DETERMINATION AND CHARACTERIZATION OF THE CARDIAC ER/SR PROTEOME

2. SPECIFIC AIMS

Cardiac sarcoplasmic reticulum (SR) has a primary role in control of cytoplasmic Ca and regulation of contraction and relaxation (1-3). Critical cardiac SR proteins involved in Ca regulation were discovered primarily due to their enrichment in microsomal membrane vesicles (4-7). Yet, our poor understanding of basic myocyte cell biology, and ambiguous relationship between SR and ER, has led to a critical need for a complete determination of the cardiac SR proteome. An ideal advance in our understanding would involve knowing the complete protein composition of both known SR subcompartments (free and junctional) as well as cardiac ER. Protocols for isolating clean and highly-enriched membrane vesicles of both junctional and free SR origin have been previously developed and published by studies (8, 9). Densities of cardiac SR membrane vesicles greatly increase when the SR Ca pump (SERCA) is activated, due to Ca concentration in vesicle lumens and precipitation with oxalate anion. The proteomic data provided by our proposed work includes the following critical resources for cardiac researchers: 1) a catalog of the most abundant proteins and protein isoforms of the SR, allowing researchers to focus their efforts on proven native protein targets, 2) a separation based upon biochemical mechanisms for enrichment of both junctional *and* free SR vesicles, producing a spectrum of SR proteins that range in their relative contributions to Ca uptake or Ca release, 3) a new template for evaluating hundreds of proteins in control and cardiomyopathic hearts, and 4) clarification of the relationship between ER and SR in the heart. To accomplish this long-term goal, we propose the following three specific aims:

1) To determine the canine SR membrane proteome

Purified junctional and free SR membrane vesicles, and crude cardiac microsomes will be isolated from canine heart tissue following Ca oxalate loading, a process that represents the actions of both SERCA and RyR activities. Protein compositions will be analyzed by label-free GeLC-MS/MS and solution-based iTRAQ analyses of tryptic digests using tandem mass spectrometry. Determinations of overall protein abundances, enrichment in SR membranes compared to microsomes, and junctional-to-free SR enrichment, will be used to characterize the expected 1100 proteins that can be detected in cardiac microsomes. A set of 354 proteins has been tentatively identified that are, on the average, 8-fold enriched in SR subfractions. An alternant approach will apply an SR purification protocol in which the ryanodine receptor is blocked by high concentrations of ryanodine, leading to selective enrichment of junctional SR membranes. Patterns of protein co-enrichments will provide important evidence for specific ER/SR subcompartments (free SR, junctional SR, rough ER, etc.), and protein abundances will predict relative functional activities in these ER/SR subcompartments. Important preliminary data will illustrate the analytical approach, and suggest that SERCA-enriched membrane vesicles contain all known ER and SR protein complexes.

2) To compare SR protein structure and content with SR from failed canine tissue samples

Many laboratories have examined relative levels of proteins from samples of heart tissue, and a consensus finding is that levels of critical proteins can differ. But no studies to date have examined failed heart tissue for changes within the entire SR proteome, an analysis that quantifies the relative levels of several hundred SR proteins at once. In addition, proteomic analyses measure protein levels semi-quantitatively, and analyses of enrichment patterns can be indicative of underlying changes in functional ER/SR subcompartmentation as part of cardiac adaptation.

3) To extend the SR proteomic analysis to samples of normal human heart tissue

While canine heart continues to serve as an important model for biochemical and physiological studies of human heart, it would be very useful and important to characterize the SR proteome of human tissue. The first step of such work involves the biochemical characterization of Ca oxalate loading and SR fractionation. Isolation of human SR subfractions on sucrose gradients will be assayed using selected SR markers established in dog heart studies. Once similar SR protein enrichments are established or characterized, we can initiate proteomic analyses.

In summary, the proposed studies will provide a growing resource of data concerning SR and ER protein structure and subcompartmentation. It will serve as an important foundation for discovery by other scientists, providing an important database of native protein structures. With strong preliminary data, these experiments are poised for timely dissemination and rapid application.

3. RESEARCH STRATEGY

A. Significance

The insufficiency of Ca release from SR is a common cause of reduced contractile strength in heart failure and other cardiomyopathies (1, 3, 10). Ca cycling in the cardiomyocyte is also critically involved in arrythmogenic Ca waves (11, 12), as well as in regulation of cardiac chronotropy (13). As such, numerous pathologies including heart failure (HF) are associated with SR protein dysfunction. Many changes in protein levels have been reported among major resident SR proteins, but strategic insights into disease remain limited. The majority of resident SR proteins have likely not yet been characterized, or even reported, as there exists no complete proteomic analysis of purified cardiac SR membranes.

With these studies, we will determine levels for all SR proteins using SR isolation techniques developed in the late 1970's (8, 9) coupled with state-of-the-art mass spectrometric methodologies. Preparations of purified SR membranes were previously described by the Applicant, and widely validated. The key enrichment step occurs by activating the biochemical actions of the SR Ca-ATPase have been described by the Applicant (9, 14, 15). This method of junctional and free SR purification has since been used in many studies to validate the distribution of certain ER and SR proteins. Proteomic analysis of these SR preparations offers a valuable source of discovery for researchers, as follows:

- ***Identifies and catalogs a complete set of cardiac SR proteins*** Cardiac researchers will have a catalog of SR proteins present in canine and human heart to consult as their investigations proceed into unchartered areas of cardiac biology. This catalog will consist of semi-quantitative data for abundant and low-abundance SR proteins and the respective isoforms present in heart, permitting researchers and clinicians to better focus translational attention on proteins that have been identified and authenticated using mass spectrometric detection.
- ***Determines protein enrichments between junctional and free SR subcompartments*** Our well-established method of biochemically isolating SR membranes is based on its standard SR Ca handling properties, those of Ca uptake and Ca release. Membrane vesicles separated by this method are a sampling of all membrane compartments that contain sufficient SERCA activity to undergo increased density. Preliminary data indicate 354 canine cardiac proteins that are enriched in such membrane vesicles. The presence of ryanodine receptors alters the otherwise sharp increases in Ca oxalate transported into membrane vesicles. A separate isolation carried out in the presence of ryanodine, will independently enrich junctional SR, further validating data from SERCA-dependent isolation.
- ***Reveals relationships between functional ER/SR compartments*** Three relatively independent biochemical parameters will be determined for every protein: its relative abundance, its enrichment in SR compared to crude microsomes, and its enrichment in ryanodine-sensitive compared to insensitive membranes. Because enrichment and abundance of individual ER/SR proteins depends upon the behavior of its membrane source, graphical analyses may resolve further SR subcompartmentation. Our preliminary results show that the isolated SR membranes contain virtually all ER functional protein complexes, with distinct enrichment patterns defined by proteins resident to those compartments.

B. Innovation

Immunoblotting is the standard method of quantifying SR protein levels in the heart. This important method, however, is generally restricted to a relatively small set of proteins needed to support a narrow hypothesis being tested. Our approach extends the determination of protein levels to the widest possible information set, wherein we analyze levels of all putative SR proteins. Given that SR is so central to cardiac function, our need for resolving the SR proteome is great. Mammalian secretory compartments have been examined from multiple tissues and species using standard membrane purification protocols (16, 17). However, the cardiac myocyte has received little research attention from non-cardiac cell biologists, leaving it to cardiac biologists, who have primarily remained focused on a small group of proteins proven to have direct effects on Ca cycling. These proteins have been the subjects of large numbers of reviews over the past decade.

