Contractile protein phosphorylation predicts human heart disease phenotypes

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Contractile protein phosphorylation predicts human heart disease phenotypes. Am J Physiol Heart Circ Physiol 304: H1644–H1650, 2013. First published April 5, 2013; doi:10.1152/ajpheart.00957.2012.—Human heart failure has been associated with a low level of thin-filament protein phosphorylation and an increase in calcium sensitivity of contraction relative to both “control” human heart tissue and tissue from small animal models. However, diverse strategies of human tissue procurement and the reliance on tissue obtained from subjects with end-stage heart failure suggest this may be an incomplete characterization. Therefore, we evaluated cardiac left ventricular (LV) biopsy samples from patients with aortic stenosis undergoing valve replacement who presented either with LV hypertrophy and preserved systolic function (Hyp) or with LV dilatation and reduced ejection fraction (Dil). In Hyp, total troponin I (TnI) phosphorylation was markedly increased and myosin light chain 2 (MLC2) phosphorylation was unchanged relative to a control group of patients with normal LV function. Conversely, in Dil, total TnI phosphorylation was significantly reduced compared with control subjects and MLC2 phosphorylation was increased. Site-specific analysis of TnI phosphorylation revealed phenotype-specific differences such that Hyp samples demonstrated significant increases in phosphorylation at serine 22/23 and Dil samples had significant decreases at serine 43. The ratio of phosphorylation at the two sites was biased toward serine 22/23 in Hyp and toward serine 43/45 in Dil. Western blot analysis showed that protein phosphatase-1 with 10.220.33.3 on July 9, 2017 http://ajpheart.physiology.org/ Downloaded from
proved the protocol (no. 06-1100) for the collection, storage, and analysis of human tissue, and all patients gave informed consent prior to collection.

Tissue extraction and gel electrophoresis. Small samples (~10 μg wet wt) 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 (42). For quantification of phosphorylation, samples were separated by 12.5% SDS-PAGE and fixed and stained with ProQ Diamond phosphoprotein gel stain (PQD; In Vitrogen). After destaining, gels were imaged with a Typhoon Gel Imager (GE Life Sciences). Gels were rinsed with water and stained with BioSafe Coomassie brilliant blue (CBB; Bio-Rad) for detection of total proteins. Phosphorylation was calculated by dividing the PQD signal by the CBB signal (see Statistical analysis).

For separation of α- and β-myosin, samples were run by modified 6% SDS-PAGE (separating acrylamide-bis ratio 1:100; resolving gel buffer pH 9.0; running gel buffer pH 8.2; β-mercaptoethanol 600 μl/l inner gel buffer). Gels were run overnight at 4°C and stained with BioSafe CBB protein stain (40).

Western blotting. For determination of phosphorylation site changes in TnI, we used phosphospecific antibodies that were generated by PhosphoSolutions (Aurora, CO) and have been carefully validated (unpublished observations). In our hands, the specificity of these antibodies for site-specific TnI phosphorylation is superior to that of other commercially available antibodies (data not shown). Gels were run as above, and proteins were transferred to polyvinylidene difluoride (PVDF). Membranes were blocked in 5% BSA for 1 h, rinsed with Tris-buffered saline-Tween 20 (TBST), and incubated in the appropriate antibody overnight at 4°C. Membranes were washed and incubated with secondary antibody for 1 h at room temperature. After washing, the proteins were visualized with enhanced chemiluminescence.

For determination of phosphatase expression, gels were run as above and proteins were transferred to PVDF. Membranes were blocked with 5% BSA for 1 h, rinsed with TBST, and incubated in primary antibody [anti-protein phosphatase (PP)1A or anti-PP2A] overnight at 4°C. Membranes were washed and incubated with secondary antibody for 1 h at room temperature. After washing, the proteins were visualized with enhanced chemiluminescence.

