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In Search of the Proteins That Cause Myocardial Stunning

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Cardiac diseases are characterized by a host of changes to the cellular processes that affect contractility of the heart. One emerging experimental approach to the study of cardiac disease, including the ischemia/reperfusion injury known as stunning, is to identify proteins that have been specifically altered or modified in the diseased state. Minimally, the identification of modified proteins will provide new diagnostic markers of myocyte injury. However, for a subset of proteins, disease-induced modification will substantively affect molecular function and contribute directly to cardiac dysfunction. Identification of these causative proteins ultimately prove beneficial to the design of new pharmacological, genetic, or peptidomimetic therapies.

The complement and status of proteins within a myocyte (its protein profile) may be altered, through gene-mediated processes, which include isoform switching and the capacity to vary levels of protein expression, or through posttranslational modification, which includes phosphorylation and proteolysis among other processes. Protein function can also be perturbed under conditions of altered cellular homeostasis, which range from oxidative stress to cellular acidosis. In chronic disease, such as heart failure, the complexity of ongoing adaptive changes to cardiac function is mirrored at the molecular level. As a result, many, possibly hundreds of, proteins are subject to altered expression and/or posttranslational modification. In the acute phase of disease, however, dysfunction often precedes gene-mediated adaptive response, which suggests that the mechanism that underlies acute cardiac injury is attributable to a select few posttranslational events. This could well be the case for myocardial stunning, the subject of the article by Thomas et al.1 in this issue of Circulation Research.

Stunning and Ischemia/Reperfusion Injury: A Continuum of Change

Stunning is a clinical syndrome, a phenomenon in which patients experience reversible contractile dysfunction of the heart following a transient episode of ischemia and subsequent reestablishment of blood flow. It is considered an acute injury, although its severity varies with the length of the ischemic episode. As such, instances of stunning may appear morphologically and functionally similar, yet need not arise necessarily from the same molecular mechanisms. In fact, it is unclear precisely which molecular events are responsible for the onset of stunning and which signaling events, if different, serve to exacerbate the dysfunction. Ischemia/reperfusion injury may result from at least two sets of protein modification pathways. The first is due to the ischemic insult, which Bolli and Marbán2 suggest acts as a “primer” for the subsequent changes caused by the second insult, the onset of reperfusion. It is noteworthy that ischemia/reperfusion injury does not cause a general breakdown of cellular processes. Rather, it is characterized by the modification of specific myofilament proteins in a number of animal models. Such modifications include the proteolysis and cross-linking of troponin I (TnI)3–5 and troponin T (TnT),5,6 alteration of α-actinin,4,7 as well as proteolysis of myosin light chain-1 (MLC1),4 desmin,7 and spectrin.7 Furthermore, other cytoplasmic proteins, such as α/β-crystallin,8,9 OSC protein4 (both of which migrate between MLC1 and MLC2), and GAPDH,8 can become associated with the myofilament during ischemia. However, of these protein modifications, far fewer occur within the reversible stage of ischemia/reperfusion injury known as stunning.

Cross-linking of TnI to the other members of the troponin complex and proteolysis of TnI are the first documented changes in the isolated rat heart model of stunning. Given that TnI is key to the control of muscle contraction and relaxation (for reviews, see References 10 and 11), it has been proposed that its modification may be responsible for stunning. The current hypothesis (see reviews in References 2 and 12) holds that TnI is selectively modified through the action of a protease and a cross-linking enzyme that are activated by the increase in cytoplasmic Ca2+ during the early stages of reperfusion. Calpain and transglutaminase are candidates for the role of proteolysis and cross-linking, respectively. Interestingly, work by Thomas et al.1 argues that there is no significant increase in TnI modification, as assessed by immunoblot, in two different in vivo swine models of stunning.1

Although ischemia is requisite for stunning, the role of reperfusion is likely to prove equally important to our understanding of altered cardiac contractility. There is evidence that the protein profile of the myocardium continues to change for considerable time after initial reperfusion. For example, Canty’s group shows that the level of hsp70 mRNA is upregulated over 60 minutes of reperfusion, although levels of TnI mRNA remain unchanged over the same period.1 These results imply an intriguing role for HSP-70 in the later stages of stunning but do not preclude the involvement of TnI, particularly in the early phase of stunning, which is characterized by posttranslational events. For example, TnI is phosphorylated in both canine and rabbit models after as little

