Substrate Modifications Precede the Development of Atrial Fibrillation After Cardiac Surgery: A Proteomic Study

Antonios Kourliouros, PhD, MRCS, Xiaoke Yin, PhD, Athanasios Didangelos, PhD, Morteza Tavakkoli Hosseini, MD, Oswaldo Valencia, MD, Manuel Mayr, MD, PhD, and Marjan Jahangiri, MD, FRCS

Department of Cardiothoracic Surgery, St. George's Hospital, and Cardiovascular Division, King's College, London, United Kingdom

Background. **Atrial fibrillation (AF) is an important cause of morbidity and mortality after cardiac surgery. The pathogenesis of AF appears to be multifactorial but little is known about the cause-effect relationship of substrate modifications with the onset of the arrhythmia. With the use of modern proteomics, this study aims to identify preexisting changes in the left atrium of patients susceptible to postoperative AF.**

Methods. **We analyzed 20 matched patients undergoing elective, first-time coronary artery bypass grafting with no history of AF. They were divided into 2 equal groups according to the development of postoperative AF. Proteomic analysis was performed in left atrial tissue obtained during surgery using two-dimensional difference in gel electrophoresis techniques. Mass spectrometry identified proteins that were differentially expressed in patients who developed AF against those who remained in sinus rhythm.**

Results. **Proteomic analysis of left atrial tissue identified 19 differentially expressed protein spots between patients who developed postoperative AF and their sinus rhythm counterparts. In patients who developed AF, proteins associated with oxidative stress and apoptosis (peroxiredoxin 1, apoptosis-inducing factor, and 96S protease regulatory subunit 8) as well as acute phase response components (apolipoprotein A-I, fibrinogen) were found to be increased. Conversely, the expression of proteins responsible for glycolysis (enolase) and pyruvate metabolism (pyruvate dehydrogenase) was reduced.**

Conclusions. **We describe protein changes that precede the development of postoperative AF and which might be suggestive of increased oxidative stress and glycolytic inhibition in the left atrium of patients predilected to AF.**

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Atrial fibrillation (AF) after cardiac surgery has been independently associated with increased early and long-term mortality [1]. Despite the development of risk stratification tools and the advent of pharmacologic and even surgical approaches for its prevention, the occurrence of postoperative AF remains considerably high [2, 3]. The pathogenesis of AF appears to be multifactorial, with electrical remodeling, structural remodeling, and inflammation playing important and interrelating roles [4]. Clinical and experimental studies have demonstrated associations between patient-related factors (including age, body mass index, underlying cardiovascular disease) [5], biomarkers derived from the peripheral circulation [6, 7], and regional modifications with the presence of AF [8, 9]. However, certain common methodological limitations hinder these studies for the delivery of definitive mechanistic associations. Presence of substrate modifications in experimental models or patients with AF cannot delineate a possible cause-effect relationship; it is often unclear whether the observed changes preexist and are responsible for the development and domestication of AF or whether they are just outcomes of the arrhythmia or the underlying cardiovascular condition that may contribute to AF [2]. Secondly, most human studies to date examine myocardial tissue derived from the right atrium [10]. It is well established that both the initiation and propagation of AF takes place predominantly in the left atrium. Therefore, left atrial studies are more likely to be reflective of the pathogenetic mechanism leading to AF. Finally, studies aiming at the structural or metabolic factors responsible for AF are often hypothesis driven and therefore target an only narrow component of pathophysiology.

The aim of this study was to identify protein modifications in the left atrium of patients in SR who developed postoperative AF when compared with patients who remain in SR. We utilized state-of-the-art cardiac proteomics that provide an unbiased and reproducible platform for the identification of protein expression differences in patients who develop AF compared with those who do not. This is to demonstrate substrate changes that precede and may contribute to AF after cardiac surgery.

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Address correspondence to Dr Jahangiri, Department of Cardiothoracic Surgery, St. George's Hospital, Blackshaw Rd, London SW17 0QT, UK; e-mail: marjan.jahangiri@stgeorges.nhs.uk.

