Thin Filament–Based Modulation of Contractile Performance in Human Heart Failure

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Background—The contribution of the sarcomere’s thin filament to the contractile dysfunction of human cardiomyopathy is not well understood.

Methods and Results—We have developed techniques to isolate and functionally characterize intact (native) thin filaments obtained from failing and nonfailing human ventricular tissue. By use of in vitro motility and force assays, native thin filaments from failing ventricular tissue exhibited a 19% increase in maximal velocity but a 27% decrease in maximal contractile force compared with nonfailing myocardium. Native thin filaments isolated from human myocardium after left ventricular assist device support demonstrated a 37% increase in contractile force. Dephosphorylation of failing native thin filaments resulted in a near-normalization of thin-filament function, implying a phosphorylation-mediated mechanism. Tissue expression of the protein kinase C isoforms \( \alpha \), \( \beta_1 \), and \( \beta_2 \) was increased in failing human myocardium and reduced after left ventricular assist device support.

Conclusions—These novel findings demonstrate that (1) the thin filament is a key modulator of contractile performance in the failing human heart, (2) thin-filament function is restored to near normal levels after LVAD support, and (3) the alteration of thin-filament function in failing human myocardium is mediated through phosphorylation, most likely through activation of protein kinase C.

Key Words: heart failure ❖ filaments ❖ heart-assist device ❖ kinases ❖ phosphorylation

Heart failure is a leading cause of death in the western world. Although advances in the treatment of this disease have resulted in modest improvements in its prognosis, the primary functional defects in the contractile machinery in human cardiomyopathy remain poorly understood. Although a small isoform shift in myosin has been identified in human heart failure, to date it has not been shown to be functionally significant. The rate of maximal myofibrillar ATP hydrolysis is markedly depressed in failing human myocardium, indicating that a significant defect in the contractile machinery is present and that the calcium-regulated interaction of myosin with the thin filament may be its underlying mechanism.

Phosphorylation of the thin-filament regulatory proteins by protein kinase A (PKA) and protein kinase C (PKC) is known to directly modulate myocardial contractile performance. Specifically, PKA phosphorylates 2 N-terminal serines on troponin I (TnI), causing a decrease in myofilament calcium sensitivity for tension with no effect on maximal activation. In contrast, activation of PKC has been shown to depress muscle fiber ATPase and force at maximal calcium activation through the phosphorylation of TnI and troponin T (TnT). Furthermore, increased PKC expression has been demonstrated in failing human myocardium. This raises the possibility that the reduction in myofibrillar ATPase in human failing myocardium is mediated by PKC-dependent phosphorylation of TnI and TnT and may represent an important mechanism underlying the contractile deficit of this disease. Whether modulation of thin-filament function induced by PKC phosphorylation results in physiologically meaningful alterations in contractile performance in human heart failure is not known.

To determine whether human heart failure is associated with an alteration of thin-filament function, the performance of native thin filaments (NTFs) isolated from human failing and nonfailing ventricles was directly assessed by use of an in vitro model of muscle contraction (in vitro motility assay). The role of phosphorylation of the thin filaments was determined by assessing thin-filament function after dephosphor-
ylation of the thin filament. These data were correlated with PKC expression levels in human myocardium and the effect of PKC activation in rat myocardium.

Methods

Human Tissue

The clinical characteristics of all human research subjects are summarized in the Table. Nonfailing tissue was obtained from 5 patients with coronary artery disease at the time of bypass surgery as previously described. These patients had no history of myocardial infarction or diabetes, no evidence of left ventricular (LV) hypertrophy, normal valve function, and normal regional and global LV function (ejection fraction, 67.5±2.5% [mean±SD]). Failing human LV tissue was obtained from 6 explanted human hearts and used for a total of 8 NTF isolations. Mean LV ejection fraction was 17.4±4.2% in this group. Failing hearts demonstrated either moderate or severe mitral regurgitation by echocardiography. In addition, paired samples were obtained from 3 patients before and after LV assist device (LVAD) support and 1 patient after LVAD support (mean LV ejection fraction, 15.0±3.5% before LVAD placement).

In the LVAD group, a total of 5 and 6 NTF isolations were performed before and after LVAD, respectively. All tissue was flash-frozen at the time of excision and stored in liquid nitrogen. Patient consent was obtained before surgery, in accordance with the Committees on Human Research at the University of Vermont and New England Medical Center.

