Altered Myocardial Thin-Filament Function in the Failing Dahl Salt-Sensitive Rat Heart
Amelioration by Endothelin Blockade

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Background—Dahl salt-sensitive rats fed a high-salt diet develop compensated left ventricular hypertrophy followed by a transition to myocardial failure. We previously reported an increase in a troponin T isoform (TnT3) and a decrease in TnT phosphorylation in failing Dahl salt-sensitive rat hearts compared with low-salt controls. The present study was undertaken to determine whether the thin filament plays a role in depression of the contractile machinery in this model.

Methods and Results—Native thin filaments (NTFs) were isolated intact from rats with compensated left ventricular hypertrophy and failing hearts and compared with age-matched controls. NTF velocity was measured as a function of free calcium in the in vitro motility assay. Maximal velocity was similar in all groups. However, NTFs from failing hearts demonstrated a reduction in calcium sensitivity compared with controls, as reflected in the pCa50 (5.88 ± 0.05 versus 6.22 ± 0.05, respectively, P < 0.001). No difference in thin-filament motility (pCa50, Vmax) was observed in rats with compensated left ventricular hypertrophy compared with controls. Protein kinase A treatment of NTFs from control and failing hearts had no effect on thin-filament calcium sensitivity. However, the endothelin receptor blocker bosentan prevented the reduction in thin-filament calcium sensitivity found in failing hearts.

Conclusions—The thin filament is a key modulator of contractile performance in the transition to failure in the Dahl salt-sensitive rat model. The alteration in thin-filament function may be mediated by an endothelin-triggered pathway potentially affecting protein kinase C signaling. (Circulation. 2003;107:630-635.)

Key Words: heart failure • endothelin • troponin • phosphorylation • motility

The functional role of the sarcomeric proteins in failing myocardium is not completely understood. Increasing evidence suggests that alterations in the contractile machinery contribute directly to the contractile deficit of heart failure. In human heart failure, myosins isolated from failing and nonfailing human ventricles exhibit similar ATPase activity, whereas myofibrillar ATPase activity is markedly reduced1,2 and contractile economy (tension-time integral/energy expenditure for cross-bridge cycling) is increased.3 This suggests that the regulated interaction of myosin with the thin filament may be affected in failing myocardium and could contribute directly to the slowed cross-bridge kinetics of this disease. Altered thin-filament composition has been reported in animal models and in human heart failure.4−6 These include troponin T (TnT) isomorph shifts and modulation of both TnT and troponin I (TnI) phosphorylation. However, a direct mechanical assessment of the functional effect of thin-filament compositional changes in heart failure has not been demonstrated. This is partly because in small-animal models, compensated hypertrophy and myocardial failure are ordinarily associated with myosin heavy chain (MHC) isoform shifts from predominantly α-MHC to predominantly β-MHC. Altered thick-filament composition in these models can potentially obscure the functional effects of thin-filament changes, because the 2 myosin isoforms differ in their functional properties.7,8

The Dahl salt-sensitive (DS) rat undergoes a predictable transition from hypertrophy to failure.9 This strain has a renin gene polymorphism and a mutation in the α1-Na+,K+-ATPase gene.10 When fed a high-salt (HS) diet, the rats show a marked increase in blood pressure because of combined pressure and volume overload, which results in compensated left ventricular (LV) hypertrophy after 5 to 6 weeks.9 After 10 to 12 weeks of HS diet, DS rats develop cardiac failure with LV dilation and contractile dysfunction.9,11 Increased endothelin receptor mRNA has been documented in this model.12 Consistent with this finding is the fact that development of failure is ameliorated by long-term treatment with the endo-
theolin receptor antagonist bosentan. Thus, an endothelin-mediated mechanism may be in part responsible for the contractile deficit in this model.

We have previously reported that the transition to failure is associated with compositional changes of the thin filament in the DS rat, specifically, a TnT isoform shift (increase in the minor, TnT3, isoform) and a reduction in bulk TnT phosphorylation. Associated with this is a reduction in maximal calcium-activated myofibrillar ATPase rate. Interestingly, the reduction in ATPase is larger than can be accounted for solely by the α- to β-MHC isoform shift observed in this model. Correspondingly, increases in contractile efficiency and economy are also out of proportion relative to the myosin isoforms. These findings are reminiscent of failing human myocardium and suggest that the regulated interaction of actin and myosin may be affected in the DS model.

