Myofilament Proteins in Genetic and Acquired Heart Disease

Alterations of myofilament proteins have long been recognized in cardiomyopathic states. A variety of mutations play a prominent role in both hypertrophic and dilated cardiomyopathy (DCM). Thus, mutations in myosin heavy chain (MyHC) that cause enhanced mechanical performance often underlie hypertrophic cardiomyopathy, whereas those causing reduced performance lead to DCM. As summarized in Table 1, post-translational modifications, most prominently isoform variations and phosphorylation state, have received considerable attention in patients with DCM. In response to experimental acquired disease in rodents, MyHC undergoes a major shift from its normally predominant α-isoform, to the β-isoform. MyHC undergoes a shift from its normally predominant α-isoform, to the β-isoform, resulting in lower myofibrillar ATPase activity and reduced α-isoform to the β-isoform. Titin, the giant protein that accounts for cardiomyocyte passive stiffness, undergoes a shift to its more compliant N2BA isoform in human DCM. Protein kinase (PKA) and PKG phosphorylate identical sites on titin. Phosphorylation of these sites is reduced in DCM, which increases stiffness and offsets the isoform shift. The net result seems to be a decrease in cardiomyocyte stiffness. Reduced phosphorylation of troponin I (TnI) and myosin regulatory light chain have also been reported in human DCM and are thought to contribute to impaired thin filament activation and contractility. Pertinent to the papers in this issue of *Circulation: Heart Failure* that are the subject of this editorial,10,11 there are few reports of the phosphorylation state of myosin-binding protein C (MyBP-C) as a regulator of myofilament function at the whole heart level. Of note, reduced phosphorylation of PKA sites on MyBP-C has been reported in human DCM.12

As shown in Table 2, post-translational modifications of myofilament proteins have also been detected in patients with heart failure with a preserved ejection fraction (HFpEF) and its frequent substrate, concentric left ventricular hypertrophy (LVH). As in DCM, changes in titin isoforms and phosphorylation result in opposite effects on titin’s stiffness, but in this case, changes in phosphorylation of titin’s PKA/PKG, as well as its PKC-α sites, are dominant and play a key role in markedly increased cardiomyocyte and myocardial passive stiffness. Using permeabilized strips of human myocardium obtained from patients with hypertension and LVH and control subjects undergoing coronary bypass grafting, all with normal EF, we recently reported that the cross-bridge dissociation rate constant is slowed and its inverse, the cross-bridge on-time (time the cross-bridge is attached and generating force), is prolonged at submaximal [Ca2+] in patients with hypertension+LVH compared with control subjects. These changes in acto–myosin dynamics serve to slow left ventricular relaxation. In association with these changes, we documented decreased phosphorylation of both TnI and MyBP-C; however, we have not yet demonstrated a cause and effect relationship between phosphorylation state and slowed relaxation in human myocardium. Nonetheless, the reports by Tong et al10 and Rosas et al11 in this issue of *Circulation: Heart Failure* are of great interest in light of results in DCM12 and our findings in patients with acquired LVH.

Current Knowledge of Myosin-Binding Protein-C

After its discovery in the 1970s, MyBP-C was recognized as being structurally important in skeletal and cardiac muscle because of its regular placement in the sarcomere at 43 nm spacing along the thick filament. Its C-terminus binds to the myosin rod and titin and accounts for about half of thick filament longitudinal rigidity. The placement of the N-terminus within the intact sarcomere is not as well delineated. It is capable of binding to both actin and the myosin S2 segment on the thick filament. Currently, there is substantial evidence that the N-terminus preferentially interacts with actin. The N-terminus of MyBP-C possesses multiple phosphorylation sites, which seem to be a natural regulator of its properties and, by extension, cardiac function. The functional importance of MyBP-C was first reported in the late 1990s when Hofmann et al removed the protein from the sarcomere and found that shortening velocity was enhanced, implicating MyBP-C as an internal viscous load and inhibitor of acto–myosin cross-bridge kinetics. Interest in MyBP-C function grew rapidly when it was found that mutations account for a substantial proportion of hypertrophic cardiomyopathies. The influence of the C-terminus in providing...
A; PKC-α; and PKG, protein kinase G.

Finally, phosphorylation of the N-terminus reduces MyBP-C extensibility,28 suggesting that interactions of the N-terminus with the thin filament are reduced in frequency and magnitude by a mechanical constraint within the protein that is enhanced by phosphorylation.

