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Development of a Novel Proteomic Approach for Mitochondrial Proteomics from Cardiac Tissue from Patients with Atrial Fibrillation

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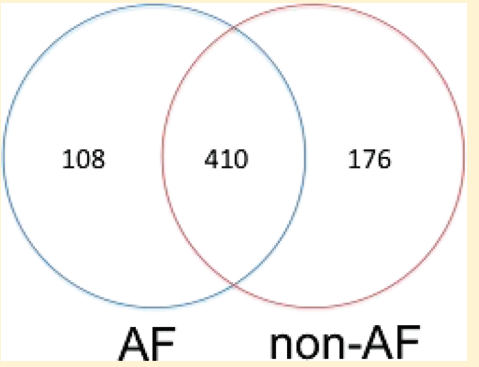
ABSTRACT: Atrial fibrillation (AF) is the most common cardiac arrhythmia affecting approximately 2.2 million Americans. Because several studies have suggested that changes in mitochondrial function and morphology may contribute to AF, we developed a novel proteomic workflow focused on the identification of differentially expressed mitochondrial proteins in AF patients. Right human atrial tissue was collected from 20 patients, 10 with and 10 without AF, and the tissue was subjected to hydrostatic pressure cycling-based lysis followed by label-free mass spectrometric (MS) analysis of mitochondrial enriched isolates. Approximately 5% of the 700 proteins identified by MS analysis were differentially expressed between the AF and non-AF samples. We chose four differentially abundant proteins for further verification using reverse phase protein microarray analysis based on their known importance in energy production and regulatory association with atrial ion channels: four and a half LIM, destrin, heat shock protein 2, and chaperonin-containing TCP1. These initial study results provide evidence that a workflow to identify AF-related proteins that combines a powerful upfront tissue cell lysis with high resolution MS for discovery and protein array technology for verification may be an effective strategy for discovering candidate markers in highly fibrous tissue samples.



KEYWORDS: atrial fibrillation, hydrostatic pressure cycling, mass spectrometry, reverse-phase protein microarray, FHL2, destrin, HSP27, CCT5, mitochondria, proteomics

INTRODUCTION

Atrial fibrillation is the most common of sustained arrhythmias encountered in clinical practice and results in significant increase of risk for stroke, premature death and heart failure. More than 2.2 million individuals in the United States are affected by atrial fibrillation with an expected prevalence of 6 million patients by 2050.[1] The disease is more common in advanced age with an approximate 10% incidence over the age of 80 years.[2] There are two general pharmacological concepts in treating atrial fibrillation; heart rate and rhythm control. The major limitation of treating patients with antiarrhythmic drugs is related to low efficiency and significant side effects. Rate control may be related to a lower incidence of drug-related complications; however, in several groups of patients remaining in atrial fibrillation, there is an increased risk of stroke, heart failure, and diminished quality of life.[3]

An alternative approach to address atrial fibrillation is the more invasive non- pharmacological treatment that consists of catheter or surgical-based ablation. Despite the fact that these interventions can yield higher success rates, there are complications related to the procedures, and although interventions seem more effective than pharmacological treatment there are many patients who do not respond to catheter and surgical ablation procedures.[4,5] These observations can be applied even to the most invasive of all nonpharmacological treatments, which is the cut and sew Maze procedure.[6]

Several studies have demonstrated that prolonged AF results in several ultrastructural changes in the atrial myocytes and atrial remodeling and changes in lipid storage/synthesis and energy/metabolism.[7−14] These structural remodelings include redistribution of nuclear chromatin, perinuclear loss of sarcomeres and sarcoplasmic reticulum, accumulation of glycogen and an increase in the number of small abnormally shaped mitochondria. The atrial myocytes show a shift toward a fetal phenotype (dedifferentiation) under such remodeling conditions.[7−14] The clinical implications of atrial tissue remodeling may include increased susceptibility to develop atrial arrhythmias. Research by us and others has revealed that an independent predictor for postoperative atrial fibrillation is the degree of mitochondrial dysfunction in response to simulated ischemia.[15,16] Our findings suggest that mitochondrial dysfunction may be related to increased susceptibility to develop atrial fibrillation. Despite extensive research, it is unclear whether mitochondrial changes are secondary to the general structural remodeling of atrial tissue or if mitochondrial dysfunction may be related to the occurrence of atrial fibrillation in the first place. Animal models have shown that during AF many small donut-shaped mitochondria can be found in atrial tissue.[17] All these observations support the fact that