The proposed research also breaks from the standard nonmuscle approach to combine powerful proteomic procedures by leveraging the actions of important Ca-handling properties of SR that define the SR organelle. As this remains the focus of heart researchers, this novel cell biological approach will mesh

SR research with the wider knowledge of ER subcompartments that continues to reveal new ER-related concepts to researchers of nonmuscle tissues.

Data generated by this work will provide the first global semi-quantitative analysis of all SR proteins along with connected ER/SR subcompartments. Important functions of ER, such as protein translation, protein translocation across the ER membrane bilayer, and N-linked glycosylation, and many others, will be viewed in a context of their relationships with important SR functional complexes. The analysis, described for a set of preliminary data, will be used to show that SR contains a wide set of protein complexes known to carry out specific functions in ER. Semi-quantitation will permit the first comparison of Ca-handling functions with other known ER functions, based upon relative abundances for the 354 SR-enriched proteins revealed thus far, along with common patterns of enrichments during SR membrane enrichment.

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C. APPROACH

C.1 Specific Aim 1: Determination of the canine SR proteome

In this section, we describe the experimental analyses that will be carried out for each sample of heart tissue. Methods discussed under this Specific Aim will be used to properly characterize the proteome for *control canine cardiac SR*. The experimental approach described here will be applied to determine the proteome of *canine SR from failed heart* (Sect. C.2), then to *human cardiac SR* (Sect. C.3) to bridge an important gap between canine and human SR protein composition. With *preliminary data* from control mongrel canine heart, we demonstrate the procedures for obtaining protein sequences, and apply an intuitive sorting of proteins that identifies 354 proteins with enrichment patterns that show them to be residents of cardiac SR subcompartments, and further resolves protein complexes into putative constituent ER/SR subcompartments.

C.1.1 Preparation of purified SR membranes

When heart tissue is homogenized using a blender, cell membranes shear into membrane vesicles (MV), constituting a microsomal fraction that can be highly enriched by differential centrifugation. In the 1960's and 70's, scientists studied the transport of $^{45}Ca^{2+}$ into microsomes to understand how this membrane fraction from heart and skeletal muscle was able to relax muscle, and eventually determined that an active Ca-ATPase was actively translocating Ca to the interior of the vesicle, reviewed in (18, 19). Over subsequent decades, analysis of microsomes and SR vesicles by SDS-PAGE analysis, protein sequencing, and later cDNA cloning, led to the identification of a dozen or so SR proteins, which remained widely studied to this day (3).

Methods for SR purification by selective Ca oxalate loading was initially developed as a method for separating sarcolemma and sarcoplasmic reticulum membranes (Fig. 1) (8). The key isolation step centers on the property of SR vesicles to specifically accumulate Ca oxalate within the lumen (20) because of their high SERCA activity and low rate of Ca leak. Following Ca oxalate loading, SR vesicles obtain an increase in density that permits their isolation on density gradients (8). The basic technique was extended in a paper by the Applicant and Larry Jones (9) that showed that two subfractions of membrane vesicles specifically seen following SERCA activation represented two subpopulations of SR membrane (Fig. 2). These two densest membrane fractions also differed sharply in sensitivity to 1 mM ryanodine (Fig. 2A). The hypothesis that these purified membrane fractions corresponded to free and junctional SR subpopulations was substantiated in ensuing years through studies of several free and junctional SR proteins and markers (4, 8, 9, 14, 15, 21-24). For example, cardiac triadin and junctin were first purified from these junctional SR vesicles, and their enrichment patterns were central to their validation as calsequestrin-2 binding proteins (5, 6, 25, 26).

Heart tissue Left ventricular tissue (posterior wall) from five control canine hearts will be analyzed. Preliminary data discussed in this proposal was acquired using fresh tissue from a single control canine heart. In previous studies, we have determined that fresh and frozen canine heart tissue yielded indistinguishable microsomal preparations, except for a greater yield of microsomes from frozen tissue. Microsome preparations typically recover only a small portion (~5%) of SR proteins present in the tissue, the remainder remaining associated with the low-speed pellet (10K x g). Frozen tissue produced an approximate 50% increase in the yield of microsomes with no discernable differences in protein patterns visualized by protein stains (Coomassie blue or Stains All), or selected immunoreactivities (preliminary data, not shown). The reasons for increased microsomal yield have not been further investigated. Use of frozen

tissue throughout this study represents a translational advantage since human tissues are generally available only in frozen form. Heart tissue from control dogs, HF dogs, and control human tissue previously obtained stored in liquid N2. These canine heart tissues (and HF tissue, *Sect. C2*) were previously analyzed for a number of biochemical indices (27).

Canine ventricular tissue will be thawed on ice (from liquid N2) for 15 min, then homogenized 1:20 in 10 mM NaHCO3 using a Polytron homogenizer. Crude microsomes (MV) will be prepared by differential centrifugation (10K x g clearing, followed by 70K x g pellet), then actively loaded with Ca oxalate and layered on a discontinuous sucrose density gradient and centrifuged for 4 h at 70K x g for 3 h using a SW27 Beckman rotor. Microsomes will be harvested from the 1.0 M sucrose layer (ρ = 1.13) and 1.5 M sucrose (ρ = 1.19) and re-sedimented for biochemical analyses. These preparations require as little as a few grams of heart tissue.

Fig. 1. Isolation and biochemical analysis of cardiac membrane vesicles (MV) *Modified from Jones et al., (8)* Fractionation of cardiac microsomes following active Ca-oxalate loading by density-gradient centrifugation (*left panel*). Ca uptake is activated by 1 mM ATP, along with Ca, Mg, and 10 mM potassium oxalate, pH 7,4 (8, 9). The increased density that results with SERCA activation (*+ATP*), drives a change in vesicular density specifically inside SR membrane vesicles. Lowest density membranes are enriched in sarcolemmal markers shown, but denser two membrane vesicle (MV) subfractions (D and E) are enriched in SERCA activity, with minimal contamination by plasma membrane vesicles (*right panel*).

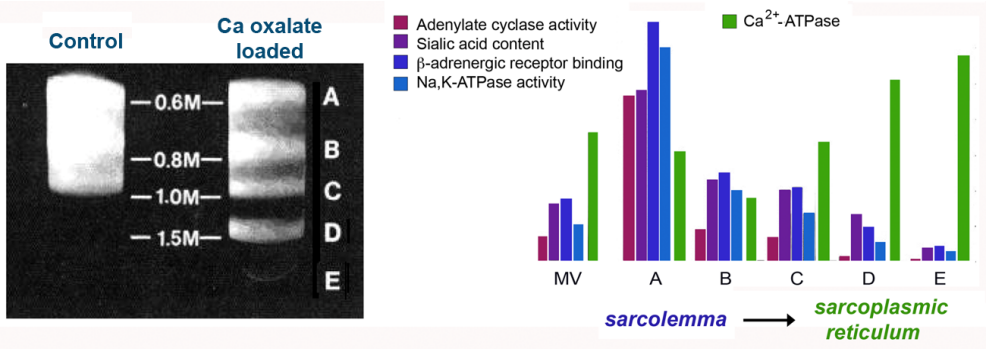
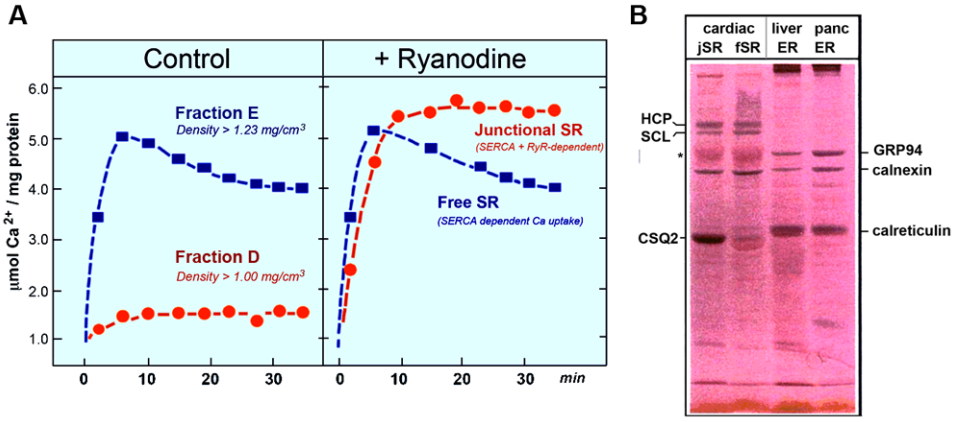