Table 1. Changes in Isoform Composition and Phosphorylation State of Myofilament Proteins in Human Dilated Cardiomyopathy

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoform Composition</th>
<th>Phosphorylation State</th>
<th>Functional Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyHC</td>
<td>↑ β/α ratio</td>
<td>NA</td>
<td>↓ myofilament ATPase activity, ↓ contractility, slowed relaxation</td>
</tr>
<tr>
<td>My ELC</td>
<td>↑ atrial isofrom in LV</td>
<td>NA</td>
<td>↓ contractility</td>
</tr>
<tr>
<td>Titin</td>
<td>↑ N2BA/N2B ratio</td>
<td>↓ PKA/G sites</td>
<td>Net ↓ cardiomyocyte passive stiffness</td>
</tr>
<tr>
<td>Troponin I</td>
<td>NA</td>
<td>↓ PKA sites</td>
<td>↓ contractility, slowed relaxation</td>
</tr>
<tr>
<td>My RLC</td>
<td>NA</td>
<td>↓ PKA sites</td>
<td>↓ contractility, slowed relaxation</td>
</tr>
<tr>
<td>MyBP-C</td>
<td>NA</td>
<td>↓ PKA sites</td>
<td>↓ contractility, slowed relaxation</td>
</tr>
</tbody>
</table>

LV indicates left ventricular; MyBP-C, myosin binding protein C; My ELC, myosin essential light chain; MyHC, myosin heavy chain; My RLC, myosin regulatory light chain; NA, not applicable; and PKA, protein kinase A.

structural rigidity is evident in the intact left ventricle, where systolic elastance is abbreviated when MyBP-C is absent.23 Absence of the protein is also associated with increased efficiency of energy conversion by the contractile machinery, consistent with a reduced viscous load.24 The MyBP-C N-terminus plays at least 3 functional roles in regulating cardiac function. The N-terminus can activate the thin filament and potentially modulate thin filament calcium sensitivity.25 It also acts to slow thin filament sliding relative to the thick filament;26 there is less slowing as phosphorylation is increased.27 Finally, phosphorylation of the N-terminus reduces MyBP-C extensibility,28 suggesting that interactions of the N-terminus with the thin filament are reduced in frequency and magnitude by a mechanical constraint within the protein that is enhanced by phosphorylation.

Examinations into the functional role of MyBP-C in the intact sarcomere reveal that the protein inhibits acto–myosin kinetics. The rate of force redevelopment after a quick stretch is elevated when MyBP-C is absent,29 implicating MyBP-C as an inhibitor of thin filament activation in response to stretch or an inhibitor of acto–myosin cross-bridge kinetics. This result is especially impressive given that mouse tissue lacking MyBP-C used by Stelzer et al30 also possessed a higher fraction of the slower β-MyHC compared with controls, yet demonstrated faster responses. We have shown that myosin cross-bridge detachment rate is inhibited by MyBP-C;30 the inhibition is more potent in β-MyHC than in α-MyHC.31 Importantly, phosphorylation of MyBP-C by PKA removes its normal inhibition of the rate of force redevelopment30 and would likewise be expected to enhance acto–myosin cross-bridge detachment rate and myofilament-dependent relaxation.

The currently recognized functions of the MyBP-C N-terminus could theoretically play a role in phosphorylation-dependent diastolic dysfunction. Any calcium-independent activation of the thin filament by MyBP-C would be expected to enhance contractile force during diastole and inhibit relaxation. The viscous load provided by MyBP-C would slow sarcomere lengthening. Finally, slowed acto–myosin cross-bridge detachment rate would also slow sarcomere lengthening independent of thin filament deactivation. Conversely, increased phosphorylation of MyBP-C would reduce activation of the thin filament, viscous load, and inhibition of acto–myosin kinetics, all of which could improve diastolic function.

Enhancement of whole-heart relaxation function by MyBP-C phosphorylation was first suggested by Nagayama et al.23 In their study, relaxation in a transgenic mouse heart expressing a nonphosphorylatable MyBP-C did not respond to adrenergic stimulation, implying that MyBP-C phosphorylation is an important determinant of cardiac diastolic reserve.

