Selective downregulation of mitochondrial electron transport chain activity and increased oxidative stress in human atrial fibrillation

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1Sheikh Khalifa bin Hamad Al Thani Center for Integrative Research on Cardiovascular Aging, Aurora Sinai/Aurora St. Luke’s Medical Centers, Milwaukee, Wisconsin; 2Patient-Centered Research, Aurora Research Institute, Aurora Health Care, Milwaukee, Wisconsin; and 3Aurora Cardiovascular Services, Aurora Sinai/Aurora St. Luke’s Medical Centers, Milwaukee, Wisconsin

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Emelyanova L, Ashary Z, Cosic M, Negmadjanov U, Ross G, Rizvi F, Olet S, Kress D, Sra J, Tajik AJ, Holmuhamedov EL, Shi Y, Jahangir A. Selective downregulation of mitochondrial electron transport chain activity and increased oxidative stress in human atrial fibrillation. Am J Physiol Heart Circ Physiol 311: H54–H63, 2016. First published May 6, 2016; doi:10.1152/ajpheart.00699.2015.—Mitochondria are critical for maintaining normal cardiac function, and a deficit in mitochondrial energetics can lead to the development of the substrate that promotes atrial fibrillation (AF) and its progression. However, the link between mitochondrial dysfunction and AF in humans is still not fully defined. The aim of this study was to elucidate differences in the functional activity of mitochondrial oxidative phosphorylation (OXPHOS) complexes and oxidative stress in right atrial tissue from patients without (non-AF) and with AF (AF) who were undergoing open-heart surgery and were not significantly different for age, sex, major comorbidities, and medications. The overall functional activity of the electron transport chain (ETC), NADH:O2 oxidoreductase activity, was reduced by 30% in atrial tissue from AF compared with non-AF patients. This was predominantly due to a selective reduction in complex I (0.06 ± 0.007 vs. 0.09 ± 0.006 nmol·min⁻¹·citrate synthase activity⁻¹, P = 0.02) and II (0.11 ± 0.012 vs. 0.16 ± 0.012 nmol·min⁻¹·citrate synthase activity⁻¹, P = 0.003) functional activity in AF patients. Conversely, complex V activity was significantly increased in AF patients (0.21 ± 0.027 vs. 0.12 ± 0.011 nmol·min⁻¹·citrate synthase activity⁻¹, P = 0.005). In addition, AF patients exhibited a higher oxidative stress with increased production of mitochondrial superoxide (73 ± 17 vs. 11 ± 2 arbitrary units, P = 0.03) and 4-hydroxynonenal level (77.64 ± 30.2 vs. 9.83 ± 2.83 ng·mg⁻¹ protein, P = 0.048). Our findings suggest that AF is associated with selective downregulation of ETC activity and increased oxidative stress that can contribute to the progression of the substrate for AF.

atrial fibrillation; humans; mitochondria; electron transport chain complexes; oxidative phosphorylation; oxidative stress; superoxide; 4-hydroxynonenal protein adducts

NEW & NOTEWORTHY

The study provides evidence of a selective downregulation of mitochondrial electron transport chain functional activity predominantly affecting complexes I and II and associated increase in oxidative stress in atrial tissue from patients with atrial fibrillation in a well-matched group of patients with respect to comorbidities and well-preserved left ventricular function undergoing open-heart surgery.

