Quantitative Proteomics of Changes in Energy Metabolism-Related Proteins in Atrial Tissue From Valvular Disease Patients With Permanent Atrial Fibrillation

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Background: The modification of cardiac energy metabolism during atrial fibrillation (AF) has been demonstrated in previous studies, indicating a close association between these 2 processes. The aim of the present study was to identify the underlying mechanisms via profiling of the expression of energy metabolism-related proteins in the left atrial appendage (LAA) of patients with AF.

Methods and Results: Isobaric tag for relative and absolute quantification-coupled 2-D liquid chromatography-tandem mass spectrometry (iTRAQ-coupled 2-D LC-MS/MS) was used to profile the expression of energy metabolism-related proteins in the LAA from valvular disease patients with sinus rhythm (SR; n=6) and AF (n=8). Using ProteinPilot 4.0, 122 energy metabolism-related proteins, consisting of 39 carbohydrate metabolism-related proteins, 22 proteins involved in lipid metabolism, 49 biological oxidation-related proteins and 12 other kinds of proteins, were identified. Most of them were key enzymes involved in energy metabolism. Moreover, most of the proteins that were expressed differently in the LAA between the AF and SR patients, and which were related to energy metabolism, were down-regulated. These results were further validated on western blot.

Conclusions: Atrial myocardium energy production in valvular disease patients is impaired during permanent AF, and this impairment in energy production may be involved in the matrix of AF formation. (Circ J 2014; 78: 993–1001)

Key Words: 2-D liquid chromatography-tandem mass spectrometry; Atrial Fibrillation; Energy metabolism; Isobaric tag for relative and absolute quantification; Proteomics
lar disease; all patients recruited into the study gave informed consent for their samples to be used. Patients with impaired glucose tolerance and diabetes mellitus were excluded from the study. The right atrial appendages (RAA) and left atrial appendage (LAA) samples were obtained from the same patient as surgical biopsies, both in patients with SR (n=6, without history of AF) and with permanent AF (n=8, documented arrhythmia >1 year before surgery), at the time of mitral valve surgery (RAA from cannulation site and LAA during ligation) following established procedures approved by the local Ethics Committee. SR patients were screened to ensure that they had never experienced AF, via direct questioning about symptoms suggestive of AF and via 12-lead electrocardiography during the preoperative review period. Routine preoperative 2-D color transthoracic echocardiography was performed for all patients. Specimens were immediately snap-frozen and stored in liquid nitrogen after they were obtained during the mitral valve surgery.

iTRAQ and 2-D LC-MS/MS

The iTRAQ technique is a high-throughput quantitative technique and a well-established way to screen the protein profile, which is widely used in basic research. The technical details of the experimental design of this section can be found in previous studies.15–16 Specimens were divided into 4 groups as follows: LAA from SR patients (SR-LAA), RAA from SR patients (SR-RAA), LAA from AF patients (AF-LAA) and RAA from AF patients (AF-RAA). The samples were pulverized under liquid-N2 into a fine powder, which was homogenized in a lysate buffer containing 8 mol/L urea, 2% 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS) w/v, 0.1% sodium dodecylsulfate (SDS) w/v and 0.1% phenylmethylsulfonyl fluoride (PMSF) w/v (Sigma-Aldrich, St Louis, MO, USA). The whole lysate was centrifuged for 30 min at 4°C, 15,000 xg, and the final supernatant was collected. The protein concentrations of cleared lysates were then determined using the 2-D Quant kit (GE healthcare, USA). Then, 1-D SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the protein composition of cleared lysates in order to confirm the validity of the lysate buffer.

The iTRAQ reagents were obtained from Applied Biosystems (Forster City, CA, USA), and the labeling was carried out following the protocol provided by the manufacturer. A set of AF-RAA lysate, AF-LAA lysate, SR-LAA lysate and SR-RAA lysate, each with 100 μg of proteins, was precipitated with acetone, and protein pellets were resuspended in dissolution buffer from the iTRAQ kit. Each sample then was reduced, alkylated, and digested with trypsin at 37°C overnight. After digestion, peptides from each sample were labeled with iTRAQ reagent 113, 114, 115, and 116 at room temperature, respectively. Labeled peptides were pooled and desalted using a SEP-PAK column (Waters, USA), lyophilized, and reconstituted in strong cation exchange (SCX) buffer (10 mmol/L KH2PO4, pH 2.6, 25% acetonitrile).