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<td>DCM indicates nonischemic dilated cardiomyopathy; ICM, ischemic cardiomyopathy; LVEF, left ventricular ejection fraction; HTN, hypertension; ACE-I, ACE inhibitor; ARB, angiotensin receptor blocker; and β agonist, dobutamine or dopamine. Duration of LVAD support is given in days.</td>
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Animal Model
PKC was nonselectively activated in isolated rat hearts by use of the phorbol ester phorbol 12-myristate 13-acetate (PMA). Specifically, hearts isolated from 8 male Sprague-Dawley rats were perfused by the Langendorff method with Krebs-Henseleit buffer. Isolated hearts were randomly divided into 2 groups: a 10 nmol/L PMA (Sigma)–treated group and a vehicle group (control). This dose of PMA has been shown previously to produce a substantial decrease in ventricular function. PMA was administered via the coronary perfusate for 20 minutes at a perfusion pressure of 90 mm Hg. The LV was rapidly dissected free and flash-frozen in liquid nitrogen. The tissue was then stored at −80°C.

Contractile Protein Isolation
Intact NTFs were isolated from frozen myocardial tissue as previously described. NTF composition was determined through SDS-polyacrylamide gel electrophoresis. Isolated rat and human NTFs were labeled with rhodamine-phalloidin at a 1:1 actin/phalloidin ratio in low-salt buffer (25 mmol/L KCl, 25 mmol/L imidazole, 5 mmol/L MgCl2, 10 mmol/L dithiothreitol [DTT], and 2 mmol/L EGTA, pH 7.4). In all in vitro motility and force experiments, chicken skeletal myosin was used, because it is more stable than cardiac myosin once isolated, providing greater consistency between the same myosin substrate and in the absence of any changes in myosin function that may be associated with failing myocardium. For in vitro studies of isometric force, the actin-binding protein α-actinin (Sigma) was dialyzed into the above low-salt buffer before use.

In Vitro Motility and Force Assays
The in vitro motility assay has been described previously. Before each experiment, myosin (200 μg/mL), equimolar actin, and 1 mmol/L ATP are centrifuged (320 000g; 20 minutes) to remove noncycling, rigor myosin bound to actin. Monomeric myosin is then adhered to a nitrocellulose-coated glass coverslip. Rhodamine-phallolidin–labeled NTFs move across the myosin surface as a function of free calcium in the presence of ATP. All experiments were performed at 30°C. Free calcium was varied in the motility solutions (pCa, 10 to 4.0). Thin-filament motility was observed with epifluorescent microscopy, recorded on videotape, and subsequently analyzed. Typically, >300 individual filament velocities were averaged to determine the mean velocity of each experiment. The distributions of the velocities around the mean were similar for the different experimental groups under the same experimental conditions. filament motility is analogous to unloaded shortening in muscle fiber preparations. Relative isotonic force was determined as previously reported by use of the actin-binding protein α-actinin as a internal load. Relative isotonic force is defined as the minimum amount of α-actinin needed to completely arrest thin-filament motility (ie, force generation by myosin and the internal load by β-actinin are balanced). Force was determined at 7 different free calcium concentrations (pCa, 10 to 5).

Dephosphorylation of NTFs
For these experiments, NTFs were isolated from 2 failing ventricles and 2 paired ventricular samples before and after LVAD. One half of the isolated NTFs were dephosphorylated with protein phosphatase 2A (PP2A), with the remaining half serving as the phosphorylated control. NTFs (0.9 mg/mL) were treated with PP2A, (Calbiochem, 22 U/mL) in 50 mmol/L Tris-HCl, 2 mmol/L MnCl2, 25 mmol/L KCl, and 2 mmol/L DTT at 4°C overnight. Dephosphorylation of NTF from nonfailing myocardium was not feasible because of the small size of the biopsy samples obtained from these patients.

Western Blot Quantification of PKC Isoforms
Protein expression levels of PKC α, β1, and β2, were determined, because these have been demonstrated previously to represent the predominant isoforms in human myocardium. Approximately 2 mg of frozen ventricular tissue was homogenized in a 10-fold volume of lysis buffer (50 mmol/L HEPES, 1 mmol/L EGTA, 150 mmol/L KCl, 1 mmol/L benzamidine, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonylfluoride, 10 μmol/L leupeptin). The protein concentration of the homogenate solution was measured by use of a protein assay (Bio-Rad) with BSA as a standard. SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed as previously described. The primary antibodies used for immunoblotting were monoclonal antibodies to PKC α, β1, and β2 (Calbiochem). Band densitometry was determined by use of the Fluor-S-Max System (Bio-Rad).

Statistical Analysis
Data are expressed as mean ± SEM unless otherwise noted. Mean motility data for each experiment were fitted to the Hill equation. Comparisons between groups were performed by unpaired t test or 1-way ANOVA with a Tukey adjustment for multiple comparisons.