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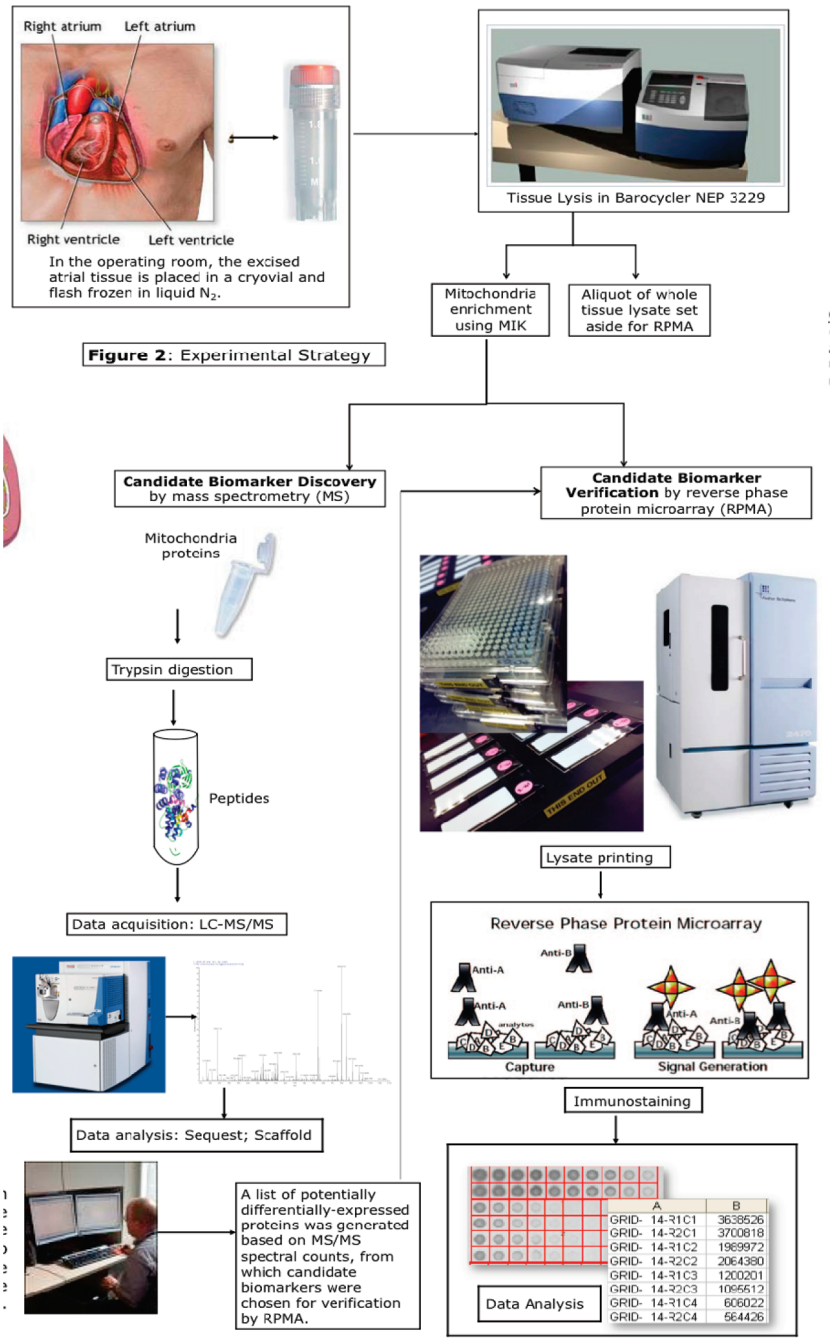

Figure 1. Description of the workflow for proteomic analysis: discovery and verification.

changes in mitochondria function and morphology are accompanied by AF development and existence.

Information regarding specific biomarkers related to atrial fibrillation is very limited, and there are only a few reports of biomarkers found specifically for AF. A recent publication[10] reported that persistent AF is associated with changes in the abundance of small molecule metabolites and proteins implicated in energy-demand pathways. We chose mitochondria for the focus of our study because of overwhelming evidence that these organelles are important in AF pathophysiology especially with regard to metabolism and lipogenesis.

The purpose of this study was to develop a novel proteomic methodological workflow to compare mitochondrial protein expression in highly fibrotic right atrial tissue in patients with and without AF for the discovery of differentially expressed mitochondrial proteins (Figure 1). This workflow consisted of:

(1) A unique study set of human right atrial tissue samples from patients with and without AF.
(2) A tissue disruption strategy that utilizes a high hydrostatic pressure cycling technology for mitochondria isolation/enrichment from highly fibrotic tissue.
(3) High resolution mass spectrometry (MS) for protein biomarker discovery.
(4) Reverse phase protein microarray (RPMA)-based verification of differentially expressed mitochondrial protein AF-related analytes using small amounts of mitochondrial lysates.

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Table 1. Patient Characteristics for Both AF and Non-AF Groups and Characteristics Specific to AF Patients

	AF	non-AF	*p* value
Age	66.5 (±12.9)	57.5 (±9.8)	0.09
Male	6 (60%)	8 (80%)	0.62
Caucasian	9 (90%)	7 (70%)	0.58
Ejection Fraction Preop	56.4 (±39.3)	52.8 (±9.5)	0.43
Left Atrium Size Preop	5.3 (±0.89)	4.1(±0.33)	0.03
Previous CV Surgery	0 (0%)	1 (10%)	1.0
	AF patients		
Long-standing AF > 1 yr	7 (70%) months 56.8 (±39.4)		
Paroxsymal AF < 7days/self-terminating	1 (10%) months 0.2		
Persistent AF > 7 days/not self-terminating	2 (20%) months 6.2 (±2.0)		
Cardioversion Pre-Op	4 (40%)		
Stand-Alone Maze	5 (50%)		
Maze/Valve	4 (40%)		
Maze/Valve/CABG	1 (10%)		