ATRIAL FIBRILLATION (AF), a rapid irregular rhythm of the atria, is associated with electrical, functional, and structural changes in the atria that promote the substrate for its recurrence and progression (36, 53, 60). The incidence and prevalence of AF increase with advancing age and aging-associated diseases such as hypertension, ischemic heart disease, and heart failure (2, 40) and contribute to increased morbidity, particularly an increased risk for stroke, heart failure, and death (25, 52). Although the pathophysiology of AF has been well characterized, the underlying mechanisms that contribute to the progression of AF in human atria have not been fully defined (33, 35, 57, 58, 60). Mitochondria, occupying 30% of cardiomyocyte volume, are critical for maintaining normal energetics of the heart, a highly aerobic organ dependent on oxidative phosphorylation (OXPHOS) for maintenance of its normal electrical and mechanical function (1, 61). Imbalance in the production of high-energy phosphate compounds and metabolic oscillations with supply-demand mismatch in adenosine 5’-triphosphate (ATP) can affect cardiac electrical activity through impact on ion channels (5a, 11, 15, 27), oxidative stress, and regulation of cell death/survival signaling (12, 17, 56, 59), which increases predisposition to arrhythmogenesis. However, information pertaining to derangement in the OXPHOS in human AF compared with a well-matched group without AF is lacking. This is important because conditions that predispose to AF—such as aging, hypertension, coronary artery disease, heart failure, or ventricular or atrial dysfunction—can by themselves contribute to mitochondrial dysfunction and must be accounted for when estimating the impact of AF on mitochondrial function (5, 11, 15, 17). Moreover, it is not clear whether reported changes in myocardial energetics and mitochondrial function (1, 27, 56) are causative or the consequence of AF or associated conditions; nor is it clear whether the OXPHOS impairment reported in some (32) but not all (5a) animal studies also occurs in the human atria (12, 37, 50). Increasing evidence has accumulated that oxidative stress plays an important role in the pathogenesis of AF (47, 17, 62) and elevated oxidative stress markers are present in patients with AF (34, 41, 43, 51). Oxidative stress can result from mitochondrial dysfunction with impairment in electron transport chain (ETC) activity (17), but this has not been systematically assessed in human atria.
The purpose of this study was therefore to assess the functional activity of the mitochondrial OXPHOS complexes I–V, expression level of their representative protein subunits, and oxidative stress, in patients with AF and comorbidity-matched patients without AF undergoing open-heart surgery.

METHODS

Patient demographics and clinical characteristics. Middle-aged and elderly patients with or without a history of AF undergoing elective open-heart surgery between July 2012 and February 2016 at Aurora St. Luke’s Medical Center in Milwaukee, Wisconsin, gave informed consent, and their atrial appendage tissues were used for this study. Patients undergoing emergency bypass surgery, requiring inotropic support, and with congenital heart disease, New York Heart Association class III and IV heart failure, systemic disorders such as infection, or severe left ventricular (LV) dysfunction (LV ejection fraction <35%) were excluded. Patients who had had prior cardiac surgery or an ablation procedure for AF management also were excluded. Patients with no history of AF were classified as the non-AF group and those with documentation of sustained AF as the AF group (25). The study was approved by the Aurora Institutional Review Board and adhered to the Health Insurance Portability and Accountability Act and Aurora Health Care patient privacy and security guidelines. The study conformed to the principles of the Declaration of Helsinki.

Processing of atrial samples. After removal from patients, the right atrial appendage tissue was immediately transferred into ice-cold Dulbecco’s phosphate-buffered saline (DPBS). Fat and connective tissue were trimmed off, and the muscle tissue was used fresh for fiber isolation or cut into pieces and frozen in liquid N2 and stored at −80°C for OXPHOS functional analysis, 4-hydroxynonenal (4-HNE) protein adducts, and proteomic assessment. Less than 5 min elapsed between removal of the tissue from the patient and freezing. If any delay occurred in collection or storage, the tissue was not used for the study.

Preparation of atrial homogenate. For functional assessment, the frozen tissue sample (~50 mg) was transferred into an ice-cold buffer (1:20 wt/vol) containing (in mM) 100 KCl, 5 MgCl2, 2 EGTA, and 50 Tris·HCl (pH 7.5) and homogenized with an OMNI Polytron homogenizer (OMNI International, Kennesaw, GA) at setting 6 on ice, as previously described, with minor modifications (55). The homogenate was centrifuged at 1,000 g for 15 min at 4°C; the supernatant was filtered through a polypropylene mesh with open size 0.125 mm, divided into aliquots, and stored at −80°C until being processed.

Polarographic measurements of NADH:O2 oxidoreductase activity. Rotenone-sensitive NADH:O2 oxidoreductase activity was measured in tissue homogenates with a Clarke oxygen electrode at 30°C in medium containing (in mM) 120 KCl, 2 KH2PO4, 1 EGTA, 10 Tris·HCl (pH 7.5), and 0.1 cytochrome c (Cyt c). To define the maximum activity of NADH:O2 oxidoreductase, 100 μM β-nicotinamide adenine dinucleotide, reduced form (NADH) was added and rotenone-sensitive activity was quantified after addition of 4 μM rotenone. The rate of NADH:O2 oxidoreductase activity was normalized to mitochondrion-specific protein citrate synthase (CS) activity and expressed as nanograms of atoms of O2 per minute per CS activity.

Assessment of mitochondrial OXPHOS complexes. The functional activity of individual OXPHOS complexes (Fig. 1) was analyzed polarographically (46) with the Infinite 200 PRO Plate Reader microplate reader (Tecan US, Research Triangle Park, NC) at 30°C. The activity was calculated as previously described (9) and normalized to CS activity.