Results
Thin-Filament Function in Failing and Nonfailing Human Myocardium
NTFs from failing myocardium demonstrated a 27% decrease in maximal calcium-activated force compared with the nonfailing group (P<0.001, Figure 1A, Figure 3A). An increase in calcium sensitivity for force was observed with NTFs from failing myocardium (pCa50, 6.31±0.02 versus 6.22±0.02 for failing and nonfailing myocardium, respectively; P=0.016) and is similar to that reported with skinned muscle fibers from failing myocardium. In contrast, calcium-velocity relations for NTFs isolated from failing myocardium demonstrated a 19% increase in maximal velocity (P=0.023) compared with nonfailing myocardium (Figure 1A, Figure 3B). No significant difference in calcium sensitivity (ie, pCa50) for velocity was detected between the 2 groups. Dephosphorylation of human failing ventricular NTF by

Figure 2. Force:pCa and velocity:pCa relations of myocardial NTFs before and after LVAD placement. NTFs isolated from human LV tissue after LVAD support (•) demonstrated higher force but lower velocity at maximal calcium activation compared with NTFs isolated from tissue before LVAD support (○). Thus, mechanical characteristics of thin filament returned back toward nonfailing profile with LVAD support (see Figure 3).
PP2A$_1$ resulted in a 32% increase in maximal force ($P<0.001$) and a 20% decrease in maximal velocity ($P<0.002$) compared with paired failing NTFs not treated with phosphatase (Figure 1B, Figure 3). Phosphatase treatment did not significantly affect the calcium sensitivity of the thin filament.

Thin-Filament Function After LVAD Support
NTFs isolated from ventricular tissue after LVAD support demonstrated a near-normalization of thin-filament function, with a 37% increase in contractile force ($P<0.001$) and a 9% decrease in maximal velocity ($P=0.001$) compared with tissue obtained before LVAD placement (Figure 2, Figure 3). No change in the calcium sensitivity of the thin filament was observed after LVAD support. Dephosphorylation of NTFs with PP2A$_1$ resulted in abolition of the differences in thin-filament function before and after LVAD placement (Figure 3). Furthermore, the dephosphorylated thin filaments functioned similarly to NTFs isolated from ventricular myocardium after LVAD support without phosphatase treatment.

Effect of PMA on Rat Thin-Filament Function
Treatment of rat myocardium with the PKC activator PMA (Figure 3) resulted in a 15% increase in maximal native thin-filament velocity compared with control rats ($P<0.03$). No significant difference in calcium sensitivity was detected between the 2 groups. NTFs from the PMA treatment group exhibited a 33% decrease in maximal calcium activated force ($P<0.001$) without altering calcium sensitivity. Thus, PMA treatment of rat myocardium resulted in strikingly similar alterations in thin-filament function as demonstrated for failing and nonfailing human NTFs and is consistent with PKC-mediated phosphorylation of the thin filament being enhanced in human cardiac failure.

PKC Isoform Expression in Human Myocardium
Protein expression of the PKC isoforms was determined by Western blotting (Figure 4). PKC $\alpha$, $\beta_1$, and $\beta_2$ isoform expression were significantly increased in failing myocardium compared with nonfailing myocardium (193%, 55%, and 124% for $\alpha$, $\beta_1$, and $\beta_2$ isoforms, respectively; $P<0.001$). Assessment before and after LVAD placement demonstrated a reduction in PKC expression (−80%, −32%, and −68% for $\alpha$, $\beta_1$, and $\beta_2$ isoforms, respectively; $P<0.001$). These reductions are similar in magnitude to the differences in expression observed in the failing and nonfailing groups.

Discussion
In the present study, we demonstrate that the thin filament is responsible for a marked depression of actomyosin force in failing human myocardium. In contrast, maximal thin-filament velocity is significantly higher in failing human myocardium than in nonfailing controls. Thin-filament contractile abnormalities were restored toward the nonfailing state with LVAD support. In addition, these changes in thin-filament function are largely reversed by dephosphorylation of the thin filament, implicating thin-filament regulatory protein phosphorylation as the major cause of the altered thin-filament function in human cardiomyopathy. These novel findings constitute direct evidence of an alteration in thin-filament function in human failing myocardium and demonstrate the critical role that the thin filament plays in the contractile deficit of this disease. Furthermore, the contractile characteristics of NTFs from PKC-activated rat myocardium were strikingly similar to failing human NTFs, suggesting that the altered thin-filament function in human failing myocardium is the result of PKC-mediated phosphorylation of TnI and TnT. Such a mechanism is supported by the increase in PKC expression in failing human myocardium and its decrease after LVAD support.

