Sarcomeres and the Biophysics of Heart Failure

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Introduction

In heart failure the heart's impaired ability to function as a pump results in poor systemic perfusion that is unable to meet the body's metabolic demands. The reduced function of the heart is brought about by either impaired cardiac contractility, as is the case in systolic heart failure, or impaired cardiac relaxation and filling, as is the case in diastolic heart failure. In either state, however, the development of impaired pump function and, thus, heart failure can be ascribed to several factors, including alterations in protein expression and function, myocyte death, and changes in signaling pathways. Of these factors, changes in the function of the sarcomere play a significant role in the development of cardiac dysfunction underlying the development of heart failure. These changes in sarcomeric properties are the result of either a change in isoform expression, post-translational modification of the sarcomeric proteins, or gene mutations linked to hypertrophic or dilated cardiomyopathy. In this chapter we will discuss the basic structure and function of the sarcomere in the context of a healthy heart, as well as the maladaptive changes that occur within the sarcomere during heart failure. We will also touch upon the

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emerging studies of genetically linked cardiomyopathies as intrinsic modulators of sarcomere function and an alternate cause of heart failure. Our hope is to provide the reader with insight into how sarcomere structure and function are altered during heart failure and how these alterations contribute to disease pathology.

The Sarcomere's Function in Normal and Failing Heart

Basic Function of the Sarcomere

The myofilaments consist of highly organized thick and thin filament proteins that facilitate, in a calcium-dependent manner, contraction and relaxation of the sarcomere (Fig. 1). Through the hydrolysis of ATP and resulting cross-bridge cycling, the cardiac sarcomere eloquently coordinates the transduction of elevated intracellular calcium into the mechanical work of contraction and relaxation necessary for proper cardiac pump function. Moreover, this critical function of the sarcomere occurs with both high fidelity and plasticity to the ever-changing physiological environment over time.

The myofilaments consist of both thick and thin filament proteins that span the sarcomere. The hexameric myosin macromolecular complex is the major component of the thick filament, accounting for roughly 30 % of the sarcomeric mass [109]. Each individual complex of myosin consists of two globular heavy chains (containing S1 and S2 regions) and two pairs of light chains (essential light chain, MLC1, and regulatory light chain, MLC2), extending from the coiled-coil tail domain to the globular N-terminal head region. Within the "rod-like" structure of the coiled-coil tail domain, neighboring myosin molecules anneal to form the thick filament backbone. Within the bare zone, or M-line, myosin molecules overlap one

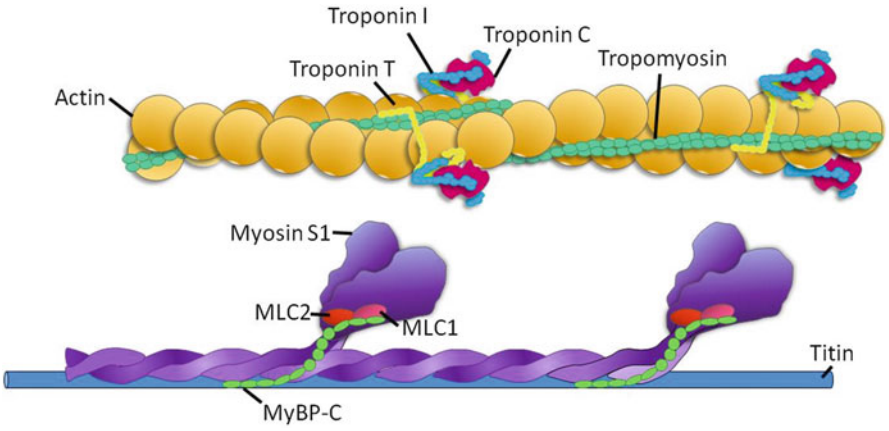

Fig. 1 Schematic representation of the thick and thin filaments of the cardiac sarcomere. *MLC* myosin light chain; *MyBP-C* myosin-binding protein-C

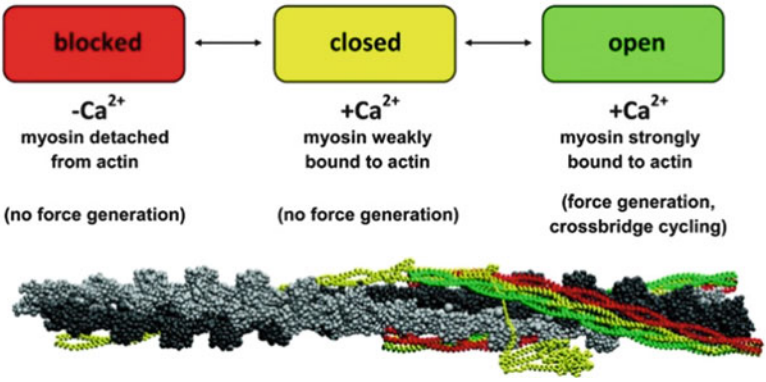

Fig. 2 The three states of tropomyosin (blocked, closed, and open) along the actin filament (modified from Manning et al. [56])

another in an antiparallel fashion, resulting in myosin heads that project from the backbone with opposite polarity. This unique organization allows for shortening of the sarcomere during cross-bridge cycling.