Fig. 2 Functional heterogeneity of cardiac SR vesicles. A (*modified from Jones and Cala (9)*) After harvesting membranes from sucrose gradients following Ca oxalate loading, one can still measure $^{45}Ca^{2+}$ transport (9). Addition of 1 mM ryanodine, a concentration that blocks the cardiac ryanodine receptor, only Fraction D exhibits a large increase in accumulated Ca oxalate, suggesting that membrane vesicle fractions D and E are comprised of junctional and free SR vesicles, respectively. **B (***modified from Cala et al. (4)*) studies carried out by the Applicant and others showed that cardiac SR subfractions contained major resident ER proteins (labeled on *left side*), along with luminal proteins that are resident to SR. Here it is shown that many can be highlighted in *blue* using the Ca-binding protein dye Stains-All. Abbreviations: HCP, histidine-rich Ca-binding protein; sarcalumenin (SCL), CSQ2, calsequestrin-2.



C.1.2 Determination of protein content, abundance, and enrichment in SR membranes

Following isolation and analysis of MV and SR subfractions by SDS-PAGE and in-gel trypsinolyisis, peptides will be analyzed by tandem mass spectrometry (MS/MS) (Fig. 3). MS/MS is a method for structure determination and analysis of molecules. Tryptic peptide ions, selected by MS1, collide with a high pressure gas and undergo fragmentation. The collision process is called collision-induced dissociation (CID). The resulting daughter ions are analyzed in MS2. MS/MS has proven extremely useful in sequence determination of peptides due to the formation of abundant daughter ions in the CID process.

Equal amounts of protein (Lowry assay) will be digested using trypsin. *In-gel digestion* begins with SDS-PAGE analysis of 20 µg each of the initial crude fraction of canine cardiac microsomes, junctional SR

(1.0 M sucrose, ρ = 1.13), and free SR (1.5 m sucrose, ρ = 1.19) in three separate lanes, and gel stained with SYPRO Ruby (Fig. 3). Each lane will be cut into 20 gel slices, destained and dried, then trypsinized. In studies using *in-solution digestion*, the same three membrane fractions will be solubilized in lysis buffer, then proteins precipitated in cold acetone. Protein pellets (25 μg) will be solubilized in 8M urea, 0.4% SDS in 250 mM TEAB. After reduction and cysteine blocking, trypsin will be added to the diluted sample solution (1:10 w/w) for overnight digestion at 37°C.

In LC-MS/MS, tryptic peptides will be separated by reverse phase chromatography (Magic C18 column, Michrom), followed by ionization with the ADVANCE ion source (Michrom), and then analyzed in an LTQ-XL mass spectrometer (ThermoElectron). For each MS scan, up to seven abundant peptide species will be fragmented with CID. Data analysis will be performed using Proteome Discoverer1.1 (Thermo) which incorporated the Mascot algorithm (MatrixScience). The NCBI database was used against mammalian protein sequences and a reverse decoy protein database will be run simultaneously for false discovery rate (FDR) determination. Secondary analysis will be performed using Scaffold (Proteome Software). A fixed modification of +57 on cysteine (carbamido methylation) and variable modifications of +16 on methionine (oxidation) and +42 on protein-termini (acetylation) were included in the database search. Minimum protein identification probability was set at ≥ 95% with 1 unique peptide at ≥ 95% minimum peptide identification probability.

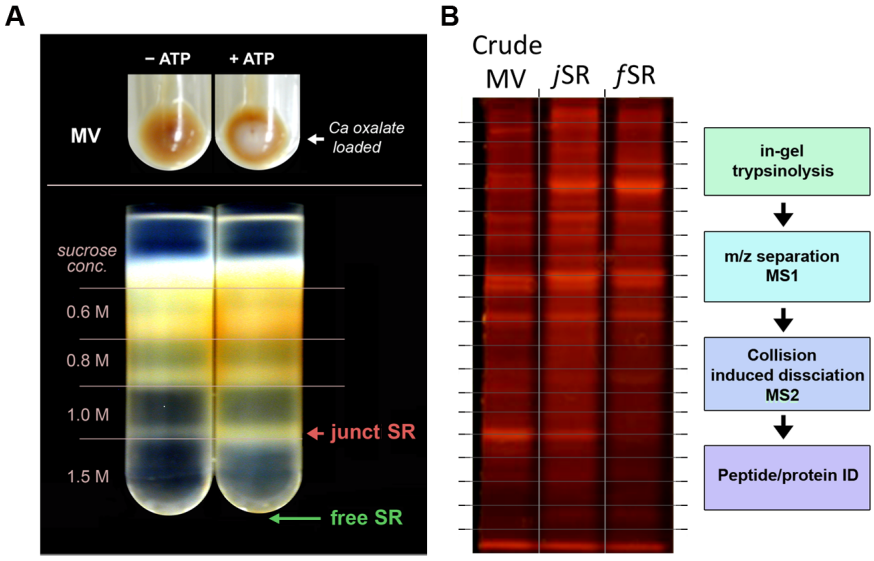


Fig. 3 Overall approach to in-gel trypsinolyisis and tandem mass spectrometry (Preliminary data)

A, *Upper half,* Crude membrane vesicles (MV) were isolated, then incubated under Ca loading conditions without ATP (-ATP) or +ATP. A white Ca oxalate precipitate inside SR vesicles appears as a whitish MV pellet, following activation of SERCA. *Lower half*, separation of vesicles on a discontinuous sucrose gradient shows banding of purified junctional SR at 1.0 M sucrose layer (ρ = 1.13), and pelleting of heavily-loaded free SR vesicles 1.5 M sucrose (ρ = 1.19) (pellet not seen in figure). **B**, Samples (20 μg) of initial MV, junctional (jSR), and free (fSR) are electrophoretically separated on a 7.5% acrylamide gels, then stained with SYPRO ruby. Subsequent steps of the mass spectrometric analysis are schematically illustrated on the right.

C.1.3 Data analyses

The types of analyses discussed in this section are somewhat unique to SR membranes because of the added biochemical separation based upon SERCA activity. The additional sensitivity to ryanodine provides a second unique separation/enrichment step that permits a unique pattern of protein enrichment characteristic of its sarcomeric role. Therefore, beyond the relative *abundance* of each SR and ER protein, we are also able to measure patterns of protein enrichment that reflect the functional ER/SR subcompartments involved in Ca homeostasis.

