Primary Role of Myofilaments in Impaired Diastolic Function in MHC-FATP Myocytes

Collectively, the data presented here demonstrate that the impaired diastolic function observed in MHC-FATP ventricles can be attributed, at least in part, to altered myofilament function. The observed increase in the  $Ca^{2+}$  sensitivity of the myofilaments, for example, could contribute to the decreased diastolic sarcomere length in transgenic myocytes in the absence of a concomitant change in diastolic  $[Ca^{2+}]_i$ . Similar changes in diastolic cell length have been observed with the addition of  $Ca^{2+}$ -sensitizing agents<sup>24</sup> and in other transgenic models that exhibit increased myofilament  $Ca^{2+}$  sensitivity.<sup>17,18</sup> It has been suggested previously that muscle relaxation can be slowed as a result of either prolonged  $Ca^{2+}$  binding to troponin C and/or altered cross-bridge cooperativity.<sup>25</sup> Both factors are known to affect the slope of the tension–pCa relation ( $n_H$ ), and either may be altered in the MHC-FATP myocytes, as suggested by the differences in  $n_H$

observed at both sarcomere lengths (Figure 6). Further studies focused on determining the molecular underpinnings of the observed changes in the  $Ca^{2+}$  sensitivity of myofilament activation and myocyte relaxation are clearly needed to further address this potentially important point. The accumulation of detrimental metabolites as a result of increased lipid uptake and usage, such as reactive oxygen species,<sup>26</sup> could contribute to the increased  $Ca^{2+}$  sensitivity in MHC-FATP transgenic animals. Although an important role for changes in MyBP-C and TnI in determining the altered properties of the myofilaments seems unlikely, we cannot rule out the possibility that phosphorylation or other posttranslational modifications of the myofilament proteins contribute to the impaired diastolic function observed in MHC-FATP cardiomyocytes. In this regard, the recent observation that acetylation of muscle LIM protein can directly affect  $Ca^{2+}$  sensitivity<sup>27</sup> raises the possibility that fatty acid dependent inhibition of histone deacetylase may contribute to the observed changes in  $Ca^{2+}$  sensitivity in MHC-FATP myocytes.

A marked increase in  $\beta$ -MHC expression was also observed in MHC-FATP ventricles. Replacement of  $\alpha$ -MHC with  $\beta$ -MHC does not sensitize the myofilaments to  $[Ca^{2+}]$ .<sup>19,28,29</sup> Therefore, the increased expression of  $\beta$ -MHC does not underlie the increased  $Ca^{2+}$  sensitivity of steady-state force development. The slower rate of ATP hydrolysis and cross-bridge cycling of  $\beta$ -MHC, however, could contribute to the slowed relaxation rate observed in MHC-FATP cells. A similar induction of  $\beta$ -MHC expression observed following thyroidectomy in rat reportedly slowed relaxation kinetics without changing myofilament  $Ca^{2+}$  sensitivity in rats, although  $Ca^{2+}$  removal was also slowed in this model.<sup>30</sup> Interestingly, an increase in  $\beta$ -MHC expression has been shown to occur during diabetes.<sup>31,32</sup> In streptozotocin-injected diabetic rats, normalizing the ratio of lipid and glucose metabolism, either by inhibiting carnitine palmitoyltransferase I and long chain fatty acid oxidation with methyl palmoixirate<sup>33</sup> or activating glycolytic metabolism by circumventing the major regulatory checkpoints with fructose feeding,<sup>34</sup> reduced the expression of  $\beta$ -MHC without changing plasma insulin or thyroid hormone levels, suggesting a direct effect of the metabolic state of the heart on MHC isoform expression. Interestingly, fructose-feeding, which increases  $\alpha$ -MHC expression, is associated with an upregulation of the class III histone deacetylase, SIRT1,<sup>35</sup> again pointing to a potential intermediate in the regulation of myofilament expression by cellular metabolism.

### Relationships to Other Models of Diabetic Cardiomyopathy

The data presented here do indicate that perturbations of cardiac metabolism alone are sufficient to generate diastolic dysfunction, which is the earliest observable sign of diabetic cardiomyopathy. Moreover, the data indicate that diastolic dysfunction is a direct result of changes in the contractile machinery. This contrasts with a number of previous reports in other models of diabetic cardiomyopathy indicating that  $[Ca^{2+}]$  cycling underlies impaired contractile performance. Streptozotocin-treated mice and rats,<sup>36–38</sup> as well as leptin-deficient (*ob/ob*)<sup>8</sup> and leptin receptor-deficient (*db/db*)<sup>9,10</sup> animals, for example, have been



reported by others to show impaired  $[Ca^{2+}]$  handling and impaired contraction. It should be pointed out, however, that these animal models also exhibit impaired systolic function, which may indicate a more advanced stage of cardiovascular disease. In addition, these mouse and rat models exhibit systemic metabolic changes, whereas metabolic derangements are limited to the myocardium in the MHC-FATP model used in the present study. The present study does not rule out the possibility that alterations in  $Ca^{2+}$  handling contribute significantly to cardiomyopathy in the presence of systemic alterations of metabolism found in db/db, ob/ob, or streptozotocin-injected rodent models. Consistent with the notion that extracardiac systems contribute to the alterations of  $Ca^{2+}$  handling, it was recently reported that blockade of the renin-angiotensin system attenuates the depression of SERCA activity in streptozotocin-injected rats.<sup>39</sup>

## Conclusion

The results of the studies detailed here indicate that altered lipid metabolism in the heart impairs myocardial relaxation and suggest that changes in the  $Ca^{2+}$  sensitivity and molecular composition of the contractile myofilaments, and not alterations in  $Ca^{2+}$  signaling, underlie the diastolic dysfunction observed in the diabetic heart. These findings do not preclude the possibility that changes in  $Ca^{2+}$  handling can and do occur in the diabetic myocardium but suggest that these events will occur later in the progression of the disease.

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## Disclosures

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