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Point/Counterpoint Troponin phosphorylation and myofilament Ca^{2+} -sensitivity in heart failure: increased or decreased?

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Abstract

Heart failure is characterised by depressed myocyte contractility and is considered to involve a complex malfunction of adrenergic regulation, Ca^{2+} -handling and the contractile apparatus. Most studies on the contractile apparatus have focused on troponin, the Ca^{2+} -dependent regulator of myofibrillar activity. Importantly, phosphorylation of troponin I secondary to beta-adrenergic receptor activation is known to induce reduced myofilament Ca^{2+} sensitivity. In muscle samples from explanted failing human hearts, troponin I phosphorylation levels are very low and Ca^{2+} -sensitivity is high. In contrast, some animal models used to study the mechanisms of heart failure give the opposite result- high levels of troponin I phosphorylation and low Ca^{2+} -sensitivity. Which is right?

Marston

The usual definition of heart failure is that the heart is incapable of pumping sufficient blood for the body's needs, therefore it is reasonable to suppose that there is a defect in cardiac muscle that powers the heart. It has long been known that there is dysfunction in the excitation-contraction coupling system that controls contractility but more recently it has been established that there is also a defect in the contractile machinery of the myofibril in failing heart[1–3]. In order to investigate the underlying mechanisms of this contractile defect, it is necessary to interrogate human tissue directly. Before we can do this we must overcome two important problems. Firstly we need to obtain human tissue from failing heart to study and more importantly obtain muscle from normal hearts as control. Secondly, it is necessary to be able to measure Ca^{2+} -sensitivity and phosphorylation levels that are representative of the heart muscle before it was removed. This precludes many techniques traditionally used with animal models.

Ca^{2+} -sensitivity was first measured in myofibrils by measuring ATPase activity[4] and subsequently several groups have measured the Ca^{2+} -sensitivity dependence of isometric

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tension in chemically skinned fibres and cell fragments[5,6]. The consensus of such studies is that Ca^{2+} -sensitivity of failing heart is greater than that of donor heart muscle. Our studies, initiated by Ian Purcell in 1998, sought to determine which components of the contractile apparatus were responsible for the higher Ca^{2+} -sensitivity in failing hearts. We used a quantitative development of the *in vitro* motility assay that allowed us to reconstitute the contractile apparatus from individual proteins for functional tests. It soon became clear that isolated troponin from donor and failing hearts, reconstituted with human tropomyosin and actin, showed the same altered Ca^{2+} -sensitivity as the whole myofibrils did in isometric tension measurements [7–9]. The central role of troponin alteration in increased Ca^{2+} -sensitivity has since been confirmed in many subsequent experiments.

It could be thought that increased Ca^{2+} -sensitivity is not what you would expect in a heart that contracts inadequately, especially since our experiments with mutations in troponin and tropomyosin that cause familial DCM showed a consistently lower Ca^{2+} -sensitivity and this was proposed to account for inadequate contractility in familial DCM [10]. On the other hand, it is well established that β -adrenergic signalling is defective in failing heart with β -receptor desensitisation, blunted inotropic response and reduced cardiac reserve. Troponin I is the main target of PKA in the contractile apparatus and reduction in troponin I phosphorylation would result in an increased Ca^{2+} -sensitivity if PKA activity was reduced [2]. Until recently, measurement of troponin phosphorylation in human heart *in situ* has been difficult. Early studies used back-phosphorylation with PKA, antibodies specific to phosphorylated or unphosphorylated forms of troponin I and separation of phosphorylated species by isoelectric focussing [5,6,11,12]. In general these studies indicated that failing heart troponin I was less phosphorylated than donor heart troponin I. These techniques are not very precise and, with the exception of isoelectric focussing, are also indirect and only address PKA phosphorylation, although it is well known that troponin I can also be phosphorylated by PKC and possibly also by PAK1.

The measurement of contractile protein phosphorylation has been revolutionised by the introduction of the phospho-protein specific gel stain, Pro-Q Diamond. By including appropriate controls and calibration we have developed Pro-Q Diamond-stained SDS-PAGE as a quantitative assay for the level of protein phosphorylation *in situ* [9]. Applied to human cardiac troponin we have determined that troponin T is phosphorylated at 3.1 molsPi/mol in both donor and failing heart muscle and that troponin I phosphorylation is 2.2 molsPi/mol in donor heart and 0.37 in failing heart. There is therefore substantially less phosphorylation of troponin I in failing heart muscle. The direct assay shows a greater reduction in phosphorylation levels than previous indirect assays.

