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Characterization and validation of new tools for measuring site-specific cardiac Troponin I phosphorylation

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Abstract

Phosphorylation of cardiac troponin I is a well established mechanism by which cardiac contractility is modulated. However, there are a number of phosphorylation sites on TnI contribute singly or in combination to influence cardiac function. Accordingly, methods for accurately measuring site-specific TnI phosphorylation are needed. Currently, two strategies are employed; mass spectrometry, which is costly, difficult and low throughput; and Western blotting using phospho-specific antibodies, which is limited by the availability of reagents. In this report, we describe a cohort of new site-specific TnI phosphoantibodies, generated against physiologically relevant phosphorylation sites, that are superior to the current commercially available antibodies: to phospho-serine 22/23 which shows a >5-fold phospho-specificity for phosphorylated TnI; to phospho-serine 43, which has >3-fold phospho-specificity for phosphorylated TnI; and phospho-serine 150 which has >2-fold phospho-specificity for phosphorylated TnI. These new antibodies demonstrated greater sensitivity and specificity for the phosphorylated TnI than the most widely used commercially available reagents. For example, at a protein load of 20 µg of total cardiac extract, a commercially available antibody recognized both phosphorylated and dephosphorylated TnI to the same degree. At the same protein load our phospho-serine 22/23 antibody exhibited no cross-reactivity with dephosphorylated TnI. These new tools should allow a more accurate assessment and a better understanding of the role of TnI phosphorylation in the response of the heart to pathologic stress.

Keywords

phosphorylation; cardiac; troponin I; antibodies; cardiovascular disease; human

1. Introduction

The nature and severity of heart failure varies from person to person and reflects the complex interactions between environmental stressors and individual physiology. The molecular mechanisms underlying this include altered intracellular/extracellular ionic

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activity, reduced force of myocyte contraction, increased β -adrenergic activity, and altered calcium handling (de Tombe, 1998; de Tombe and Solaro, 2000; Bristow, 2003). Clinically, cardiac dysfunction is classified mainly as hypertrophic with preserved ejection fraction or dilated with reduced ejection fraction (Chatterjee, 2012). However, in reality, cardiac dysfunction is not binary but rather reflects a continuum from compensated to decompensated heart failure (Walker et al., 2013). Our lab is exploring the hypothesis that unique patterns of cardiac troponin I (TnI) phosphorylation and dephosphorylation define distinct points along this continuum (Walker et al., 2013). To this end, we have developed new tools to more precisely define and quantify these biochemical events and in this manuscript we describe three site-specific phosphoTnI antibodies that we anticipate can be used to stage patients' cardiac dysfunction.

2. Materials and Methods

2.1 Tissue extraction

Tissue was obtained from the left ventricles of all animals (either control, transgenic or subjected to experimental myocardial infarction, as previously described (Walker et al., 2010)). Animals were anesthetized and the heart was rapidly removed. Hearts were cleaned, weighed and the left ventricles were removed. The ventricles were homogenized in 25 volumes of the appropriate assay buffer (see below) and centrifuged for 5 min at 14,000 X g at 4°C. The protein concentration of the supernatant was measured using a Nanodot 2000. Samples were stored at -80°C until use. For experiments using a "standard sample" ventricles from 5 individual hearts were removed, flash frozen in liquid nitrogen and pulverized in a liquid nitrogen cooled stainless steel mortar and pestle to create a fine powder. The powdered hearts were mixed and homogenized as described above. Human cardiac muscle samples were prepared in a similar fashion from biopsy specimens collected in the operating room that were fast frozen in liquid N₂ immediately following excision and stored at -80 °C (Walker et al., 2013).

2.2 Experimental solutions

Assay buffer composition: phosphatase assay buffer: 100mM Tris-HCl (pH 7.5), 4mM DTT, 6.2mM EDTA, and 0.5mM MnCl₂; kinase assay buffer: 75mM HEPES, 40mM MgCl₂, 0.5mM CaCl₂, 5 mM ATP, 0.2 μ M okadaic acid, and protease inhibitors; isoelectric focusing buffer: 8M Urea, 2.5M thiourea, 4% CHAPS, 2mM EDTA, 1 mM DTT, 2 mM TBP, and protease inhibitors.