Further regulation of myosin structure and function occurs via the myosin-associated proteins, myosin-binding protein C (MyBP-C) and titin. MyBP-C collars the myosin head and acts to both tether the cross-bridge to the tail region, promoting greater order of the myosin heads along the thick filament [62], and connecting myosin to the cytoskeletal proteins through titin binding. As a consequence of binding to MyBP-C, myosin's kinetics is constrained and cross-bridge formation is extended [50, 103, 104]. Titin is a giant filamentous protein that extends from the Z-disk to the center of the sarcomere, where it interacts with myosin within the M line [31, 60]. Titin also provides elasticity and is further responsible for most of the passive tension within the sarcomere [29, 30].

Actin monomers, self-assembled into a filamentous structure, form the major portion of the thin filament and are critical for enhancing myosin ATPase activity during cross-bridge cycling. The functional unit of the thin filament comprises seven actin monomers, one tropomyosin (Tm) dimer, and one troponin complex that include the calcium-binding subunit, troponin C (TnC), the inhibitory subunit, troponin I (TnI), and the tropomyosin-binding subunit, troponin T (TnT). This multimeric structure allows for regulation of sarcomeric activation and cross-bridge cycling in a calcium-dependent manner. The Tn complex is crucial for binding calcium and allowing for the regulation of cross-bridge attachment, which it does by modulating Tm's position on the actin thin filament.

During diastole, intracellular calcium concentrations are low and the binding of calcium to the regulatory site on TnC is not favored. Furthermore, in this state the Tn complex acts to prevent actomyosin formation via Tn–Tm interactions that form an ordered structure along the actin filament. This structure promotes the positioning of Tm along the actin groove, resulting in either blockage of myosin binding to actin ("blocked" state) or weak cross-bridge attachment ("closed" state) [61]. As suggested by the three-state model of muscle contraction (Fig. 2), such positioning of Tm does

not allow for significant force generation and the requirement of ATP for myosin binding and hydrolysis is minimal. It is also possible that the Tn complex itself may block the interaction between actin and myosin directly [111].

During systole, intracellular calcium levels rise promoting the binding of calcium to TnC. This binding induces a conformational change within the Tn complex resulting in strong binding of TnI, both the inhibitory region and the C-terminal domain, to TnC. As a result, TnI is released from actin and the interaction between TnT and tropomysin becomes significantly weaker [102]. This change in TnT–Tm interaction facilitates the movement of Tm into the "open" state. In this state, myosin-binding sites on actin are exposed allowing for the strong binding of myosin cross-bridges to actin, which greatly enhances actomyosin ATPase activity leading to cross-bridge cycling and force generation [21, 52, 61]. The formation of strong, force generating cross-bridges also promotes the binding of additional cross-bridges and enhances calcium binding to TnC [82]. This interdependence results in a relationship between calcium concentration and isometric force that is very steep and shows a highly cooperative character.

The interaction between the thick and thin filament is dependent on both the intracellular milieu, as well as the state of each protein within the sarcomere. Moreover, the functional interaction of these proteins involves multiple mechanisms, including allosteric, steric, and cooperative activation. It is this complex relationship within the sarcomere that makes the transition of the myofilaments from the relaxed state to the contractile (or activated) state highly sensitive to regulation at multiple points. Moreover, alterations in sarcomeric function can occur via regulation of practically any of the sarcomeric proteins. Of note, in the context of heart failure, chronic stress, indeed, causes alterations in a majority of the sarcomeric proteins, as will be discussed in subsequent sections of this chapter.

The Cross-Bridge Cycle and Dynamics

The cross-bridge cycle is the means by which the heart couples the hydrolysis of ATP to positive work production and produces force. It is driven by several thermodynamically favorable reactions. Cross-bridge cycling is dependent upon the ability of the myocyte to maintain sufficient levels of reactants (MgATP and H_2O) and products (MgADP, P_i, and H^+), thereby generating continual force production. A simplified schematic of the critical steps involved in cross-bridge cycling are shown in Fig. 3.

As mentioned in section "Basic Function of the Sarcomere," the myosin globular head domain contains an S1 region where both nucleotide binding and subsequent hydrolysis occurs (Fig. 3, Step 1). At this point, myosin remains bound to the hydrolysis products, MgADP and inorganic phosphate (P_i), with some myosin S1 heads binding weakly to actin. According to the three-state model of thin filament activation, this state of weakly bound myosin with a strongly bound nucleotide represents the "closed" state [65]. Further isomerization of the myosin head results