At this point it is necessary to consider whether these measurements are physiologically meaningful. The first question to tackle is whether our donor heart samples are a satisfactory control. We have studied at least 11 different hearts, sourced from hospitals in Britain and Australia and collected from 1998–2005. We have been surprised at the consistency of functional properties in our donor samples. For example the standard deviation of EC_{50} determined with troponin from 5 different heart samples was 11% and for troponin T and troponin I phosphorylation it was 7 and 17 % respectively. Thus as a control for comparison with failing heart, donor heart is quite satisfactory. We believe that donor heart is also substantially normal, based on measurements of protein phosphorylation. When human donor heart is compared with flash-frozen guinea-pig or mouse heart, the levels of myosin binding protein C-, troponin T and troponin I phosphorylation are found to be the same [9].

The end-stage failing hearts also yield proteins with consistent properties. Thus we found EC_{50} of failing heart was significantly lower than donor heart, not only within a paired assay ($p < .0001$) but also between measurements of troponin from different hearts ($p < .0001$) [13].

This has also been the experience of other investigators using explanted heart muscle from patients with end-stage failure. We have also found that troponin from hearts with other pathologies (hypertrophic cardiomyopathy and familial dilated cardiomyopathy) show quite different patterns of EC₅₀ shift and troponin phosphorylation level from each other and also from failing heart. Thus, as predicted from *in vitro* experimentation, the molecular phenotype of acquired heart failure, familial dilated cardiomyopathy and hypertrophic cardiomyopathy are clearly distinct [14,15]. These results have convinced us that we are investigating a real phenomenon.

Are the higher Ca²⁺-sensitivity and lower troponin I phosphorylation related? Van der Velden [5] showed, using heart samples with different degrees of failure, that there was a correlation between pCa₅₀ and troponin I phosphorylation as measured by isoelectric focussing; however a similar correlation has also been shown for MLC-2 phosphorylation [16]. The definitive experiments linking Ca²⁺-sensitivity and phosphorylation involve manipulation of the troponin I phosphorylation level. PKA treatment of donor and failing heart muscle resulted in decreased Ca²⁺-sensitivity for failing heart and made failing and donor heart Ca²⁺-sensitivities indistinguishable. Conversely, using the *in vitro* motility assay, phosphatase treatment of troponin increased the Ca²⁺-sensitivity of donor troponin more than failing heart troponin and made donor and failing heart indistinguishable. The critical evidence that troponin I phosphorylation by PKA is responsible for the contractile defect in failing heart muscle was obtained in the experiments of Messer et al, who exchanged recombinant troponin I (PKA-phosphorylated or unphosphorylated) into native human troponin. Exchange of troponin I (phosphorylated or unphosphorylated) made the Ca²⁺-sensitivity of donor and normal heart troponin indistinguishable. Moreover, the shift in Ca²⁺-sensitivity and the difference in crossbridge turnover rate characteristic of failing heart could be precisely reproduced by exchanging either phosphorylated or unphosphorylated troponin I into native troponin from both failing and non-failing heart [9].

The point arising from this discussion is that repeated and fully-controlled experimentation shows that in failing heart myofibrillar Ca²⁺-sensitivity is higher than in non-failing heart (EC₅₀ is decreased 2–3 fold) because the level of troponin I phosphorylation is very low, being 0.37 molsPi/mol total phosphorylation and 0.18 molsPi/mol at the PKA sites, serine 23 and 24. This abnormality of the thin filaments makes a significant contribution to the phenotype of heart failure in concert with reduced phosphorylation of MyBP-C and phospholamban and associated abnormalities of EC-coupling.

de Tombe

Heart failure is a clinical syndrome that results from compromised cardiac pump performance that has, at its basis, a reduction in the contractile function of the cardiac myocyte. Despite modern treatment strategies, the prognosis of end-stage heart failure is dismal, rivalling and sometimes even exceeding that of many incurable malignancies, such as for example lung cancer. The global incidence of end-stage heart failure is growing steadily, now reaching epidemic proportions in the developed world. Furthermore, the only cure that is currently available is cardiac transplantation; however, apart from other serious drawbacks of this approach, donor availability is unlikely to ever fulfil the ever growing need. Because of these considerations, intense research programs have been established in various laboratories around the world with a focus to elucidate the cellular and molecular mechanisms that underlie reduced cardiac myocyte contractile function in heart failure.

