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Recent proteomic advances in cardiac cells

Parveen Sharma[1], **Jake Cosme**[1], and **Anthony O. Gramolini**[1]

[1]Department of Physiology, University of Toronto, Toronto, Ontario, M5G 1A8, Canada

Abstract

Cardiovascular diseases (CVDs) are the major source of global morbidity and death and more people die annually from CVDs than from any other cause. These diseases can occur quickly, as seen in acute myocardial infarction (AMI), or progress slowly over years as with chronic heart failure. Advances in mass spectrometry detection and analysis, together with improved isolation and enrichment techniques allowing for the separation of organelles and membrane proteins, now allow for the in-depth analysis of the cardiac proteome. Here we outline current insights that have been provided through cardiovascular proteomics, and discuss studies that have developed innovative technologies which permit the examination of the protein complement in specific organelles including exosomes and secreted proteins. We highlight these foundational studies and illustrate how they are providing the technologies and tools which are now being applied to further study cardiovascular disease; provide new diagnostic markers and potentially new methods of cardiac patient management with identification of novel drug targets.

1. Introduction

Cardiovascular diseases (CVDs) are the major source of global morbidity and death and more people die annually from CVDs than from any other cause. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths [1]. Functionally, heart disease is the inability of the heart to pump sufficient blood to meet the metabolic needs of the body. This disease can occur quickly, as seen in acute myocardial infarction (AMI), or progress slowly over years as with chronic heart failure (HF) [2]. Considering the global health burden of cardiac disease, a greater understanding of the molecular basis of cardiac function will help guide the development of novel diagnostic and therapeutic strategies. In order to improve patient care clinicians require innovations in medical diagnostics that can identify early disease as well as identify novel drug targets that can be used for therapeutics. Techniques such as cDNA and oligonucleotide microarrays make it possible to undertake rapid, global transcriptomic profiling of mRNA expression. However, we and others have found that presence of RNA does not always correlate with the presence of the protein [3–5] and in particular studies have suggested that RNA expression maybe an unreliable predictor of cell-surface protein [5, 6], thereby impeding the identification and discovery of potential membrane embedded drug targets. These caveats can now be overcome by proteomic based studies which provide essential insight into changes in total

Address correspondence to: Anthony Gramolini PhD, Department of Physiology, 112 College Street, Rm 307, University of Toronto, Canada, Tel: 416-978-5609, Fax: 416-581-7629, anthony.gramolini@utoronto.ca. Parveen Sharma, PhD, Department of Physiology, 112 College Street, Rm 307, University of Toronto, Canada, Tel: 416-978-5609, Fax: 416-581-7629, parveen.sharma@utoronto.ca.

protein complement during disease as well as insight into post-translation modifications (PTMs) of proteins which are responsible for some of the key biological changes in the function and regulation of proteins. Furthermore, recent technical advances in proteomics and methodologies developed to enrich for membrane proteins will now allow us to investigate cardiac muscle to an unprecedented depth. These technologies provide not only greater scientific insight into cardiac muscle and related diseases, but will also help to develop additional markers of disease progression and even identify novel therapeutic targets to increase our ability to manage cardiac patients. In this review, we outline progress made in these fields and highlight innovative technologies of cardiac research which could potentially improve patient diagnosis and therapies.

2. Proteome and subproteome insight into the heart

The majority of cardiac proteomic research has been carried out using 2 dimensional gel-based approaches (2-DE) in which proteins are separated in two dimension according to their charge properties (isoelectric point) [7] under denaturing conditions and then their relative molecular mass (M_r) by SDS-PAGE [3, 8, 9]. This methodology remains one of the core techniques used in proteomic and, indeed, has played a central role in providing insights into not only the biology of the normal heart [10, 11], but also elucidating markers of disease [12, 13]. Inherent with the application of 2-DE is its ability to detect PTMs which can cause changes in the isolelectric point and/or the molecular weight of the modified protein, which are readily detected. However, the major limitation of 2-DE to display complete proteomes is the limited dynamic range of 2-DE, with an estimated maximum dynamic range of 10^4 magnitude [3, 14], compared with the very high dynamic range of protein abundance, estimated at 10^6 for cells and tissues [3] and 10^{12} for plasma [3, 15].