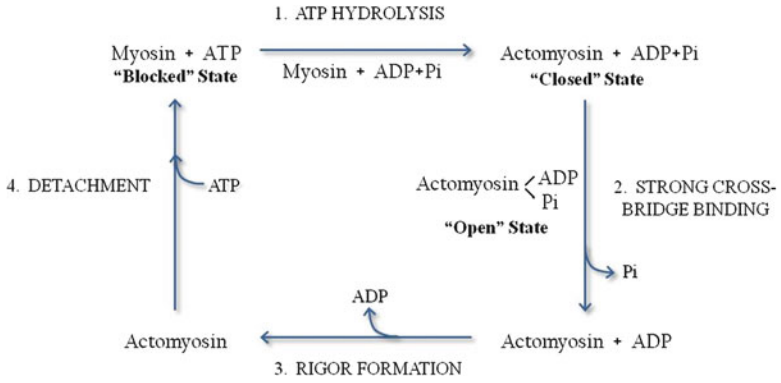

Fig. 3 A schematic of the cardiac cross-bridge cycle (adapted from Katz [46], *Physiology of the Heart*, Lippincott Williams & Wilkins)

in stronger binding to actin and a weakening of the associated nucleotide to form the "open" state. It is this transition from the closed to the open state that is regulated by the binding of calcium to TnC and subsequent movement of Tm away from myosin-binding sites along the actin thin filament [37, 66]. Following formation of the open state, inorganic phosphate is quickly released from the actomyosin complex (Fig. 3, Step 2). At this step along the cross-bridge cycle, the potential energy generated from ATP hydrolysis is transferred to the myosin "lever arm" and harnessed to produce the power stroke, which drives sliding of the thick and thin filament past one another [90, 112]. Under steady-state isometric force, ADP release from the actomyosin complex is rate-limiting and results in the formation of a rigor cross-bridge (Fig. 3, Step 3). At this point, given the high local concentration of MgATP, myosin undergoes rapid nucleotide binding followed by detachment of the myosin head from actin (Fig. 3, Step 4).

The precise biophysical changes that govern cross-bridge cycle dynamics have long been studied. For example, optical trapping technique allows for investigating and mathematically defining the force generated by a single myosin motor, the kinetics of the cross-bridge cycle, and how they change during different physiological and pathological conditions [112]. In these experiments, myosin undergoes transition from weak to strong actin binding and generation of a unitary force (F_{uni}) in a single step. This is proportional to the measured unitary displacement (d) (Fig. 4). In a normal cross-bridge cycle detachment of myosin from actin requires the release of ADP ($t_{-\text{ADP}}$) and the binding of ATP ($t_{+\text{ATP}}$). Thus, the duration of strong binding of the cross-bridges, or t_{on}, is a summation of $t_{-\text{ADP}}$ and $t_{+\text{ATP}}$. Moreover, once the length of time for a single cross-bridge cycle, t_{cycle} is obtained, the duty ratio (duty ratio $= t_{\text{on}}/t_{\text{cycle}}$) can also be determined. Kinetic measurements of d, t_{on}, and t_{cycle}, allow for determination of the average force (F_{ave}), which is proportional to the F_{uni} and the percentage of time myosin spends in strong binding state (i.e., the duty ratio). At the single molecule level myosin sliding velocity (V_{max}), measured using the in vitro motility assay, is proportional to d/t_{on} [44].

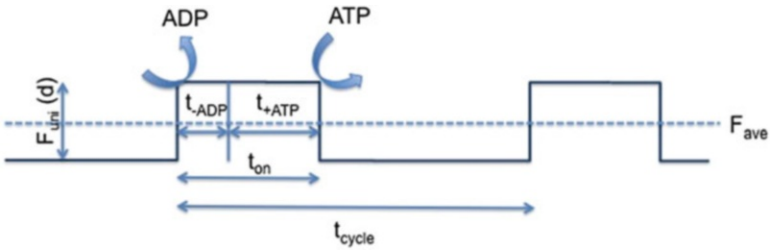

Fig. 4 A kinetic model of myosin unitary steps observed during the strong binding of cross-bridges to the actin thin filament. The upward deflection of the solid line reflects a weak-to-strong binding transition between myosin and actin during an optical trapping experiment, while the downward deflection of the line demonstrates dissociation of myosin from actin (modified from Tyska and Warshaw [112])

Interestingly, changes in several of these defined parameters can occur simultaneously having either additive or compensatory effects. For example, it has been demonstrated that F_{ave} is similar in V1 (αMyHC) and V3 (βMyHC) despite longer t_{on} and t_{cycle} in V3 because F_{uni} and the duty ratio are similar between the two isoforms [81, 105, 106]. Therefore, changes in any of these parameters will be important in determining changes in the cross-bridge dynamics that influence the dynamics of cardiac function.

Modulation of the parameters that define cross-bridge dynamics can be both calcium-dependent and calcium-independent. MgATP binding and the resulting detachment of myosin from actin (t_{on}) determines the maximal velocity of the cross-bridge cycling. The regulation of this process is calcium-independent and depends on the ability of the myocyte to maintain high levels of MgATP and low levels of MgADP, P_i, and H^+ [60]. In a state of energy deprivation, as observed in end-stage heart failure, the altered velocity of cross-bridge cycling becomes a significant contributor to the overall contractile dysfunction [33]. Maximal force production (F_{avg}) is determined by calcium-regulated mechanisms that include the number of rigor cross-bridges formed and the time these cross-bridges spend in the rigor state (duty ratio). Any observed change in contractile function associated with the sarcomere ultimately alters cross-bridge dynamics (kinetic properties of the cross-bridge cycle). These properties include changes to the velocity of shortening, the duty cycle, and/or the unitary force generated by the strongly bound cross-bridges, intrinsically related to the state of both the thick and thin filament. In the context of heart failure, evidence has supported alterations in the state of both the thick and thin filament as a major contributor to the associated contractile dysfunction. Such alterations can arise via extrinsic factors, such as changes in kinase activity and, thus, the post-translational state of the myofilament, and from intrinsic factors, such as changes in myofilament protein expression. In the following sections we will discuss how the changes in sarcomeric protein expression and post-translational state observed in heart failure alter cross-bridge dynamics and contribute to cardiac dysfunction.