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Material and Methods

Patient population

We analyzed left atrial tissue from 20 patients undergoing elective first-time coronary artery bypass surgery with the use of cardiopulmonary bypass. All patients were in SR before surgery and had no history of atrial or other arrhythmias, presence of permanent pacemaker, or receiving any antiarrhythmic medications. They were divided in 2 groups; 10 patients who remained in SR postoperatively and 10 patients who developed new-onset AF during hospital stay. None of these patients had history of diabetes mellitus, chronic obstructive pulmonary disease, and previous cerebrovascular accident, and no patient was on steroids. There was also no evidence of heart failure (left ventricular ejection fraction < 0.50) or valvular disease. All patients were on aspirin (75 mg) up to 7 days preoperatively, and received routine statin therapy with simvastatin (20 mg or 40 mg) for at least 30 days before surgery. Standardized anesthetic, perfusion, and surgical protocols were followed [11]. This included early postoperative administration (within 24 hours) of β-blockers unless contraindicated due to severe bradycardia or hypotension. No other specific agent was routinely administered aiming solely at AF prophylaxis. In order to analyze similar groups according to cardiovascular risk profile, medications, and operative variables, and with an observed incidence of postoperative AF of 30%, we selected our patients from a cohort of 120 patients from whom we had complete data and obtained left atrial tissue. This study was approved by the regional ethics committee, received institutional approval from the research and development office, and all patients gave written informed consent.

Routine postoperative heart rhythm assessment was achieved with continuous telemonitoring for the first 72 hours after surgery and with 4-hourly clinical examinations thereafter. Twelve-lead electrocardiograms were also obtained and assessed daily. Postoperative AF was defined according to the Society of Thoracic Surgeons National Cardiac Surgery Database guidelines and definitions, as new-onset AF requiring treatment, and this does not include recurrence of AF that had been present preoperatively [5]. In our study we only included patients in the analysis for whom postoperative AF was sustained for more than 15 minutes from diagnosis and did not terminate spontaneously. Patients who developed AF were treated with amiodarone (intravenous or oral, after appropriate loading) while correcting electrolyte and acid-base imbalances. In cases of hemodynamic compromise due to new-onset AF, or where amiodarone was not effective in heart rhythm control, electrical cardioversion was performed. Statistical analysis was performed using the Fisher exact test for dichotomous data, the χ^2 test for contingency tables, and the t test for normally distributed continuous variables (SPSS 17.0; SPSS Inc, Chicago, IL).

Tissue Sampling and Preparation

Left atrial tissue was obtained from the left atrial appendage during cardiopulmonary bypass and just after cardioplegic arrest of the heart, and the atrial incision was oversewn with 4.0 Prolene. Sample comprised of approximately 0.5×1.0 cm myocardial tissue that was immediately snap frozen and stored in liquid nitrogen. Atrial tissue was fully solubilized in two-dimensional electrophoresis (2DE) lysis buffer (9.5M urea, 2% w/v CHAPS, 1% w/v DTT and 0.8% w/v Pharmalyte pH 3-10). Urea and nonionic detergents were used to unfold most proteins to their fully random configuration. Protease inhibitors were added into the mix, as some proteases remain active despite high urea concentrations in the lysis buffer. Lysis buffer, 1mL, was added to 100 mg of tissue. Samples were then homogenized with specialized lysing matrix particles (Lysing Matrix D, MP Biomedicals, Solon, OH) in a high-speed benchtop homogenizer (FastPrep 120; Krackeler Scientific Inc, Albany, NY). Samples were acidified with 0.1M hydrogen chloride and protein concentration was estimated using the Bradford assay with the bovine serum albumin standard curve. Prior to 2DE, samples were further processed using a commercial cleanup kit (ReadyPrep, Bio-Rad). This process involved quantitative precipitation and concentration of proteins within the samples, while potential substances that could interfere with Isoelectric focusing/2DE such as ionic detergents, salts and lipids, were washed away. The proteins were then resuspended in DIGE lysis buffer (8M urea, 4% w/v CHAPS, 30 mM trisCl, pH = 8.5) and stored at -80°C until further analysis.