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Statistical analysis. Differences in total phosphorylation (SDS-PAGE) were determined by dividing the PQD signal for each protein either by the CBB signal for itself [myosin binding protein C (MyBP-C), Tnl, and MLC2] or by the CBB signal for the actin/TnT (for TnT normalization, as we were unable to effectively measure TnT alone). Similarly, the ratio of α- to β-MHC was determined by densitometry of the CBB-stained gel, and percent α-MHC was expressed as the α signal divided by the total (α + β). Site-specific phosphorylation was quantified by densitometric analysis as the ratio of the phosphospecific signal to the total protein signal. All data are expressed as means ± SE. Comparisons between groups (control, Hyp, Dil) were made by one-way ANOVA followed by Fisher’s least significant difference test. A value of P < 0.05 was considered significant.

RESULTS

Table 1 describes the clinical characteristics of the patients studied. The three groups of patients were of slightly varying ages (control 55 ± 1 yr, Hyp 64 ± 6 yr, Dil 61 ± 3 yr; P = 0.184), but this was not statistically different. Aortic valve area as determined by echocardiography was slightly greater in the patients with hypertrophy, and ventricular volumes were significantly larger and ejection fraction was lower in the patients with LV dilation (ejection fraction in control 66 ± 2%; Hyp 66 ± 3%; Dil 43 ± 5%; P < 0.001). The control patients were slightly younger and had slightly larger ventricular cavitory dimensions relative to those with hypertrophy, and wall thickness was greater in those with LV hypertrophy (not shown). Two of the control patients were taking beta-blocker therapy, whereas one of the aortic stenosis patients with hypertrophy and two of those with dilation were on beta-blocker therapy. All of the aortic stenosis patients in both groups had fibrocalcific disease of the aortic root, and none had a congenitally bicuspid valve.

Table 1. Clinical characteristics of patients studied

<table>
<thead>
<tr>
<th></th>
<th>n (M/F)</th>
<th>Age, yr</th>
<th>LV EDD, cm</th>
<th>LV ESD, cm</th>
<th>AVA, cm²</th>
<th>EF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 (4/1)</td>
<td>55 ± 1</td>
<td>5.5 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>NL</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>Hyp</td>
<td>6 (4/2)</td>
<td>64 ± 6</td>
<td>4.6 ± 0.2*</td>
<td>3.0 ± 0.2</td>
<td>0.71 ± 0.1</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>Dil</td>
<td>4 (3/1)</td>
<td>61 ± 3</td>
<td>6.0 ± 0.3</td>
<td>4.9 ± 0.4*</td>
<td>0.56 ± 0.1</td>
<td>43 ± 5*</td>
</tr>
</tbody>
</table>

Values are means ± SE for n patients. Control, normal left ventricular (LV) function; Hyp, LV hypertrophy and preserved systolic function; Dil, LV dilation and reduced ejection fraction (EF); M, male; F, female; EDD, end-diastolic dimension; ESD, end-systolic dimension; AVA, aortic valve area; NL, normal. *P < 0.05 vs. control.

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decrease in calcium sensitivity of the myofilament, and an increase in rates of fiber shortening, or the sites more commonly associated with PKC activation at serine 43/45, which are associated with a decrease in peak tension development and velocity of fiber shortening as well as decreased calcium sensitivity. As shown in Fig. 2, the Hyp group had increased phosphorylation of the serine 22/23 sites, but phosphorylation at the serine 43 site was not different from the control group. Conversely, in the Dil group, phosphorylation of the serine 22/23 sites was not different from control, and the serine 43 site was significantly reduced. Interestingly, in these samples, TnI was visualized as an immunoreactive doublet. This has been shown previously both in animal models and in human samples (22) and is ascribed to COOH-terminal proteolysis of TnI. However, this has not been confirmed in this study because of the very limited nature of these tissues and may