2.3 Phosphorylation/Dephosphorylation of Native TnI

Dephosphorylated cardiac homogenates were prepared by incubating mouse cardiac extract (500 μ g) prepared from a "standard sample" with shrimp alkaline phosphatase (Sigma P9088) (135 units/mL) at room temperature for 2 hours. Phosphorylation of cardiac proteins was performed by incubating 500 μ g of dephosphorylated cardiac homogenate with 4000 U/mL PKA (Calbiochem) for 2 hr at 37°C.

2.4 Gel electrophoresis and western blotting

For 1-dimensional SDS-PAGE, samples were mixed 1:1 with 2x sample buffer (120 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.02% bromophenol blue, 5% 2-mercaptoethanol) and proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis. For isoelectric focusing, samples were mixed 1:1 with isoelectric focusing (IEF) buffer containing 8 M urea, 2.5 M thiourea, 4% CHAPS and 5 mM EGTA, and phosphorylated TnI was separated as previously described (Kobayashi et al., 2005; Scruggs et al., 2006). Briefly, samples were focused on 1-dimensional urea-glycerol isoelectric focusing gels using mixture of ampholytes (5–8;3–10). After separation, samples were

transferred to nitrocellulose membranes and blocked for 1 hour at room temperature with 5% BSA in Tris-buffered saline with 0.1% Tween (TBST). After blocking, membranes were incubated with primary antibody overnight at 4° C. Membranes were washed, incubated in secondary antibody for one hour at room temperature, and washed again. Protein bands were visualized using enhanced chemiluminescence. For normalization, the membranes were stripped and re-probed with a pan-TnI antibody. Relative phosphorylation was determined by dividing phospho signal by total TnI signal.

2.5 Antibodies

All antibodies were diluted to appropriate concentrations in TBST. Anti-cardiac TnI (phospho) antibody (Abcam AB-8291), 1:50000; phospho ser22/23 (PhosphoSolutions 9058), 1:1000; phospho ser43 (PhosphoSolutions 9057), 1:2000; phospho ser150 (PhosphoSolutions 9095), 1:1000; panTnI (Fitzgerald, 10-1359), 1:5000; Anti-Rabbit IgG (whole molecule)–Peroxidase Conjugate (SIGMA A-9169), 1:50000; Anti-Mouse IgG (Fab specific)–Peroxidase Conjugate (SIGMA A-2304), 1:50000.

3. Results

We examined the specificity of a commercially available phosphospecific antibody generated against the s22/23 sites of TnI using mouse heart homogenates that were dephosphorylated with shrimp alkaline phosphatase. Homogenates were separated by 1-dimensional isoelectric focusing as previously described (Kobayashi et al., 2005; Scruggs et al., 2006), transferred to nitrocellulose and probed with commercially available antibodies generated to either total TnI (A, panTnI (Fitzgerald)) or phospho22/23 TnI (B, p22/23, (Abcam)). As shown in Figure 1, phosphatase treatment of the extract resulted in near complete dephosphorylation of TnI as indicated by the collapse of upper (phosphorylated) bands into a single, unphosphorylated band. However, the Abcam phospho s22/23 antibody reacts quite strongly with unphosphorylated TnI as demonstrated by its continued reactivity with TnI in the presence of phosphatase.

We generated three new antibodies against known phosphorylation sites in troponin, serine 22/23, serine 43 and serine 150. Antibodies were generated against phosphorylated peptides corresponding to amino acids surrounding each of these regions and were purified using the appropriate phosphopeptide. To verify the specificity of each, we used the phosphopeptides to block the antibody before western blot (Figure 2). Each antibody was completely blocked with its corresponding peptide but showed no decrease in affinity with the phosphopeptide for the alternate sites. Additionally, we attempted to use mutant recombinant troponin I (S to A substitution to render each site non-phosphorylatable or S to D to mimic phosphorylation) in order to demonstrate specificity, but the PhosphoSolutions antibodies are generated against natively phosphorylated peptides and did not recognize pseudophosphorylated recombinant proteins (data not shown).