Proteomic Analysis

The sequence of analytical steps and the summary of important methodological parameters is as follows:

2D FLUORESCENCE DIFFERENCE GEL ELECTROPHORESIS. DIGE dyes (CyDye DIGE Fluor minimal dyes, GE Healthcare) were used to label proteins with fluorochromes so that accurate detection and quantification of protein abundance following 2DE could be performed (Ettan DIGE). Each different protein sample from a given pair (left atrial tissue of postoperative AF vs SR patient group) was labeled with CyDye. These fluorochromes attach to proteins through an amide bond to the ε amino group of lysine, without replacing the isoelectric point (PI) of the protein. Subsequently, the 2 different protein samples of this paired comparison, were mixed together to run firstly on the same IEF and second on SDS PAGE (sodium-dodecylsulphate–polyacryamide gel electrophoresis). As a result, proteins from 2 different samples were run in the same gel and could be visualized independently due to the differential wavelength emission with a fluorescent scanner. To increase the confidence of subsequent matching between different gels from different paired comparisons, an internal standard was created by mixing half proportion of all different samples in the experiment and labeled with Cy2. The same protein amount of the internal standard as individual samples were added into each 2DE gel. After protein labeling,

proteins from different atrial samples within the same pair and the same amount of internal standard were pooled together.

ISOELECTRIC FOCUSING AND SDS PAGE. We performed IEF of proteins in the PI region of 3-10, which was previously shown to provide a good separation of protein extracts [8]. After overnight rehydration the IPG strips (GE Healthcare) were transferred into the IPGphor unit. Voltage was gradually increased from 150V to 8,000V. The IPG strips were then washed and placed on the upper edge of the SDS PAGE gels. Finally, gels were run in Tris-Glycine SDS running buffer for 8 to 10 hours at 100 Watts.

DECYDER ANALYSIS. For the purpose of differential analysis and screening of peptides arising from the 2 different experimental groups specialized software was used (DeCyder 7.0, GE Healthcare). Through the automatic detection of protein spots, user variation is minimized while quantitative information and matching statistics can also be provided. After automatic matching, we manually performed cross-detection to confirm spots already automatically matched, but more importantly to correct mismatched spots and add unmatched spots, which should be potentially matched with others. By specifying certain match and group statistic parameters, a list of matched proteins was displayed after applying the filter ($p < 0.05$, ratio > 1.2 or ratio ≤ 1.2). Once DyCyder analysis was completed a copy of silver stained gel with the spots of interest clearly marked was produced to serve as a template for manual spot picking, which was carried out just prior to tryptic digestion and mass spectrometry.

TRYPTIC DIGESTION AND TANDEM MASS SPECTROMETRY. In-gel digestion with trypsin was performed according to published methods [12] modified for use with an Investigator ProGest (Genomic Solutions, Ann Arbor, MI) robotic digestion system. After tryptic digestion, peptides were separated by liquid chromatography on a reverse-phase column (Acclaim PepMap100 C18, Dionex) and applied to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher). Spectra were collected from the Orbitrap mass analyzer using full ion scan mode over the mass-to-charge (m/z) range of 450 to 1,800. The MS-MS scans were performed on each ion using dynamic exclusion. Database searches were performed using SEQUEST version 28 (Bioworks Browser 3.3.1 SP1, Thermo Fisher Scientific) and X! Tandem (version 2007.01.01.2).

Carboxyamidomethylation of cysteine was chosen as a fixed modification, and oxidation of methionine was chosen as a variable modification. The mass tolerance was set at 1.5 amu for the precursor ions and at 1.0 amu for fragment ions. Two missed cleavages were allowed. Scaffold (version 2.6, Proteome Software Inc, Portland, OR) was used to calculate the spectral counts and to validate MS-MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm [13]. Protein identifications were accepted if they could be established at greater than 99.9% probability with at least 2 unique peptides.