One-dimensional chromatographic separation of the pooled samples was performed on a Shimadzu 20 AD HPLC system (Shimadzu, Japan). Tryptic digested and labeled peptides were fractionated on cation exchange liquid chromatography using a 2.1×100-mm, 5-μm, 200-Å, column (The Nest Group, MA, USA) with a linear gradient of 0–350 mmol/L KCl (25% v/v acetonitrile, 10 mmol/L KH2PO4, pH 2.8) for 60 min at a flow rate of 200 μl/min and 20 fractions were collected. Each of the fractions was then dried, dissolved in 0.1% formic acid and then loaded onto a reversed phase (RP) column (ZORBAX 300SB-C18 column; 5 μm, 300Å, 0.1×150 mm; Microm, USA) and the RPLC gradient was 5–35%. The flow rate used for separation on the RP column was 300 nl/min.

The LC eluent was subjected to positive ion mode for nanoflow electro-spray analysis using a Triple TOF 5600 system (Applied Biosystems) in information-dependent acquisition (IDA) mode. MS spectra were acquired across the mass range of 400–1,500 m/z in high-resolution mode (>3,000,000) using a 250-ms accumulation time per spectrum. A maximum of 15 precursors per cycle was chosen for fragmentation from each MS spectrum with a 100-ms minimum accumulation time for each precursor and dynamic exclusion for 20s. MS/MS spectra were recorded in high sensitivity mode (resolution >15,000) with rolling collision energy on and iTRAQ reagent collision energy adjustment on.

Data Analysis

Protein identification and relative iTRAQ quantification were done using ProteinPilot 4.0 (Applied Biosystems) using the Paragon algorithm as the database search engine. All reported data were based on 95% confidence for protein identification as determined by Protein Pilot (Prot Score ≥1.3). The confidence levels of the altered expression of proteins were calculated by Protein Pilot as P-values, which allowed the results to be evaluated based on the confidence level of protein expression changes, not only by the magnitude of the changes. The raw peptide identification results from the Paragon Algorithm (Applied Biosystems) were further processed using the Pro Group Algorithm (Applied Biosystems) within ProteinPilot before final display. The Pro Group algorithm can remove redundant hits and comparative determine the amounts of the components so that the minimal set of justifiable identified proteins can be found. In addition, P<0.05 was significant for protein quantification. To designate significant changes in protein expression, fold-changes >1.2 or <0.8 were set as the cut-offs. Both of the 2 repeated MS results reached the setting parameter standard of the protein database.

We performed this analysis twice.

Western Blot Analysis

The iTRAQ protein expression results were validated on western blot. Total protein extracts used for western blot analysis were obtained from the aforementioned experiments for LC-MS/MS analysis. Samples containing 50 μg of total proteins were separated using 12% SDS-PAGE and transferred onto PVDF membranes by electro-blotting. Membranes were then probed with the primary antibody anti-cytochrome c oxidase subunit 5B (anti-cox5B; Sigma-Aldrich) at 1:800 dilution. HRP goat anti-mouse IgG antibody (Proteintech) was used as the second antibody at a dilution of 1:3,000. SupersignalWest solutions (Thermo Pierce) were used. The data represent 1 single experiment.

Statistical Analysis

Clinical characteristics of the SR and AF patients are expressed as mean±SEM. Mann-Whitney test was used to determine statistical differences between SR and AF patients. P<0.05 was considered statistically significant.

Results

Patient Characteristics

We collected LAA samples from mitral valve disease patients under SR or in permanent AF. The left atrium (LA) size for the AF patients was bigger than that of the SR patients, and mitral valve area was smaller in the permanent AF patients than in...
SR patients (Table 1). There were no differences in age, body weight, body mass index, fasting blood glucose, total cholesterol, triglycerides, ejection fraction or severity of mitral valve regurgitation between the 2 groups of patients (Table 1).