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as 15 minutes of ischemia.\textsuperscript{13,14} Subsequent reperfusion further increases phosphorylation in the in vivo canine model.\textsuperscript{15} In another study, increased TnI phosphorylation persists for several days after coronary artery occlusion.\textsuperscript{16} The length of the ischemic and reperfusion periods is particularly important, since it has yet to be established whether there is any overlap between events triggered by ischemia and those due to reperfusion. For instance, TnI undergoes similar extents of proteolysis whether rat hearts have been subjected to 60-minute ischemia alone or 15-minute ischemia followed by 45-minute reperfusion.\textsuperscript{4} Detailed time-course studies of both ischemia and reperfusion are therefore key to our understanding, given the dynamic nature of the protein modifications.

Whereas the stunned myocardium, by definition, is devoid of necrosis or apoptosis, recent studies indicate that the processes that lead to apoptosis,\textsuperscript{17,18} and presumably necrosis, may be already underway. The viability of a myocyte may therefore result from a battle between competing life and death signals. The protein profile of a cell would then reflect whether the cell would commit, on further injury, to an apoptotic or necrotic pathway. Clearly, it will be important to determine which circumstances lead to each cell fate. A detailed model of stunning therefore must address the issue of the temporal and spatial sequence of conflicting signals within the myocardium. Differences between experimental stunning protocols, animal models, and analytical techniques further complicate efforts to obtain such a model.

\section*{Experimental Strategies for the Identification of Modified Proteins}

It is becoming clear that stunning can have many faces, in no small part owing to differences between animal models. One of the points at issue is whether proteolysis of TnI is a hallmark of stunning. Work by Canty’s group shows that TnI is degraded in two porcine models of stunning under control conditions, but that there is little or no change in the quantity of TnI degradation during ischemia/reperfusion.\textsuperscript{1} However, other work with swine has demonstrated that troponin isolated from stunned hearts can confer altered function when reconstituted with skeletal muscle, clearly implying that at least one of the troponin subunits has been modified.\textsuperscript{19} In other species, TnI is modified specifically. In an in vivo canine model of stunning, there is little proteolysis of TnI under control conditions, although TnI forms a high molecular weight covalent complex following ischemia.\textsuperscript{20} Studies using a rat Langendorff reperfusion model show that after 15-minute ischemia and 45-minute reperfusion, TnI was degraded to TnI\textsubscript{1–193}, and that the cleavage product represented 24\% of total TnI. Covalent complex formation accounted for an additional 17\% of total TnI.\textsuperscript{5}

The simplest interpretation of these differences is that the animal models differ with respect to levels and/or activity of protease and transglutaminase. The data also suggest that different mechanisms of stunning may be at play in each animal model. To discriminate among these possibilities, it is imperative that we evaluate the technical aspects of these studies critically. At issue, in addition to the identity of the modified protein(s), is exactly how much protein must be modified to affect cardiac function? Studies of other cardiac diseases may provide clues. Tardiff et al\textsuperscript{21} showed that expression of mutant TnT, to levels <5\% of the total TnT in transgenic mice, was sufficient to cause contractile dysfunction and sudden death. Thus, quantification of myofilament modification will be essential if we are to dissect the causes of stunning away from mere markers.

Much of what is known about the complement and status of proteins in stunning and ischemia/reperfusion injury is derived from immunoblot analysis. Because the cost and time required for systematic characterization of the myocardial proteins by immunoblot analysis are prohibitive, researchers confine their study to a few antibodies, pursuant to particular hypotheses.