MATERIALS AND METHODS

Clinical Study Set and Sample Collection

Following the approval of the study by the Internal Review Board, informed consent was obtained from each study participant. Right atrial appendages were obtained from 20 patients; 10 from patients with AF undergoing the Maze procedure for persistent atrial fibrillation, and 10 from non-AF patients undergoing any other type of cardiac surgery (candidates for coronary artery bypass or valve surgery) (Table 1). The total AF patient population consisted of 60% males and 40% females with a mean age of 66.5 ± 12.9 years, while the total non-AF patient population consisted of 80% males and 20% females with a mean age of 57.5 ± 9.8. The tissue was collected before the patients were placed on the heart-lung machine. Following collection, the atrial tissue samples were cut and placed in cryovials, which then were deposited immediately in liquid nitrogen to minimize protein degradation. The second and third tissue pieces were stored in formalin and gluteraldehyde, respectively. The frozen atrial samples were transported on dry ice and stored at −80 °C until analysis.

Mitochondria Enrichment

For mass spectrometry (MS) and reverse phase protein microarray (RPMA) analyses, atrial tissue samples were lysed for mitochondria enrichment using a novel hydrostatic pressure cycling technology (Figure 1) (Pressure Biosciences, South Easton, MA).[18] This technology uses high pressure cycling technology to pulverize tissue specimens and is useful especially for highly fibrotic samples such as primary human atrial biopsy samples, which are very difficult to homogenize. The mitochondrial fractions from the resulting homogenates were produced using several centrifugation steps and a commercially available mitochondrial isolation buffer (BioChain Institute, Hayward, CA) supplemented with protease inhibitors (aprotinin, pepstatin, leupeptin, Pefabloc) and 1 mM sodium orthovanadate as a protein phosphatase inhibitor. As suggested by the kit manufacturer, the homogenate then was centrifuged at 600× *g* for 10 min at 4 °C, and the resultant supernatant was centrifuged at 12 000× *g* for 15 min at 4 °C. The resultant mitochondrial enriched pellet was resuspended in 8 M urea and sonicated for 6 min at room temperature to create a cellular lysate that is compatible with subsequent MS and RPMA analysis. Validation of the quality of the mitochondrial lysate preparation protocol was performed by RPMA measurement of two mitochondrial proteins: voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT), and two cytosolic proteins: calreticulin and alpha tubulin. The analysis revealed strong staining of the VDAC and ANT only in the mitochondrial-enriched fraction and calreticulin and alpha tubulin only in the cytoplasmic fraction (data not shown).

Mass Spectrometry

Mitochondrial proteins enriched from tissue were solubilized, reduced and alkylated, digested with trypsin, and the resultant peptide mixture was desalted. To each original mitochondrial protein sample bovine beta casein was added as an internal protein standard. A 10 μg aliquot of the mitochondrial protein digest was analyzed by online liquid chromatography−electrospray ionization−tandem mass spectrometry (LC−MS/MS) using a high resolution LTQ-Orbitrap mass spectrometer (Figure 1) (Thermo Scientific, San Jose, CA). The digest was loaded onto a homemade LC column consisting of fused silica packed with C18 resin with an integrated laser-pulled tip. The peptides were eluted at 200 nL/minute with a linear binary solvent gradient (A: 0.1% formic acid, B: 0.1% formic acid, 80% acetonitrile) in 100 min. The mass spectrometer was operated in a data-dependent mode in which each full MS scan was followed by nine MS/MS scans, one for each of the nine most abundant peptide ions selected from the MS scan, in which the selected peptide ions were collisionally dissociated and the fragment ions detected. The MS data were searched against a combined forward/reversed human protein database using the SEQUEST algorithm with the parameters of fully tryptic peptide sequences, static cysteine carbamidomethylation, and variable methionine oxidation. The search results were filtered to yield high confidence peptide identifications (maximum false discovery rate (FDR) of ∼1%). The Scaffold program (Proteome Software Inc.) was used to compare peptide and protein relative abundances based on the number of assigned MS/MS spectra (spectral count approach. FDR is the expected incorrect assignments among the accepted assignments. This approach is based on the use of the target-decoy database search strategy, and the decoy sequences are formed by reversing the sequences from the target database. Calculating a false discovery rate (FDR) in Scaffold consists of counting total number of reverse matches and dividing by the number of forward matches. We selected for a FDR of 1% in our search results, which is a measure of incorrect assignments within the data set. The database matches were filtered in Scaffold using 95% probability for both proteins and for peptide assignments and a minimum of 2 peptides per protein. These probabilities are the result of Peptide and Protein Prophet algorithms that are used by Scaffold. In addition, peptide sequences that are contained in multiple proteins (homologous families) are consolidated to those proteins that then yield the most concise and highest probability matches, as according to the principle of parsimony. The peptide and protein identifications and peptide abundances were confirmed by manual evaluation of the MS data. In our initial studies, we analyzed mitochondrial proteins of right atrial tissue from 10 AF versus 10 non-AF patients.