Proteomics

Data from iTRAQ labeling and LC-MS/MS contained 1,023 unique proteins from the cardiac tissues, including well-known markers associated with the cytoskeleton, energy metabolism, and cardiac cytoprotection. We used the cut-off 1.2-fold for over-expression and 0.8-fold for under-expression. A total of...
Among the 12 other kinds of proteins, however, 3 of them (aldehyde dehydrogenase, nicotinamide phosphoribosyltransferase and glutamate dehydrogenase 1) were upregulated and only 3-hydroxyisobutyrate dehydrogenase was downregulated in the AF-LAA. These proteins are involved in ethanol oxidation and amino acid metabolism.

**Desmoplakin, COX5b and Heat Shock Protein \( \beta \)-1**

The 1,023 unique identified proteins were associated with the cytoskeleton, energy metabolism, and cardiac cytoprotection, three of which were desmoplakin, COX5b and heat shock protein \( \beta \)-1 (HSP\( \beta \)). Western blot was done to further validate the distribution of these 3 typical proteins between the LAA from patients with mitral valve disease in SR and those in permanent AF. **Figures 1–3** show that the changes in protein level on western blot were generally consistent with the variations on LC-MS/MS results. All proteomics experiments were performed twice to validate the reliability of iTRAQ. The 2 iTRAQ results for desmoplakin, COX5b and HSP\( \beta \) were consistent. Thus, we believe that the present proteomics data are reliable.

**Discussion**

**Main Finding**

The main finding of this proteomics study is that most of the differently expressed energy metabolism-related proteins identified in the AF-LAA were downregulated. Also, most of them were key enzymes in energy metabolism. This suggests that the energy production status of atrial myocytes in valvular disease patients during permanent AF is impaired.

**Significance of the LA in AF**

AF is usually initiated and maintained in the LA.\(^4\) The LA is far from being a simple passive transport chamber. There is convincing evidence of an important pathophysiological association between LA remodeling and AF.\(^4\) LA remodeling includes structural, functional, electrical, metabolic, and neurohumoral changes that occur in response to several pathologic processes.\(^4,18\) Moreover, a recent microarray study using a pig model of AF showed that the gene changes are more pronounced in the LA than the RA, with considerable overlap in the gene response.\(^20\) Another previous study compared differences in

**Table 3. Lipid Metabolism-Related Proteins Expressed Differently in AF-LAA vs. SR-LAA**

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession</th>
<th>114:115(^*)</th>
<th>GN</th>
<th>Function</th>
<th>Expression</th>
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<tr>
<td>Long-chain-fatty-acid–CoA ligase 1</td>
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<td>ACSL1</td>
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<td>Very long-chain specific acyl-CoA dehydrogenase, mitochondrial</td>
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<td>P49748</td>
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<td>ACADVL</td>
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<td>2,4-Dienoyl-CoA reductase, mitochondrial</td>
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<td>( \beta )-oxidation</td>
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<td>3,2-Trans-enoyl-CoA isomerase, mitochondrial</td>
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<td>P42126</td>
<td>0.685488</td>
<td>DCI</td>
<td>( \beta )-oxidation</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>sp</td>
<td>P49327</td>
<td>0.654636</td>
<td>FASN</td>
<td>Fatty acid biosynthetic</td>
</tr>
<tr>
<td>Fatty acid-binding protein, epidermal</td>
<td>sp</td>
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<td>Fatty acid binding</td>
</tr>
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\(^*\)SR-LAA was labeled with reporter tag 115, AF-LAA with reporter tag 114. Abbreviations as in Table 2.
## Table 4. Biological Oxidation-Related Proteins Expressed Differently in AF-LAA vs. SR-LAA