Findings of the Current Studies

Tong et al10 and Rosas et al11 have exploited transgenic mouse MyBP-C phosphomimetic lines, in which the 3 PKA sites (ser273, ser282, and ser302) are replaced by either alanine (t3SA), rendering them nonphosphorylatable, or aspartic acid (t3SD), simulating 100% phosphorylation, as well as mice with wild-type MyBP-C (tWT), to expand our knowledge of the physiological and pathophysiologic roles of MyBP-C phosphorylation. Along with Copeland et al13 and our report,15 their results support the idea that changes in MyBP-C phosphorylation play a significant role in DCM and HFpEF. Understanding MyBP-C as a phosphorylation-dependent regulator of human diastolic function is important because acto–myosin cross-bridges are never fully disengaged in human myocardium during diastole.15,32

Tong et al10 explored the role of MyBP-C phosphorylation in 2 key inotropic mechanisms, the Bowditch effect or force-frequency relation (FFR), in which increases in stimulation frequency increase inotropy and the response to adrenergic stimulation (dobutamine). These combine to generate the myocardial response to exercise. Both the FFR and adrenergic responsiveness are markedly depressed in DCM.15,34 Moreover, some patients with DCM also demonstrate frequency-dependent incomplete relaxation and diastolic dysfunction.15 We reported similar frequency-dependent diastolic dysfunction in excitable myocardial strips obtained from patients with LVH and normal EF.32 Previously, calcium-modulated kinase 2δ, whose activity is frequency-sensitive, was thought to play a key role in the FFR by phosphorylating the ryanodine receptor.
at increasing heart rates, resulting in increased Ca\textsuperscript{2+} delivery to the myofilaments.\textsuperscript{36,37} Tong et al\textsuperscript{10} show that t\textsuperscript{3}SA mice with nonphosphorylatable MyBP-C have a virtually undetectable FFR and, in vitro experiments, that the predominant mechanism of the positive FFR is phosphorylation of MyBP-C by calcium-modulated kinase \( \delta \) at its ser302 site. Intracellular Ca\textsuperscript{2+} transients were similar in t\textsuperscript{3}SA and t\textsuperscript{WT} mice as frequency was increased, implying that changes in Ca\textsuperscript{2+} delivery are less important than MyBP-C phosphorylation. They also show that dobutamine’s inotropic effect is likely primarily caused by MyBP-C phosphorylation because it is largely absent in t\textsuperscript{3}SA mice and does not seem to be importantly related to Ca\textsuperscript{2+} delivery. PKA-mediated effects of dobutamine were shown to be caused by phosphorylation of all 3 sites. PKA also phosphorylates TnI,\textsuperscript{38} which results in myofilament calcium desensitization and, in contrast to MyBP-C, reduced force generation at submaximal \([\text{Ca}^{2+}]_o\). The authors propose that MyBP-C phosphorylation increases force by virtue of changes in the protein that modulate acto–myosin interaction and increase the number of force generating cross-bridges. These novel results should lead to a re-evaluation of our thinking about the mechanisms of these fundamental inotropic responses.

Rosas et al\textsuperscript{11} focus on the lusitropic effects of MyBP-C phosphorylation and, by inference, its potential role in HFpEF. Both phosphomimetic models and t\textsuperscript{WT} mice were used to explore the full range of MyBP-C phosphorylation. They show that MyBP-C phosphorylation has major positive lusitropic effects in the intact left ventricle assessed with echocardiographic–Doppler techniques and direct measurement of the time constant of relaxation. Excitable myocardial strips from t\textsuperscript{3}SA mice displayed positive lusitropic effects in the absence of differences in intracellular Ca\textsuperscript{2+} transients that could provide an alternative explanation. Analogous to Tong et al,\textsuperscript{10} the positive lusitropic effect was enhanced at increasing contraction frequencies. Previously, physiological modulation of lusitropy in mammalian myocardium has been thought to result largely from the combination of altered intracellular Ca\textsuperscript{2+} dynamics\textsuperscript{40} and TnI phosphorylation.\textsuperscript{36} Taken together, an important implication of these papers is that MyBP-C phosphorylation is a key player in the coordinated response to increased frequency and adrenergic activation, resulting in increased force and presumably power generation and more rapid relaxation, that is, an optimal response to exercise and other physiological stresses.

Tong et al\textsuperscript{10} previously studied the calcium sensitivity of isometric force in permeabilized strips and found no differences between t\textsuperscript{3}SA and WT mice. This is reminiscent of our findings in humans in whom this relationship was similar in LVH and controls, despite the fact that cross-bridge dissociation kinetics were slowed at submaximal \([\text{Ca}^{2+}]_o\) in LVH strips. This suggests that similar directional changes in acto–myosin kinetics are present in human LVH myocardium with hypophosphorylated MyBP-C and mouse myocardium with unphosphorylated MyBP-C. Remarkably, mice with unphosphorylated MyBP-C demonstrated concentric left ventricular remodeling typical of many patients with HFpEF along with signs of circulatory congestion.