Reverse-Phase Protein Microarray

The RPMA format immobilizes an individual test sample in each array spot (Figure 1), and has been described.[19,20]

Table 2. Differentially Expressed Proteins ($p < 0.01$) between AF and Non-AF Right Atrial Tissue Specimens[a]

protein	number of assigned MS/MS spectra		comparative analysis	
	non-AF right	AF right	*p*-value	% difference (AFR-nAFR)
Crystallin alpha B	32	143	0.000045	100
Desmin	38	109	0.0018	65
Acyl-coenzyme A dehydrogenase	28	112	0.0000012	75
Glyceraldehyde 3-phosphate dehydrogenase	120	69	0.0071	−74
Gelsolin isoform b	89	51	0.0022	−74
Chaperonin containing TCP1 subunit5 (CCT5)	0	11	0.0035	100
Destrin	2	19	0.0098	89
Four and a half LIM domain 2 (FHL2)	5	46	0.0051	89
Heat shock protein 27 (HSP27)	0	17	0.00025	100
Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0	26	0.000064	100
Decorin isoform a preproprotein	2	69	0.0015	97
Phosphofructokinase, platelet	1	22	0.0099	95
3-oxoacid CoA transferase 1 precursor	3	38	0.00045	92
Acetyl-coenzyme A acyltransferase 2	6	64	0.0000094	90
Tu translation elongation factor, mitochondrial	14	111	0.0000017	84
Succinate-CoA ligase, GDP-forming, alpha subunit	5	30	0.000063	83
Coagulation factor XIII A1 subunit precursor	5	27	0.0019	81
Succinate-CoA ligase, ADP-forming, beta subunit	4	21	0.000012	81
Eukaryotic translation initiation factor 4A isoform 2	5	24	0.000027	80
Peroxiredoxin 6	4	12	0.0018	65
Aconitase 2 precursor	49	143	0.00048	66
Clathrin heavy chain 1	28	74	0.0018	62
2,4-Dienoyl CoA reductase 1 precursor	26	68	0.00029	62
Pyruvate kinase 3 isoform 1	29	71	0.000060	59
Aspartate aminotransferase 2 precursor	23	53	0.0055	58
Isocitrate dehydrogenase 2 ($NADP_+$), mitochondrial precursor	58	138	0.000050	58
Eukaryotic translation elongation factor 1 alpha 2	18	42	0.0023	57
Cysteine and glycine-rich protein 3	52	114	0.0021	54
Vitronectin precursor	27	57	0.00056	53
Tubulin, beta, 2	74	150	0.000029	51
ATP synthase, H_+ transporting, mitochondrial F_1 complex, alpha subunit precursor	194	358	0.0010	46
Erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked) isoform 3	128	41	0.0010	−212

[a] Proteins identified for further validation are highlighted in italics.

The enriched mitochondrial lysates were printed on nitrocellulose-coated slides (Whatman, Inc., Sanfort, ME) and subjected to RPMA analysis using a model 2470 Arrayer (Aushon BioSystems Ins., Billerica, MA) outfitted with 350 μm pins. Each sample was printed in triplicate two-point dilution curves. Blocked slides were incubated with antibodies for the 4 candidate protein biomarkers identified by MS as being differentially expressed. Staining for presence of primary antibody was performed using an automated stainer (Dako Cytomation, Carpinteria, CA). Catalyzed Signal Amplification System kit (Dako Cytomation, Carpinteria, CA) and fluorescent IRDye 680 Streptavidin (LI-COR Inc., Lincoln, NE), were used as the detection system. Stained slides were scanned with NovaRay Image Acquisition Software (Alpha Innotech, San Leandro, CA). A representative slide from the print run was stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) and visualized with NovaRay Image Acquisition Software (Alpha Innotech, San Leandro, CA) to measure the total protein concentration of each spot, which was used as a normalization control.

Acquired images of each slide were analyzed using MicroVigene software (Vigenetech, Carlisle, MA) that finds spots, performs local background subtraction, subtraction of nonspecific binding using a slide exposed to all components except for the primary antibody, averages replicates and normalized each sample for the total protein value. RPMA values then were subjected to supervised analysis using two-tailed *t* test or Wilcoxon rank sum depending on normalcy of the data distribution using JMP 5.1 software (SAS, Cary, NC).

RESULTS

Discovery of Candidate Mitochondrial Associated Biomarkers Using High Resolution Mass Spectrometry

The MS analyses of mitochondrial-enriched fractions from the atrial tissue samples of 10 AF patients and 10 non-AF subjects yielded identification of approximately 700 proteins in the aggregate. Analysis of peptide/protein relative abundances based on spectral counts yielded several potentially differentially expressed