C.1.3.1 *SR protein abundances*

Quantitation of relative protein abundances is based upon recovery of tryptic peptide spectra. The useful (semi-)quantitiation of relative protein abundances requires an approximate normalization to protein length, dividing spectra by protein molecular weight (MW) (Fig. 4). Evaluations of protein enrichments between two fractions, on the other hand, are independent of MW, and can reflect components of a single protein complex or co-enrichment in the same vesicles. These features are illustrated for SERCA and its binding partner *phospholamban* for which tryptic peptide spectra will differ greatly, attain a more semi-

quantitative relative enrichment once corrected for molecular weight, and exhibit similar enrichment in free SR compared to starting MVs (Fig. 4).

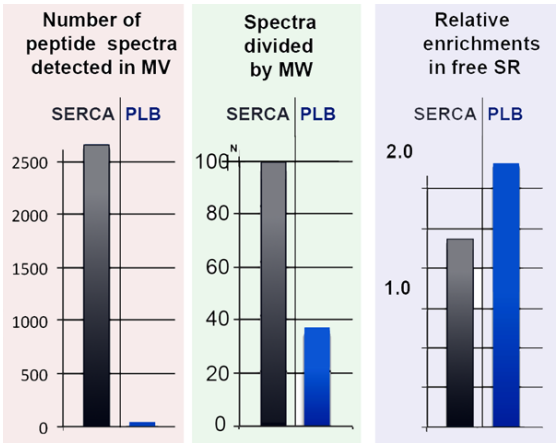
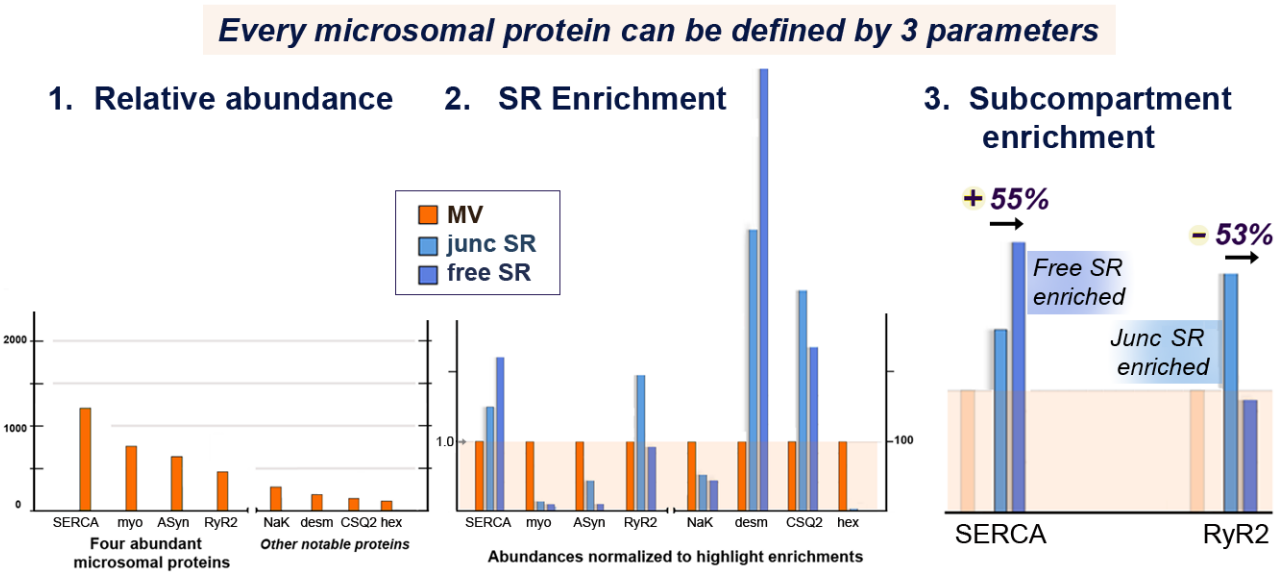


Fig. 4 (Preliminary data) ***SERCA2*** generates the most peptide spectra of all other membrane proteins, roughly 65-fold more than were identified for phospholamban (PLB) (*left plot*). However, correcting for the vast difference in molecular weight (MW) for SERCA and PLB (110 kDa versus 6 kDa), leads to relative levels that are more similar. Meanwhile, enrichment of SERCA and PLB proteins in SR (compared to crude MV) is even more similar between the two proteins. Abundance of a protein is independent of its enrichments, and enrichments are likely to be a better indication that two proteins reside in the same subcompartment.

C.1.3.2 *SR protein enrichments*

For every protein identified by MS/MS analysis of tryptic peptides, it is its enrichment in the denser sucrose gradient layers that define it as an *SR-enriched protein* (Fig. 5). If a population of SR (or ER) in the starting microsomal fraction (MV) is completely recovered in membrane vesicles at densities $\rho > 1.13$, it would theoretically enrich roughly 5-fold, assuming that the denser membranes contain about 20% of the protein in the starting material, as previously suggested (9). This is only an approximation, however, since a low abundance ER/SR subcompartment might exhibit greater levels of Ca oxalate accumulation than the average SERCA-containing vesicle. Based upon preliminary data (discussed in the next section, Sect C.1.3.3), we will use a value of $SR_{total}/MV = 2.0$ as cutoff for determining a microsomal protein as derived from SR. Based upon a graphical analysis of SR protein abundance and enrichment (discussed in Sect. 1.3.3.2 *below*), a more inclusive set of proteins from which to sort will maximize the potential for discovery, whereas our graphical analyses should aid in the invalidation of non-SR proteins. Empirically, we found that a 2.0 cutoff captured every SR protein of which we are aware.

Fig. 5 Three independent indices of SR proteins derived from MS/MS analyses of MV and SR protein tryptic digests Our analyses will generate 3 data points for every microsomal protein: its relative abundance (*panel 1*), its enrichment in SR (percent recovered in more dense layers) (*panel 2*), and enrichment in free SR compared to junctional SR (*panel 3*). Vales are shown for selected proteins: Abbreviations, *myo*, myosin; *ASyn*, ATP synthase; *NaK*, Na,K-ATPase; *desm* desmin; *hex*, hexokinase. Only bona fide SR proteins show enrichment (*panel 2*), so that non-SR proteins in crude membranes are removed from consideration. A requirement that a protein be at least 2-fold enriched in SR ([jSR+fSR]/MV) selects 354 out of 1100 (see Fig. 6). Plotting proteins based upon their enrichment in free SR compared to junctional SR sorts *positively* enriched (free SR) from *negatively* enriched (see Fig. 7).



C.1.3.3 *Sorting of cardiac microsomal proteins based upon these empirical-determined parameters (Preliminary data)*

C.1.3.3.1 Preliminary Data: Abundance versus enrichment in SR

For each protein identified in cardiac microsomes, its SR enrichment (SR_{total}/MV) will be a key determinant of its identity as an SR protein. Here, SR_{total} refers to the sum of levels in junctional and free SR. Using a cutoff of SR_{total}/MV = 2.0, generates a set of 354 proteins (of 1100 proteins present in MV) that exceed this cutoff value (Fig. 6). Thus, about one-third of microsomal proteins (354/1100) were enriched > 2.0 fold in these preliminary data. Further sorting and analysis of these 354 SR proteins are discussed below, where we extend our sorting to a *third* parameter, enrichment in free SR compared to junctional SR.