Function of the Sarcomere in the Failing Heart

Over half a century ago, the first study was published which demonstrated a reduction in myofibrillar ATPase activity in the failing heart, suggesting a role for the myofilament in the development of heart failure [1]. We now know that many changes occur within the sarcomere to cause reduced cardiac function. There are three primary mechanisms that account for alterations in sarcomeric function observed during heart failure: changes in gene expression, protein proteolysis, and post-translational modification of the myofilament proteins. Of these, the role of protein proteolysis in cardiac dysfunction associated with heart failure appears to be minimal and may only occur as an initial response to ischemia-reperfusion injury [18, 64, 94]. Our focus in this section is on the alterations in both gene expression and the post-translational state of the sarcomeric proteins that have been shown to contribute to the reduced cardiac function in heart failure. While studies of these changes have provided sufficient evidence to suggest the sarcomere is a significant contributor to contractile dysfunction, one must also take into consideration the effects of such alterations in the context of an altered intramyocellular environment. Here we focus exclusively on how changes in sarcomeric protein composition and post-translational state directly relate to depressed cardiac function.

Changes in Composition of Sarcomeric Proteins and Their Biophysical Consequences

Thick Filament and Associated Proteins

Myosin Heavy Chains

The human myocardium expresses two isoforms of MHC: α-MHC and β-MHC. These two isoforms display 93 % amino acid identity [67], but show different biophysical properties. The isoform expression of MHC is different in atria and ventricles and changes in hypertrophy, heart failure, and other diseases. Non-failing human atria express about 90–100 % of α-MHC, but this amount decreases to 50–55 % in heart failure [91, 96, 113], whereas human non-failing ventricles express small (0–15 %) amounts of α-MHC [8, 68]. In hypertrophy and heart failure, the amount of α-MHC in the ventricle decreases to 0–4 % [70, 91, 96]. Although this observed shift in MHC isoform expression is relatively small, the two isoforms differ in ATPase activity, actin filament sliding velocity, and power output. These differences may still have a significant effect on the function of the sarcomere. In humans this is a complex task, not only because access to human samples is limited but also because in hypertrophy and heart failure there are dynamic changes in the level of myofilament proteins making it difficult to separate their individual effects. Thus, animal models of hypertrophy and heart failure are

often employed to both circumvent these limitations and provide a longitudinal system for study.

It is well documented that α-MHC exhibits a several-fold higher actin-activated ATPase activity [55] and higher actin filament sliding velocity [35]. Moreover, the force generated by different cardiac preparations is dependent on the levels of expression of MHC isoforms. Herron et al. [39] reported that the force generated by myocytes expressing α-MHC is about 3 times higher than the force generated by myocytes expressing β-MHC. The rates of force development and unloaded shortening velocity are also shown to be increased in preparations expressing α-MHC [25]. Moreover, cross-bridge cycling kinetics is depressed linearly with decreased expression of α-MHC [93]. The power output, measured in skinned rat myocytes and isolated working heart preparations, is also linearly related to MHC content and decreases as expression of β-MHC increases [49]. A similar reverse relationship was observed between unloaded shortening velocity and β-MHC expression [49]. Very interesting data were published by the same group that compared loaded velocities, power output and peak normalized power in myocytes expressing either 0 or 12 % α-MHC. Peak normalized power output was 52 % greater in myocytes expressing 12 % vs. 0 % α-MHC [40]. These data suggest that even small shifts in α-MHC expression, as has been observed in human heart failure, may have a significant effect on overall heart function.

Myosin Light Chains

In addition to the shifts in MHC isoforms in hypertrophic and failing human hearts, there are reports regarding the shift in both the isoforms and the level of expression of essential and regulatory light chains. In the human heart, three isoforms of regulatory light chains (atrial, ALC-2, ventricular-a, VLC-2a, and ventricular-b, VLC2-b) and two isoforms of essential light chains (ventricular, VLC-1, and atrial, ALC-1) have been identified (for further review, see [38, 72, 75]). In the normal human heart the ratio of MLC1/MLC2 is 1:1 [57], but while it may change in pathological conditions the reported data are not consistent. Morano et al. [74] reported no change in MLC1/MLC2 ratio in failing hearts, but recently, Li et al. [54] have presented data indicating that the expression of MLC-2 is down-regulated in heart failure and the degree of the down-regulation is associated with the class of heart failure (New York Heart Association stages II–IV). Also, Margossian et al. [57] found that in idiopathic dilated cardiomyopathy the ratio of MLC1/MLC2 varied from 1:0.1 to 1:0.69. This decreased level of MLC2 was linked to the presence of an active protease and associated with a decrease in V_m of actin-activated myosin ATPase but no changes in the rates of ATP binding to myosin were detected.