The activity of complex I (NADH-decyldiubiquinone oxidoreductase) was measured from decreased absorbance of NADH in incubation medium containing 10 mM KH2PO4 (pH 8.0) supplemented with 50 μM 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzo-

![Fig. 1. Measurement of mitochondrial oxidative phosphorylation complex activity.](http://ajpheart.physiology.org/DownloadedFrom)
complex I is downregulated in human atrial fibrillation

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quinone (Qo) as an electron acceptor, 100 μM NADH as an electron donor, and 2 μM antimycin A to block the activity of downstream complexes. The addition of 4 μM rotenone allowed quantification of the rotenone-sensitive activity of the enzyme (Fig. 1). The extinction coefficient 6.81 mM⁻¹·cm⁻¹ for NADH at 340 nm was used to quantify the functional activity. The activity of complex II (succinate-decylubiquinone 2,6-dichlorophenolindophenol reductase) was measured in an incubation media containing 50 mM KH2PO4 (pH 7.4) supplemented with 10 mM succinate as a donor, 50 mM 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q2), and 75 mM 2,6-dichlorophenolindophenol (DCPIP) as an acceptor of electrons, in the presence of 4 μM rotenone and 2 μM antimycin A (Fig. 1). Baseline activity of complex II was determined with 5 mM 2-thienyl-trifluoroacetic acid (TFFA), a selective inhibitor of succinate dehydrogenase. The extinction coefficient 21 mM⁻¹·cm⁻¹ for DCPIP at 600 nm was used to quantify the functional activity of the complex. Complex III (ubiquinol-cytochrome c reductase) activity was determined in an incubation buffer containing (in mM) 10 KCl, 5 MgCl2·2H2O, 20 imidazole, 5.76 MgCl2·6H2O, and 50 MES (pH 7.1), supplemented with 14.3 phosphocreatine, 6.56 MgCl2·6H2O, and 50 MES (pH 7.1), as previously described (24). Coomassie Brilliant Blue G-250 was added to the samples to a final concentration of 0.05%. The samples were separated with NativePAGE Novex precast 3–12% Bis-Tris minigels and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked in 3% nonfat milk dissolved in Tris-buffered saline containing 0.1% Tween 20 and immunoblotted with primary mouse MitoProfile Total OXPHOS human antibody cocktail against complex I subunit nicotinamide adenine dinucleotide hydrogen dehydrogenase (ubiquinone) (NDUFB8), complex II 30 kDa (SDHB), complex III core protein 2 (UQRC2), complex IV subunit I (MTCO2X), and complex V α-subunit (ATPSA). The secondary goat anti-mouse IgG-HRP antibody was used against the primary OXPHOS human antibodies. Proteins were visualized by SuperSignal West Pico chemiluminescent substrate and monitored with UltraQuant v6.0 software in molecular imaging systems (UltraLum, Claremont, CA).

Quantification of protein expression of representative subunits of OXPHOS complexes by Western blot. Frozen atrial tissue (30–50 mg) was homogenized in an ice-cold RIPA lysis buffer with protease/phosphatase inhibitors and centrifuged at 12,000 g for 10 min at 4°C. Tissue extracts were separated by SDS gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose membrane with the iBlot dry blotting system (Thermo Fisher Scientific, Waltham, MA). The membrane was blocked in 5% nonfat milk dissolved in Tris-buffered saline containing 0.2% Tween 20 and immunoblotted with primary mouse MitoProfile Total OXPHOS human antibody cocktail against NDUF8B, SDHB, UQRC2, MTCO2X, ATPSA, anti-Cyt c, and glyceroldehyde-3-phosphate dehydrogenase (GAPDH-14C10) HRP-conjugated rabbit monoclonal antibody against Cyt c and GAPDH. The secondary goat anti-mouse IgG-HRP antibody was used against the primary OXPHOS human and anti-Cyt c antibodies. Proteins were visualized by SuperSignal West Pico chemiluminescent substrate and monitored with UltraQuant v6.0 software in molecular imaging systems (UltraLum). Densitometric evaluation of protein bands was performed with ImageJ software (National Institutes of Health) http://rsb.info.nih.gov/ij/). Bands corresponding to the mitochondrial complexes were normalized to the density of respective GAPDH and Cyt c bands.

