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ORIGINAL PAPER

Tissue procurement strategies affect the protein biochemistry of human heart samples

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Abstract The ability to analyze the biochemical properties of human cardiac tissue is critical both to an understanding of cardiac pathology and also to the development of novel pharmacotherapies. However current strategies for tissue procurement are not uniform and are potentially biased. In this study we contrasted several commonly used approaches for tissue sampling in order to determine their impact on contractile protein biochemistry. Not surprisingly our results show that different tissue handling strategies have the potential to produce a wide variation in the phosphorylation and proteolysis of selected contractile proteins. However this was not uniform: phosphorylation of troponin I (TnI) and myosin light chain 2 (MLC2) varied significantly depending on approach whereas changes in desmin and myosin binding protein C (MyBP-C) were relatively unaffected. Moreover, some strategies increased whereas others reduced TnI phosphorylation, suggesting a dynamic balance between kinase and phosphatase activities. Overall, procurement strategies that involved maintenance of tissue in cardioplegia solution deviated most dramatically from prompt and rapid tissue immersion in liquid nitrogen.

Keywords Heart · Phosphorylation · Sample handling · Protein biochemistry

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Introduction

While it seems obvious, the study of progressive human heart disease requires access to human cardiac tissue. Murine and other experimental animal surrogates have been extraordinarily useful in defining biochemical pathways that are altered in disease states (Molkentin and Robbins 2009) but ultimately these abnormalities need to be confirmed in human samples. Identifying gross morphologic changes such as cardiocyte hypertrophy, progressive cell loss, and fibrosis is a relatively straight forward undertaking, but discerning more subtle changes such as post-translational modification of contractile proteins or changes in signaling molecules in human heart tissue which might be appropriate and potentially tractable targets of novel therapy has proven to be more elusive (Jweied et al. 2007). This is in part reflective of the ante mortem characteristics of "control" hearts which are generally unused donor hearts that may well have been exposed to neurologic trauma and/or excessive adrenergic stimulation (either physiologic or pharmacologic) but is also reflective of the strategies used for tissue procurement of diseased hearts, which often reflect the vagaries of the operating room as well as diverse protocols for tissue collection. These range from in situ perfusion with cardioplegia solution followed by cell isolation in Krebs-Henseleit digestion solution (Weisser-Thomas et al. 2003), excision of large pieces of tissue from explanted hearts, maintenance in iced cardioplegic solution and subsequent freezing in liquid N_2 (van Dijk et al. 2009), to rapid flash freezing of intraoperative (Jweied et al. 2005) or endomyocardial (van Heerebeek et al. 2006) biopsy without any intermediate steps. In order to assess the impact of tissue procurement strategy on the biochemical characteristics of explanted cardiac tissue, as well as to develop an optimized

protocol to capture post-translational modifications of target proteins, we subjected muscle samples collected from explanted hearts to several different procurement strategies, varying the duration of time at room temperature or on ice prior to rapid freezing in liquid nitrogen as well as varying the buffer conditions. Our results suggest that these variables can have a significant and potentially confounding impact on protein biochemistry.

Methods

Heart procurement methods

Failing human hearts were obtained from five patients with end-stage heart failure (New York Heart Association Class IV) who were cardiac-transplant recipients. The failing hearts were procured in the O.R. immediately following explantation. Upon excision, each heart was immediately placed in an ice-cold solution of MOPS Buffer where dissection began promptly. Seven tissue samples ($\sim$0.5 g each) were dissected from the free wall of the left ventricle near the apex of the heart. After the dissection was complete, each tissue was placed in one of the seven experimental handling conditions: (1) Immediately flash frozen in liquid nitrogen; (2) submerged in MOPS buffer and placed on ice for 60 min before being flash frozen; (3) submerged in MOPS buffer and placed at room temperature for 60 min before being flash frozen; (4) submerged in cardioplegia and placed on ice for 60 min before being flash frozen; (5) submerged in cardioplegia and placed at room temperature for 60 min before being flash frozen; (6) placed in a dry tube and placed on ice for 30 min before being flash frozen; and (7) placed in a dry tube and placed at room temperature for 30 min before being flash frozen. The time required to receive the heart from the surgeons, to dissect the heart, and to place the heart in the appropriate experimental handling condition was under 20 min.