To overcome these restrictions, subcellular fractionation methods have been developed to reduce sample complexity. These methods include differential centrifugation, flow cytometery, immune-based isolation, membrane protein enrichment strategies and/or density gradient isolation of organelles such as the nucleus or mitochondria. These isolation methods are now routinely used together with 2DE based studies, or more recently, 1D gel SDS coupled to LC-MS. Removing the 2DE gel also removes one further major caveat with 2DE which is that membrane proteins are usually underrepresented because of their poor solubility in the isoelectric-focusing sample buffer. With this in mind, Franklin *et al* used subcellular gradient fractionation to isolate murine cardiac nuclei followed by further fractionation into acid soluble proteins, chromatin bound molecules, and nucleoplasmic proteins and identified a nuclear proteome of 1048 proteins many of which isolated uniquely to one sub-fraction in the nucleus [16]. They identified 142 integral membrane proteins, the majority of which were exclusive to the nucleoplasmic fraction. The authors further used high mass accuracy techniques to identify peptides that mapped to a total of 54 histone variants, 17 of which were identified by at least 1 unique peptide. This study provided subcellullar localisation information of proteins to cardiac nuclei under normal conditions, and layed the foundation for analysis of potential protein trafficking and re-distribution under diseases conditions.

As another example of fractionation applied in cardiac muscle, several studies have assessed mitochondrial proteomes. Mitochondria are essential for cell survival both not only because of their role as metabolic energy providers, but also as regulators of programmed cell death. Mitochondria are double-membrane containing organelles with many membrane embedded proteins, two features that provide a unique challenge concerning solubility. For instance, early 2-DE analysis coupled to MALDI using human placenta identified only 46 proteins [17]. However, more recent work by Zhang *et al,* who carried out differential centrifugation to enrich for mitochondrial proteins in the presence of detergent to aid solubility, applied 1D SDS-PAGE coupled to LC/MS/MS that resulted in the identification of 940 distinct mitochondrial proteins; 480 proteins of which had not been identified previously [18]. Emerging technologies have also allowed for a detailed analysis of the phosphorylation state of cardiac mitochondria. For instance, Deng *et al* employed titanium dioxide (TiO_2) beads to enrich for phosphorylated peptides in isolated mitochondria and in their studies identified 236 phosphorylation sites in 203 unique phoshoproteins, in a diverse range of pathways including ion balance, proteolysis and apoptosis [19].

These studies will provide the essential foundations of knowledge for both cardiac nuclear and mitochondria proteomes, not only providing key information on the total cardiac organellular protein complements and potential PTM sites, but highlight crucial techniques that can provide an in-depth look at cellular organelles. These initial studies will ultimately allow further studies of investigation into changes that occur at an organelle level in disease proteomes originating from models of cardiac distress.

3. Enrichment of membrane proteins

Approximately 50% of all current drugs target membrane protein signalling however relatively little is known about this class of proteins. The investigation of cell-surface proteins has proven to be difficult for several reasons including their relative low abundance and their hydrophobic nature, decreasing their solubility in aqueous media. Kislinger *et al* [20] carried out subcellular fractionation of multiple tissue types including the mouse heart and coupled it to tandem mass spectrometry to an impressive depth identifying a total of 1652 cardiac proteins, however they note that proportionally fewer plasma membrane proteins were identified than were expected relative to the predicted proteome. The use of methods of cell-surface protein enrichment such as chemical capture-based approaches such as silica-bead coating, biotinylation and glyco-capture of cell surface proteins can now be coupled to mass spectrometry to provide a greater investigation of this class of protein. These enrichment techniques have already been implemented to successfully identify novel membrane and membrane associated proteins in a variety of cells and tissues including cardiac tissue. For instance glyco-capture of rat cardiac proteins identified a total of 1556 *N*-linked glycosylation sites representing 972 protein groups were identified of which more than 650 have a predicted transmembrane domain. ProteinCenter analysis of the revealed 76.9% of identified proteins showed that were predicted to localize to the membrane [21]. These studies carried out by Parker *et al* further showed an alteration in the abundance of glycoproteins involved in cardiac remodelling following ischemia and reperfusion providing further essential insight into the mechanism of cardiac dysfunction.