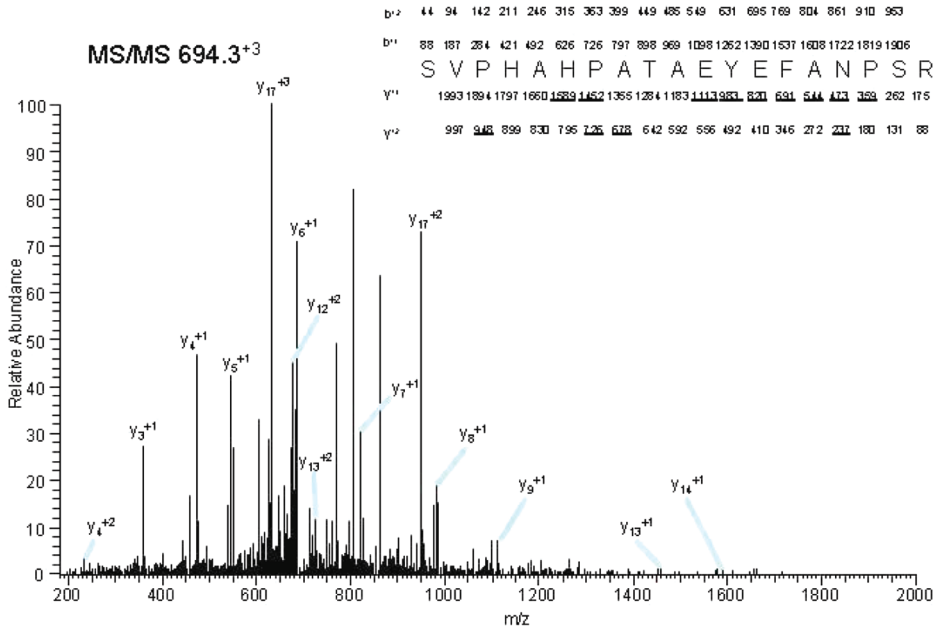

Figure 2. MS/MS spectra for an HSP27 peptide. The precursor b and y fragment ion masses and the annotated, assigned amino acid sequence are shown. For the y ions the corresponding peaks in the spectrum are labeled.

proteins (p-value <0.01):

(1) Twenty-eight proteins were more abundant in AF versus non-AF tissue samples, and
(2) Four proteins were more abundant in non-AF versus AF tissue samples.

Label-free mass spectrometry techniques combined with spectral counting and statistical comparative analysis methods have been shown to yield data that shows promising reproducibility for differential protein expression measurements.[21] The statistically significant (p < 0.01) differentially abundant (AF right atrium tissue vs non-AF right atrium tissue) proteins from the mitochondrial-enriched isolate and the associated spectral counts are shown in Table 2. Note that each spectral count value shown in Table 2 is the sum of all the MS-MS spectra identified per protein in all of the analyzed samples. It is worthwhile to note that several of the above AF vs non-AF candidate biomarker proteins are consistent with results of a recent report, for example, Crystallin alpha beta and desmin.[10] However, our results indicate an opposite relative abundance of glyceraldehyde 3-phosphate dehydrogenase (non-AF > AF), which might be due to our targeting of the mitochondrial protein fraction (vs total proteins, which was the focus of published work). Our strategy has yielded more potentially differentially abundant proteins, and in particular mitochondrial proteins, which may allow greater biochemical insights into the underpinning pathophysiology of a disease and future research to focus more on the metabolism/energy disorder.

Verification of Differentially Expressed Mitochondrial Associated Proteins Using Reverse-Phase Protein Microarray Analysis

We next sought to verify the differential expression of a subset of the proteins identified by MS in order to enhance the significance of the findings. Four proteins, CCT5, HSP27, destrin and FHL2 (in bold italics, Table 2) were chosen for further analysis based on two criteria: (1) the analytes had been implicated in atrial function and pathophysiology[23−33] and (2) validated commercially available antibodies were available for specific recognition of the selected proteins, which would provide for facile verification of differential abundance using immunoassay-based techniques. Prior to immunoassay validation of the MS results, we manually verified the amino acid sequences, obtained from the SEQUEST searches, of the tryptic peptides detected corresponding to the four selected proteins. As shown in Figure 2 for an HSP27 peptide, the peptide sequence was assigned correctly. To exemplify the differential expression of the selected proteins between the AF and the non-AF samples, we chose the MS/MS spectra of FHL2 and produced the reconstructed ion current chromatograms in an AF sample (Figure 3, panel C), and in a non-AF sample (Figure 3, panel D). The figure highlights the absence of a peak with m/z of 746.85 corresponding to FHL2 in panel D. The elution time was expanded in panels C and D to show the presence of m/z peak of interest in AF and its absence in the non-AF sample. The MS/MS spectrum in both cases was manually examined to ensure the correct peptide to peak assignments. The Venn diagram (Figure 3, panel E) shows that out of a total of approximately 700 proteins identified in the sample sets, 410 proteins were found in both AF and non-AF samples, while 108 proteins were present only in the AF samples and 176 were present mainly in the non-AF samples. On the basis of these results, we next performed RPMA analysis of the samples in order to confirm the MS data. As shown in Figure 4, RPMA analysis of relative expression levels of these 4 proteins from the mitochondrial isolates of the 20 samples used in MS discovery revealed a significantly elevated expression of the proteins from the right atria of patients with AF (p = 0.0003 for CCT5, p = 0.002 for destrin, p = 0.013 for FHL2, and p = 0.05 for HSP27), which confirmed the MS results.