To delineate the role of the thin filament during the transition from compensated hypertrophy to failure in the DS rat, thin-filament function was assessed with an in vitro motility assay. The relation between pCa and unloaded velocity of intact native thin filaments (NTFs) isolated from cardiac myofibrils from hypotrophied and failing Dahl rats was compared with age-matched controls. The confounding effect of myosin isoform shifts was eliminated by use of purified skeletal muscle myosin in the assay. Furthermore, the effect of chronic endothelin receptor blockade on thin-filament function was also delineated.

Methods

Animal Model

The Institutional Animal Care and Use Committee of the University of Vermont approved the procedures. At 6 weeks of age, DS rats (Brookhaven National Laboratory, Upton, NY) were divided into populations receiving either HS (8% NaCl) or low-salt (LS, 0.3% NaCl) diet. Each group was divided into subgroups that were killed at either 6 or 12 weeks after starting the diet. Thus, rats were divided into 4 groups by diet and duration: HS for 6 or 12 weeks (HS-6 [n = 4] and HS-12 [n = 6]) or LS for 6 or 12 weeks (LS-6 [n = 4] and LS-12 [n = 6]). The rats received a lethal injection of sodium pentobarbital (90 mg/kg IP) at the predetermined times, after which a midline thoracotomy was performed and the heart was removed. The LV was rapidly dissected free, briefly rinsed in Krebs solution, and flash-frozen in liquid nitrogen. The tissue was then stored at −80°C and subsequently used for in vitro motility studies.

Bosentan Treatment

Eight male DS rats and 4 male Dahl salt-resistant rats (control group) were used (Eisai Company, Tokyo, Japan). At 6 weeks of age, all rats were fed HS diet for the remainder of the protocol. After 5 weeks of diet, DS rats were randomly treated with either oral bosentan (100 mg · kg⁻¹ · d⁻¹, Actelion Ltd, n = 4) or vehicle (failing heart group, n = 4). After 11 weeks of HS (6 weeks of drug treatment), all rats were killed, and ventricular tissue was stored as above.

Contractile Proteins

Myosin was isolated from chicken skeletal muscle as previously reported. NTFs were isolated from frozen LV and labeled with rhodamine-phalloidin. Isolated NTF composition was determined by SDS-PAGE, with protein stoichiometry quantified by densitometry (Figure 1). Immunoblotting was performed on tissue homogenates and isolated NTFs to determine whether proteolytic cleavage of TnI occurred during the transition to failure and/or during NTF isolation. Whole-tissue homogenates (400 µg) determined from tissue weight) and NTFs (20 µg) were run on a 12% polyacrylamide gel. Immunoblotting was performed as previously described using 2 monoclonal mouse anti–cardiac TnI antibodies (clones 7F4 and C5, with corresponding epitopes on rat cardiac TnI amino acid residues 191 to 198 and 87 to 93, respectively; Advanced ImmunoChemical).

Protein kinase A (PKA)-mediated phosphorylation of NTFs isolated from 6 failing hearts (HS-12) and 4 age-matched controls (LS-12) was assessed as follows. One half of the NTFs isolated from hearts were treated with PKA (Sigma) as previously described and analyzed with paired, untreated NTFs in the motility assay as described below. In separate experiments, reconstituted thin filaments containing predominantly dephosphorylated troponinT were treated with PKA. Last, the PKA treatment protocol was repeated using [γ-32P]ATP and myofibrils isolated from HS-12 and LS-12 hearts. The back-phosphorylation method was used to quantify the in vivo phosphorylation state of PKA phosphorylation sites (ser 23 and ser 24) on TnI in the failing and nonfailing states as previously reported.

In Vitro Motility Assay

In vitro motility of thin filaments in a calcium-regulated system was performed as previously described. In brief, myosin molecules were adhered to a nitrocellulose-coated coverslip. Rhodamine-phallolidin–labeled thin filaments were observed by epifluorescence microscopy to be moving across the myosin-coated surface. Free calcium was varied in the motility solution (pCa 10 to 4.5), and the velocity of thin filaments as a function of pCa was determined. Typically, >250 individual filament velocities were averaged to determine the mean velocity-pCa data point for each experiment. Data from each heart were combined within each experimental group to generate a mean velocity-pCa relation. The data were fit by use of the Hill equation.