Determination of 4-hydroxynonenal protein adducts. The level of 4-HNE was measured in tissue homogenate by the OxiSelect HNE adduct competitive enzyme-linked immunosorbent assay (ELISA) with a commercial kit. Briefly, frozen atrial samples were homogenized in ice-cold PBS buffer with 0.1% BSA and centrifuged for 10 min at 10,000 g. The 4-HNE level was assessed in supernatant according to manufacturer’s recommendations.

Preparation of permeabilized fibers. Myofibers were isolated as previously described by Anderson et al. (3). Briefly, the right atrial appendage tissue was transferred into ice-cold buffer containing (in mM) 7.23 K2EGTA, 2.77 CaK2EGTA, 20 imidazole, 20 taurine, 5.7 ATP, 14.3 phosphocreatine, 6.56 MgCl2·6H2O, and 50 MES (pH 7.1), cut into small strips (6 × 3 mm), and incubated with collagenase type I for 30–45 min at 4°C. Then pieces were washed with fresh buffer and carefully trimmed of connective tissue and fat. Fibers were mechanically separated along the longitudinal axis and permeabilized with saponin (30–50 μg/ml) for 30 min at 4°C. After permeabilization, myofibers were washed in ice-cold buffer containing (in mM) 110 K-Mes (pH 7.4), 35 KCl, 1 EGTA, 5 KH2PO4, 3 MgCl2·6H2O, and 0.02 blebbistatin with 5 mg/ml BSA and remained in the buffer until analysis at 4°C.

Analysis of mitochondrial superoxide production in myofibers. Mitochondrial superoxide production was measured in isolated and permeabilized myofibers in a buffer containing (in mM) 110 K-Mes (pH 7.4), 35 KCl, 1 EGTA, 5KH2PO4, 3 MgCl2·6H2O, and 0.02 blebbistatin with 5 mg/ml BSA and supplemented with 100 μM ADP, 5 mM glucose, and 1 U/ml hexokinase to keep mitochondria in an

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energized and phosphorylating state (3). The level of superoxide production in the myofibers was determined as a change in fluorescence intensity of MitoSOX Red (ΔF/F₀ = 510/580), a mitochondrial superoxide-sensitive indicator, in response to 10 μM antimycin A exposure by time-lapse laser scanning confocal fluorescence microscopy (Olympus FV1200). Frames were acquired 30 s apart with a ×10 objective. The change in the level of superoxide production was quantified as a difference in MitoSOX Red fluorescence intensity before and after antimycin A addition when intensity reached plateau.

Materials. Chemicals for myofiber isolation and biochemical assays, including blebbistatin and cytochrome-c oxidase kit (CYTOCOX1), were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s PBS and collagenase type I were purchased from Lonza BioWhittaker (Walkersville, MD). BCA protein assay kit was from Thermo Fisher Scientific (Waltham, MA). Western blot, 1D BN-PAGE reagents, MitoSOX Red, and Hoechst 33342 were purchased from Life Technologies (New York, NY). Human Mitofluor Total OXPHOS antibody cocktail was from MitoSciences (Eugene, OR). Anti-Cyt c antibody was purchased from Biolegend (San Diego, CA). Secondary goat anti-mouse IgG-HRP antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and GAPDH (14C10) HRP-conjugated rabbit monoclonal antibody and protease/ phosphatase inhibitors were from Cell Signaling Technology (Danvers, MA). OxiSelect HNE adduct competitive ELISA kit was purchased from Cell Biolabs (San Diego, CA).

Statistical analysis. An initial step in the analysis entailed frequency counts and proportions for categorical variables, while continuous variables were summarized as the mean, SE, median, and quartiles. Association among categorical variables was performed with the χ²-test of association and Fisher’s exact test, while the correlation coefficient was used to assess associations among continuous variables. A comparison between non-AF and AF groups was performed with a two-sample t-test. All tests were performed at a 5% level of significance, and statistical analysis was performed with SAS software (version 9.4, SAS Institute, Cary, NC) and SigmaPlot (version 12.3, SYSTAT Software, San Jose, CA).

RESULTS

Clinical characteristics of patients. Right atrial appendage samples were obtained from 62 patients (n = 33 for non-AF vs. n = 29 for AF). The clinical characteristics of non-AF and AF patients are summarized in Table 1. The groups were well-matched for the presence of the risk factors for AF or its complications. There were no significant differences in age, sex distribution, body mass index (BMI), history of chronic obstructive pulmonary disease, coronary artery disease, heart failure, stroke, hypertension, diabetes, left atrial volume index, type of surgery performed, or medications.