DISCUSSION

Previous work by others and us indicated an association between mitochondrial dysfunction in response to ischemia and postoperative atrial fibrillation.[16] Alterations in mitochondrial

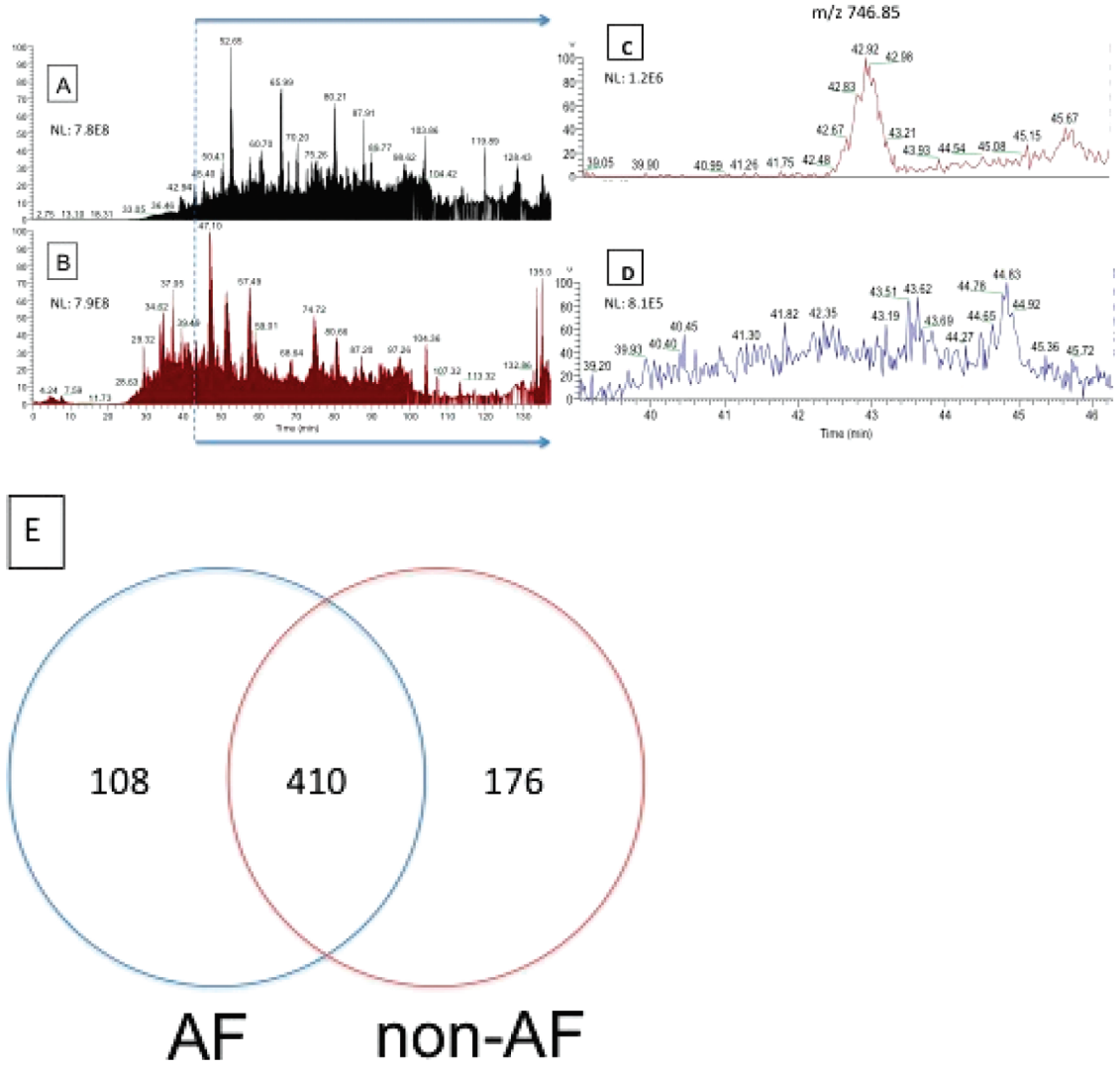

Figure 3. Total ion current and reconstructed ion current for FHL2 compared between an AF and a non-AF sample. (A) FHL2 TIC in an AF sample and (B) that in a non-AF sample. (C and D) RIC of FHL2 at a m/z of 746.85 in an AF and a non-AF sample, respectively. The elution time was expanded in panels C and D to further show the presence of m/z 746.85 peak at 42.92 min in panel C and its absence in panel D. (E) Venn diagram of the total number of proteins identified in the AF and non-AF samples. The proteins in the overlapping section were present in both sample sets.

oxidative phosphorylation have been shown to contribute to pathogenesis of atrial fibrillation, also.[22] On the basis of this previous work, we postulated that mitochondrial enriched isolates would serve as a rich source of AF-associated proteins that could be identified with a proteomic strategy. However, because isolation and enrichment of mitochondria from highly fibrotic clinical tissue samples such as cardiac atrium can be difficult, we developed a method that combined a unique high pressure cycling lysis technology with mass spectrometry and protein microarray analysis to yield a novel discovery and verification strategy (Figure 1).