**Limitations**

Although both papers provide important new insights into the physiological and pathophysiological significance of MyBP-C phosphorylation, it is important to keep in mind some limitations. First and foremost, caution should be exercised in extrapolating these results to human myocardium. Rodent and human myocardium differ greatly with respect to Ca\textsuperscript{2+} dynamics. Thus, there are marked differences in their dependence on sarcomemal Ca\textsuperscript{2+} transport versus sarcoplasmic reticulum Ca\textsuperscript{2+} release for myofilament activation.\textsuperscript{41} Moreover, rodent myocardium is characterized by much more rapid acto–myosin kinetics than human myocardium.\textsuperscript{42,43} In view of the latter and the fact that MyBP-C normally functions as a brake on acto–myosin interaction, species-dependent quantitative differences in the effects of MyBP-C phosphorylation are likely. With respect to the FFR, the frequencies studied by Tong et al\textsuperscript{10} were well below the physiological range for mice. Moreover, mice have much smaller percent changes in heart rate with activity compared with humans. In contrast to these results in mice, there is strong, albeit circumstantial evidence in human myocardium that increased Ca\textsuperscript{2+} delivery to the myofilaments plays a major role in the positive FFR.\textsuperscript{33,44,45}

Another limitation is that the phosphomimetic substitutions targeted only the PKA sites on MyBP-C. As discussed in the articles,\textsuperscript{10,11} MyBP-C has multiple non-PKA phosphorylation sites that can potentially modulate myofilament function in a complex fashion both independently and in conjunction with PKA.

**Implications for Human Disease and Future Directions**

Because reduced MyBP-C phosphorylation has been reported in both human DCM and LVH, these papers may have important implications for our understanding of the pathophysiology of heart failure. It is important to note that our measurements of MyBP-C and TnI phosphorylation in human myocardium\textsuperscript{15} from patients with LVH were not site-specific. If reduced MyBP-C phosphorylation plays a significant role in impaired inotropic responses and slowed/incomplete relaxation in human DCM and HFpEF, it will be important to quantify site-specific phosphorylation in larger numbers of patients and attempt to establish cause and effect. Currently, mass spectroscopic methods offer the most precise quantitative estimates of site-specific phosphorylation,\textsuperscript{46} whereas myofilament/myofibril protein reconstitution methods using genetically modified proteins\textsuperscript{47} can provide in vitro mechanistic insights in human myocardium analogous to those in transgenic animals. Because TnI and other myofilament proteins in addition to MyBP-C are targets for PKA and various other kinases, changes in myofilament function in acquired human disease are undoubtedly exceedingly complex and, a priori, unlikely to be dominated by phosphorylation of a single protein.

Despite these caveats, the important contributions of Tong et al\textsuperscript{10} and Rosas et al\textsuperscript{11} should spark additional investigations into the significance of MyBP-C phosphorylation in human disease, especially HFpEF, for which pharmacological treatments have been disappointing. If such studies support an important role for reduced phosphorylation of PKA sites on
MyBP-C in HFpEF, this would lead to an evaluation of therapeutic strategies that could modulate phosphorylation of these sites. In this regard, it is of interest that PKA/PKG sites on titin are also hypophosphorylated.\textsuperscript{13,14} Thus, β-adrenergic blockers, which are administered to many patients with HFpEF, could potentially worsen diastolic dysfunction by exacerbating hypophosphorylation of both proteins.

To our knowledge, whether PKA sites on MyBP-C are also targets for PKG has not been studied. If they are, this would offer the possibility of treating HFpEF by manipulation of NO availability to increase phosphorylation of both titin and MyBP-C, which would be expected to have dual effects, a decrease in passive myocardial stiffness, and speeding of relaxation. Obviously, improved endothelial function would be an additional benefit. Unfortunately, the recently reported Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Heart Failure with Preserved Ejection Fraction (RELAX) trial of the effects of the phosphodiesterase V inhibitor sildenafil\textsuperscript{48} on maximal exercise capacity in HFpEF did not show benefit. Nitrates are an alternative that might accomplish the same goal of increasing both titin and MyBP-C phosphorylation. The results of the ongoing Nitrate's Effect on Activity Tolerance in Heart Failure with Preserved Ejection Fraction (NEAT) trial of the effects ofisosorbide mononitrate on exercise capacity in HFpEF\textsuperscript{49} are therefore eagerly anticipated.

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