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<th>Protein name</th>
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<th>Function</th>
<th>Expression</th>
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<td>Creatine kinase M-type</td>
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<tr>
<td>Creatine kinase B-type</td>
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<td>Creatine kinase, sarcomeric mitochondrial</td>
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<td>P17540</td>
<td>0.586138</td>
<td>CKMT2</td>
<td>Decrease</td>
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<td>NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 5</td>
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<td>Q16718</td>
<td>0.275423</td>
<td>NDUFAS5</td>
<td>Decrease</td>
</tr>
<tr>
<td>NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial</td>
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<td>0.394457</td>
<td>COX5B</td>
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<td>Electron transfer flavoprotein subunit β</td>
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<td>ATP synthase subunit d, mitochondrial</td>
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<td>Electron transfer flavoprotein subunit α, mitochondrial</td>
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<td>ETFA</td>
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<td>Cytochrome b-c1 complex subunit Rieske, mitochondrial</td>
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<td>ATP synthase-coupling factor 6, mitochondrial</td>
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<td>0.625173</td>
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<td>Chaperone activity of bc1 complex-like, mitochondrial</td>
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†SR-LAA was labeled with reporter tag 115, AF-LAA with reporter tag 114.
NADH, reduced nicotinamide adenine dinucleotide. Other abbreviations as in Table 2.
Figure 1. (A) Western blot of desmoplakin expression in the left atrial appendage from atrial fibrillation (FL) patients and from those in sinus rhythm (SL); (B, C) relative levels measured from (B) western blot and (C) iTRAQ. (D) Comparison of the 2 iTRAQ results. FL:SL: relative level >1.2, up-regulation; relative level <0.8, down-regulation. iTRAQ, iTRAQ-coupled 2-D LC-MS/MS.

Figure 2. (A) Western blot of cytochrome c oxidase subunit 5B (COX5b) expression in the left atrial appendage from atrial fibrillation (FL) patients and from those in sinus rhythm (SL); (B, C) relative levels measured from (B) western blot and (C) iTRAQ. (D) Comparison of the 2 iTRAQ results. FL:SL: relative level >1.2, up-regulation; relative level <0.8, down-regulation. iTRAQ, iTRAQ-coupled 2-D LC-MS/MS.
ed, including glucose-6-phosphate isomerase and 6-phospho-fructokinase. Moreover, many significantly downregulated proteins were key enzymes of glycolysis and the tricarboxylic acid cycle process. This indicates that the main condition of carbohydrate catabolism in AF patients is downregulation. A previous proteomics study found that the downregulation of LA proteins responsible for glycolysis (enolase) and pyruvate metabolism (pyruvate dehydrogenase), may precede the development of AF after cardiac surgery.22 Another proteomics and metabolomics analysis found that malate dehydrogenase, the α-subunit of the E1 component of pyruvate-DH, and α- and β-enolase expression were increased at 24-h and decreased at 2-week ventricular tachypacing.23 Those changes were related to the congestive heart failure-associated atrial remodeling and suggested that metabolic remodeling may contribute to the AF substrate.23 α-enolase is also downregulated in human permanent AF.24 Thus, early increased expression is likely a response to increased metabolic needs, and longer-term decreased expression, an energy-saving adaptation.23 Mayr et al, however, noted upregulation of triosephosphate isomerase in RAA from AF patients.25 The expression of isocitrate dehydrogenase is not different between AF and SR patients.26 In the present study, compared to the upregulated proteins, we found many other carbohydrate metabolism-related proteins were downregulated in AF patients, which may contribute to the impaired energy production con-

![Figure 3.](image)

**Figure 3.** (A) Western blot of heat shock protein β-1 (HSPβ1) expression in the left atrial appendage from atrial fibrillation (FL) patients and from those in sinus rhythm (SL); (B, C) relative levels measured from (B) western blot and (C) iTRAQ. (D) Comparison of the 2 iTRAQ results. FL:SL: relative level >1.2, upregulation; relative level <0.8, downregulation. iTRAQ, iTRAQ-coupled 2-D LC-MS/MS.
conditions during AF. Given that optimal cardiac function under normal and pathological conditions is dependent upon glycolysis and pyruvate oxidation, the decreased carbohydrate metabolism may also contribute to the decreased atrial contraction function in AF patients.

Among the differently expressed proteins involved in lipid metabolism, 8 were downregulated and only 1 protein was upregulated, suggesting that downregulation was the main trend in lipid metabolism in the atria of AF patients. Moreover, those downregulated proteins were closely related to each other, especially long-chain-fatty-acid-CoA ligase 1, which may play a key role in the regulation of lipid metabolism. In addition, the key functions of those proteins in fatty acid oxidative metabolism may also have a significant effect on the energy supply of cardiomyocytes. For example, very-long-chain acyl-CoA dehydrogenase in the atrial tissue of AF patients may seriously disturb myocardial function.