These results provide insights into the changes in protein expression that occur in the mitochondrial atrial tissue of patients with AF. From an approximate total of 700 proteins identified, statistical analysis of the spectral count differences revealed 32 proteins, or 5% of the total, with statistically different abundances in the 10 AF vs 10 non-AF samples ($p < 0.01$). The results suggest that AF development manifests in remodeling of the atrial proteome. Many of the differentially expressed proteins are consistent with other reports, such as of Crystallin alpha beta and desmin.[10]

Confirmation of the MS spectral count results was accomplished by RPMA analysis, an immunoassay-based method, of 4 (CCT5, HSP27, destrin and FHL2) of the 32 proteins identified by MS as being differentially expressed in the atrial tissue of patients with AF. These analytes were chosen for further analysis based on their known importance in energy production and regulatory association with atrial ion channels. FHL2 has been identified as a potential HERG (*human ether-a-go-go-related gene*) partner.[23] The alpha subunit of potassium channel HERG is required for the rapid component of the cardiac delayed rectifier current. FHL2 as a specific adaptor protein can couple metabolic enzymes to sites of high energy consumption in the sarcomere through interaction with titin/connectin.[24] FHL2 mutations have been shown to affect its binding to N2B and to two regions of titin, leading to impaired recruitment of metabolic enzymes to the cardiac sarcomere and hence to cardiac failure. FHL2 expression and localization are preserved in human left

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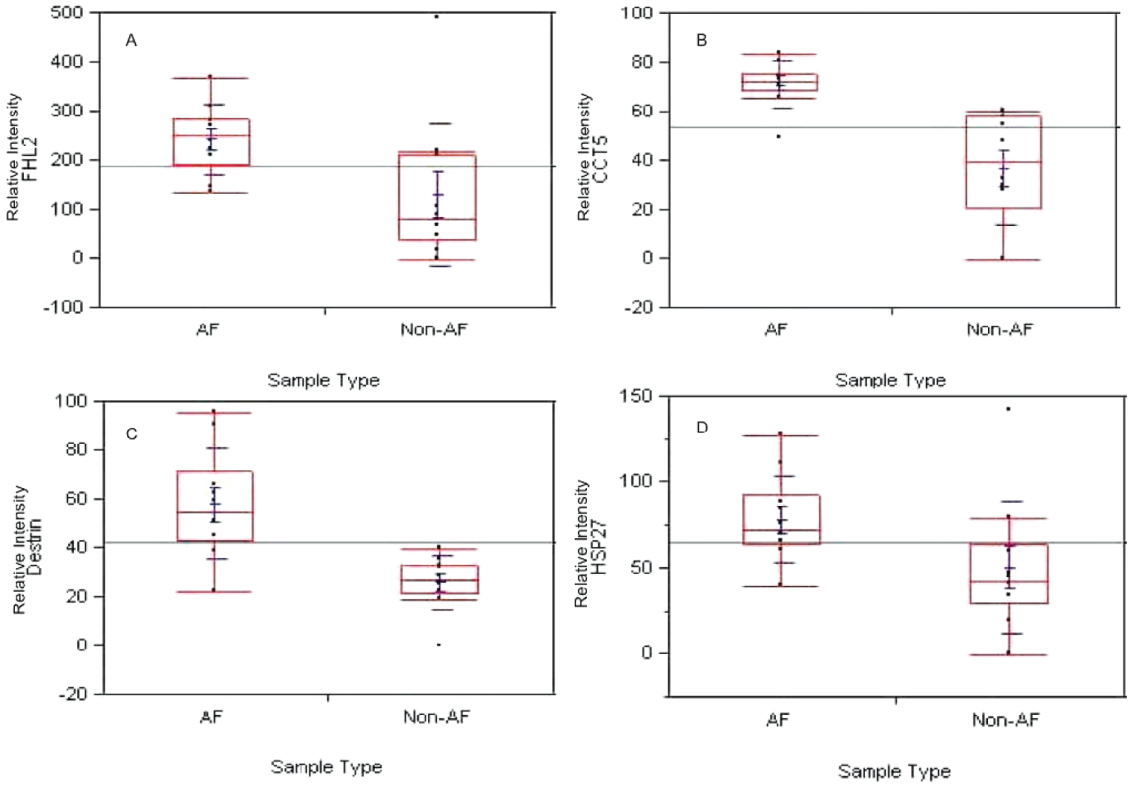

Figure 4. Box plots of RPMA measurements of 4 proteins found MS to be elevated in the enriched mitochondrial preparations of right atrial tissue from patients with AF (left) and non-AF (right). (A) FHL2, (B) CCT5, (C) Destrin, and (D) HSP27. All results are statistically significant ($p \leq 0.05$). Relative RPMA measurements are shown on the *y*-axis.

ventricular hypertrophy but disrupted in failing cardiomyocytes.[25] Another LIM domain-containing protein is FHL1, which is a novel chaperone for atrium-specific Kv1.5 channels with a potential role in atrial arrhythmogenesis.[26] In general, LIM domain proteins shuttle between the nucleus and cytosol and interact with transcription factors to regulate gene expression. There is emerging evidence that LIM domain proteins mediate the communication between the nuclear and plasma membrane compartments.[26] FHL1 interacts with human Kv1.5 in the plasma membrane. The FHL1-related current phenotype closely resembles that of I_{Kur} in atrial myocytes,[26] suggesting that FHL1 is a major regulator of atrial I_{Kur}. I_{Kur} is an atrium-selective ion current with the potential of being a promising drug target for therapy of atrial arrhythmias without concomitant adverse effects in the ventricles. Therefore, FHL proteins affect atrial function and morphology.