Cardioplegia composition (in mM): KCl (20.0), $MgCl_2$ (17.0), $CaCl_2$ (2.0), Procaine hydrochloride (1.0), bicarbonate (25.0), pH 7.4. MOPS buffer composition (in mM): NaCl (140.1), KCl (4.7), Na_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (1.6), d-glucose (5.6), MOPS (2.0), EDTA (0.02), pH 7.4.

Gel electrophoresis

Small samples of the left ventricle were homogenized in 8 M urea, 2.5 M thiourea, 4% CHAPS, 10 mM EDTA and a cocktail of protease and phosphatase inhibitors. For quantification of phosphorylation, samples were separated by 12% SDS-PAGE, fixed and stained with ProQ Diamond Phosphoprotein Gel Stain (Invitrogen) (Messer et al. 2007; Verduyn et al. 2007). After destaining, gels were imaged using a Typhoon 9410 Gel Imager (GE Lifesciences). The gels were rinsed with water and stained with BioSafe Coomassie Blue (BioRad) for detection of total proteins. Phosphorylation was calculated by dividing the PQD signal for each protein of interest by the CBB signal for the essential myosin light chain (MLC1). We used MLC1 as a normalization factor since there was no change in either quantity or phosphorylation of this protein (Walker et al. 2010). For determination of serine 22/23 phosphorylation of Troponin I, Western blotting with a phospho-specific antibody (Cell Signaling) was employed. Briefly, after electrophoresis, the proteins were transferred to PVDF membranes and the non-specific binding sites were blocked with 5% BSA for 1 h. The membranes were rinsed with TBST and incubated in the appropriate antibody overnight at 4°C. Membranes were washed and incubated with secondary antibody for one hour at room temperature. After washing, the proteins were visualized using enhanced chemiluminescence. For normalization, after visualizing the S22/23 signal, membranes were stripped and re-probed using a pan-troponin I antibody (Fitzgerald). Phospho-troponin I (S22/23) was calculated as phospho signal/total protein signal. Antibodies: anti-TnI (total) was from Fitzgerald, Inc. (used at 1:2500); anti-pS22/23 Troponin I was from Cell Signaling (used at 1:1000); anti-MyBP-C was from Santa Cruz (sc-67353 used at 1:1000); anti-sarcomeric actin was from AbCam (used at 1:1000); secondary anti-mouse and anti-rabbit were from Sigma (used at 1:50,000).

Statistical analysis: Variation in protein phosphorylation was determined by One-way ANOVA. If significance was found amongst the group, individual sample conditions were subjected to Students *t* test (compared to FF sample). $P < 0.05$ was considered statistically significant.

Results

Samples from five independent explanted failing hearts were collected and subjected to a variety of sample handling techniques. In all cases, a small piece of the left ventricle was immediately excised and flash frozen in liquid nitrogen. This sample was considered to be representative of the in situ condition and phosphorylation levels were normalized to the flash frozen phosphorylation levels. Following flash freezing of the initial sample, small pieces ($\sim$1 cm^3) of the left ventricle were excised and placed in one of six other conditions: (1) in cardioplegia at room temperature, (2) in cardioplegia on ice, (3) in MOPS buffer at room temperature, (4) in MOPS buffer on ice, (5) in a small sample vial without liquid at room temperature, or (6) in a small sample vial on ice. After 30–60 min all

samples were rapidly frozen in liquid nitrogen and stored at −80°C until analyzed.

Figure 1a shows representative ProQ Diamond staining for phosphoprotein analysis and subsequent coomassie brilliant blue staining of the same gel for analysis of total protein levels of one failing explanted heart subjected to these sample handling conditions. There was no significant effect of sample handling on the phosphorylation levels of myosin binding protein C or desmin, however MLC2, TnT and TnI all demonstrated significant changes in phosphorylation depending on the sample handling method employed (Fig. 1a, b). The most significant dephosphorylation was of MLC2 in the samples that were maintained in cardioplegia on ice, a condition commonly used when collecting human samples. In this condition, MLC2 phosphorylation was 85.2 ± 4.0% of the flash frozen samples. We confirmed this finding using two-dimensional electrophoresis of selected samples (data not shown). Quantification of the 2-D gels showed that MLC2 phosphorylation varied from a total phosphorylation of 55% in the flash frozen samples to about 40% in the samples that were maintained in cardioplegia on ice, again a difference of approximately 15%, similar to what was seen using traditional SDS-PAGE. Phosphorylation of TnT was significantly reduced in cardioplegia either at room temperature or on ice. Surprisingly, phosphorylation of TnI was significantly increased in both the cardioplegia and MOPS buffers that were maintained at room temperature and in all cases the samples maintained at room temperature in either buffer or dry showed less dephosphorylation that those held on ice. While protocol dependent changes in overall phosphorylation of TnI were evident, these did not reflect site-specific changes at the N-terminal