Functional activities of OXPHOS complexes. The activity of rotenone-sensitive NADH:O₂ oxidoreductase, representing the overall activity of the mitochondrial ETC, was significantly lower in atrial tissue from patients with AF compared with tissue from non-AF patients (142 ± 6 vs. 201 ± 20 ng atoms O₂-min⁻¹.CS activity⁻¹, P = 0.03) (Fig. 2). To dissect out the specific sites responsible for the diminished ETC activity in patients with AF, the functional activities of individual complexes I–IV as well as the activity of complex V were analyzed in patients without and with AF. The functional activity of the complexes was normalized to CS activity, a mitochondrial-specific matrix protein, which was not significantly different between the two groups (323 ± 31 vs. 356 ± 37 nmol-min⁻¹-mg NCP⁻¹ in non-AF and AF, respectively, P = 0.49). There was significant reduction in activity of mitochondrial complex I (AF 0.06 ± 0.006 vs. non-AF 0.09 ± 0.007 nmol-min⁻¹-1-CS activity⁻¹, P = 0.02) and II (AF 0.1 0.012 vs. non-AF 0.16 ± 0.012 nmol-min⁻¹-1-CS activity⁻¹, P < 0.01) activities in AF patients (Fig. 3). The functional activities of complexes III (AF 0.11 ± 0.018 vs. non-AF 0.15 ± 0.021 nmol-min⁻¹-1-CS activity⁻¹, P = 0.16) and IV (AF 0.55 ± 0.08 vs. non-AF 0.51 ± 0.06 nmol-min⁻¹-1-CS activity⁻¹, P = 0.67) were not significantly different between the two groups (Fig. 3). However, complex V activity was increased in the AF group (AF 0.21 ± 0.027 vs. non-AF 0.12 ± 0.01 nmol-min⁻¹-1-CS activity⁻¹, P < 0.01). Since the mean value for BMI in both non-AF and AF groups was >30 kg/m² (obese group by the World Health Organization classification) (4), the impact of BMI on OXPHOS complexes also was separately analyzed and no correlation between functional activity of the complexes and BMI was observed (Fig. 4).

### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>nAF (n = 33)</th>
<th>AF (n = 29)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>68 ± 2</td>
<td>71 ± 1</td>
<td>0.26</td>
</tr>
<tr>
<td>Range</td>
<td>48–84</td>
<td>53–81</td>
<td></td>
</tr>
<tr>
<td>Male, %</td>
<td>39 (13)</td>
<td>38 (11)</td>
<td>0.91</td>
</tr>
<tr>
<td>Comorbidities, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>27 (9)</td>
<td>38 (11)</td>
<td>0.37</td>
</tr>
<tr>
<td>Hypertension</td>
<td>82 (27)</td>
<td>83 (24)</td>
<td>0.92</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>79 (26)</td>
<td>62 (18)</td>
<td>0.15</td>
</tr>
<tr>
<td>Smoking</td>
<td>58 (19)</td>
<td>41 (12)</td>
<td>0.20</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>12 (7)</td>
<td>14 (4)</td>
<td>0.45</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>94 (31)</td>
<td>83 (24)</td>
<td>0.07</td>
</tr>
<tr>
<td>Heart failure</td>
<td>9 (3)</td>
<td>24 (7)</td>
<td>0.11</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>12 (4)</td>
<td>7 (2)</td>
<td>0.49</td>
</tr>
<tr>
<td>Obstructive sleep apnea</td>
<td>9 (3)</td>
<td>10 (3)</td>
<td>0.87</td>
</tr>
<tr>
<td>Stroke</td>
<td>3 (1)</td>
<td>10 (3)</td>
<td>0.24</td>
</tr>
<tr>
<td>Valve disease</td>
<td>42 (14)</td>
<td>52 (15)</td>
<td>0.46</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>33 ± 1</td>
<td>28 ± 1</td>
<td>0.78</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>60 ± 2</td>
<td>58 ± 2</td>
<td>0.36</td>
</tr>
<tr>
<td>LV diastolic dimension, cm</td>
<td>4.5 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>LV systolic dimension, cm</td>
<td>3.1 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Intraventricular septum thickness, cm</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.47</td>
</tr>
<tr>
<td>LV posterior wall thickness, cm</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.4</td>
<td>0.82</td>
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<tr>
<td>Left atrial volume index, ml (range)</td>
<td>31 ± 3 (16–58)</td>
<td>43 ± 4 (25–75)</td>
<td>0.12</td>
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<tr>
<td>Medications, %</td>
<td></td>
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<tr>
<td>β-Blockers</td>
<td>72 (24)</td>
<td>83 (24)</td>
<td>0.35</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>18 (6)</td>
<td>31 (9)</td>
<td>0.24</td>
</tr>
<tr>
<td>ACE I/ARB</td>
<td>45 (15)</td>
<td>48 (14)</td>
<td>0.82</td>
</tr>
<tr>
<td>Diuretics</td>
<td>49 (16)</td>
<td>69 (20)</td>
<td>0.10</td>
</tr>
<tr>
<td>Statins</td>
<td>70 (23)</td>
<td>48 (14)</td>
<td>0.12</td>
</tr>
<tr>
<td>Insulin</td>
<td>15 (5)</td>
<td>24 (7)</td>
<td>0.37</td>
</tr>
<tr>
<td>OHA</td>
<td>15 (5)</td>
<td>14 (4)</td>
<td>0.88</td>
</tr>
<tr>
<td>Insulin and OHA</td>
<td>12 (4)</td>
<td>17 (5)</td>
<td>0.57</td>
</tr>
<tr>
<td>Type of cardiac surgery, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CABG alone</td>
<td>58 (19)</td>
<td>41 (12)</td>
<td>0.20</td>
</tr>
<tr>
<td>CABG and valve surgery</td>
<td>39 (13)</td>
<td>31 (9)</td>
<td>0.49</td>
</tr>
<tr>
<td>Valve replacement without CABG</td>
<td>3 (1)</td>
<td>17 (5)</td>
<td>0.06</td>
</tr>
<tr>
<td>Other</td>
<td>0 (0)</td>
<td>10 (3)</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as % of total number with number of patients (n) in parentheses, except for age, body mass index, and echocardiography parameters, which are presented as means ± SE. LV, left ventricle; ACE I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor antagonists; OHA, oral hypoglycemic agents; CABG, coronary artery bypass grafting; AF, atrial fibrillation group; nAF, non-atrial fibrillation group.
of complex III, as assessed by MitoSOX Red fluorescence in superoxide production in response to antimycin A, an inhibitor of complex III activity in patients with AF compared with age- and comorbidity-matched non-AF patients (AF 77.64 \pm 30.02 \text{ ng/mg protein} vs. non-AF 9.83 \pm 2.83 \text{ ng/mg protein}, P = 0.048; Fig. 7A). Mitochondrial superoxide production in response to antimycin A, an inhibitor of complex III, as assessed by MitoSOX Red fluorescence in permeabilized myofibers, was more than sixfold higher in AF compared with non-AF patients (AF 73 \pm 17 \text{ vs. non-AF 11 \pm 2 \text{ arbitrary units}}, P = 0.03; Fig. 7, B and C).

DISCUSSION

The major finding of our translational study utilizing human cardiac tissue is that a selective reduction in the mitochondrial ETC activity is present in the atria of patients with AF undergoing open-heart surgery compared with a well-matched group of patients without AF. The strong reduction in activity within the ETC was predominantly observed at the level of complexes I and II, but the assembly of the complexes was not affected by AF. The reduction in complex I functional activity was associated with reduced protein expression of the NDUFB8 subunit, while the expression level of the SDHB subunit of complex II did not change. The functional activity of complexes III and IV and the expression of their representative protein subunits were not significantly different between AF and non-AF groups. Complex V activity was increased in AF patients over non-AF patients. The overall lipid peroxidation, as reflected by 4-HNE protein adduct level, as well as antimycin A-induced mitochondrial superoxide production was higher in atrial tissue from AF patients. The 4-HNE level directly correlated with mitochondrial superoxide production in AF patients. The decrease in NADH:O2 oxidoreductase activity, representing the overall ETC activity in patients with AF, was not related to reduction in the mitochondrial content, as reflected by similar activities of CS activity in AF and non-AF patients.