Alteration in Thin-Filament Function in Human Heart Failure
Alterations at the contractile machinery level are increasingly being recognized as substantial contributors to the contractile function of the myocardium.
deficit of myocardial failure. At the contractile protein level, such alterations must be ascribable to changes in myosin function or alterations in the regulation of actomyosin function by the thin filament. Direct assessment of myosin isolated from failing and nonfailing hearts has not demonstrated significant differences in function. The fact that myofibrillar ATPase is depressed in human cardiomyopathy but isolated myosin ATPase is unchanged suggests that the regulated interaction of myosin with the thin filament is altered, leading to depressed myofibrillar ATPase and contributing to the contractile deficit of this disease. This hypothesis was validated in the present study, in which NTFs isolated from failing human myocardium exhibited a marked decrease in maximal isometric force.

Skinned fibers and myocytes isolated from human failing myocardium have demonstrated smaller reductions in isometric tension than reported in the present study. The differences in magnitude between fiber and in vitro motility experiments could be accounted for, in part, by (1) the use of skeletal myosin in this study, (2) the shift in the Frank-Starling relation found in failing human cardiac fibers, and (3) changes in myocardial elasticity with myocardial failure. In the motility assay, the confounding issues of sarcomere length and fiber elasticity are eliminated because analysis of contractile function is derived directly from purified NTFs and myosin. Consistent with the findings of the present study, overexpression of PKC in a transgenic mouse model resulted in the induction of cardiac failure, with altered myofilament function being implicated in this transformation. Furthermore, recent fiber data demonstrate the importance of specific amino acid residues on TnI and TnT in PKC-mediated depression of contractile force.

Reversal of Failing Thin-Filament Dysfunction: Implications of PKC-Mediated Phosphorylation
Increased myocyte shortening has been demonstrated after LVAD support. In the present study, the thin filament was identified as a significant contributor to the observed improvement of contractile function. Furthermore, the fact that thin-filament function can revert back to a nearly normal state with LVAD support underscores the plasticity of the contractile machinery in human heart failure. The factors that may lead to the thin-filament remodeling after LVAD placement include a decrease in systemic neurohormonal signaling and a decrease in LV wall stress that is associated with the hemodynamic unloading of the LV. Both of these factors most likely contribute to the reduction in PKC signaling after LVAD placement.

The fact that the changes in thin-filament function are largely reversed with dephosphorylation indicates that although other factors may affect thin-filament function, this posttranslational modification is the principal mediator of altered thin-filament function in human myocardial failure. Although phosphorylation of several thin-filament proteins could possibly be responsible for the demonstrated alterations in thin-filament function, TnT and TnI are the most likely targets of kinase activity, because these 2 thin-filament proteins are known to contain multiple phosphorylation sites. The changes in thin-filament function in failing human myocardium and after LVAD support strongly correlate with tissue PKC expression, providing a credible link between kinase expression and thin-filament function.

PKC and Myosin Cross-Bridge Kinetics and Molecular Mechanics
Similar to failing myocardium, PMA treatment of rat myocardium resulted in an increase in NTF velocity and a decrease in isometric force at maximal calcium activation. Although the effect on unloaded shortening and isometric force may initially seem inconsistent, these results can be accounted for by a 2-state myosin cross-bridge model first proposed by Huxley. In this model, the myosin cross-bridge is either strongly bound to actin in a force-generating state or
detached from the thin filament. In the context of the model, unloaded shortening velocity is inversely proportional to the attachment time (\( t_{on} \)) whereas force is proportional to attachment time (\( t_{on} \)) divided by the cross-bridge cycling time \([t_{cycle}]\). Perturbations that increase the rate of cross-bridge detachment from the thin filament would cause a decrease in \( t_{on} \) and ultimately give rise to increased velocity but decreased force. An increase in cross-bridge cycle time and/or a decrease in the number of cross-bridges interacting with the thin filament is also probably contributing to the reduction in force observed, which is supported by the previously reported reduction in myofibrillar ATPase in failing human myocardium.\(^5\)

In summary, these novel data implicate the phosphorylation of the thin filament as a key modulator of contractile performance in the failing human heart. The large changes in PKC expression associated with the alteration of human thin-filament function, coupled with the effect of PKC activation on rat myocardial thin-filament function, strongly suggest that elevated levels of PKC in the failing human heart directly affect thin-filament function. On the basis of the fact that angiotensin II blockade (a principal treatment of heart failure) is known to reduce intracellular PKC signaling,\(^27\) our findings provide new mechanistic support of this therapy at the molecular level. Furthermore, considering the reversibility of this defect, our findings provide a potential target for future therapies in the treatment of human heart failure.

Acknowledgments

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References