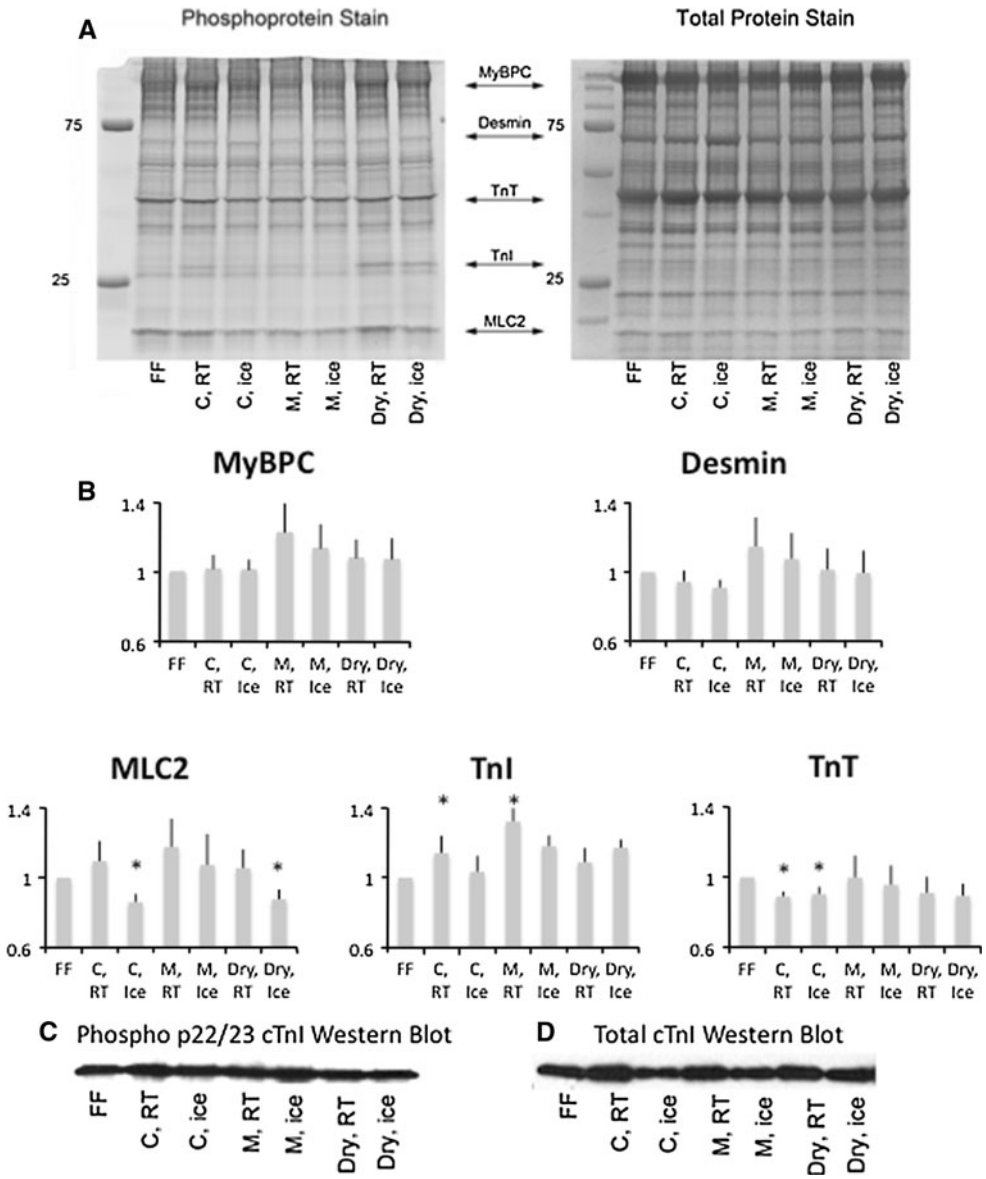

Fig. 1 Representative SDS–PAGE of human LV samples handled under a variety of conditions. **a** ProQ Diamond staining demonstrates phosphoprotein signal, and Coomassie Brilliant Blue staining of the same gel for total protein signal. **b** Summary data from five explanted failing hearts. Normalized signal was derived by dividing the ProQ Diamond signal by the total protein signal for the essential myosin light chain. All samples were normalized to the flash frozen sample for the same heart. *Asterisk* denotes $P < 0.05$ compared to FF. **c** Western blot using a site specific phosphoantibody demonstrating that phosphorylation of TnI at S22/23 is not affected by procurement protocol. **d**. Western blot for total TnI as a loading control

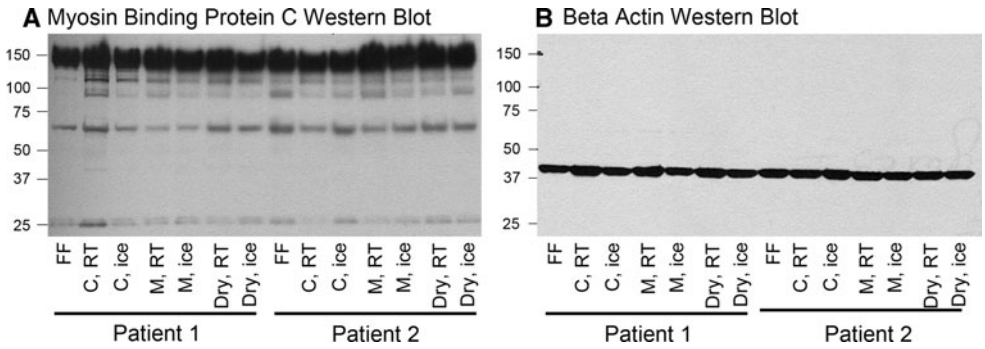

Fig. 2 Representative Western blot for Myosin Binding Protein C (MyBP-C). **a** Samples were separated on 8% SDS-PAGE and probed with an antibody generated again the N-terminal of MyBP-C. Samples from two independent hearts are shown. **b** The same membrane was stripped and reprobed with an anti-sarcomeric-actin antibody as a loading control

PKA dependent site. Phosphorylation of the putative PKA dependent site at S22,23 was constant regardless of protocol (Fig. 1c, d) suggesting that other sites, for example S43/45 and T144, were far more labile.

We also analyzed myosin binding protein C proteolysis and found a 25 kDa fragment that was immunoreactive and possibly represents the C1C2 calpain cleavage product (Kunst et al. 2000; Sadayappan et al. 2006) (Fig. 2), however, there was no systematic effect of sample handling on the proteolysis of MyBP-C with the fragment ranging from approximately 1 to 10%. This fragment was seen both in control hearts and in explanted failing hearts and the extent of proteolysis in general appeared to be less in the non-failing heart tissue (data not shown). However, we saw a number of other immunoreactive fragments of varying sizes with the MyBP-C antibody, bringing into question the specificity of this antibody. As with the smaller fragment, though, the extent of proteolysis was not reflective of the tissue handling protocols.

Discussion

In the present study we chose to focus on contractile proteins, in part because of the central role these elements play in modulating cardiac contractility and also because there is a rich body of literature suggesting that post-translational modification of the contractile elements are influenced by disease progression and in turn, impact the contractile performance of cardiac muscle. By taking biopsy sized pieces of the left ventricle from a single heart in the OR and then subjecting them to variable tissue preservation strategies we were able to directly determine the effect of these strategies on the biochemical characteristics of the contractile proteins. Our aim using this approach was not to define characteristic changes in protein phosphorylation in the failing heart since these are likely influenced by intercurrent therapy, adrenergic activity and disease duration, etc., as well as by reference to a control group but rather to determine the effects of relatively brief post-mortem sample handling on muscle biochemistry.