Table 1. Baseline Characteristics and Intraoperative Variables Between Patients Who Developed Postoperative AF and Patients Who Remained in Sinus Rhythm (SR)

Variables	Postoperative AF (n = 10)	Postoperative SR (n = 10)	p Value
Age	69	65	0.33
Gender (male)	10	8	0.47
Hypertension	8	6	0.63
β-blockers	7	7	1.00
ACEIs/ARBs	5	6	1.00
Calcium channel blockers	4	1	0.30
CCS III	2	4	0.72
NYHA III-IV	2	3	0.77
Previous MI	6	6	1.00
Left main stem disease	4	4	1.00
Number of grafts (>3)	6	4	0.76
CPB time (minutes)	88	72	0.12
Cross-clamp time (minutes)	57	45	0.07

ACEI = angiotensin-converting enzyme inhibitors; AF = atrial fibrillation; ARB = angiotensin receptor blockers; CCS = Canadian Cardiovascular Society; CPB = cardiopulmonary bypass; MI = myocardial infarction; NYHA = New York Heart Association; SR = sinus rhythm.

Results

Characteristics between patients who developed postoperative AF and patients who remained in SR are shown in Table 1. These 2 selected groups appear similar in most baseline characteristics, some of which are known to be associated with postoperative AF (such as β-blocker and angiotensin-converting enzyme inhibitor use, severity of symptoms, and previous myocardial infarction). There was a trend for prolonged cross-clamp and cardiopulmonary bypass times in the AF group, which was not found to be statistically significant. All patients in the postoperative AF group reverted to normal SR after administration of intravenous amiodarone and remained free of AF during their routine 6-week and 3-month follow-up.

Proteomic analysis of left atrial tissue in patients who developed postoperative AF compared with those who remained in SR detected a maximum of 1,404 spots of which 1,015 were matched to the master gel. Of these, 19 spots were differentially expressed between the 2 groups. These spots are shown in Figure 1. A characteristic 2D DIGE image is shown in Figure 2. Mass spectrometry identified the corresponding proteins, which are shown in Table 2. Thirteen proteins were found upregulated in atrial tissue of patients who developed AF, and 6 proteins

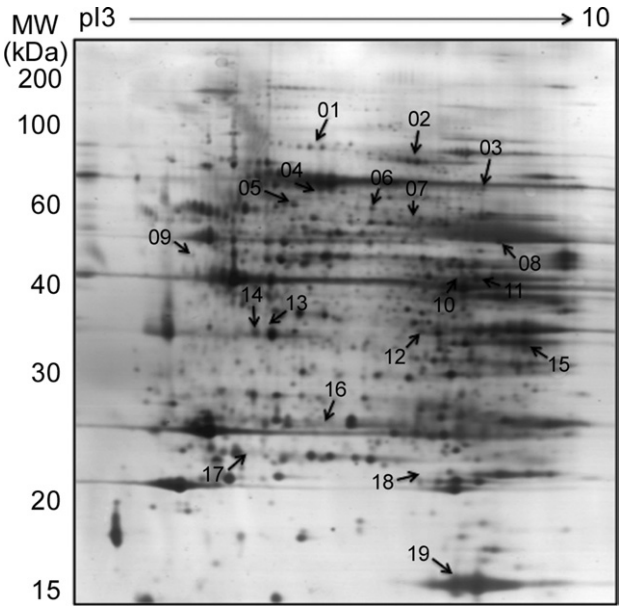

Fig 1. High-resolution two-dimensional electrophoretic left atrial proteomic analysis of postoperative sinus rhythm versus atrial fibrillation groups. (MW = molecular weight.)

were found to be downregulated. Acute phase response components (apolipoprotein A-I, fibrinogen, and transferrin) were found upregulated in patients who were predilected to postoperative AF. Conversely, the expression of proteins responsible for glycolysis (enolase) and pyruvate metabolism (pyruvate dehydrogenase) were found reduced in patients who developed postoperative AF. Oxidative stress and apoptosis-related proteins were found to be upregulated in patients prone to postoperative AF. Such proteins include peroxiredoxin 1, apoptosis-inducing factor, and 96S protease regulatory subunit 8. Finally, some other proteins such as myosin-7, creatine kinase, and desmin, which did not fall into any of the analyzed canonical pathways, were also found differentially expressed and may have an association with postoperative AF.