Destrin is a mammalian 19-kDa protein that rapidly depolymerizes F-actin in a stoichiometric manner. It is known that under stress conditions such as heat shock, reorganization of actin cytoskeletons is mediated by proteins such as destrin. Destrin is an isoprotein of cofilin which regulates the actin cytoskeleton in various eukaryotes. Dephosphorylation of destrin has been observed upon stimulation of several cell types.[27] This protein was first reported in 1985 by Muneyuki to be capable of rapidly depolymerizing F-actin as if it destroyed the filaments, thus the name destrin.[28] A gene expression study of a connexin43 (Cx43) null mouse heart showed overexpression of destrin in the Cx43 null mouse heart.[29] However, this protein has not been implicated in an AF study before; therefore, this is the first association of destrin with AF and cardiac function. It has been shown by our MS and RPMA results that this protein is differentially expressed between AF and non-AF patients, being more abundant in the right atria of AF patients. Identification of destrin as a differentially abundant protein suggests involvement of actin cytoskeleton machinery in cardiomyopathy and AF in particular.

HSP27 is required for the development of CNS, skeletal and cardiac muscles. The HSP chaperonins seem to mediate their protective effects by maintaining mitochondrial function and integrity as well as capacity for ATP generation, which is crucial for survival of cardiac myocytes undergoing ischemia/reperfusion injury.[30] As shown in animal models, chaperonins prevent toxic protein aggregation by binding to partially unfolded proteins, thus preventing atrial remodeling. These proteins have been shown to attenuate the promotion of AF from paroxysmal AF to chronic, persistent AF in both human and animal experimental models.[31] It has been shown that the induction of heat shock proteins (Hsp72 and Hsp27) by hyperthermia and/or geranylgeranylacetone protects the heart against atrial remodeling.[32] Induced heat shock responses (including induction of Hsp72 and Hsp27) may prevent newly developed AF and delay the progression of paroxysmal AF to persistent AF. In our study, this protein was shown to be more abundant in the right atria of AF patients than in the right atria of non-AF patients.

The chaperonin-containing TCP-1 protein plays a vital role in folding cellular cytoskeletal proteins that are intimately involved in cell structure, division and locomotion. CCT-containing chaperonins provide a free-energy contribution from their ATP cycle, which drives actin to fold from a stable, trapped intermediate I_3, to a less stable but productive folding intermediate I_2.

Up to now CCT containing chaperonins have not been mentioned in any AF studies, therefore, this is the first report of CCT5 associated with AF. Our results reveal that protein folding defects may underpin a large part of the pathophysiology of AF because HSP27 also was found to be elevated in the right atrium of AF patients compared with the right atrium of non-AF patients. Molecular chaperones such as CCT5 and HSP27 play a critical role in the folding of many proteins, and CCT5 transcript expression has been found to be upregulated in p53-mutated tumors,[33] which is consistent with the biological importance of protein folding and normal cellular homeostasis.

The link between mitochondria dysfunction and cardiomyopathy has been the subject of many studies. Our results suggest that mitochondrial and mitochondrial-associated proteins are involved and affected during the onset of AF; therefore, studying mitochondrial proteins may be important in our quest for AF biomarkers.

In conclusion, we describe herein a novel strategy for AF biomarker discovery based on the postulate that mitochondrial dysfunction may have an important physiological role in AF. While this study should be considered exploratory in scope and used a relatively small number of patient samples, several candidate proteins were identified, and these may be useful for diagnostic, prognostic or predictive purposes if validated in larger sample cohorts with clinical follow up. RPMA verification of differential expression of a subset of the MS discovered proteins reveals the potential for our analysis to produce potentially important protein biomarkers that are associated biochemically with AF pathophysiology and reveal overt changes in mitochondrial function, energy balance and remodeling. Current efforts are underway to validate these specific proteins in larger tissue study sets as well as examine the potential for these tissue markers to be liberated into the circulation (e.g., cardiac troponin) as markers for AF disease detection, disease and therapeutic monitoring through the analysis of serum study sets from the same patient cohort used for this study. AF is a complicated, heterogeneous disease, and the tissue study sets used herein reflect this underpinning heterogeneity. Of interest, however, is that in spite of the hard-wired disease heterogeneity we have identified several candidate markers that appear to transcend these physiological differences and point to a potential common mitochondrial dysfunction.

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ABBREVIATIONS:

MS, mass spectrometry; RPMA, reverse-phase protein microarray; HSP27, heat shock protein 27; FHL2, four and a half Lim domain; CCT5, chaperonin-containing TCP1; AF, atrial fibrillation.

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