In normal human heart the expression of ALC-1 is restricted to the atria, although it can be reexpressed in the ventricles in the context of different disorders. Patients with dilated cardiomyopathy expressing variable amounts (2.4–10.3 %) of ALC-1 showed increased myofilament calcium sensitivity that correlated with the

amount of ALC-1 expression [75]. However, van der Velden showed no significant correlation between ALC-1 expression and calcium sensitivity in human end-stage donor samples [115]. One possible explanation for the difference between the two studies is the etiology of failure in the donor samples. In the study by van der Velden, the correlation was conducted using primarily samples taken from ischemic heart disease patients, whereas samples from patients with dilated cardiomyopathy were used in the former study. In addition it has been reported that increased expression of ALC-1 in skinned fibers prepared from human hearts with congenital heart diseases resulted in increased detachment rate and the rate of force development [76]. The increased cross-bridge kinetics was partially due to weaker interactions between the N-terminal domain of ALC-1 and actin [73]. The functional role of these changes has yet to be established, although studies using different TG mouse models strongly support the importance of the myosin light chain isoforms on cardiac function [13, 84].

Myosin-Binding Protein-C

As mentioned previously (see section "Basic Function of the Sarcomere"), MyBP-C is a critical component of the sarcomere and an important regulator of cardiac function. Three isoforms are expressed in muscle, but only one isoform, MYBPC3, is found in cardiac myocytes (for review, see [47]). Currently, no known changes in isoform expression of MyBP-C have been identified.

Titin

Titin is the largest protein in mammals and is often referred to as the third filament of striated muscle. It is responsible for the determination of passive tension and is a major sensing and signaling molecule. Two major isoforms, both which show distinct biophysical properties, are expressed in the human heart and their relative amounts change in heart failure. For a complete discussion of titin, see Chap. 10.

Thin Filament Proteins

Troponin Complex

Troponin is a trimeric complex that comprises TnT (Tn-tropomyosin), TnI (Tn-inhibitory), and TnC (Tn-calcium). TnT plays both a modulatory and structural role within the thin filament and binds to both Tm and TnI-TnC. Cardiac TnT is encoded by a single gene encoding multiple isoforms: one adult isoform (TnT3) and three fetal isoforms (TnT1, TnT2, and TnT4). While TnT3 is normally the only isoform expressed in the adult human heart, tissue samples from failing hearts have

revealed the re-induction of the fetal isoforms [2, 69]. Moreover, Barton et al. reported expression of the slow skeletal muscle TnT gene in end-stage heart failure [4]. The functional significance of the expression of different TnT isoforms in human heart failure is not clear. It has been shown that human cTnT isoforms affect the calcium sensitivity of force development and the ability to inhibit the actomyosin ATPase activity, but no differences in maximal actin-Tm-activated myosin ATPase activity were observed [27]. Myofilaments reconstituted with human TnT1 and TnT2 isoforms that showed increased calcium sensitivity when compared with the TnT3 and TnT4 isoforms [27].

TnI and TnC are expressed as cardiac isoforms in the human heart. While changes in the ability to activate or inhibit actomyosin activity have been observed in the presence of ssTnI [28], ssTnI is only expressed during development [58] and there are no reports of re-expression of ssTnI in heart failure. The same is true for cTnC, in which expression levels appear to remain unaltered during heart failure.

Actin

The human heart expresses two actin isoforms: skeletal muscle α-actin and cardiac α-actin that only differ by two amino acid substitutions and a transposition [6, 97, 117]. The healthy human heart contains about 20 % of skeletal muscle α-actin [117] and in pathological conditions the same [6, 97, 117] or higher levels of skeletal muscle α-actin have been reported [108]. Recently, Copeland et al. reported that end-stage failing hearts express 53 % skeletal muscle α-actin compared to 21 % in normal heart [19]. However, they did not find any differences in the motility assay between those two actin isoforms. These functional data are not surprising, since the only difference between the two actin isoforms is two amino acid substitutions and one transposition of amino acids [118].

Tropomyosin

Tropomyosins are a family of actin-binding proteins encoded by four different genes. The adult human heart expresses predominantly α-Tm [86], but also β- and κ-Tm [24, 87]. Interestingly, in heart failure and dilated cardiomyopathy the expression level of κ-Tm increased by twofold [87], while β-Tm levels were reduced (personal communication from Dr. David Wieczorek). κ-Tm is structurally less stable and binds more weakly to actin as compared to α-Tm. When expressed in Tg mice, it results in decreased systolic and diastolic function with decreased myofilament sensitivity to calcium [87] suggesting that its altered expression in human failing hearts could contribute to the cardiac dysfunction.

Post-translational Modifications of Sarcomeric Proteins in Heart Failure

Sarcomeric Protein Phosphorylation

In the healthy heart, protein–protein interactions within the sarcomere are finely tuned by extrinsic signal transduction and subsequent post-translational modifications. Under pathological conditions, however, where the extrinsic signal is continually present, such post-translational modifications are no longer able to sufficiently tune contractile performance and these modifications become deleterious. The activities of several protein kinases and phosphatases have been shown to be altered in heart failure, resulting in changes in the post-translational state of many sarcomeric proteins. More recent studies have also provided evidence for a functional role of oxidative stress in modifying the post-translational state of sarcomeric proteins, contributing to contractile dysfunction.