Therefore, to examine the specificity of each new antibody for phosphorylated TnI, increasing amounts of dephosphorylated cardiac extracts (determined to be dephosphorylated using IEF as in Figure 1) were separated by SDS-PAGE, transferred and western blotted with each antibody. As shown in Figure 3, each new antibody recognized phosphorylated TnI with much greater sensitivity than dephosphorylated TnI. Quantification of reactivity (Figure 4) shows that the commercially available antibody exhibited virtually identical labeling of the phosphorylated and dephosphorylated TnI. In contrast the new antibody raised against phospho-serine 22/23 specific (9058) from PhosphoSolutions, demonstrated marked phosphospecificity. That is, at protein loads ranging from 20 to 50 µg the antibody generated a strong signal with the phosphorylated lysate and exhibited little recognition of dephosphorylated TnI at any protein load. The antibody generated against

phospho-s43 (9057) had some cross-reactivity with dephosphorylated TnI, but had greater than 2-fold higher reactivity with phospho-s43. An antibody generated against phospho s150 demonstrated high specificity for phosphorylated TnI, with only slight recognition of dephosphorylated TnI at higher protein loads.

To further show phospho-specific recognition, we used dephosphorylated cardiac extracts and re-phosphorylated them using protein kinase A which has been shown to preferentially phosphorylate cardiac TnI at s22/23. Extracts were then separated by 1D-IEF and western blotted. Figure 5 shows strong recognition of both the upper, phosphorylated and lower, dephosphorylated TnI band by Abcam-8291. In contrast, the phospho-serine 22/23 antibody from PhosphoSolutions demonstrated intense labeling of the phosphorylated upper band compared the weak recognition of the dephosphorylated lower TnI band. SDS-PAGE separation and western blot analysis of the re-phosphorylated extracts demonstrate quantitative differences in site-specific recognition of the 22/23 site by PhosphoSolutions 9058 but no difference with Abcam-8291 (Figure 6).

In order to show in-situ site-specific recognition of phosphorylated TnI, we used cardiac extracts from control (sham-operated) mice and mice that were given myocardial infarcts by ligation of the left anterior descending artery. The details of these samples have been previously published (Walker et al., 2010), and we have previously demonstrated time-dependent changes in the phosphorylation of TnI at both the s22/23 sites and the s43 site. Examination of the same samples described in our previous publication using the new anti-p22/23 antibody confirms our earlier findings (Figure 7). That is, using the PhosphoSolutions antibody we were able to confirm the temporal phosphorylation changes in troponin I following myocardial infarction. However the magnitude of the changes identified using the current antibody is far greater than we previously reported (data not shown). To date, we have tested the new antibodies and have been able to demonstrate phosphospecific recognition of bovine, pig, rat and mouse (Figure 8) TnI. All antibodies are also immunoreactive with human TnI (data not shown). Other species have not yet been tested.

4. Discussion

Phosphorylation of cardiac contractile proteins, and in particular, cardiac TnI is an important modulator of sarcomeric function and more recently, site-specific phosphorylation has been implicated both as a target of distinct signaling pathways and as a determinant of cardiac contractile performance (Solaro, 2002; Solaro et al., 2013). We (Walker et al., 2010; Walker et al., 2013) and others (van der Velden, 2011; Guggilam et al., 2013) have suggested that the various stages of heart failure can be characterized by a sarcomeric protein phosphofingerprint. Given this, reagents that can reliably determine phosphorylation patterns have great value both as stage-specific biomarkers and also by providing a functional read-out of cardiac performance and response to treatments.

In this paper, we describe three novel phosphospecific TnI antibodies that are highly selective for serine 22/23, serine 43 and serine 150. These three sites were selected as they have been implicated in stage and signaling pathway specific cardiac adaptations: Serine 22/23 is generally felt to be a PKA dependent site and phosphorylation at this site results in both increased cross-bridge cycling and reduced Ca^{2+} - sensitivity (Noland et al., 1989; Noland et al., 1995; Solaro, 2002); serine 43 is strongly associated with PKC activation and phosphorylation at this site is associated with a reduction in maximal force and reduced cross-bridge cycling (Noland et al., 1995; Noland et al., 1996); Serine 150 has been implicated in AMPK signaling and phosphorylation is associated with an increase in Ca^{2+} - sensitivity (Nixon et al., 2012). TnI harbors other potential phosphorylation sites that may be

prone to phosphorylation, however defining patterns of TnI phosphorylation using these three reagents will provide insight into the signaling pathways activated in the heart at different disease stages. For example, we have shown that persistent phosphorylation at the S22/23 site is associated with adaptive cardiac hypertrophy (Walker et al., 2010; Walker et al., 2013) whereas dilated heart failure with a diminished contractile reserve is characterized by a reduction in phosphorylation at serine 22/23 and a relative increase in phosphorylation at serine 43. These same phenotypes are generally recapitulated in transgenic mouse models.