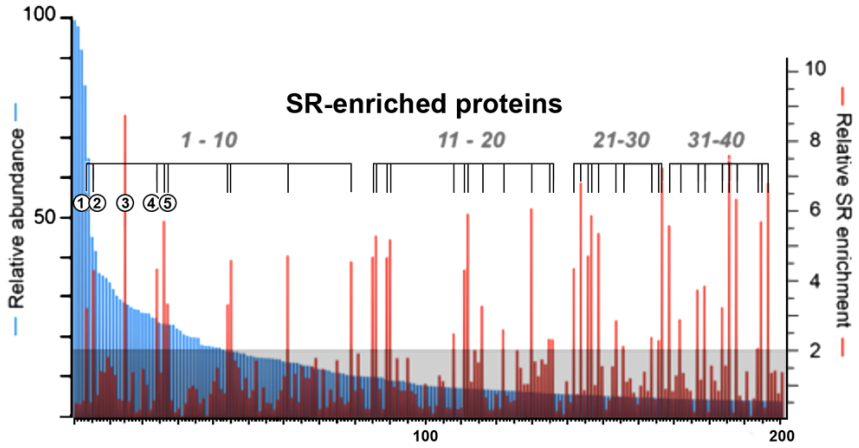


Fig. 6 Abundance versus enrichment in SR Relative abundances (*smooth curve of blue values*) *and* relative SR enrichment (SR/MV, *randomly spaced red values*) for the first 200 (of 1103 proteins discovered in MV). Values of relative SR enrichment > 2.0 extend above the shaded area. Of the 200 proteins shown here, only 40 (see top scale) qualify as SR enriched. The same analysis applied to all 1103 proteins generates a list of 354 SR-enriched proteins. The first 5 SR proteins highlighted in red (designated with circles) are: SERCA, sarcalumenin, desmin, calsequestrin-2, and phospholamban. The first 3 most abundant proteins (in blue) are ERGIC-53, isocitrate dehydrogenase, and pyruvate dehydrogenase kinase-2; however, these 3 proteins were strongly de-enriched in subsequent steps of SR membrane purification. Protein abundances have been factored for molecular weight (cf. Fig. 4).

C.1.3.3.2 Preliminary Data: Subcompartment enrichment of SR proteins

Just as we originally reported based upon ryanodine sensitivity (9), our SR purification protocol generates two new membrane subfractions that correspond to junctional and free SR subcompartments, based upon relative levels of several standard SR markers. Distribution of just the known major SR and ER proteins in terms of free SR enrichment shows how this parameter divides proteins into free SR enriched and junctional SR enriched (Fig. 7).

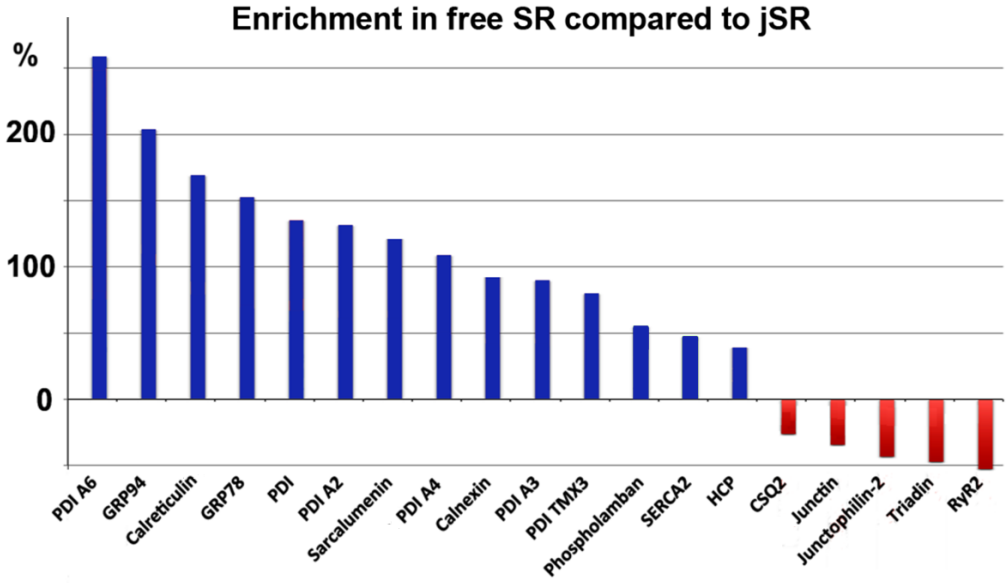


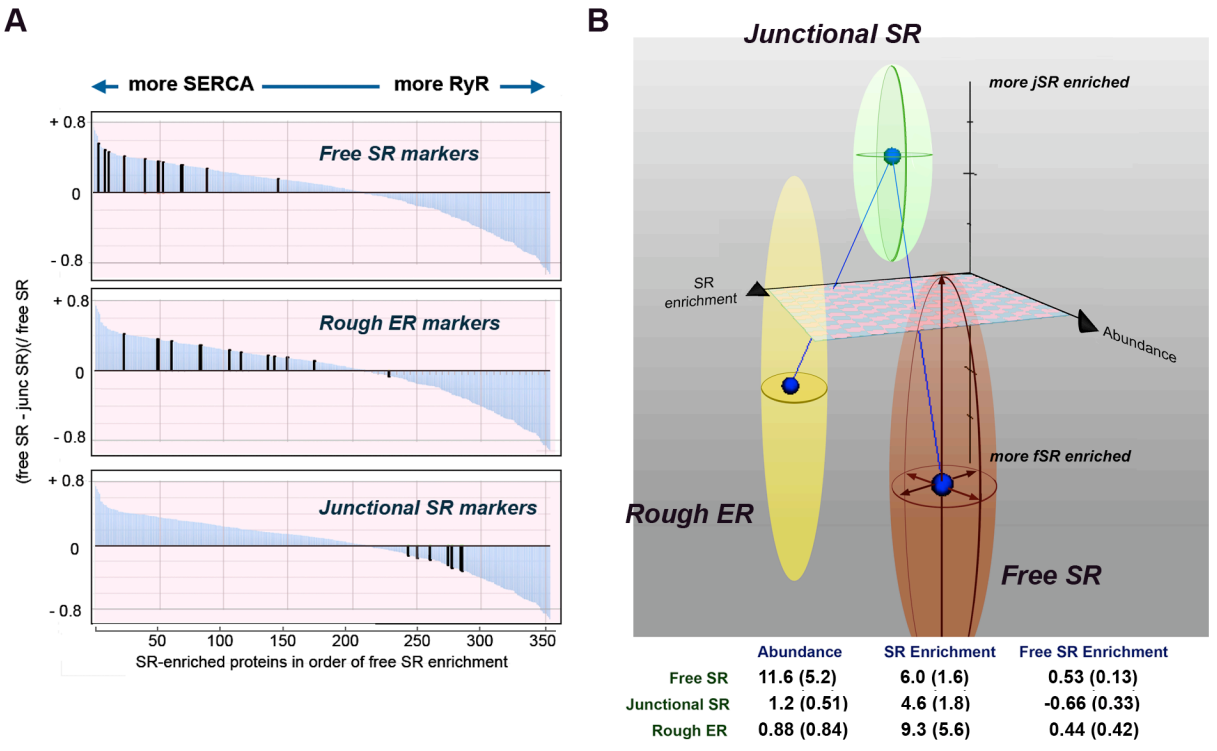
Fig. 7 Free SR (fSR) enrichment compared to junctional SR (jSR) enrichment When SR protein levels are calculated as follows, (jSR-fSR)/ jSR, an interesting pattern is seen. All previously identified jSR proteins are negative values, and all known fSR proteins are positive values, as expected if the protocol enriches the two subcompartments as intended.