Elevations in the level of catecholamines during the development of early heart failure result in increased β-adrenergic signaling, however, over time, chronic exposure leads to β-adrenergic receptor desensitization and reduced expression [10–12, 34]. As a result of depressed β-adrenergic signaling, generation of cAMP is greatly reduced in end-stage heart failure and PKA activity is low. This has significant effects on the sarcomere, as several of the sarcomeric proteins are known to be phosphorylated by PKA leading to altered cardiac dynamics. Interestingly, it has been shown that the etiology of the disease may also determine the degree to which PKA signaling is reduced [115]. Besides changes in PKA activity observed during heart failure, PKC activity is also altered. The initiation of hypertrophic remodeling causes activation of PKC within the myocardium, most notably activation of PKCβ and PKCα isoforms [9].

It appears that the reduction in PKA activity also plays a role, at least in part, in altering protein phosphatase activity in heart failure. One of the downstream targets of PKA is Inhibitor 1 (I-1), whose activity is dependent on phosphorylation by PKA. The main function of I-1 is to prevent protein phosphatase 1 (PP1) activity. Therefore, as a consequence of reduced PKA activity and, thus, reduced I-1 phosphorylation, PP1 activity is enhanced in the failing heart [77]. Studies in skinned myocytes from human failure samples suggest that the reduced PP1 expression in heart failure contributes directly to alterations in the myofilament response to calcium through changes in the phosphorylation state of MLC-2 and cTnI [114]. Finally, it appears that the activity of PP2A, while important in balancing the phosphorylation state of sarcomeric proteins in the healthy heart, does not change during heart failure [77].

Phosphorylation of Thin Filament Proteins: cTnI and cTnT

The putative PKA sites within the cTnI protein sequence (Ser-23 and Ser-24) are located within the N-terminal extension of cTnI that is unique to the cardiac isoform. This region is important for the interaction of cTnI with the regulatory domain of cTnC, found in its N-lobe, and for modulation of TnC's calcium-binding affinity. In the unphosphorylated state, the N-terminal extension remains highly flexible and binds to TnC, enhancing calcium-binding affinity. Consequently, the responsiveness of the myofilament to calcium is increased. Indeed, myofilament calcium sensitivity is increased in human failure samples and is returned to control levels upon PKA treatment [115, 121]. This effect was shown to be linked to reduced cTnI phosphorylation [115].

TnI can also be phosphorylated by PKCs and in some cases the sites of phosphorylation are isozyme-dependent. The majority of PKC isoforms expressed in the heart (PKCα, βI and βII, δ, ε, and ζ) target cTnI at Ser-43 and Ser-45 and alter ATPase rate with little to no effect on calcium sensitivity. However, PKCδ was shown to preferentially phosphorylate the PKA sites on cTnI and alter calcium sensitivity [80] in addition to reducing ATPase rate. Serine 43 and 45 of cTnI are located in the region of cTnI that participates in intermolecular binding to the C-lobe of cTnC. Phosphorylated cTnI at Ser-43/-45 promotes the blocked state of thin filament activation reducing actomyosin affinity and as a result, the calcium-stimulated ATPase rate is reduced [80]. This post-translational modification is likely to play a role in maladaptive remodeling during the transition from early to late stages of heart failure, as evidenced by the up-regulation of several PKC isoforms (ε, α, β) in end-stage heart failure [9, 48, 78, 100].

While phosphorylation of cTnI at Ser-43 and Ser-45 by PKCs appears to have the greatest consequence on contractile function of the failing myocardium, it has been shown that phosphorylation at threonine 144 may also play a significant role. Using the in vitro motility assay, Burkart et al. were able to demonstrate that the effects of PKC phosphorylation of cTnI on actin–myosin interactions exhibited site specificity, inasmuch as reconstituted preparations with cTnI pseudo-phosphorylated at Ser-43, Ser-45, and Thr-144 displayed both a decrease in maximal sliding speed and calcium sensitivity, while preparations with cTnI pseudo-phosphorylated only at Thr-144 displayed a reduction in calcium sensitivity without changes in sliding speed [14]. This implies that phosphorylation of Thr-144 is necessary for regulation of myofilament calcium sensitivity. Indeed, it is possible that the introduction of a negative charge within the inhibitory peptide region of cTnI, where Thr-144 is located, would be likely to alter the ability of cTnC to bind calcium and augment the calcium responsiveness of activation. Also of interest is the fact that the cardiac isoform of TnI is the only isoform that contains a phosphorylatable residue at position 144 (Pro-144 in slow skeletal and fast skeletal TnI). This may provide an alternative means for cTnI to regulate contractility in cardiac muscle, where the ability to recruit additional motor units for regulation is lacking.