The contractile strength, contractility if you may, of the cardiac myocyte is determined by *i)* the amount of activator calcium ions delivered each beat to the contractile machinery that is located in the sarcomeres, and *ii)* the response of the contractile proteins to this activator

calcium[17]. There is ample evidence to suggest that heart failure is associated with altered calcium cycling, in particular, reduced calcium loading into and release from the sarcoplasmic reticulum (SR)[18]. The mechanisms that may contribute to this phenomenon have been proposed to be either a malfunction of the SR SERCA calcium pump, increased leakage of calcium from the SR, or a combination of these two factors. In fact, this notion has gained sufficient ground to prompt the idea of gene therapy, an approach where SERCA DNA is directed to the failing heart with the eventual goal to increase calcium loading of the SR so as to improve activator calcium delivery to the cardiac sarcomere; clinical trials putting this concept to the test are currently underway (Dr. Harding at imperial college; cf. ClinicalTrials.gov # NCT00534703).

That the reduced contractile strength of the failing heart might also be caused by altered contractile protein function was hinted at, biochemically, in a study reported in 1962 by the late Dr. Alpert[19], a former editor of this journal. At the time, Norm was employed in my current department (recently, a door was serendipitously uncovered that still carries his name; to this date, we have still not removed it). In that study, Norm found reduced myofibrillar ATPase activity in human heart failure; later work showed that purified acto-myosin ATPase is perfectly normal, a finding that is strongly suggestive of a defect in thin filament regulation. In contrast, early work on calcium responsiveness of myofilament force revealed no changes between skinned muscles preparations obtained from non-failing donor hearts or end-stage explanted hearts. More recent work, however, rather consistently reports myofilament calcium sensitivity of force that is lower in donor-derived material compared to that in explanted heart failure material. In addition, maximum calcium saturated force development, when appropriately normalized to cross-sectional area size, is consistently reported to be lower in heart failure, albeit not always statistically significant in the individual reports (for a recent review see [20]). These results contrast with findings in small rodents by others and us that show a reduction in myofilament Ca^{2+} sensitivity, maximum force development, and cross-bridge cycle kinetics[21,22]. Thus, altered myofilament function in heart failure can present itself in the form of an increase or decrease in the concentration of activator Ca^{2+} that is required to reach 50% of maximum activation (Figure 1, left panel) or a decreased level of maximum activity (Figure 1, right panel). It is interesting that there is apparently no dispute regarding the decrease in maximum activity of the various myofilament functional parameters that have been studied (i.e. force, ATPase activity, sliding velocity, etc), so for the purpose of this discussion we can more or less safely put that issue to rest. There is a third parameter, the Hill coefficient, which indexes the steepness of the Ca^{2+} dose response curve, which is a reflection of the level of cooperativity of the contractile machinery. Although cooperativity of activation may well be affected in heart failure, it is a rather difficult parameter to accurately measure in practice; it requires very precise control of sarcomere length and many repeated measurements in the steep portion of the dose-response curve (see for example[23] where this issue was the specific focus of study for more details).

There is no reason to suspect that the reported human Ca^{2+} sensitivity results are somehow incorrect; the studies were all well performed, many of the conditions such as the solutions employed are all generally very similar between the studies (but see section below). So why then would myofilament Ca^{2+} sensitivity in heart failure yield such disparate results between small rodent studies and the human studies? I believe that there are several potential reasons for this. First, and foremost, one needs to consider what one calls a control. We have recently discussed this issue in detail in this journal[24], so I won't repeat the arguments excessively. Basically, donor heart derived contractile proteins material appears to be phosphorylated in a manner consistent with protein-kinase A activation in comparison to explanted heart failure heart proteins. This finding is consistent with *i*) catecholamine surge due to brain damage, *ii*) inotropic support of donors prior to harvesting of the heart (the last organ to be removed from donors who's heart is not used for transplantation for technical reasons), and *iii*) down-

regulated beta-receptors and reduced beta-adrenergic response in heart failure. In addition, pharmacological treatment of end-stage heart failure patients is likely to be extensive and varied, and also likely very different from the treatment history of donors. Of interest in this regard, we have recently found decreased myofilament Ca^{2+} sensitivity in patients suffering from type-2 diabetes mellitus compared to patients without diabetes[25]. In that study, small biopsies were obtained during bypass surgery from the epicardial surface of the heart from both groups under identical conditions. Incidentally, we also found a decrease in maximum force development, albeit not significant. There have also been reports of increased myofilament Ca^{2+} sensitivity in animal models of cardiac disease, both large animal (dog, pig), and small rodents (mouse)[20]. In general, however, these were relatively short duration studies (induction of cardiac disease in the course of weeks), compared to long-term studies in our studies (7–8 months duration). Thus, it may well be that heart failure is associated with early increase in myofilament Ca^{2+} sensitivity that reverts to decreased Ca^{2+} sensitivity at end-stage heart failure, but this needs further study.