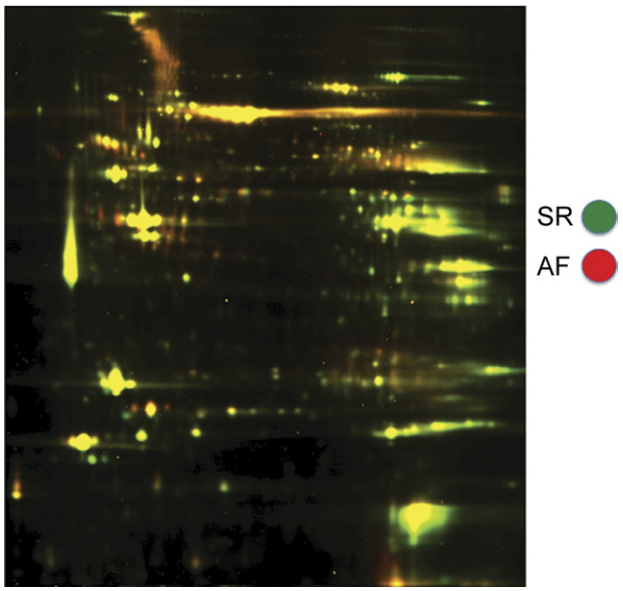

Fig 2. Two-dimensional difference in gel electrophoresis image of sinus rhythm (SR) versus atrial fibrillation (AF) group comparison.

Comment

Proteomic analysis of left atrial tissue from patients in SR, some of whom developed postoperative AF, provides a potential platform for the identification of factors that may predispose to the arrhythmia. One of the main criticisms of previous experimental studies examining patients with a history or presence of AF versus SR counterparts is that they failed to delineate whether a certain finding (ie, increased inflammation or fibrosis in AF) is a cause of the arrhythmia, the result of the arrhythmia, or just a manifestation of an underlying condition [4]. Furthermore, the use of tissue from the right atrium only limits these studies further as there is convincing evidence to suggest that AF originates and propagates from the left atrium, and that there are also significant functional and structural differences between the right and the left atria. In this study we utilized modern proteomic techniques in the analysis of left atrial tissue of patients in SR at baseline, and identified changes specifically associated with the development of postoperative AF.

Acute Phase Response

In addition to its role as a major component of high-density lipoprotein in lipid metabolism, apolipoprotein A-I is also an acute phase reactant, which was found to be upregulated in patients who developed postoperative AF. Conversely, serum amyloid P component, which has also been associated with inflammation, was reduced in AF patients. Fibrinogen is also an acute phase reactant that was found upregulated in postoperative AF patients. Once again, the increased synthesis and secretion of the 3 polypeptide chains of fibrinogen is an additional effect to its main role in coagulation by forming fibrin through enzymatic cleavage with thrombin. Finally, transferrin was increased in AF implicating iron metabolism and inflammation in its pathogenesis.

Glycolysis and Pyruvate Metabolism

Enolase expression was found to be reduced in patients who developed postoperative AF, affecting the dehydration of 2-phosphoglycerate to phosphoenolpyruvate at the final stages of glycolysis. Pyruvate dehydrogenase beta was also reduced in AF patients affecting pyruvate decarboxylation and the link between glycolysis and citric acid cycle. In glycolysis the production of fructose from D-glucose requires the energy through ATP (adenosine triphosphate). However, fructose can also be produced by sorbitol, which can be synthesized by glucose with the action of aldose reductase, which was found decreased in the AF group. Malate dehydrogenase, which catalyzes the oxidation of malate to oxaloacetate

Table 2. Protein Changes in Patients Who Developed Postoperative Atrial Fibrillation Versus Patients Who Remained in Sinus Rhythm