The adult cardiac isoform of TnT (TnT3) contains four identified sites of phosphorylation by PKC, Thr-197, Ser-201, Thr-206, and Thr-287, all located in

the C-terminal region of the protein. This region of TnT aids in the control of actin–myosin interactions in a calcium-dependent manner via interactions with cTnI and TnC. Specifically, changes in the C-terminal region can be propagated across the TnT structure and alter N-terminal interaction of TnT with Tm, thereby playing a direct role in modulating the calcium-dependent actomyosin ATPase activity [85]. It has been suggested that in end-stage congestive heart failure PKCα induces hyperphosphorylation of TnT, with little change in the early stages of failure [5]. Moreover, one study using detergent skinned mouse papillary muscles showed that PKCα-dependent phosphorylation of cTnT significantly depressed actomyosin ATPase rate, calcium sensitivity, and cooperativity of the myofilament and maximal tension development. They also demonstrated that Thr-206 appears to be the critical site for TnT regulation of reduced maximal tension, as mimicking phosphorylation (by glutamic acid substitution) at this site alone reduced isometric tension and calcium sensitivity while pseudo-phosphorylation of the other sites had no effect on these parameters [107]. Despite these findings, earlier studies suggest that the phosphorylation of cTnT alone cannot account for the full reduction in actomyosin ATPase rate [79] but rather, that a reduced affinity of TnT for actin-Tm potentiates the calcium-regulated myosin binding.

Phosphorylation of Thick Filament Proteins: MLC-2, MyBP-C, and Titin

MLC-2 exists in the human myocardium in two isoforms, LC-2 and LC-2*, distinguished by their increasing acidity and, therefore, difference in their isoelectric points. Both forms are targeted for phosphorylation by PKC, PKA, and myosin light chain kinase (MLCK) at Ser-15, while rodent MLC-2 is also phosphorylated at Ser-19. These two residues are located at the N-terminal end of MLC-2, a portion of the light chain that interacts with the C-terminal end of myosin's lever arm. Introduction of negative charges in this interacting region were shown to promote disorder of the helical array of myosin heads within the lattice structure, shifting them closer to the thin filament [53]. More recently, human MLC-2 was shown to exist in three predominate forms—the unphosphorylated species, a singly phosphorylated species, and a phosphorylated/deamidated species [99]. The deamidation occurs at the Asn adjacent to Ser-15, resulting in a switch from Asn to Asp and introduction of a negative charge at this residue (either position 14 or 16). The authors were unable to detect any phosphorylation at Ser-19 in the human samples.

Functionally, phosphorylation of MLC-2 serves to aid in basal cardiac contraction and ejection via alterations in myosin cross-bridge kinetics [95, 98]. Scruggs et al. demonstrated that ablation of basal MLC-2 phosphorylation reduced tension cost (ATPase activity/force produced), a measurement of the rate of cross-bridge detachment, without altering calcium sensitivity [98]. The lack of a change in myofilament response to calcium observed was consistent with the initial study characterizing this mouse model [95]. However, Morano et al. demonstrated

increases in myofilament calcium-dependent tension development with MLC-2 phosphorylation, due to an increase in the rate of weak-to-strong cross-bridge transition [71]. Despite the discrepancy in calcium-sensitivity changes, these studies all concluded that MLC-2 phosphorylation is important for cardiac contractility. In human heart failure, it has been shown that the state of MLC-2 phosphorylation, but not MLC-2*, is reduced [114]. Although this reduction would be suspected to decrease calcium-dependent force production in the failing heart, the authors found the opposite to be true. They attribute the observed increase in calcium sensitivity to the greater functional effect of cTnI dephosphorylation on the myofilament properties. Despite this apparent masking of the functional role of MLC-2 phosphorylation in cardiac failure, the same authors showed that further dephosphorylation of MLC-2 by PP1 in failure has a much greater effect on the response of the myofilament to calcium than it does in the healthy heart. This was proposed to be a possible "last resort" mechanism that the myocardium employs to improve diastolic function in end-stage failure [114].

The level of MyBP-C phosphorylation has also been shown to be reduced in heart failure, with unphosphorylated MyBP-C serving as the predominate species and only a small portion existing in the mono-phosphorylated form [20, 45]. This reduction in the phosphorylation state of MyBP-C is likely due to a reduction in PKA activity, as PKA can phosphorylate MyBP-C at Ser-273, Ser-282, Ser-302, and Ser-307. However, MyBP-C is also a substrate of PKC phosphorylation and, therefore, the idea of increases in MyBP-C phosphorylation throughout cardiac remodeling cannot be excluded. In fact, in a mouse model of dilated cardiomyopathy caused by cardiac-specific PKCε overexpression, it was shown that MyBP-C phosphorylation at Ser-302 was significantly increased and likely contributed to cardiac dysfunction [122]. All of the phosphorylatable residues on cardiac MyBP-C are located within the M domain of the protein. This region interacts with both titin and myosin, restricting myosin movement [43]. More recently, Weith et al. demonstrated that MyBP-C also interacts with actin through the M domain and acts as a viscous load against filament sliding [120]. Upon phosphorylation by PKA, MyBP-C's ability to interact with actin was weakened resulting in greater myosin motility observed using the in vitro motility assay. It has also been shown that phosphorylation of MyBP-C causes an extension of the myosin head away from the myosin backbone [119], increasing the ATPase rate [63] and enhancing both contraction and relaxation. Interestingly, the post-translational state of MyBP-C also appears to affect protein stability. Following ischemia-reperfusion, MyBP-C was shown to be dephosphorylated, promoting its degradation [23, 94]. What effect this has on the myocardium during failure is not fully understood, but one would expect it to likely contribute to the observed reduction in the actomyosin ATPase rate.