Statistical Analysis

Values are expressed as mean±SEM unless otherwise noted. Statistical significance was determined for the fitted parameters of the Hill equation by ANOVA with a Tukey adjustment for multiple comparisons.

Results

Thin-Filament Composition

We previously determined the isoform composition and bulk phosphorylation state of TnT in failing DS rat. There are 2 TnT isoforms in the adult rat, TnT3 and TnT4, with TnT3 isoform being predominantly phosphorylated at ser 23 and ser 24. In the failing heart, there is an increase in TnT3 and a decrease in TnT4. In the DS rat, there is a similar increase in TnT3, but the decrease in TnT4 is not as pronounced. This suggests that the decrease in TnT4 is not as significant in the failing DS rat as in the failing human heart.

Figure 1. SDS-PAGE of isolated NTF demonstrating full representation of regulatory proteins and actin. A small amount of myosin contamination is present.
increasing from 16.7±2.0% in the control group to 22.5±2.2% in the HS-12 (mean±SD; P<0.001). In addition, a decrease in TnT phosphorylation was detected in the failing group (27.0±5.1%) compared with controls (51.4±9.9%; P<0.001). Western blotting of whole-tissue homogenates demonstrated a small amount of TnI degradation (≈2%) in all study groups.

**Thin-Filament Function**

The maximal calcium-activated velocity of each group was similar to the velocity of purified, unregulated actin filaments under similar experimental conditions. LS-6 and HS-6 rats demonstrated a calcium sensitivity and cooperativity of activation similar to that demonstrated by pCa50 and Hill coefficient (Table 1, Figure 2). LS-12 rats exhibited a calcium sensitivity and cooperativity similar to that of the two 6-week groups. However, in the failing HS-12 rats, a significant decrease in calcium sensitivity was observed compared with the control group (P<0.001; Table 1, Figure 3). The difference in failing NTFs represents a near doubling of the free [Ca$^{2+}$] required to achieve half-maximal activation.

Treatment with PKA did not alter the calcium sensitivity of NTFs isolated from HS-12 rats compared with untreated HS-12 NTFs (pCa50, 5.91±0.04 versus 5.90±0.03, respectively, P=NS; Figure 4A). Treatment of LS-12 NTFs with PKA also had no significant effect on thin-filament calcium sensitivity (pCa50, 6.18±0.05 versus 6.19±0.05 for PKA-treated and untreated, respectively, P=NS; Figure 4B). In contrast, phosphorylation by PKA of reconstituted thin filaments containing dephosphorylated troponin caused a dramatic increase in thin-filament calcium sensitivity compared with untreated thin filaments (pCa50, 6.44±0.06 versus 6.11±0.02, respectively; P<0.0001; Figure 4C). PKA back-phosphorylation of Dahl rat cardiac myofibrils with [$\gamma$-32P]ATP revealed that TnI-PKA sites (ser 23/24) were ≥70% phosphorylated in vivo, with no difference between LS-12 and HS-12 hearts (70±4% and 76±4%, respectively, P=NS).

**Effects of Bosentan Treatment**

Experiments in bosentan-treated rats confirmed the consistency of the thin-filament changes in this model. Specifically, maximal calcium-activated velocities for all of the study groups were not different (Table 2, Figure 5). Similarly, cooperative activation of the thin filaments was not different as assessed by the Hill coefficient (Table 2). As above, the failing group demonstrated reduced calcium sensitivity compared with the control group (P<0.001; Table 2, Figure 5). No reduction in calcium sensitivity was observed in the bosentan-treated group. Thus, long-term bosentan treatment prevented the loss of thin-filament Ca$^{2+}$ sensitivity observed in DS rats receiving a HS diet.

**Discussion**

In the present study, intact NTFs isolated from failing DS rats manifested decreased calcium sensitivity of unloaded velocity in vitro compared with age-matched, nonfailing controls. These changes cannot be ascribed to myosin isofrom shifts because skeletal muscle myosin was used in the motility assay. This constitutes the first direct evidence of an alteration in thin-filament function in failing myocardium. In contrast, no change in thin-filament function was identified in compensated, hypertrophied hearts compared with either control group (LS-12 and LS-6). The reduction in calcium sensitivity observed in the HS-12 thin filaments is temporally associated with previously reported alterations in TnT isoform expression and reduction in TnT phosphorylation. These data strongly suggest that thin-filament compositional changes in the Dahl rat contribute causally to the altered cross-bridge cycling that occurs during the transition to failure in this model. This correlation is further supported