ID	Protein Name	Protein Accession Numbers	MW	Number of Unique Peptides	Number of Unique Spectra	Number of Total Spectra	Percentage of Total Spectra	Percentage Sequence Coverage	*t* Test	Average Ratio
Upregulated proteins										
1	Myosin-7	MYH7_HUMAN	223,086	6	6	9	0.52%	3.77%	0.049	1.44
2	Serotransferrin	TRFE_HUMAN	77,032	20	23	53	2.85%	29.10%	0.041	1.28
3	Apoptosis-inducing factor 1, mitochondrial	AIFM1_HUMAN	66,884	4	5	8	0.88%	7.18%	0.023	1.27
4	Myosin-7	MYH7_HUMAN	223,086	7	7	15	1.12%	4.39%	0.03	1.24
5	Myosin-7	MYH7_HUMAN	223,086	7	8	14	0.75%	3.93%	0.031	1.46
6	Dihydropyrimidinase-related protein 3	DPYL3_HUMAN	61,946	7	8	13	1.12%	18.80%	0.041	1.35
7	Fibrinogen beta chain	FIBB_HUMAN	55,911	3	3	7	0.70%	8.35%	0.0012	1.34
8	ATP synthase subunit alpha, mitochondrial	ATPA_HUMAN	59,734	17	22	81	3.73%	30.40%	0.019	1.21
10	26S protease regulatory subunit 8	PRS8_HUMAN	45,609	9	9	23	1.08%	24.60%	0.013	1.23
11	Creatine kinase, sarcomeric mitochondrial	KCRS_HUMAN	47,488	17	23	59	2.36%	37.50%	0.043	1.22
15	Maltase dehydrogenase, mitochondrial	MDHM_HUMAN	35,486	11	12	47	2.53%	37.30%	0.029	1.2
17	Apolipoprotein A-I	APOA1_HUMAN	30,761	6	6	13	1.60%	19.90%	0.033	1.27
19	Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	17,995	4	4	13	1.30%	23.60%	0.034	1.87
Downregulated proteins										
9	Gamma-enolase	ENOG_HUMAN	47,252	3	4	13	0.98%	9.68%	0.023	−1.21
12	Aldose reductase	ALDR_HUMAN	35,836	8	9	18	0.97%	24.40%	0.0051	−1.33
13	Desmin	DESM_HUMAN	53,519	5	5	10	0.74%	11.70%	0.043	−1.43
14	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	ODPB_HUMAN	39,215	10	12	32	2.92%	26.70%	0.012	−1.23
16	Serum amyloid P-component	SAMP_HUMAN	25,370	6	7	18	0.97%	27.40%	0.018	−1.29
18	Peroxiredoxin-1	PRDX1_HUMAN	22,093	4	4	10	0.70%	23.60%	0.026	−1.24

Protein accession numbers = unique identifier of a protein within Universal Protein Resource (UniProt) knowledgebase. MW = protein molecular weight; Number of unique peptides = the number of unique peptides on which the protein identification was based; Number of unique spectra = the count of spectra that match different peptides (even if the peptides overlap) or match 2 different charge states of the same peptide, or match both a peptide and a modified form of the peptide; Number of total spectra = sum of spectra identified in each protein; Percentage of total spectra = the percentage of total spectra in the entire sample represented by the spectra that contributed to this protein identification; Percentage sequence coverage = percentage of sequence of the full-length protein covered by the unique peptides that were identified.

during the final step of the tricarboxylic acid cycle, was increased in patients who developed postoperative AF.

Oxidative Stress and Apoptosis

Peroxiredoxin 1, which was decreased in the AF group, is an important enzyme in redox regulation by catalyzing hydrogen peroxide and alkyl hydroperoxide reductions. In addition, peroxiredoxin 1 has been implicated in the protection against apoptosis. Apoptosis-inducing factor causes nuclear disassembly and induces the production of apoptogenic proteins cytochrome C and caspase 9 from the mitochondria. Apoptosis-inducing factor, as well as 96S protease regulatory subunit 8 that is also involved in intracellular degradation, were found to be increased in the left atrium of patients who developed postoperative AF.