The major findings of the study were that tissue procurement strategy matters and that rapid freezing and the use of MOPS based buffers rather than extended exposure to cardioplegia solutions appears to optimize phosphorylation status and to minimize post-mortem proteolysis. The impact of this was significant: we saw a 14% increase in troponin I phosphorylation, a 8% decrease in troponin T phosphorylation and a 18% decrease in MLC2 phosphorylation in tissues that were kept either in cardioplegia when compared to samples that were immediately flash frozen in liquid nitrogen. Interestingly, in our current study, the samples maintained at room temperature in cardioplegia or dry more closely resembled the flash frozen sample than those maintained on ice. This is possibly due to discordance in the temperature dependence of the phosphatases and kinases active at the myofilament. For example, it has been previously shown in smooth muscle that the myosin light chain phosphatase (IV) demonstrates an extremely high Q_{10} value, whereas the Q_{10} of other phosphatases including the skeletal muscle myosin phosphatase 1 and protein phosphatase 2Ac is much lower (Mitsui et al. 1994). While there are no data available regarding the cardiac phosphatase(s) temperature dependence, it is likely that there are a number of myofilament bound kinases and phosphatases (Yin et al. 2010) that have differing temperature dependencies. In addition, we assessed MyBP-C proteolysis which has been postulated to play a physiologically important role in the modulation of contractility in heart failure, both in experimental animals (Sadayappan et al. 2006) and in man, and while we confirmed that this cleavage does occur in the heart failure specimens, this was less dependent on procurement strategy and varied by approximately 10% between conditions, an extent of change that could mask or amplify a statistical difference between experimental and control hearts. This finding is consistent with the study by Shin et al. (Shin et al. 2000) describing dramatic differences in the sensitivity of PKC isoforms to proteolytic degradation depending on sample handling and processing.

The importance of these results is evidenced by a review of the literature and in particular the controversy describing

troponin I phosphorylation changes in progressive heart failure (Marston and de Tombe 2008). While the emerging consensus appears to be that overall TnI phosphorylation is reduced in the end-stages of human heart failure (Hamdani et al. 2008; van der Velden et al. 2003; Messer et al. 2007), the extent to which this is true is by no means unanimous and is reflective of multiple and fairly poorly described tissue procurement strategies, some of which (in particular those which involve prolonged exposure to cardioplegic solutions) would appear to be optimized to enhance phosphatase activity. In the absence of overall consensus on the magnitude of change, there has been considerable attention focused on the specific sites of phosphorylation within the molecule (Kooij et al. 2010b; Solaro and van der Velden 2010) with most attention focused on the putative PKA sites at serine 22,23 and a second site at serine 43,45 (targeted in vitro by PKC). The literature would suggest that the ratio of phosphorylation of these sites changes as function of disease severity and that decreased phosphorylation of the predominant PKA site and increased phosphorylation at the serine 43,45 site is potentially important in advanced heart failure (Kooij et al. 2010a), albeit these findings are far more evident in rodent than in human models of disease (Walker et al. 2010; Belin et al. 2007; van der Velden et al. 2006). In fact in human heart tissue, there are recent data to suggest that basal phosphorylation of TnI and its reduction in heart failure is dominated by the behavior of the PKA site (Ayaz-Guner et al. 2009; Zabrouskov et al. 2008). In the present study, we found that phosphorylation at the S22,23 site was relatively unaffected by procurement protocol, even in the face of overall changes in total TnI phosphorylation. This might suggest that the less predominant sites are more vulnerable to site-specific dephosphorylation.

Phosphorylation of myosin binding protein C and proteolytic cleavage is also influenced by disease state and likely influences actin myosin interactions. Increases (Yuan et al. 2006) and decreases (Barefield and Sadayappan 2010; Jacques et al. 2008) in overall protein phosphorylation have been reported in heart disease, although most models of human heart failure have shown a decrease (Copeland et al. 2010; El-Armouche et al. 2007). Decreased phosphorylation of the molecule at serine 282 and 302 (both PKA sites) has been postulated to accelerate proteolysis (Barefield and Sadayappan 2010) and to depress myofilament function either via haploinsufficiency or through the generation of a "poison peptide" fragment. As with TnI, our data suggest that there is artifactual variability in the extent of MyBP-C phosphorylation in end-stage heart tissue. Moreover, the abundance of the C1C2 cleavage product which has functional significance in animal models and reconstituted fibers (Kunst et al. 2000) is also affected. This is not surprising when one considers that a likely mediator of the proteolytic cleavage of MyBP-C is mitochondrial calpain and the activity of this enzyme is highly influenced by mitochondrial integrity, intracellular calcium and by redox state (Kar et al. 2010). However, the variability seen in MyBP-C cleavage was not significant, suggesting that changes in the proteolytic product may indeed be reflective of physiologic changes associated with heart failure.