Apart from the difficulties associated with the study of human cardiac tissue, there are several other issues that need to be taken into account. First, although perhaps not overly crucial in explaining the different results, it appears that many studies on skinned human myocardium have been performed in the absence of protease inhibitors and anti-oxidants. It has now become clear that some contractile proteins are quite susceptible to proteolytic breakdown, in particular the giant molecule titin[26]. Titin has been implicated modulation of myofilament length dependent activation and, thus, depending on the sarcomere length at which studies are performed (usually long sarcomere length) this could affect the outcome of the studies[27]. Likewise, it is now recognized that oxidative modulation of contractile proteins affects myofilament function[28]. Sarcomere length and origin of the tissue, that is, epicardium or endocardium has also emerged as an important determinant of myofilament Ca^{2+} sensitivity and myofilament length dependent activation[22]. Clearly, there is room for improvement in standardization of procedures that should help to clarify whether technical issues are the cause of the observed differences between the studies. Exchange of tissue (biopsy) material between the various investigative groups is another approach that should certainly be explored in the future.

Nevertheless, what is clear is that myofilament dysfunction plays a significant role in the depression of cardiac pump performance in heart failure. There is also ample evidence that this is related to, at least in part, inappropriate protein phosphorylation, be it PKA or PKC mediated. There is agreement that that maximum force development is depressed in heart failure, a phenotype that is consistent with PKC upregulation in heart failure[21,29]. Further work needs to be done to resolve whether myofilament Ca^{2+} sensitivity is increased or decreased. I believe this can be best accomplished in animal models of heart failure, under well-controlled conditions and standardized techniques (origin of tissue, pharmacological treatment, solution compositions, anaesthesia, etc). In our studies in small rodents[21], we found increased Tn-I phosphorylation, but in a rather subtle way: by using a non-equilibrium gel electrophoresis approach[30], we found a shift in the distribution of TnI phosphorylation towards a state of multi-site phosphorylation (up to 5 sites phosphorylated). Our biophysical structure-function relationship studies strongly suggest that PKC mediated phosphorylation of either TnI or TnT leads to reduced myofilament Ca^{2+} sensitivity and reduced maximum force[17]. Replacement of troponin by recombinant or non-failing in heart failure myofilaments restored decreased Ca^{2+} sensitivity, as did phosphatase treatment[21]. Together, these data strongly suggest increased PKC mediated contractile protein phosphorylation in experimental heart failure causing reduced myofilament Ca^{2+} sensitivity and reduced maximum myofilament force development. A final thought to consider is the fact that changes in the distribution of phosphorylation between various target sites during the transition to end-stage heart failure may be of paramount importance, but yet will be difficult to measure using the relatively crude

assays that are currently available. That is, which sites of phosphorylation and which contractile protein(s) is (are) the culprit we do not know. The best approach to solve this question would be to apply quantitative mass spectrometry of phosphor-proteins, but the technology has not yet been developed to a level sufficient to provide unambiguous results; future improved proteomic analysis of phosphoproteins in cardiac disease will certainly help resolve the issues discussed here.

It should be stressed that resolution of the current controversy is very important; our findings in the laboratory form the basis of future developments of new therapeutic strategies to combat human heart failure (for example, myofilament Ca^{2+} sensitizing agents).

Marston

It is evident that there is a consensus that Ca^{2+} -sensitivity is inversely related to troponin I phosphorylation and reasonable agreement that the sites phosphorylated by PKA are involved in this effect. The argument is thus over whether the animal models give a more accurate representation of human heart failure than failing human heart tissue itself. Looked at from this point of view there should be no contest, however there is a great deal of intellectual capital invested in animal models of heart disease and in my opinion this has inhibited researchers from asking the difficult questions. There is no way a rat, mouse or guinea-pig, sitting in a cage and protected by legislation from most harmful activities, can reproduce either the size of the human heart, or the multiple effects of the stress and aging that patients are subjected to on the way to developing heart failure. The usual defence of animal models is to attack the use of human tissue. A favourite referee's tactic is to cite confounding factors such as 'catecholamine storm' to discredit the use of donor heart as a control, despite the continuing evidence that donor hearts do provide a uniform population. Searching the literature for work on 'catecholamine storm' reveals it is mostly anecdotal and the few large animal definitive studies actually show it to be a transient phenomenon and so not relevant to donors kept on life support after brain death.