Recently, intravascular silica-bead perfusion of mouse placenta followed by shotgun proteomics identified 1181 plasma membrane proteins at the blood tissue interfaces; 171 of which were enriched at the maternal blood-trophoblast interface, and 192 at the fetal blood-endothelial interface. Many of these proteins were found upregulated in placental disease and were shown to be unique predictors of 3 sub-types of preeclampsia and can now be used as potential diagnostic tools for sub-type analysis [22]. A large-scale proteomic study of the cell surface and surface associated proteins of four stem cell lines that ultimately give rise to the mouse fetus, placenta or yolk sac were investigated by cell surface biotinylation coupled to MuDPIT based mass spectrometry [5]. These biotinylation studies led to the identification of a total of 3432 proteins many of which were cell type unique and allowed the separation of cell types using flow assisted cell sorting. These techniques described above provide methods used to carry out successful large-scale proteomic based studies of cardiac membrane enrichment in both normal and disease conditions. Large-scale cardiac membrane proteins analysis will not only increase our current knowledge of cardiac membrane and membrane associated proteins but may allow the identification of novel drug targets as well as diagnostic biomarkers.

4. The search for an early stage CVD detection marker

An optimal biomarker is defined as a protein that is easily measurable in a short period of time ideally in an easily accessible, non-evasive body fluid such as urine or blood, elevation of which would provide the clinicians with diagnostic information and aid in the medical decision making process [23]. Currently, there are four markers that have sufficient evidence of clinical utility to be recommended for regular clinical use [24]. These markers include: Cardiac troponin I and T which are used as a gold standard to diagnose acute myocardial infarction (MI); B-type natriuretic peptides (BNP and NT-proBNP) which are used to aid in the diagnosis of chronic as well as acute heart failure; C-reactive protein (CRP) [25] and D-dimer, which are known inflammatory markers associated with ischemic heart disease [26]. Advances in detection of existing biomarkers is greatly improving patient diagnosis with new high sensitivity cardiac troponin I and T assays greatly improve patient diagnosis [27], however the caveat inherent with current biomarkers remains that they detect late stage cardiovascular disease.

We and others have recently carried out high throughput proteomics studies in order to identify early cardiac disease biomarkers. We have previously reported that an Arg-9 to Cys mutation in the human gene phospholamban (PLN-R9C) leads to the early onset of dilated cardiomyopathy in affected patients, typically commenced during adolescence leading to deterioration in cardiac function leading to crisis and mortality [28]. We investigated a mouse model carrying the PLN-R9C mutation and carried out exhaustive gel-free protein profiling and parallel microarray-based mRNA screening techniques to examine temporal changes in the global expression patterns during disease progression in the cardiac ventricular muscle of R9C mutant animals as compared with age-matched healthy controls [29]. We followed disease progression from early to late stage in this mouse model and identified 467 upregulated proteins many of which showed significant increase at an early stage and were shown to present in previously determined human plasma and urine proteomes. Several of the markers identified in this earlier study are currently under

validation in a patient cohort with initial promising results. For example, Askevold *et al* have independently identified one of the high probability markers found in this study, namely secreted frizzled related protein 3 (sFRP3) and further validated this protein in a patient cohort and found high serum levels to predict poor patient outcomes [30].

Myeloperoxidase (MPO) is an enzyme being investigated as an early indicator of cardiovascular disease, with mass spectrometry being used to identify its mode of action. MPO is hemeprotein that catalyses the conversion of chloride and hydrogen peroxide to hyperchlorite and is released into the extracellular fluid by activated neutrophils and macrophages during inflammation [24, 31]. MPO together with metalloproteases degrade the collagen layer of an atheroma leading to erosion or rupture of plaques. MPO has been linked to plaque instability although it may not be specific to cardiac disease since activation of neutrophils and macrophages may occur due to infections or non-cardiac disease linked inflammations [24]. However, several clinical trials have indicated increased levels of MPO as an early indicator of coronary heart disease [32, 33] in patients before detection by conventional methods such as coronary angiography [24, 34] and before the detection of cardiac troponin [35]. Mass spectrometry has been used extensively to identify targets of MPO [36, 37] and recent multiple reaction monitoring (MRM) based mass spectrometry evidence suggests that chlorination of apolipoprotein A-I by MPO may contribute to generation of a dysfunctional form of HDL *in vivo.* This, in turn prevents the cardioprotective effect of HDL which normally removes excess cholesterol from macrophages in the artery wall in a process termed reverse cholesterol transport [38]. These studies not only identify MPO as an early detectable biomarker but also provide a mechanism of action and potentially identify an avenue for therapeutic design.