Mitochondria are the major source of ATP that provides the energy to support myocardial contraction and maintenance of ionic homeostasis and cellular integrity (21, 22, 61). Under physiological conditions, >90% of energy production required for heart function comes from mitochondria and is dependent on activity of the multisubunit complexes forming the ETC (complexes I–IV) that generates the proton–motive force driving ATP synthesis through complex V. The activities of the ETC and ATP synthesis are tightly regulated to match ATP supply and demand in the heart during physiological workload (22). Any alteration in mitochondrial function is therefore expected to reduce the energetic reserves and disturb homeostatic balance during stress, resulting in impairment of cardiac electrical and mechanical functions (1, 5, 10, 11, 48, 59) and,
through regulation of cell death and fibrosis, contributes to the
development of the substrate for AF and its progression with
aging and aging-associated diseases (39, 46, 49). Reduction in
mitochondrial function has been demonstrated previously in
patients with AF; however, the confounding effect of aging or
disease conditions such as heart failure, stroke, mitral valve
diseases, history of chronic obstructive pulmonary disease,
coronary artery disease, hypertension, obstructive sleep apnea,
chronic renal failure, diabetes, or obesity on mitochondrial
function was not evaluated. Our study assessed differences in
the activity of the OXPHOS complexes after careful matching
of patients with and without AF for age, sex, and comorbid-
ties, known factors that can alter mitochondrial function (22,
44, 49). Since patients in our study population were predomi-

Fig. 4. Correlation between functional activity of oxidative phosphorylation complexes and body mass index (BMI). ○, non-atrial fibrillation (nAF); ●, atrial fibrillation (AF). Functional activity is expressed in nanomoles per minute per citrate synthase activity. *P < 0.05.

Fig. 5. Separation and identification of mitochondrial complexes from patients without and with atrial fibrillation. A: representative gel of separated mitochondrial oxidative phosphorylation (OXPHOS) complexes I–V in native form by 1-dimensional BN-PAGE electrophoresis. B: identification of OXPHOS complexes after transferring proteins from BN-PAGE gel to PVDF membrane with MitoProfile Total OXPHOS human antibody cocktail. C and D: Western blot of individual subunits of OXPHOS complexes I–V using MitoProfile Total OXPHOS human antibody cocktail (C) and densitometry of bands from immunoblot corresponding to mitochondrial complexes normalized to density of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band (D). MW, molecular weight of the marker; nAF, without atrial fibrillation (n = 11); AF, with atrial fibrillation (n = 11); C I–C V, complexes I–V. Data are means ± SE. *P < 0.05.
nant obesity (BMI > 30), we additionally performed correlation analysis between the mitochondrial complexes and BMI and found that the correlation was not significant.

The reduction in functional activity of complex I in AF was associated with a significant reduction in protein expression level of the NDUFB8 subunit in complex I. Mitochondrial complex I is the largest component of the ETC, consisting of 45 protein subunits. Of these, 7 are encoded by mitochondrial DNA and 38 by nuclear genome (13). The main function of complex I is oxidation of NADH, transferring electrons from NADH to the ETC for ATP production. In addition, it has been proposed that complex I plays an essential role in the assembly of respirasomes that bring together ETC subunits, providing structural interdependence to the individual OXPHOS complexes important in channeling electron flow, controlling superoxide production (13, 38), and sensitivity of mitochondria toward mitochondrial permeability transition pore opening (28). Alterations in expression of genes and protein subunits of complex I lead to disassembly, instability of the complex, and an increase in reactive oxygen species (ROS) production, thus resulting in cardiac diseases, cardiomyopathy (7, 18), and heart failure (28). The BN-PAGE data indicate no difference in mobility of the complexes, suggesting that the structure of the complexes is not altered and a selective reduction in complex I and II activity might be due to other alterations. A reduced expression level of the NDUFB8 subunit points to the functional importance of this protein in AF, which has been reported previously to be reduced in human atria with aging and in patients with obesity (42). In our study we accounted for differences in age, diabetes, BMI, and other comorbidities.

Patients with AF had reduced functional activity in complex II, but this was not associated with expression level of SDHB subunit. Complex II is a mitochondrial membrane-bound component of the Krebs cycle. It is composed of four nuclear encoded subunits that transfer electrons from FADH2 to coenzyme Q and downstream complexes for ATP synthesis and involved in ROS production (14). The decrease in functional activity of complex II without changes in protein expression level has been previously described in a rabbit model of pressure-overload hypertrophy (22). In streptozotocin-induced diabetic rats, significant decline in the cardiac OXPHOS complex I and II enzyme activity was observed without changes in complex III, IV, and V activity (30). This was associated with increased oxidative stress, lipid peroxidation, and 4-HNE gen-
eration in cardiac mitochondria similar to our observation in AF patients that could be the result of reduced electron utilization along the ETC. The precise mechanism of impairment of the ETC complexes activity is not known. Proposed mechanisms include the reduction of the ETC activity due to posttranslational modifications of subunits as a result of oxidation (17, 20, 29) or carbonylation and/or alteration of mitochondrial DNA (12, 31, 32, 56) that can promote energy impairment or susceptibility to cell death and fibrosis contributing to the substrate for arrhythmogenesis or progression of AF (1, 5, 11, 20, 29). However, this needs to be further confirmed in human atrial tissue.