Fig. 2. Representative Western blots of phosphorylated TnI. Samples were separated on 12.5% SDS-PAGE and probed with antibodies generated against phosphoserine 22/23 TnI (A) and phosphoserine 43 TnI (C). Blots were re-probed with a pan-TnI antibody (B and D). Phosphorylation and total TnI levels were quantified with ImageJ, and the normalized intensities (phospho signal/total protein signal) for each group are shown in the histograms on right. n = 5 (control), 6 (Hyp), and 4 (Dil). *P < 0.05 vs. control; **P < 0.05 vs. Hyp.
represent either proteolysis or other posttranslational modifications. Both bands demonstrated NH$_2$-terminal immunoreactivity and were included in the quantitation of phosphorylation. We were unable to detect changes at serine 150, a putative AMP kinase site in either group (data not shown). These results together with those from the phosphoprotein stain clearly demonstrate that the patterns of contractile protein phosphorylation differ strikingly in these two phenotypic models of ventricular remodeling.

Since contractile protein phosphorylation reflects not only selective activation of regulatory kinases but also phosphatase-mediated dephosphorylation, we investigated whether there were differences in the abundance of the major myofilament protein phosphatases, PP1A and PP2A, between Hyp and Dil. These data are shown in Fig. 3, and they demonstrate that relative to control hearts there is a clear reduction in PP1A in Hyp and a reduction in PP2A in Dil.

Finally, since the hallmark of pathological hypertrophy in small animals as well as in humans is a transcriptional shift from expression of the fast ATPase α-MHC gene and protein to the slower β-MHC (V1 to V3), we established the relative abundance of these two protein isoforms. As has been seen previously in human heart disease, the predominant isoform expressed was the slow V3, ββ-homodimer, which accounted for >95% of the total MHC in both control and diseased hearts as shown in Fig. 4. There was no difference among the three groups.

DISCUSSION

It has long been appreciated that there are two distinct myocardial responses to a sustained pressure overload: The first, which can be viewed as a functional adaptation, consists of ventricular hypertrophy, normalized wall stress, and preserved overall contractility, whereas the second, which is functionally maladaptive, consists of ventricular dilation, increased wall stress, and depressed contractility. While these can be easily defined phenotypically, establishing the nature of the associated biochemical changes and whether they are similar or distinct has remained an open question. This study addresses this issue in the context of clinical aortic stenosis and clearly demonstrates that the patterns of myocardial contractile protein phosphorylation (in particular of TnI and MLC2) in the hypertrophic and dilated patterns of myocardial response are both different and distinct.

Before discussing the specific biochemical changes seen, it is important to comment on the magnitude of overall contractile protein phosphorylation in human heart disease. Several studies have suggested that levels of phosphorylation are low in human heart relative to small animal models and have therefore questioned the importance of these changes in the overall regulation of contraction in human heart disease (13, 18, 36, 37). However, it has also been shown that even modest increases in troponin phosphorylation can have a significant impact on ventricular function (17). Given this, it is important to contrast the patient population and sampling technique employed in this study with these previous reports. In general, prior studies showing low levels of phosphorylation have been
done with muscle samples obtained from end-stage myopathic hearts at the time of transplant, hearts that were subjected to extraordinary adrenergic support (likely in the context of \( \beta \)-receptor desensitization) prior to excision. Extrapolating from this to the present circumstance, namely, hearts that were by no means end-stage (and in fact would be predicted to substantially recover after aortic valve replacement) is likely misleading, and indeed the relative robust levels of phosphorylation seen in the present study support this conclusion. Moreover, studies that have taken muscle from nontransplant hearts (for example, during CABG) have generally employed extensive cardioplegia prior to tissue sampling in order to facilitate cell isolation, a strategy that would be predicted to result in substantial protein dephosphorylation. When we have compared samples collected with the strategy of minimal cardioplegia and flash freezing in the OR with these other approaches, we have generally seen far more robust contractile protein phosphorylation (41).