The discrepancies between animal models and the human results are large and need to be resolved. In order to believe the argument that human tissue results are artefactual due to degradation and oxidation in the sample it is necessary to generate the same artefact in the animal model tissue by subjecting them to similar treatments. In fact, discussions about donor hearts are a distraction from the main point of this debate. It really does not matter what the phosphorylation level is in donor hearts because in failing human heart muscle the level of troponin I phosphorylation is extremely low, consequently Ca^{2+} -sensitivity is high. If there is no phosphorylation, arguments about which sites are phosphorylated become irrelevant.

de Tombe

We agree that maximum myofilament activity may be depressed in heart failure, which is a good outcome of this discussion. With regards to myofilament Ca^{2+} sensitivity and contractile protein phosphorylation, it appears that we agree to disagree. In particular, this is so when it comes to the matter as to whether the human samples are representative of the state of the failing human heart, and more importantly, the state of a normal heart. The problem is simply that there is no good control material; patients that are on the donor list are virtually always kept on inotropic support to sustain blood pressure following brain death, which quite likely leads at a minimum to PKA activation, but probably the activation of a host of other signal processes as well. The treatment of the heart failure patients is varied, *and*, very different from that of the donor non-failing patient. Couple to that down-regulated beta-receptors in the failing heart, and we have a problem. There is no doubt in my mind that all of this will lead to very different post-translational modifications of the contractile proteins in the control group versus the heart failure group. Thus, treatment of the subject under study has a tremendous influence

on biochemical and biophysical readouts, as was already discussed at great detail by Solaro et al in the original description of the myofilament desensitizing impact of beta-adrenergic stimulation[31]. Interpretation and extrapolation of this kind of data is only possible under controlled experimental conditions, and such conditions are more likely met in experimental animal studies than in human studies. Ideally large animals more similar to humans should be used, but this has only rarely been the case owing to the difficulty of maintaining ischemia long-term and to the absence of genetic models of human disease. On the other hand, while mouse models have considerable advantages in both of these instances, the mouse at rest is already hyper-contractile and has relatively limited dynamic range in response to adrenergic interventions. So, should we stop studies of biophysical function and biochemical analyses of myofilaments in human heart failure? Of course not! However, results should be viewed with a careful eye towards the problems that I outlined above, and certainly, therapeutic strategies should not be based on the human results alone. Nevertheless, detailed proteomic and functional analysis of cardiac sarcomere in heart failure (be it human or experimental animal derived) should continue in full force to gain clues as to what may cause contractile failure in this syndrome, new therapeutics developed, tested in experimental animal models and then hopefully move to trials and treatment in human patients. We disagree on the path taken, but we do agree on the ultimate desired outcomes.

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Biographies

Pieter de Tombe is Professor of Physiology and Biophysics, Medicine (Cardiology) and Bioengineering, University of Illinois at Chicago since 1996. His research is focused on myofilament function in health and disease with a special focus on the molecular mechanisms

that underlie impaired cardiac function in heart failure and the cellular mechanisms that underlie length sensing of the muscle sarcomere. His studies range from cardiac pump function in the intact organism to studies at the molecular level in the regulation of sarcomere dynamics in single isolated myofibrils. Recent work by the de Tombe laboratory has indicated that post-translational alterations in cardiac troponin, most likely phosphorylations, play an important role in the depressed myofilament function that is seen in heart failure. Other recent work by the laboratory has also implicated troponin, and in particular troponin-I, as an important protein involved in the molecular signal transduction pathways by which sarcomere length modulates myofilament calcium responsiveness.

Steven Marston is Professor of Cardiovascular Biochemistry and has been at the National Heart and Lung Institute division of Imperial College London and its predecessors since 1981. His research career has been devoted to investigating molecular mechanism of muscle contraction and its regulation by Ca^{2+} , with a particular interest in vascular smooth muscle and cardiac muscle. His recent research has concentrated on cardiomyopathies, both acquired heart failure and the genetic myopathies, hypertrophic cardiomyopathy and dilated cardiomyopathy. His research group investigates mechanisms of regulation at the single filament level by in vitro motility assays and also in more organised systems including human heart muscle and transgenic mouse models of cardiomyopathies. This approach recognizes the fact that animal models alone are often inadequate representations of human disease whilst investigation on human tissue has practical limitations and the state of tissue examined may not be physiological. By applying a common range of experimental techniques to both animal and human heart resolution of these problems becomes more likely.

Figure 1.
Possible manifestations of myofilament function alterations in heart failure.