We mention, only in brief, alterations to the post-translational state of titin demonstrated in human failure. The reader is referred to Chap. 10 for a more in-depth discussion. Dephosphorylation of titin at Ser-469 within the N2B region of titin's spring element has been shown to occur during failure, resulting in increased passive stiffness [7, 51]. The increased passive stiffness can be returned

to healthy, control levels following PKA treatment [59, 116], suggesting that the reduced β-adrenergic signaling associated with failure is likely the cause for the depression in titin phosphorylation. Moreover, it seems that elevations in passive stiffness are not a result of weakly bound cross-bridge cycling as BDM treatment, which inhibits actomyosin interaction, did not reduce the passive stiffness observed in failing cardiomyocytes [7].

Sarcomeric Protein Oxidation

While it is well-known that oxidative stress occurs in heart failure [42, 88, 101], more recently increases in reactive oxygen species (ROS) have been linked to modifications of the sarcomere. Modifications include oxidation, *S*-glutathionylation, and carbonylation. Of the sarcomeric proteins, actin, Tm, and titin all have been shown to be modified by ROS during heart failure. Oxidation of actin was associated with reduced contractility via a reduction in the maximal force production and a depression in the force-frequency relationship [22]. Cys-374 is likely the modified residue, as oxidation at this site is associated with reduced ATPase activity and sliding velocity [22]. Actin was also shown to be carbonylated in human heart failure patients, as was Tm. These two modifications, aling with Tm disulfide cross-bridge formation strongly correlated with the associated cardiac contractile impairment [15]. Oxidation of Tm, presumably at Cys-190, has also been shown to occur in mice following MI [3]. Cys-190 is located within the TnT-interacting region of Tm and oxidation at this site is likely to decrease contractility through altered Tn–Tm interaction, as well as affecting Tm flexibility during the development of heart failure [16, 17, 41]. The same study by Avner et al. that demonstrated Tm oxidation also showed increased *S*-glutathionylation of a high molecular weight protein that they suggest is likely titin [3]. Interestingly, in vitro studies have pointed to three residues in titin that contain disulfide bridges under oxidative conditions. These modifications were localized to the N2B region of titin and resulted in increases in passive stiffness due to a reduction in the extensibility of titin [32].

Several other oxidative modifications have been observed within the sarcomere but their role in cardiac pathology remains unexplored. Myosin has been shown to be *S*-glutathionylated at Cys-400 and Cys-695 both within the myosin head region, and Cys-947 resulting in reduced ATPase activity [83]. Acutely, following an MI, myosin heavy chain was shown to be oxidized at Cys-707 and Cys-697. This was associated with reduced sliding velocity, suggestive of reduced unloaded shortening velocity of the cross-bridge. In addition, following the acute phase post-MI, MyBP-C carbonylation was shown to be increased [89]. Whether these acute, early changes in sarcomere protein oxidation remain present later on in heart failure progression are yet unknown.

Intrinsic Modulation of Sarcomere Function by Genetically Linked Mutations

To this point we have discussed the maladaptive changes to the sarcomere that occur during heart failure, in the presence of chronic extrinsic stressors. However, our understanding of the mechanisms underlying cardiac dysfunction associated with heart failure have broadened in recent years to include maladaptive changes to the sarcomere and cross-bridge dynamics caused by genetically linked mutations, or the so-called intrinsic stressors. Such genetically linked mutations are most frequently associated with mutations in sarcomeric proteins and lead to either hypertrophic or dilated cardiomyopathies (HCM or DCM, respectively). In part due to the complexity of the cardiac contractile apparatus, the precise mechanisms whereby mutations in sarcomeric proteins cause human cardiomyopathies are likely to be vast (for recent reviews, see [26, 36, 109, 110]). In the context of the preceding discussion, it is interesting to note that some of the proposed alterations in sarcomeric structure caused by thin filament mutations are predicted to change either the accessibility of the post-translational substrate site and/or the physiologic response to phosphorylation. Ongoing longitudinal studies of genotyped cohorts will continue to provide crucial data regarding the role of sarcomeric mutations in the progressive remodeling that occurs in many of the genetic cardiomyopathies [92].

Summary

Collectively, the modifications in both protein expression and post-translational state modulate the contractile state of the heart via direct effects on the biophysical properties of the cardiac sarcomere. The overall complexity of both the sarcomeric complex and the resultant patterns of cardiac ventricular remodeling observed in heart failure create a seemingly endless array of potential downstream pathogenic mechanisms. Coupling a deeper understanding of the primary biophysical causes of changes in contractile function to a more complete understanding of the resultant pathogenic ventricular remodeling that occurs over time will allow for both significant advances in disease management and new points of therapeutic intervention.

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