Oxidative phosphorylation is the principal process by which ATP is formed. The changes in enzymes that were involved in respiratory chain complexes may seriously impair energy production in LA myocytes.

Myocardial bioenergy deficits were associated with atrial electrical instability, structural and tonic remodeling and may constitute an additional component of the substrate for AF in congestive heart failure. In fact, a variety of modifications in energy metabolism during AF have been observed. Most studies that identified AF as a high-energy-demand state were based on short episodes of AF, while others that suggested impaired energy production or consumption during AF tended to reflect chronic AF. The 2 previous proteomics studies, however, were focused on the upregulated proteins in the atrium of AF patients. In the present study, we found that few proteins were upregulated and many proteins were downregulated in the LA of AF patients. Western blot was done to further validate the iTRAQ results. The changes in COX5B seen on western blot were generally consistent with those determined on proteomics analysis. Thus, we believe that the application of quantitative proteomics based on iTRAQ technology is effective in evaluating LA tissues.

The complex COX, which is localized to the inner mitochondrial membrane, is the terminal enzyme complex of the mitochondrial electron transport chain and plays an important role in the biosynthesis of ATP. COX5b is the most conserved subunit among the nuclear-encoded subunits of COX and appears to be a single, unique, polypeptide in all tissues. COX5b is highly expressed in the tissues with higher O2 consumption such as the heart, kidney, and brain. COX5b may also play a regulatory role in COX activity. Cyclic adenosine monophosphate-dependent phosphorylation by protein kinase A of COX5b could regulate the intra-mitochondrial ATP/ADP ratio and then allosterically influence the COX activity. Therefore, COX5b likely plays a crucial role in COX assembly and regulation, and consequently in the maintenance of mitochondrial integrity and function. Previous studies suggested that COX5b was specifically upregulated under anaerobic conditions of low oxygen tension, and could be highly expressed in a hypoxic environment in rapidly growing cancer cells. We have confirmed that COX5b in the LAA of AF patients is downregulated compared to SR patients. This means that the increased intra-atrial pressure in LAA and AF could affect the expression of COX5B in the atrium, which may lead to impaired function of the respiratory chain and influence energy production. Besides carbohydrate and lipid, other metabolites such as some amino acids and aldehydes may also influence energy status. Glutamate dehydrogenase 1 is a mitochondrial matrix enzyme that catalyzes the oxidative deamination of glutamate to α-ketoglutarate and ammonia. Aldehyde dehydrogenase could oxidize aldehydes to generate carboxylic acids for use in the heart. In the present study, both glutamate dehydrogenase 1 and aldehyde dehydrogenase were upregulated in the LAA of AF patients. Such strengthening in the catabolism of other metabolites may serve as compensation for the impaired energy metabolism.

Influence of Larger LA on Metabolism

Enlargement of the LA is usually related to LA structural remodeling, and adverse structural and histological changes of the LA are often associated with sustained and prolonged AF. A recent proteomics study found that many energy metabolism proteins were expressed differently in the LAA of AF patients compared to SR patients. Although atrial growth was correlated with only 1 energy metabolism protein, the influence of LA size on energy metabolism should not be neglected. Meanwhile, valvular heart disease often leads to AF through changing gene, mRNA and even ion-channel protein expression. Given that an acute-stretch AF model suggested a specific modification in atrial myocyte energy, we should also consider that valvular heart disease itself may affect the proteomics results.

Study Limitations

The main limitation was the small number of patients, due partly to the low number of patients with mitral valve disease in SR. The small subject group means that some differences in clinical characteristics between the SR and AF patients may not have been identified. In addition, it is possible that the SR patients may develop paroxysmal AF. Further large-scale studies are needed to analyze protein expression in the LAA in AF patients to confirm these results.

Conclusions

Most of the differently expressed energy metabolism-related proteins in the LAA of AF patients were downregulated, indicating that energy production in the atria of myocardium of valvular disease patients during permanent AF is impaired. To our knowledge, this is the first proteomics analysis of permanent AF using iTRAQ-coupled 2-D LC-MS/MS, and it may provide comprehensive insights into the mechanisms of energy metabolism in permanent AF.

Acknowledgments

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