C.1.3.2.1 Preliminary Data: ER/SR compartmentation as viewed graphically (based upon biochemical properties of individual proteins

Besides developing an important data set for future investigations, graphical representations of proteins will reflect functional subcompartmentation, based upon the three biochemical parameters determined for every SR protein (Preliminary data, Table I). Putative ER/SR subcompartments can be visualized as 3-D volumes with boundaries defined by the respective variances (Fig. 8). Three of the most well characterized ER/SR subcompartments are viewed as cleanly separated graphical clusters. Rough ER proteins are relatively low in abundance, relatively high in SR enrichment, and free SR enriched over junctional SR. Meanwhile, free SR and junctional SR are also cleanly delineated (Fig. 8).

This graphically defined ER/SR subcompartmentation may provide a useful construct with which to predict subcompartmentation of novel SR-enriched proteins. The positions of these clusters in 3-D space may also represent a useful, and potentially functional, perspective from which to assess changes in HF tissue, or to compare canine versus human functionality. Addition of ryanodine to microsomes during Ca oxalate loading will only shift the position of the junctional SR cluster, causing bona fide junctional SR proteins to co-enrich with free SR membranes (cf. Fig. 2A).

Fig. 8 Graphical analyses of SR proteins With 3 relatively independent parameters determined for each protein in cardiac microsomes, one could evaluate proteins using one or more combinations of these parameters. **A**, Proteins plotted in order of highest-to-lowest free SR enrichment (light blue curves, also compare Fig. 7). ***Free SR marker***s indicate values for ER resident proteins that contain a C-terminal KDEL retention sequence (known to reside in free SR (4)), ***Rough ER markers*** that include the translocon channel protein complex (sec61α, TRAPα, signal peptidase, and oligosaccharide transferase (OST) subunits), and ***Junctional SR markers*** that include ryanodine receptor-2 (RyR), calsequestrin-2, triadin, junctin, and junctophilin-2. This sorting effectively sorts proteins from high SERCA/low RyR to low SERCA/high RyR. **B**, schematic representation of how these same 3 ER/SR subcompartments would distribute in 3-D space. Blue spheres represent the means of the values for SR abundance, SR enrichment, and free/junctional SR enrichment (Z-axis). The colored spheres represent roughly the variance for the 3 parameters. Note that panel A is essentially a projection of panel B, *without abundance*. Arrows shown inside Free SR cluster indicate the standard deviation calculated for the three variables (see Table under panel B with mean values including S.D. in parentheses). The graphical representation shows, for example, how rough ER proteins are less abundant than SR proteins, but somewhat similar to free SR proteins in overall SR enrichment, and free-to-junctional SR enrichment. Variances (S.D.) can be employed to predict subcompartmentation of novel ER/SR proteins. Several additional functional clusters can be generated. Plots will be generated using Mathematica (Wolfram).



	Abundance	SR Enrichment	Free SR Enrichment
Free SR	11.6 (5.2)	6.0 (1.6)	0.53 (0.13)
Junctional SR	1.2 (0.51)	4.6 (1.8)	-0.66 (0.33)
Rough ER	0.88 (0.84)	9.3 (5.6)	0.44 (0.42)

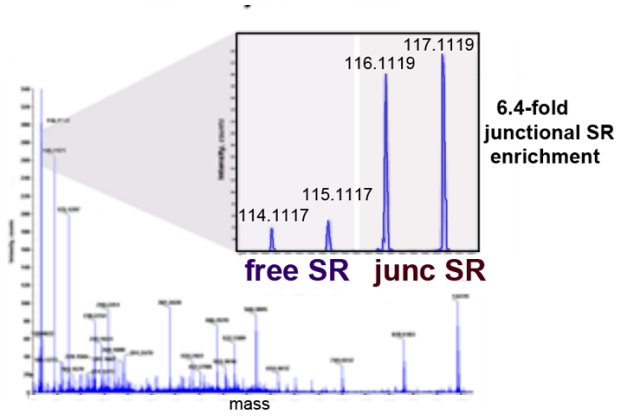
Table I Top 25 SR proteins (and select others) out of 354 SR-enriched proteins, based upon total abundance in SR. Also listed is molecular weight (MW) in kDa, total abundance in starting microsomes (MV) , SR enrichment (jSR +fSR)/MV, and free SR (fSR) enrichment (jSR-fSR/fSR), a value that is negative for junctional SR (jSR) proteins.

Order	PROTEIN	MW	Total MV abundance*	Total SR abundance	SR enrichment	Free SR enrichment
1	SERCA2	110	10.40	35.90	3.45	0.38
2	desmin	53	3.63	34.40	9.48	0.42
3	sarcalumenin	54	5.68	26.29	4.63	0.59
4	phospholamban	6	2.96	18.27	6.18	0.40
5	calsequestrin-2	47	3.15	14.74	4.69	-0.35
6	NADH-cytochrome b5 reductase 3	34	2.95	10.60	3.59	0.28
7	NADPH--cytochrome P450 reductase	7	2.10	10.38	4.96	0.32
8	ADP-ribosylation factor-like 6-interacting protein 5	22	1.76	8.97	5.10	0.46
9	CDP-diacylglycerol--inositol 3PtIns-transferase	11	2.11	7.55	3.57	0.55
10	vesicle-assoc memb protein-associated protein B	23	1.30	7.44	5.72	0.47
11	fat storage-inducing transmembrane protein 2	27	1.19	6.65	5.60	0.57
12	alpha-crystallin B chain	30	1.35	6.64	4.91	0.45
13	GRP78	20	1.30	6.58	5.05	0.13
14	junctin	72	1.23	6.21	5.04	0.66
15	SERCA1	23	0.94	5.99	6.40	-0.42
16	transmembrane protein 109	110	0.75	5.50	7.37	0.37
17	memb-assoc progesterone receptor component 2	26	0.84	5.49	6.57	0.06
18	vimentin isoform 12	30	0.64	4.99	7.82	0.33
19	malate dehydrogenase, cytoplasmic isoform 1	54	0.58	4.87	8.37	0.54
20	peptidyl-prolyl cis-trans isomerase B	36	0.74	4.70	6.35	-1.89
21	peroxisomal multifunctional enzyme type 2	24	0.95	4.43	4.65	0.14
22	ankyrin-1	80	0.28	4.32	15.40	-1.74
23	dehydrogenase/reductase SDR fam. member 7C	18	0.73	4.25	5.80	0.09
24	vesicle-assoc memb protein-assoc protein A	35	0.44	4.12	9.34	0.32
25	calcium-binding protein p22	22	0.43	4.02	9.32	0.30
37	GRP94	28	0.55	4.06	7.36	0.45
41	malectin	93	0.53	3.04	5.74	0.70
42	calnexin	32	0.33	2.79	8.47	0.24
43	rab-2A	68	0.41	2.78	6.79	0.53
46	alpha-actinin-2	24	1.32	2.69	2.03	-0.15
56	junctophilin-2	104	0.60	2.49	4.15	0.15
57	signal peptidase complex subunit 2	46	0.69	2.13	3.07	-0.59
58	translocon-associated protein subunit delta	25	0.21	2.08	9.72	0.28
63	ryanodine receptor 2	19	0.52	2.04	3.95	-0.16
64	long-chain fatty acid transport protein 1	565	0.78	1.94	2.48	-1.01
86	caveolin-1	71	0.35	1.94	5.51	0.29
93	FKBP12.6	21	0.65	1.59	2.46	-1.12
119	TRAPα	12	0.32	1.52	4.73	-0.17
142	calreticulin	32	0.16	1.09	7.02	0.42
160	triadin	48	0.10	0.88	8.48	0.70
171	caveolin-2	78	0.12	0.75	6.16	-0.94

* arbitrary units, total number of peptide spectra divided by MW; other abundances calculated similarly

C.1.4 Quantitative proteomic analysis by iTRAQ labeling

A widely accepted quantitative approach to determining levels of CSQ2 in SR preparations employs iTRAQ methodology (28, 29). With iTRAQ, tryptic peptides of SR proteins from multiple samples (up to eight) are separately labeled in solution using specialized reporter groups that result in a single Dalton shift, then analyzed together as a single sample (Fig. 9). Beginning with our same three samples MV, junctional SR, and free SR gradient fractions, we will compare levels of multiple SR proteins to independently evaluate their relative levels following in-solution tryptic digestion (Sect. C.1.2). Use of this technology in each specific aim will permit a high degree of validation of relative levels, often capable of 100-200 validations in a single iTRAQ experiment. Using this separate experimental approach of in-solution protease digestion provides quantitation that independent of protein and peptide recoveries between two or more samples. It also provides critical validation of particular differences found in control versus diseased heart tissue, discussed below for Specific AIms 2 and 3. Statistical significance for label-free GeLC and iTRAQ data will be determined using ANOVA.