5. Exosomes as biomarkers of disease

Many cells are capable or releasing secretory membranous vesicles which have been characterised by their size and mode of secretion. Exosomes are vesicles of 40–100nm diameter which are secreted the endosomal compartment and released via fusion of multivesicular bodies with the plasma membrane in comparison to shedding vesicles which range from 100–1000nm result from direct release from the plasma membrane [39, 40]. Recent studies have shown that exosomes are released from multiple cell types, contain protein and RNA species, and have been exploited as a novel reservoir for disease biomarker discovery. The molecular content, including proteins, of exosomes are heavily dependent on the tissue/cell-type from which it is derived. With the unveiling of more exosome-proteome studies, it is becoming clear that exosomes from diverse origins contain a conserved set of proteins as well as a subset of cell type/tissue specific proteins [41]. It has been shown that many disease conditions including cancer, alter the protein complement of the cargo contained within vesicles as well as increase the secretion of exosomes into bodily fluids including into the blood, urine and acities [42] of patients allowing them to be differentiated from normal secretions by the cell and so this avenue is now being investigated as a potential source of biomarkers [43–46].

Recent reports have shown shed microvesicles and exosomes released from cardiomyocytes, cells which were not thought of as secretory cells previously [47, 48]. Gupta *et al* showed

exosome mediated secretion of HSP60 from adult cardiomyocytes, levels of which were tripled upon mild hypoxia [49]. Waldenstrom *et al* focused on the mRNA content of exosomes released from cardiomyocytes under normal conditions and identify 1520 mRNA by microarray analysis [48]. Quantitative proteomics on the endothelial exosome identified 1354 proteins of which 19 had altered abundances due to *in vitro* stressors such as TNF-α activation, hypoxia, and high levels of mannose or glucose. Through microarray analysis, 1992 mRNA transcripts were also identified in the endothelial exosome with 21 of them being altered in abundance due to either hypoxia or TNF-α activation [50]. Although these studies focused on the contents of these vesicles, they open up the field for an in-depth proteomic analysis of cardiac exosomes both under normal and disease conditions. Such studies become an essential field of investigation when we consider that the contents of microvesicles and exosomes have already influenced cardiac research, particular with respect to microRNAs. MicroRNA (miRNA) has been found in recent research to be a close regulator of messenger RNA (mRNA) translation and found to be protected from degradation by their encapsulation into exosomes [51, 52]. MiRNA are short non-coding oligonucleotides of approximately 20–26 nucleotides in length with approximately 650 identified in the human genome [53]. Their mechanism of regulating mRNA translation involves the miRNA interacting with the 3′ untranslated region of the target mRNA, leading to target degradation or gene silencing [54]. There has been evidence supporting the use of miRNA as viable circulating biomarker for myocardial injury. Plasma miR-208 was shown to increase following isoproternol-induced myocardial injury in rat models and that their time-dependent changes in concentrations were similar to that of cardiac troponin I, a biomarker currently in use for assessing myocardial injury [55]. In consideration that miR-208 was found to be cardiac-specific, its use in tandem with cardiac troponin I and cardiac troponin T levels would improve specificity to myocardial injury as troponin assays are also used in assessing renal failure and disease [56].

6. Systems-biology approach to biomarkers

Another comprehensive system-biology approach to understanding disease, as well as aiding in biomarker discovery, has recently started to emerge and mature [57–59]. In the ‘omics’ era, as a community we now are able to extract large protein datasets from disease conditions and compare them accordingly to normal samples. Stringent filtering methods allow the identification of a subset of the most statistically differentially expressed proteins and the rest although significantly altered in disease tend to get left behind. However, analysis of these dataset as a whole may provide us with more detailed analysis regarding the mechanism of cardiovascular dysfunction. For instance, a systems biology approach has provided essential information of a multi-subcellular complex of PKCε an enzyme whose activation is known to be cardioprotective and enhance resistance to myocardial ischemia/reperfusion [60, 61]. Ping *et al* carried out co-immunoprecipitation of PKCε from cardiac tissue followed by proteomic analysis and identified 36 binding partners of PKCε including known signalling molecules such as Src and Lck tyrosine kinases and mitogen-activated protein kinases (p38 MAPKs, JNKs, and ERKs), and upstream modulators of PKCε, such as PI3 kinases and their substrate, PKB/Akt. However, they also identified novels roles of regulation of PKCε in the modulation of iNOS, eNOS, COX-2, Hif-1a, heme oxygenase-1,