In summary, this manuscript should serve to interject a cautionary note into the discussion of subtle protein modifications in end-stage heart disease. While it is overwhelmingly likely that alterations in signaling pathways that characterize progressive heart failure are reflected by altered protein biochemistry, capturing and quantifying these changes is not trivial and the strategy used for tissue procurement can introduce unforeseen bias. Based on data presented here, we would propose that the optimal (although likely still imperfect) strategy for human myocardial tissue collection (when biochemical factors are an experimental focus) ought to minimize cardioplegia exposure, and should involve very rapid (<5 min) freezing and storage in liquid nitrogen.

References

Ayaz-Guner S, Zhang J, Li L, Walker JW, Ge Y (2009) In vivo phosphorylation site mapping in mouse cardiac troponin I by high resolution top-down electron capture dissociation mass spectrometry: Ser22/23 are the only sites basally phosphorylated. Biochemistry 48(34):8161–8170

Barefield D, Sadayappan S (2010) Phosphorylation and function of cardiac myosin binding protein-C in health and disease. J Mol Cell Cardiol 48(5):866–875

Belin RJ, Sumandea MP, Allen EJ, Schoenfelt K, Wang H, Solaro RJ et al (2007) Augmented protein kinase C-alpha-induced myofilament protein phosphorylation contributes to myofilament dysfunction in experimental congestive heart failure. Circ Res 101(2):195–204

Copeland O, Sadayappan S, Messer AE, Steinen GJ, van der Velden J, Marston SB (2010) Analysis of cardiac myosin binding protein-c phosphorylation in human heart muscle. J Mol Cell Cardiol 49(6):1003–1011

El-Armouche A, Pohlmann L, Schlossarek S, Starbatty J, Yeh YH, Nattel S et al (2007) Decreased phosphorylation levels of cardiac myosin-binding protein-C in human and experimental heart failure. J Mol Cell Cardiol 43(2):223–229

Hamdani N, de Waard M, Messer AE, Boontje NM, Kooij V, van Dijk S et al (2008) Myofilament dysfunction in cardiac disease from mice to men. J Muscle Res Cell Motil 29(6–8):189–201

Jacques AM, Copeland O, Messer AE, Gallon CE, King K, McKenna WJ et al (2008) Myosin binding protein c phosphorylation in normal, hypertrophic and failing human heart muscle. J Mol Cell Cardiol 45(2):209–216

Jweied EE, McKinney RD, Walker LA, Brodsky I, Geha AS, Massad MG et al (2005) Depressed cardiac myofilament function in human diabetes mellitus. Am J Physiol Heart Circ Physiol 289(6):H2478–H2483

Jweied E, deTombe P, Buttrick PM (2007) The use of human cardiac tissue in biophysical research: The risks of translation. J Mol Cell Cardiol 42(4):722–726

Kar P, Samanta K, Shaikh S, Chowdhury A, Chakraborti T, Chakraborti S (2010) Mitochondrial calpain system: an overview. Arch Biochem Biophys 495(1):1–7

Kooij V, Boontje N, Zaremba R, Jaquet K, dos Remedios C, Stienen GJ et al (2010a) Protein kinase c alpha and epsilon phosphorylation of troponin and myosin binding protein C reduce Ca^{2+} sensitivity in human myocardium. Basic Res Cardiol 105(2): 289–300

Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM et al (2010b) Effect of troponin I ser23/24 phosphorylation on Ca^{2+}-sensitivity in human myocardium depends on the phosphorylation background. J Mol Cell Cardiol 48(5):954–963