Our analysis shown here, carried out for CSQ2, measured a 7-fold relative enrichment in junctional versus network SR samples (Fig. 9). GeLC analysis produced a difference of 1.3-fold (Table I). The discrepancy was unusually high for calsequestrin compared to 80 other proteins so far examined.

Fig. 9 Preliminary iTRAQ analysis of CSQ2 levels One of four separate iTRAQ reagents having identical iso-charge/mass ratios were used to individually treat 2 different free SR and 2 different junctional SR samples. Samples were mixed, and CSQ2 peptides were analyzed by LC-MS/MS. iTRAQ moieties generate CSQ2 peptides differing by 1.000 Da (isotopic difference). Simultaneous analyses of all junctional SR and free SR permit determination of all protein differences between two or more samples.

C.1.5 Expected outcomes

Based upon our preliminary data and analysis, our proteomic data will greatly extend the known protein structure of cardiac SR, as well as its possible functional connections. Decades of data generated by immunoblotting of these and other cardiac membrane fractions have principally examined only a handful of proteins. While very important for SR and for cardiac function, this small group of abundant proteins is shadowed by a huge repertoire of roughly 354 proteins. A large proportion of these proteins are highly enriched in SR. Abundances may range, but the findings of multiple members of a single known complex, support the idea that *cardiac ER (aka SR) is the same organelle as in nonmuscle, but with high levels of Ca-handling proteins; as opposed to the idea that cardiac ER and SR are separate subcompartments*. Much additional analysis of the basic proteome of the canine heart ER/SR should clarify the component activities of multiple subcompartments, while identifying the precise protein isoforms that exist in the heart.

The list of cardiac SR proteins will be an exciting resource for the cardiac research community. Interspersed with well-studied SR Ca-handling protein complexes will be proteins that reflect important new functions of cardiac ER/SR, including proteins that serve as the very “building blocks” of ER and SR tubules and cisternae. The data will also establish relative levels of newly localized complexes involved in functions known outside of cardiomyocyte biology, but new to cardiac biologists. Many researchers will use these data to provide critical support for their hypotheses.

C.1.6 Alternative outcomes and potential pitfalls

It will be important to analyze membrane fractions from control sucrose gradients (absent SERCA activation) to assure that all proteins are generated by the activity of SR Ca2+-ATPase. This work has not been carried out, although earlier work has shown that all major bands seen by SDS-PAGE and protein staining are seen only after Ca oxalate loading (S. Cala and L. Jones, personal observations and Ref (9)). Our LC-MS/MS analysis will be carried out on samples prepared from “no ATP” controls to determine bands

that are enriched on sucrose density gradients naturally. This could include mitochondrial fragments and peroxisomes. Proteomic analyses of these cellular compartments have recently been reported (30, 31), which along with our data will be used to test the validity of true SR co-localization.

In addition to the required analysis form control sucrose gradients, *two* new techniques will be applied to elucidate possible contamination from non-SR membranes. First, as shown in Fig. 2, addition of 1 mM ryanodine during the Ca oxalate loading step specifically activates loading of putative junctional SR compared to other membranes, including putative free SR membranes. We have previously used such a ryanodine-dependent density-shift to purify junctional SR (14, 15). Converting junctional SR vesicles from density of 1.13 to a density of 1.19 by ryanodine will be a powerful determinant of validity.

A second technique useful for validating SR subcompartmentation will be use of thapsigargin during Ca oxalate loading, to assure that SERCA alone is responsible for the density shifts and subsequent SR protein isolation. Thapsigargin is a potent and irreversible inhibitor of SERCA isoforms (32).
Our preliminary data show slight to moderate SR enrichments of a small set of mitochondrial proteins. Interestingly, the putative mitochondrion-SR tether mitofusin-2 (33), while present in cardiac MVs (380th most abundant cardiac MV protein overall), did not show significant enrichment in SR vesicles (SRtotal/MV=1.3; our cutoff is 2.0). We have also found very large enrichments of a large set of peroxisomal proteins in the ρ= 1.13 layer, but whether this density requires Ca oxalate or can be enriched by ryanodine-dependent Ca loading will require further analysis.

The data discussed in this proposal are, therefore, directed at determining SR content as defined by SERCA content and Ca oxalate accumulation. While it will be possible that select ER/SR subcompartments could be excluded from these protocols, our preliminary data and previous publications (4, 14, 15, 25) suggest that SR membranes are quantitatively recovered in these two new high-density membrane vesicle fractions, along with major luminal ER proteins. SR-enrichment of every known protein complex associated with rough ER, suggests that cardiac SR and cardiac ER and synonymous terms and concepts.

C.2 Specific Aim 2: Comparison of SR proteome from control and failed canine heart tissue

When investigators compare protein levels in two or more tissue samples, they will generally normalize measured levels to a control protein not expected to change. Our proteomic approach will essentially employ hundreds of controls, in that changes will be evaluated for large numbers of proteins, most of which will remain unchanged.

Heart samples Hearts were previously obtained and analyzed from animals placed into heart failure by two different methodologies (27), as well as from control dogs. Ischemia-induced HF was induced by multiple sequential intracoronary embolizations with microspheres were performed in mongrel dogs 1-3 weeks apart over 6 months, and were discontinued when left ventricular (LV) ejection fraction was ≤35, and animals were sacrificed after 3 additional weeks. For tachypacing-induced, LV dysfunction was induced by rapid ventricular pacing in mongrel dogs at >180 beats/min for >4 weeks to produce HF (ejection fraction ≤ 35%), and animals were generally sacrificed within 1-3 weeks. Left ventricles were cut into small pieces before freezing in liquid N2.

Samples from each of five failed canine heart samples will be evaluated to compare SR protein levels with five control (normal) samples. These heart tissue samples were recently used to determine glycosylation changes in calsequestrin-2 (27). In that study, a common dramatic pattern of change was seen, whether the underlying cardiomyopathy was induced by tachypacing or ischemia (27). We will evaluate the entire set of 1100 microsomal proteins, with focus on 354 SR-enriched proteins identified in the control heart tissue. We expect to find the very same set of SR-enriched proteins, for which levels in total SR protein/levels in MV > 2.0 (cf. Fig. 6). Therefore, beyond a simple change in levels, we will determine whether the same set of (354) proteins exhibit SR enrichment, and whether changes found for individual proteins point to a wider change in ER/SR subcompartment (cf. Fig. 8). Where t-tests and ANOVA statistical tests are applied, we will seek consultation from University statisticians.