The presence of a proinflammatory state in the left atria of patients who developed postoperative AF further supports the inflammatory hypothesis in its pathogenesis. In addition, it is in accordance with the previous analyses of epicardial adipose tissue and circulating cytokines highlighting the instrumental contribution of local factors against the dominant theory of a generally augmented systemic inflammatory status [9]. As oxidative stress and inflammation often coexist, it is not surprising that peroxiredoxin 1, an enzyme also responsible for the regulation of cytokine-induced peroxide levels, was found decreased in patients who were in SR at the time but developed postoperative AF. This finding complements some of our previous work where peroxiredoxin 1 was found decreased in patients with persistent AF, and suggests that oxidative stress is in effect a proarrhythmogenic condition [8].

The identification of a state of glycolytic inhibition in patients in SR who developed postoperative AF demonstrates that metabolic dysfunction may precede the development of the arrhythmia by creating a substrate where inflammation and oxidative stress contribute and possibly trigger AF. Interestingly, such a metabolic turn with a decrease in the glycolytic enzyme enolase was observed in an animal model of cardiomyopathy [14], while skeletal muscle β-enolase deficiency due to a mutation has been implicated in clinically significant myopathy [15]. Cardiomyocytes under oxidative stress and energy depleted are prone to degradation and apoptosis, which is in line with the observed over-expression of the apoptosis inducing factor 1 and 26S protease subunit alpha. Once again apoptotic activity, which is a dominant feature of established AF [16], is also found in SR patients predisposed to AF, establishing the potential temporal relationship between apoptosis, oxidative stress, and the arrhythmia.

Metabolic dysfunction and dysregulation of the cardioprotective properties of the left atrium were observed in patients with chronic AF compared with patients in SR, all in the context of operated mitral disease [17]. As the patient population comprising the AF group was older and exhibited significantly greater left atrial dimensions, it is very difficult to delineate whether the proteomic changes are attributable to the arrhythmia or to the different disease characteristics and hemodynamic profile between the 2 groups. It is apparent, however, that there was an upregulation of many energetic metabolic enzymes, such as enoyl CoA hydratase, pyruvate dehydrogenase E1, and ATPase β-chain isotype 2, in both the left and right atrial appendages of patients in permanent AF compared with their SR counterparts. The authors explain this activated metabolic state as an attempt of the chronically fibrillating atria to counterbalance for possible preexisting energy depletion and increased energy demand, a hypothesis that we also endorse as seen in a previous combined proteomic-metabolomic study by our group [8]. Finally, comparison of the left and right atrial appendages demonstrated mainly over-expression of cytoskeleton-related proteins such as actin and desmin isotypes. This may be reflective of the different physiological function of the left atrium and its more developed contractile apparatus.

Study Limitations

Despite the use of gold-standard proteomic techniques in this study, it is recognized that very small or large proteins, hydrophobic or very basic and short-lived proteins, may not be accurately represented on 2D gels. In addition, with the extraction of whole tissue lysates identification of extracellular matrix proteins, which only form a minor fraction of the total protein concentration, can be obscured by the more abundant cellular ones. As a result, the important role of extracellular matrix and, therefore, of preexisting structural remodeling in AF pathogenesis, cannot be accurately identified with this proteomic experiment. Although some of the findings of this proteomic analysis complement our previous metabolomic work in AF [8], further validation with analytical techniques such as western blotting may be necessary to ascertain true protein expression differences. Furthermore, identification of preexisting factors leading to postoperative AF does not establish a cause-effect relationship and more studies are needed to investigate the functional relevance of the observed protein changes. In contrast to experimental animal studies, human proteomic experiments carry the inherent variability between subjects, which we tried to minimize by carefully selecting the 2 groups and by analyzing proteins that were present in at least 75% of patients in each group. Despite the use of robust protocol for patient matching, there was a trend for prolonged duration of aortic cross-clamp in the AF group and the potential for unknown confounders. Finally, we included only patients undergoing isolated coronary artery bypass grafting with good left ventricular function, limiting the generalizability of these findings in different populations and cardiac pathologies.

Conclusions

Preexisting alterations in the left atrial substrate, consistent with increased inflammatory state, oxidative stress, apoptosis, and glycolytic inhibition, precede and may be responsible the development of AF after cardiac surgery.

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