### Table 1. Native Thin-Filament Motility and Troponin I Phosphorylation in Hypertrophied and Failing Rat Myocardium

<table>
<thead>
<tr>
<th>Group</th>
<th>pCa50</th>
<th>Vmax (µm/s)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Salt</td>
<td>6.31±0.08</td>
<td>4.9±0.4</td>
<td>2.5±0.7</td>
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<tr>
<td>High Salt</td>
<td>6.26±0.07</td>
<td>5.2±0.2</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>12 Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Salt</td>
<td>6.22±0.05</td>
<td>4.7±0.2</td>
<td>2.7±0.5</td>
</tr>
<tr>
<td>High Salt</td>
<td>5.88±0.05*</td>
<td>4.7±0.1</td>
<td>2.0±0.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean of fit to the Hill equation ±SEM. Vmax indicates maximal calcium-activated thin-filament velocity; pCa50, negative log of the calcium concentration at which NTF velocity is half maximal. Hill coefficient reflects the slope of the transition.

*$^0.0001$ compared to other treatment groups.
by the fact that endothelin blockade with bosentan using a treatment protocol that preserves global LV function also prevented changes in thin-filament function. Our results thus implicate the thin filament as a key modulator of contractile function during the transition from hypertrophy to failure.

Mechanisms of the Transition From Hypertrophy to Failure

The DS model of combined pressure and volume overload is well suited to assess the progression from compensated hypertrophy to failure. In this model, relatively modest alterations in calcium homeostasis are reported during the transition to failure, consisting of a reduction in peak cytosolic calcium levels and a slowed rate of sarcoplasmic reticular calcium uptake. In contrast, papillary muscles from the failing Dahl rat exhibit profoundly depressed isometric tension that seems out of proportion to the reported alterations in calcium handling. This suggests that the contractile deficit in these failing hearts may be primarily the result of alterations in the contractile machinery. Our earlier work documented alterations in contractile efficiency and economy, myofibrillar ATPase, and cross-bridge kinetics and the aforementioned changes in TnT isoforms and phosphorylation, which correlate temporally with the transition to failure. The present study suggests that an alteration of the thin filament may be an important mechanism underlying these transitional changes in the contractile machinery.

Altered Thin-Filament Activation in Myocardial Failure

The velocity of thin filaments from failing DS rats demonstrated a decrease in calcium sensitivity. In skinned-fiber experiments, failing DS rats exhibited an increase in calcium sensitivity for isometric force compared with age-matched controls. The combination of increased calcium sensitivity for force and decreased calcium sensitivity for velocity can be accounted for by a 2-state cross-bridge model in which the myosin cross-bridge is either attached to the thin filament and generating force or detached. In the context of this model, isometric force is directly proportional to the cross-bridge attachment time, whereas velocity is inversely proportional to the cross-bridge attachment time. Perturba-

Table 2. Parameters of Native Thin-Filament Motility From Control, Failing, and Bosentan-Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>pCa50</th>
<th>Vmax (µm/s)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=4)</td>
<td>6.16±0.05</td>
<td>5.2±0.2</td>
<td>2.47±0.35</td>
</tr>
<tr>
<td>Failing (n=4)</td>
<td>5.86±0.06*†</td>
<td>5.0±0.1</td>
<td>2.28±0.56</td>
</tr>
<tr>
<td>Bosentan (n=4)</td>
<td>6.18±0.02</td>
<td>5.0±0.2</td>
<td>2.93±0.81</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Vmax indicates maximal calcium-activated NTF velocity; pCa50, negative log of the calcium concentration at which NTF velocity is half maximal. Hill coefficient reflects the slope of the transition. *P<0.01 compared with Control; †P<0.01 compared with bosentan.

Figure 4. A, Effects of PKA treatment of isolated NTFs from a failing heart (HS-12) compared with untreated NTFs from same heart. B, Effects of PKA treatment of isolated NTFs from a nonfailing heart (LS-12) compared with untreated NTFs from same heart. C, pCa:velocity relation of reconstituted thin filaments treated with PKA compared with dephosphorylated controls, demonstrating a significant effect of PKA phosphorylation on thin-filament calcium sensitivity.