SR protein changes in failed tissue compared to control heart tissue are expected to include the following:

1) *Changes in levels of a few individual proteins*, but without similar changes for other proteins having similar enrichment patterns (e.g., SERCA changes in the absence of equivalent changes in other free SR proteins). This is the type of data that is typically generated using immunoblotting. One advantage of our approach will to generate data for all 354 SR-enriched

proteins simultaneously. The change in levels of all 354 proteins will scatter above and below zero, but will establish an average basal change from which to visualize and determine a wide view of change for a few specific proteins. This approach will allow a unique quantitative assessment of global SR protein change in cardiomyopathy. T-tests and ANOVA will be used to gauge the significance of changes.

2) *Changes in the enrichment patterns of ER/SR subcompartments.* Our analysis allows us to determine whether there is change in the enrichment patterns of large sets of protein data. We can determine the variances in levels of proteins that are presumed or determined to derive from the same ER/SR compartment (see Fig. 8). The hypothesis that such enrichment patterns can change, reflecting cardiomyopathic adaptation, should be tested since global changes in cardiomyocyte SR function are widely described (3).
3) *Changes in ER metabolic functional groups and interactomes* Further mathematical sorting and graphical visualization schemes are anticipated, as data is acquired. Future informatics analyses will factor associations based upon common ER/SR functions, such as Ca^{2+} homeostasis, Ca^{2+} release, Ca^{2+} uptake, signaling, lipid biosynthesis, mitochondria, etc. Identification of ER/SR interactomes might also highlight novel changes in HF (34). Such a complete analysis has recently been published for the mitochondrial proteome (31). The ability to combine such abundance and enrichment patterns with functional ER/SR protein interactomes may provide additional perspectives into human myocardial disease. Post-hoc t-tests may be used to assess the possible effects of HF on changes described in 1) and 2).

C.3 Specific Alm 3: Comparison of SR proteome from human heart and canine heart

Canine heart has served as an important model for biochemical and physiological studies of human heart. Knowledge of the SR proteome of human tissue would advance progress in translational research by providing protein markers for disease that tie to the canine model. At the same time, bridging the important structural divide between these two mammalian tissues can add to the understanding of the functional importance of single proteins and protein complexes present in myocardial tissue (16, 31, 34). The first step of such work involves the basic demonstration that putative junctional and free SR subfractions can be isolated from frozen human heart tissue. Purification of human SR subfractions on sucrose gradients will be assayed using selected SR markers established in dog heart studies (4, 8, 9, 14, 15, 21-23). Once SERCA-activated Ca oxalate loading, and ryanodine-receptor dependent leakage is established, we can initiate proteomic analyses.

Heart samples Human heart tissue from The National Disease Research Interchange (NDRI)/The National Resource Center (NDRI#0050260), is currently stored in liquid N2. Post-mortem hearts were certified free of major human pathogens and free of major (overt) heart disease. Data provided by NDRI includes age, sex, major known disease, limited health information, and prescribed medicines. Hearts were received frozen, and thawed enough to generate small pieces for subsequent freezing in liquid N2. Samples from each of five human heart samples will be roughly age matched within available limits, and SR proteomic analyses performed as described in Sect. C.1.

The first component of this specific aim is to demonstrate that human cardiac microsomes can be Ca oxalate loaded to achieve changes in SR vesicle density, and that higher-density membrane vesicles can be isolated, as well as fractioned into ryanodine-sensitive and ryanodine-insensitive fractions. While such uptake assays generally the original work required an assay of $^{45}Ca^{2+}$-uptake, establishment of several subcompartment marker proteins, we will instead characterize membrane enrichments based upon SDS-PAGE, Stains-All protein staining, and immunoblotting data (4, 8, 9, 14, 15, 21-23).

Samples of frozen human left ventricular tissue will be homogenized, and crude microsomes isolated as performed for canine left ventricular tissue. Ca oxalate loading and sucrose density gradient-centrifugation will be carried out, vesicle isolated, and equal amounts of MV will be compared with fractions collected in 1.0 M and 1.5 M sucrose, as before. Immunoblotting will be performed to determine whether marker proteins such as SERCA, calsequestrin-2, and TRAPα are enriched as expected. We expect enrichment patterns that are similar to membranes prepared from canine heart tissue based upon early work on different mammalian species (for example, ref. (35)).

Following alignment and assignment of proteins to peptide spectra, we will begin to assemble comparisons of individual proteins in the two species. We are expecting a highly similar proteomic structure for the two species. We will compare individual proteins in ways similar to those that will be developed for

control versus cardiomyopathic canine heart tissue (Sect. C.2, *above*). This approach includes both comparisons of all SR-enriched proteins (354 in dog) and clustering into enrichment patters with its potential indications of functional subcompartmentation. When differences in structure, content, or enrichment patterns of SR proteins occur, the degree of variation will be assessed using as baselines the entire protein dataset.

C.4 Replicate determinations and immunological validation

Proteomic analyses require both biological replicates as well as technical replicates. Therefore, in addition to the analysis of SR samples prepared from five different animals, three replicate analyses will be carried out from at least one membrane preparation for each experimental group (canine control, canine ischemic HF, tachypacing HF, and human control). We will determine by ANOVA whether variances due to proteomic analysis are sufficient for routine analyses. When quantitative iTRAQ analyses differ from GeLC approaches, ANOVA tests will be applied to draw statistical conclusions based upon the variances and means from the two types of data.

C.5 Future directions and time line

With our first LC-MS/MS proteomic analysis underway, and substantial progress towards understanding methods of data analysis (outlined above), we will first complete the initial analysis along with the analyses of critical negative control samples, to establish and publish the basic information. We describe below new links to the complete dataset on the website of the WSU Library server to assure ready availability to all researchers. As part of this primary analysis, we will carry out a number of immunoblots and Immunohistochemical stainings of newly identified, or newly discovered enrichments, to verify by immunoblotting the presence of previously uncharacterized SR proteins and their distribution among SR subfractions. Establishment of the first fully characterized canine cardiac SR proteome, validated by a second control sample analysis and 3 technical replicate datasets, with control MS/MS determinations on samples recovered in the absence of ATP, and samples treated with ryanodine, will establish a strong base line proteomic dataset within the first 12-18 months of the project. Analysis of failed heart will take another year, with analyses aimed at comparisons with the dataset for control dog heart. The last 18 months will be devoted to determining the proteome in human heart, clearly characterizing its similarities and differences from canine SR.

A long-term plan for this research is to extend our findings from canine heart failure to human myopathic heart tissue where it can validate new markers for diagnosis and further investigation. The understanding and knowledge that will result from this proposed work will expedite this goal, and provide a wealth of critical data that will be an important contribution to efforts of many laboratories.

Resource sharing plan

The cardiac SR proteome data produced by the project will be made freely and publicly available for the use of investigators in cardiac biology and medicine. Mass spectrometric and peptide/protein identification data will be made available by depositing the data to the ProteomeXchange via EBI/PRIDE, a mature NIH-funded database. Software tools freely available through EBI/PRIDE will allow researchers to locate, visualize, and download the data from the project. Deposit of these results will take place prior to the acceptance for publication of the main findings of the project. Unpublished data and project publications will also be freely and publicly shared with researchers through DigitalCommons@WayneState, the Wayne State University institutional repository. These data will be made available to researchers in .pdf or .xls format as they are created, once validated. Entry for the project in DigitalCommons@WayneState will also refer to the project files stored on the EBI/PRIDE site, thereby increasing visibility and access to the mass spectrometer data. The data on both repositories (EBI/PRIDE and DigitalCommons@WayneState) will be documented using MIAPE-compliant metadata as defined by the Proteomics Standards Initiative of the Human Proteome Organization to enable unambiguous interpretation and validation of the data by subsequent users.

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