Kunst G, Kress KR, Gruen M, Uttenweiler D, Gautel M, Fink RH (2000) Myosin binding protein C, a phosphorylation-dependent force regulator in muscle that controls the attachment of myosin heads by its interaction with myosin S2. Circ Res 86(1):51–58

Marston SB, de Tombe PP (2008) Troponin phosphorylation and myofilament Ca^{2+}-sensitivity in heart failure: increased or decreased? J Mol Cell Cardiol 45(5):603–607

Messer AE, Jacques AM, Marston SB (2007) Troponin phosphorylation and regulatory function in human heart muscle: dephosphorylation of ser23/24 on troponin I could account for the contractile defect in end-stage heart failure. J Mol Cell Cardiol 42(1):247–259

Mitsui T, Kitazawa T, Ikebe M (1994) Correlation between high temperature dependence of smooth muscle myosin light chain phosphatase activity and muscle relaxation rate. J Biol Chem 269(8):5842–5848

Molkentin JD, Robbins J (2009) With great power comes great responsibility: using mouse genetics to study cardiac hypertrophy and failure. J Mol Cell Cardiol 46(2):130–136

Sadayappan S, Osinska H, Klevitsky R, Lorenz JN, Sargent M, Molkentin JD et al (2006) Cardiac myosin binding protein C phosphorylation is cardioprotective. Proc Natl Acad Sci USA 103(45):16918–16923

Shin HG, Barnett JV, Chang P, Reddy S, Drinkwater DC, Pierson RN et al (2000) Molecular heterogeneity of protein kinase C expression in human ventricle. Cardiovasc Res 48(2):285–299

Solaro RJ, van der Velden J (2010) Why does troponin I have so many phosphorylation sites? Fact and fancy. J Mol Cell Cardiol 48(5):810–816

van der Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, Owen VJ et al (2003) Increased Ca^{2+}-sensitivity of the contractile apparatus in end-stage human heart failure results from altered phosphorylation of contractile proteins. Cardiovasc Res 57(1):37–47

van der Velden J, Narolska NA, Lamberts RR, Boontje NM, Borbely A, Zaremba R et al (2006) Functional effects of protein kinase C-mediated myofilament phosphorylation in human myocardium. Cardiovasc Res 69(4):876–887

van Dijk SJ, Holewijn RA, Tebeest A, Dos Remedios C, Stienen GJ, van der Velden J (2009) A piece of the human heart: Variance of protein phosphorylation in left ventricular samples from end-stage primary cardiomyopathy patients. J Muscle Res Cell Motil 30(7–8):299–302

van Heerebeek L, Borbely A, Niessen HW, Bronzwaer JG, van der Velden J, Stienen GJ et al (2006) Myocardial structure and function differ in systolic and diastolic heart failure. Circulation 113(16):1966–1973

Verduyn SC, Zaremba R, van der Velden J, Stienen GJ (2007) Effects of contractile protein phosphorylation on force development in permeabilized rat cardiac myocytes. Basic Res Cardiol 102(6):476–487

Walker LA, Walker JS, Ambler SK, Buttrick PM (2010) Stage-specific changes in myofilament protein phosphorylation following myocardial infarction in mice. J Mol Cell Cardiol 48:1180–1186

Weisser-Thomas J, Piacentino V 3rd, Gaughan JP, Margulies K, Houser SR (2003) Calcium entry via na/ca exchange during the action potential directly contributes to contraction of failing human ventricular myocytes. Cardiovasc Res 57(4):974–985

Yin X, Cuello F, Mayr U, Hao Z, Hornshaw M, Ehler E et al (2010) Proteomics analysis of the cardiac myofilament subproteome reveals dynamic alterations in phosphatase subunit distribution. Mol Cell Proteomics 9(3):497–509

Yuan C, Guo Y, Ravi R, Przyklenk K, Shilkofski N, Diez R et al (2006) Myosin binding protein c is differentially phosphorylated upon myocardial stunning in canine and rat hearts—evidence for novel phosphorylation sites. Proteomics 6(14):4176–4186

Zabrouskov V, Ge Y, Schwartz J, Walker JW (2008) Unraveling molecular complexity of phosphorylated human cardiac troponin I by top down electron capture dissociation/electron transfer dissociation mass spectrometry. Mol Cell Proteomics 7(10): 1838–1849