The maintenance of overall TnI phosphorylation in the Hyp group and, more importantly, the apparent redistribution of phosphorylation along the molecule so as to favor the already predominant NH2-terminal serine sites is consistent with other animal models of hypercontractility and hypertrophy without dilation. One model in particular, first described by MacGowan et al. (17a), in which the serines in TnI at 43,45 were mutated to alanines (and rendered nonphosphorylatable) underscores the reciprocal functional relationship between these sites. These animals showed an increase in both +\( \tau \) and \(-dP/dt\) that was associated with an apparent increase in phosphorylation of the NH2-terminal serines in concert with the lack of any change in phosphorylation at the serine 43/45 sites (23). Over time, it might be predicted that the adrenergic stimulation would result in \( \beta \)-receptor downregulation and a gradual decline both in contractility and in phosphorylation at the NH2-terminal (PKA dependent) serines (2, 3, 6), which is what is seen in the Dil group. Of course, there are several other potential phosphorylation sites along the molecule that we are unable to evaluate given the limited sample size and biochemical reagents available, and how these contribute to overall contractility is speculative.

The changes in the steady-state phosphorylation levels of TnI in the Hyp group and the Dil group are consistent with those seen both in animal models of heart failure and in other human circumstances in which an overall decline in total phosphorylation has been described (18, 42). The decrease in phosphorylation at the PKA-dependent serine 22,23 sites might well reflect downregulation of \( \beta \)-adrenergic signaling or activation of specific phosphatases (see below) and would certainly be consistent with reduced cross-bridge cycling and slower rates of both contraction and relaxation. The overall decline in phosphorylation at serine 43, the putative PKC site, is somewhat surprising as we have previously shown relative preservation of phosphorylation at this site in end-stage cardiomyopathy patients (1), although these studies employed a different antibody. Indeed, cardiac restricted transgenic overexpression of calcium-independent isoforms of PKC has been shown to be sufficient to induce a dilated cardiomyopathy that is associated with an increase in PKC-dependent, TnI-dependent phosphorylation and a decrease in both maximal force development and the calcium sensitivity of cardiac muscle contraction (11, 32). However, it is striking that the relative ratio of phosphorylation at serine 22/23 and serine 43 is biased toward serine 22/23 in Hyp and toward serine 43 in Dil, clearly supporting the hypothesis that a change in myocyte signaling characterizes the transition from a hypertrophic, hypercontractile phenotype to a dilated hypokinetic phenotype. The findings related to MLC2, and the lack of an effective change in the other surveyed contractile proteins, are both striking and somewhat unexpected. MLC2 has generally been viewed as quite important in regulating contractility in smooth muscle but less important in cardiac muscle. However, phosphorylation of MLC2 (generally felt to occur at serine 15) has been shown to increase cardiac myofilament calcium sensitivity (and transgenic replacement with a nonphosphorylatable MLC2 isoform eliminates the MLCK-mediated left shift in the tension-calcium relationship), with only a modest effect on tension development (20, 21, 30). It has also been shown that the gradient of tension development across the myocardium correlates with an analogous gradient of MLC2 phosphorylation (7). Our group and others have also shown that ablation of MLC2 phosphorylation is associated with a reduction in calcium-activated tension development (but not calcium sensitivity) and also with an increase in phosphorylation of other contractile proteins including TnI and MyBPC (31). Given this, it is attractive to imagine that as the ventricle dilates and wall stress increases across the ventricular wall, there is a parallel increase in MLC2 phosphorylation that would not be seen in the Hyp phenotype, in which wall stress is, if anything, slightly reduced.

The absence of a significant change in TnT and MyBPC phosphorylation is noteworthy. Both molecules have been shown to be phosphorylation targets (of both PKA and PKC),
and in reconstituted fibers and in transgenic models phosphorylation of these molecules appears to have significant functional effects (5, 23, 29, 38). However, our data suggest that these biochemical modifications are not relevant in these human disease models, especially not when contrasted with the striking findings related to TnI and MLC2.