Figure 5. Summary of calcium:velocity relationship in control (n=4), failing (n=4), and bosentan-treated (n=4) groups. Dotted regression and indicate control group; solid regression and bosentan group; and dashed regression and bosentan-treated group. A rightward shift in pCa:velocity relation is observed in failing group, indicating a decrease in NTF calcium sensitivity. There is no significant difference in calcium sensitivity between control and bosentan-treated groups.
tions that decrease the rate of cross-bridge detachment from the thin filament would cause an increase in cross-bridge attachment time, ultimately giving rise to increased force but decreased velocity. Thus, in the failing DS rat, compositional changes at the level of the thin filament may cause a decrease in the rate of cross-bridge detachment at submaximal calcium concentrations. This would translate into a decrease in velocity but an increase in force at transitional calcium concentrations, ie, a decrease in pCa50 for velocity but an increase in pCa50 for force.

The altered thin-filament function identified in the failing DS rat could be the result of several molecular alterations, including proteolytic degradation, a change in isoforms, or alterations in thin-filament protein phosphorylation. Western blotting of TnT and TnI (the 2 thin-filament proteins most susceptible to proteolytic degradation) did not demonstrate significant degradation in the failing heart. The altered TnT isoform expression reported in the failing Dahl rat is another potential mechanism by which thin filaments could exhibit a decrease in calcium sensitivity. However, in a direct comparison of the 2 adult beef cardiac TnT isoforms, no functional difference was elicited. Consideration of these results and the small magnitude of the shift in the Dahl rat argue against a major role for the TnT isoform shift.

These negative results suggest that the change in thin-filament function in the failing DS rat may result from modulation of thin-filament phosphorylation. An alteration in adrenergically mediated phosphorylation of TnI by PKA is one possibility. PKA is known to phosphorylate serines 23/24 near the N-terminus of TnI, resulting in a reduction in pCa50 for force and ATPase activity. NTFs treated with PKA demonstrated no change in calcium sensitivity or velocity in either the failing group or age-matched controls. This suggests that a change in PKA-mediated phosphorylation is not responsible for the reduced calcium sensitivity in the failing DS rat. Consistent with this finding, back-phosphorylation studies showed that the PKA-phosphorylatable sites of TnI are largely (>70%) and equally phosphorylated in both failing and control hearts. In contrast, PKA treatment of dephosphorylated thin filaments caused a marked increase in calcium sensitivity of velocity, indicating that the assay is sensitive to PKA-mediated phosphorylation.

Phosphorylation of TnI and TnT by PKC is also known to affect contractile function. Whereas nonspecific activation of PKC results in depression of maximal actomyosin ATPase, the PKC effects on myofilament function are highly isoform-specific. At least one isoform (PKC-ζ) affects half-maximal calcium activation with no effect on maximal activation. Although there are currently no published data on PKC isoform expression in the DS model, PKC-β and PKC-ε expression are increased in a rat model of pressure-overload hypertrophy. In addition, PKC-β expression is increased in human heart failure, and a transgenic mouse overexpressing PKC-β develops a dilated cardiomyopathy. However, the direct effects of PKC-ε or PKC-β on the ability of the myofilament to shorten and generate force is not well understood. Elucidation of these effects is of great interest in potentially accounting for the thin-filament defect in failing Dahl rats.

Normalization of thin-filament function by bosentan treatment also supports a PKC phosphorylation mechanism, because endothelin is known to activate PKC. Moreover, this suggests that the effects of endothelin on PKC are isoform specific. The concept that isoform-specific as opposed to nonspecific PKC activation is important during the transition to failure is also consistent with the observation that bulk TnT phosphorylation is reduced in the failing DS rat. This situation is analogous to human heart failure, in which there is both an increase in PKC-β expression and a decrease in bulk TnI phosphorylation. Thus, a decrease in bulk TnT phosphorylation could mask isoform-specific and opposite phosphorylation effects at specific sites on TnT (as well as TnI) that result in decreased thin-filament calcium sensitivity.

In summary, by isolating NTFs from hypertrophied, failing, and control DS rats, we have identified a thin-filament defect that causes a reduction in calcium sensitivity of unloaded velocity that is temporally correlated with the transition from hypertrophy to failure. In light of the fact that there is currently no satisfactory explanation for depressed cross-bridge cycling in human heart failure, these results raise the possibility that similar alterations occur in patients. Furthermore, correction of this defect may at least in part explain the efficacy of endothelin blockade in experimental heart failure.

Acknowledgments

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References


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