Of the three antibodies described, all have a high level of specificity and the S22/23 antibody in particular is far superior to a widely used commercially available antibody which shows a significant degree of cross-reactivity with non-phosphorylated TnI, a fact which is not well appreciated by scientists who have made use of this reagent. Whether this is responsible for some of the confusion that inhabits the literature (van der Velden et al., 2003; Messer et al., 2006; Messer et al., 2007; Hamdani et al., 2008) in this field is not immediately evident. However, the blotting conditions that are routinely used may only imprecisely distinguish the phosphorylated and the non-phosphorylated form of TnI. To see if we had inadvertently contributed to this confusion, we repeated a previous study describing temporal changes in TnI phosphorylation following experimental MI in a mouse model. Results using the newer antibody were directionally similar although quantitatively distinct (Figure 7).

While these new reagents are potentially very useful, it is worth emphasizing that their ability to distinguish between phosphorylated and non-phosphorylated TnI is still imperfect, particularly at relatively low protein concentrations. Given this, we hope that the details provided in this paper will provide a methodological roadmap for investigators who wish to use them in the future, with the strong caveat that utilizing a range of protein concentrations is advisable.

Finally, the antibodies were all generated against consensus sequences that are highly conserved across species. We have tested them in both large and small animals and found excellent specificity. This would suggest utility in high throughput staging of clinically evident cardiac disease.

5. Conclusions

Sensitive methods to determine site-specific changes in troponin I phosphorylation are essential to furthering our understanding of cardiac maladaptation as TnI is a major regulator of cardiac contractility in vivo and changes in phosphorylation regulate its function. However, the available tools for quantitatively measuring TnI phosphorylation have resulted in disparate data. Here, we describe three novel phosphospecific TnI antibodies that are highly selective for serine 22/23, serine 43 and serine 150. These tools will help provide a more accurate assessment and a better understanding of the role of TnI phosphorylation in the response of the heart to pathologic stress.

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Figure 1.

Mouse ventricular homogenates were treated with alkaline phosphatase and proteins were separated by 1-dimensional isoelectric focusing, transferred and blotted with either a pan-specific TnI antibody or with phosphoTnI antibody from Abcam. Note that the lowest band (corresponding to unphosphorylated TnI) is well recognized by the phosphoTnI antibody).

Figure 2.
Western blots of mouse cardiac extracts using site-specific phosphoantibodies and their appropriate/inappropriate peptide controls.

Figure 3. Mouse ventricular heart homogenates treated with or without alkaline phosphatase were separated by SDS-PAGE, transferred and blotted with a variety of commercially available (A) or newly generated (B–D) phosphospecific antibodies.

Figure 4.
Quantitation of antibody signals in the presence and absence of phosphatase.

Figure 5.

Dephosphorylated mouse ventricular homogenates were treated with protein kinase A and proteins were separated by 1-dimensional isoelectric focusing, transferred to PVDF and western blotted with either the phosphoTnI antibody from Abcam or the newly generated p22/23 TnI antibody. Note that the lowest band (corresponding to unphosphorylated TnI) is well recognized by the phosphoTnI antibody from Abcam, but shows much less cross-reactivity with the new antibody.

Figure 6.

Dephosphorylated mouse ventricular heart homogenates treated with or without protein kinase A were separated by SDS-PAGE, transferred to PVDF and blotted with (A) phosphoTnI antibody from Abcam or (B) the newly generated p22/23 TnI antibody. C and D; quantitation of phosphospecific antibody signals.

Figure 7.

Representative samples from sham-operated (S) or infarcted (MI) mice. Cardiac extracts were blotted with phospho-s22/23 demonstrating marked changes in phosphorylation of TnI at serines 22/23. Membranes were re-probed with a total TnI antibody.

Figure 8. Representative samples of bovine, pig, mouse, and rat. Cardiac extracts were blotted with phosphoantibodies (A–C) or with a pan-TnI antibody. Cross-species reactivity was found with each antibody.