A remarkable finding that emerges from this study is the directionally opposite changes in the associated phosphatases PP1A and PP2A, which may well be key players in the distinct phosphoprotein fingerprints and mechanical sequelae that are seen. In mammalian heart, two major phosphatases have been identified, PP1A and PP2A. The former serves as a negative regulator of contractility (4, 24) (and has previously been felt to be functionally significant because of its specificity for phospholamban). Overexpression of inhibitors of PP1A (and subsequent decreases in PP1A activity) results in increases in cardiac function (16, 25), a picture that is completely consistent with that seen in the Hyp group. PP2A, on the other hand, is a positive regulator of contraction, and increased PP2A activity has been shown to increase the calcium sensitivity of cardiac contraction (33), so it would be predicted that a reduction in PP2A, as is seen in Dil, would be associated with reductions in overall muscle contractility. Both PP1A and PP2A can be found in functional complexes with regulatory kinases, and it is likely that their activities are coordinately regulated (27, 34, 43). The precise targets of the action of these phosphatases can only be inferred from the present data, although it is certainly attractive to postulate that they influence the contractile protein alterations described, either directly as myofilament-docked proteins or indirectly via activation or inhibition of the relevant regulatory kinases.

The final biochemical interface assayed in this study was the isof orm distribution of MHC. A shift from the fast to the slower ATPase enzyme has long been implicated in the transition from a normal heart to one that is hypertrophic and/or failing, and indeed increased expression of β-MHC mRNA in human hearts strongly correlates both with heart failure and failing, and indeed increased expression of MHC isoform distribution of MHC. A shift from the fast to the regulatory kinases.

In conclusion, our data suggest that the phenotypically diverse responses to chronic pressure overload seen in patients with aortic stenosis are reflective of changes in contractile protein phosphorylation (in particular of TnI and MLC2) and parallel changes in phosphatase expression. Given the clear functional consequences of these contractile protein changes, it would seem likely that the characteristic “phosphofingerprints” seen might well be characteristic of acquired human hypertrophic and dilated cardiomyopathy in general. We suggest that the combinatorial pattern of myofilament protein phosphorylation and cardiac function are tightly linked, so that cardiac disease is a continuum of changes within the myocardium rather than distinct disease states. Figure 5 summarizes this idea; the transition from a diastolic dysfunction (with preserved ejection fraction) to a systolic dysfunction (evidencing a reduced ejection fraction) is the result of multiple changes in the myofilament proteins. The combinatorial phosphorylation found in an individual patient may reflect the functional status of the myocardium at a particular stage of cardiac disease. Moreover, it would be of great interest to see whether these patterns could help predict the myocardial response to valve replacement or, more generally, to unloading therapy and/or response to pharmacological therapies. Further investigation will be required to answer these questions; however, tissue samples from patients who have recovered from valve surgery have been difficult to acquire. Our preliminary data in patients with dilated cardiomyopathy do suggest that preserved global levels of TnI phosphorylation are associated with clinical response to beta-blocker therapy.

REFERENCES


Author contributions: L.A.W., D.A.F., and P.M.B. conception and design of research; L.A.W. and D.A.F. performed experiments; L.A.W. analyzed data; L.A.W. and D.A.F. drafted manuscript; L.A.W. and D.A.F. interpreted results of experiments; L.A.W. prepared figures; L.A.W. and P.M.B. drafted manuscript; L.A.W. and P.M.B. edited and revised manuscript; L.A.W., D.A.F., and P.M.B. approved final version of manuscript.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.A.W., D.A.F., and P.M.B. conception and design of research; L.A.W. and D.A.F. performed experiments; L.A.W. analyzed data; L.A.W. and P.M.B. interpreted results of experiments; L.A.W. prepared figures; L.A.W. and P.M.B. drafted manuscript; L.A.W. and P.M.B. edited and revised manuscript; L.A.W., D.A.F., and P.M.B. approved final version of manuscript.