Quantification of proteolysis, covalent complex formation, and phosphorylation by immunoblot analysis is paramount to understanding the mechanisms of contractile dysfunction, yet is fraught with difficulty, due largely to issues of antibody specificity and sensitivity. Specificity refers to the capacity of an antibody to discriminate between binding epitopes. Sensitivity is a function of binding affinity between an antibody and its cognate protein and may change if the antibody binds to a protein whose epitope differs marginally in composition, whether through substitution of an amino acid, phosphorylation, or proteolysis. We have recently found several examples of anti-TnI antibodies whose affinities are affected by these parameters. First, the C5 antibody, among others, displays lower sensitivity for rabbit and human TnI than for the rat isoform, which is likely due to amino acid substitution within the binding epitope. These antibodies also display reduced affinity (and therefore sensitivity) for porcine and canine forms of TnI, likely for the same reason. Second, it is widely held that if TnI proteolysis is not detected with a given antibody, TnI is not degraded. However, absence of evidence is not necessarily evidence of absence. Implicit is the assumption that antibodies have the same affinity for the cleaved TnI as they do for intact TnI. In our laboratory, we have found that antibodies vary greatly with respect to their affinity for TnI fragments. Specifically, we have observed that the antibody 8I-7 binds the degradation product TnI\textsubscript{1–193} with greater affinity than several antibodies, including C5. Therefore, if TnI undergoes proteolysis in the porcine model of Thomas et al,\textsuperscript{1} it would likely remain undetected with such antibodies.

In any case, surely the process of electroblotting, the process by which protein is transferred from gel to nitrocellulose or polyvinylidene difluoride membrane, has been long established. Are we then safe in the assumption that the proteins are transferred uniformly? Unfortunately, the efficiency of transfer is dependent on many factors, including the pH of the transfer buffer, length of transfer, as well as the intrinsic properties of the protein itself. This is particularly troublesome for studies of TnI (pI >9) and its degradation products, because proteolytic fragments of TnI have different isoelectric points and therefore transfer with varying efficiency. Furthermore, linearity of signal from secondary antibody, length of exposure, and method of quantification all have an impact on the interpretation of data. SDS-PAGE followed by either Coomassie Blue or silver staining has also been used to quantify myofilament changes, although the
practice is perilous. Many proteins and their fragments comigrate on a gel, making unambiguous identification and quantification of a particular protein difficult. One study, for example, has shown that the proteolytic product TnI1–193 comigrates with MLC1.⁴

An alternative approach to the issue of protein modification is to use a broad-scope screening method to observe as many proteins as possible. In this approach, currently termed “proteomics,” 2-dimensional electrophoresis is used to resolve proteins (and their modified products), which can then be characterized by mass spectrometry.²²–²⁴ The identity of promising candidate proteins can then be confirmed by sequence analysis, and the nature of the protein change must then be determined. One of the advantages of this approach is that it is generally free of bias, although certain proteins do confound 2-dimensional electrophoresis (due to issues of solubility, high pl, and cellular abundance). Furthermore, just as the field of genomics has driven the development of new techniques in molecular biology, the study of the proteome will spur advances in protein separation and characterization technologies. Finally, the proteomic approach will prove powerful for the documentation of the subtle and complex changes that occur along the continuum of cardiac injury.

Proteomic and traditional immunoblotting approaches will continue to yield more information about the nature and degree of posttranslational modifications that comprise cardiac stunning. In time, the combination of experimental strategies will resolve how many mechanisms of stunning are at play and whether they diverge between species. To identify the proteins that cause stunning, research will undoubtedly begin to focus on the functional consequences of protein modifications at both the physiological and biochemical levels. Studies of transgenic mice should help resolve the issue of which specific protein modifications cause contractile dysfunction. These studies will also address the extent of modification required to cause this phenotype. How closely will the phenotypes of the transgenic mice match that of clinical stunning? Interestingly, studies of novel transgenic mice expressing low levels of TnI1–193 display systolic and diastolic dysfunction in addition to reduced maximum Ca⁺⁺-activated force, recapitulating stunning in the clinical setting.²³ Biochemical analysis will continue to delineate the precise protein–protein interactions or functions that lead directly to contractile dysfunction. Finally, the ultimate test will be to determine which protein modifications occur in humans and to assess whether the modifications correlate with the development of ischemic heart disease.

References


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