HSPs, and aldose reductase. This systems approach provided new avenues of research of PKCε regulation in novel pathways and subcellular localisations [60–62]. Dewey *et al* have also used this approach identified from gene co-expression data a subset of network modules that distinguished cardiac hypertrophy from heart failure [57]. Disparate mechanisms of cardiac stress invoke a common pathway of cardiac malfunction and so identifying a common core of upregulated pathways will not only provide a panel of biomarkers but information on the mechanisms involved in disease.

Analysis tools such as STRING [63, 64], Cytoscape [65] and KEGG[66] have greatly enhanced our ability to monitor protein-protein interactions. These tools aid in the identification and visualisation of network-based interactions. These programmes observe proteins as nodes and the interaction as edges to those nodes. They allow the identification of key proteins that have a large number of interactions termed central hubs which are more likely to be essential to organism survival [67] as well as networks of functional modules which give vital information of pathways involved in normal and disease state.

7. Quantification of biomarkers

Discovery based proteomics studies tend to amass large quantities of data and provide many candidates which are potentially differentially regulated between a normal and disease condition. These candidates are then subjected to extensive validation using conventional biochemistry techniques such as western blotting, ELISA and immunofluorescence. However, the bottleneck here remains the availability of high grade antibodies and many novel proteins may get overlooked because of the unavailability of suitable detection reagents. Here, advances made in MRM based mass spectrometry are providing not only an alternative in the lab but also providing high grade quantification in the clinic. This method allows the recognition of proteins based on predicted transitions of prototypic peptides in triple quadrople instruments. In this way multiple proteins (10–100) can be detected simultaneously from one sample allowing the rapid identification of a panel of proteins and biomarkers. However, the major caveat in using MRM based analysis remains the dynamic range of proteins present in blood. Serum is a highly complex biological fluid in which the concentrations of all proteins span 12 orders of magnitude, ranging from albumin and immunoglobulins milligrams per milliliter to many current biomarkers of clinical relevance which present in nanograms per milliliter amounts [68, 69]. However, recent studies have reported that the with the depletion of the top 12 most abundant proteins in serum combined with limited peptide fractionation by strong cation exchange (SCX) improves detection limits by up to 1000 fold when compared to direct sample analysis [70]. Using these techniques Keshishian *et al* then used a MRM based assay to detect 6 known cardiac biomarkers from patient samples undergoing alcohol septal ablation treatment for hypertrophic obstructive cardiomyopathy [69]. Although the detection levels of these proteins remain lower by MRM than those detected by conventional immunoassay methods it provides evidence that multiplex assays are possible with MRM detection analysis. With the advent of better separation methods and more sensitive mass spectrometry technology, MRM based clinical detection may reduce time and handling errors in the analysis of panels of biomarkers, in particular novel biomarkers which may not have highly established antibodies and reagents for conventional assays from patient samples. These tools may

become increasingly valuable when we consider how panels of proteins identified from a systems-biology approach will aid patient diagnosis.

Conclusion

This review highlights insights that have been learned through cardiovascular proteomics and foundational studies that have been initiated under normal conditions. These studies have developed innovative technologies that now can be used to further study cardiovascular disease; provide new diagnostic markers and potentially new methods of cardiac patient management with identification of novel drug targets. The prospects of proteomic analysis of cardiovascular diseases may lie in the under-represented subproteomes of the extracellular space. These subproteomes, which include the ECM and secreted factors, will contribute to the understanding of pathological proteomic changes during disease. Treating cardiovascular disease requires understanding of the diseased cardiac cell in its pathological environment. Applying the established technology the proteomes of the cardiac extracellular space will hopefully create a more clear picture of cardiovascular disease by complementing the established work of subcellular cardiovascular proteomics.

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