In our study, oligomycin A-sensitive ATPase activity was increased in atrial tissue from patients with AF, which might be a compensatory mechanism to maintain ATP production with reduced ETC activity as previously shown in a pacing-induced AF sheep model (7) to match the ATP production with consumption.

Our findings are in line with the recent report of reduction in mitochondrial function in atrial myocardial fibers from patients with metabolic syndrome who developed postoperative AF, providing a link between mitochondria and AF (37). Specifically, ADP-stimulated mitochondrial respiration supported by complex I-dependent substrates was significantly decreased in patients who subsequently developed postoperative AF, and the reduced mitochondrial respiration was an independent predictor of postoperative AF. In contrast, ADP-stimulated complex II-dependent respiration was not different but activity of complex III was reduced. These findings in human tissue, however, are not universal in other models of atrial dysfunction. In a canine model of AF after pacing-induced heart failure, a reduction in the activities of complexes III and V, without change in the activity of complexes I, II, and IV, was reported (32). Similarly, no change was reported in the activities of complex IV, ATPase, or NADH oxidase in a goat model of chronic AF (5a). Adenine nucleotides and their degradation products did not change significantly during AF (5a). In human mitochondria from heart failure patients, the ETC activity and mitochondrial function also were reported to be preserved (19). The exact cause for the discrepancy in the functional activity of the OXPHOS complexes in reported studies is not known, but it could be related to the specific substrate (structural and electrical remodeling) for AF or triggering factors, coexisting conditions, and species-dependent effects of AF on cardiac energetics.

Impairment in complex I and II activity was associated with increase in mitochondrial superoxide production and 4-HNE protein adduct level in our AF patients, in agreement with other studies indicating enhanced level of oxidative stress with elevated markers, 4-HNE (30), superoxide, diacrine-reactive oxygen metabolites (43), oxidized glutathione, oxidized thiols (41), peroxynitrite, nitric oxide synthase, and NAD(P)H oxidase (29, 47). Mitochondria from AF patients exhibited more than sixfold higher production of superoxide to antimycin A treatment compared with non-AF patients (Fig. 7, B and C). Increased oxidative stress in AF patients explains the higher level of 4-HNE (Fig. 7A) and is consistent with reduced functional activity of complexes I and II. This suggests that increased mitochondrial ROS production may underlie the oxidative stress in AF. Enhanced ROS production can predispose to myocardial injury and arrhythmogenesis (1). The relative contribution of other mitochondrial and cytosolic ROS sources contributing to AF progression is beyond the scope of the present study.

A serious limitation in studying pathophysiology at a molecular level in human tissue, particularly from the heart, is the variability in patient demographics and comorbidities, as well as in the amount of tissue obtained from a patient and the site from where the tissue was taken. This variability makes assessment difficult. Absence of a true control group also is challenging, as biopsies from healthy human hearts cannot be obtained. Previous studies have included patients with variable extent of myocardial or valvular dysfunction and comorbidities. Therefore, in the present study, we closely matched the baseline characteristics of the AF and non-AF groups of patients with respect to age, sex, myocardial dysfunction, and comorbidities known to affect mitochondrial function to clarify the effect of AF on the OXPHOS in human atrial tissue. Special care was taken to collect tissue in a standardized manner, avoiding any delays that can affect mitochondrial function. Our sample size of 62 patients is small, but the data presented are from human atrial tissue, which is difficult to obtain, especially when we apply strict inclusion and exclusion criteria to minimize the confounding effects of conditions such as severe heart failure, reduced ejection fraction, presence of comorbidities, and atrial enlargement. These conditions are known to affect mitochondrial function, and therefore patients with and without AF should be matched. We believe our careful matching of the AF and non-AF groups in terms of baseline demographics, comorbidities, cardiac structure and function, and medication is a major strength. This allows differentiation of the impact of AF on mitochondrial function, which distinguishes our study from others (12, 37, 50) that have not accounted for these important clinical differences that could affect mitochondrial bioenergetics. In our study, the observed mitochondrial dysfunction with reduced ETC activity and increased oxidative stress in AF patients is not proof of a cause-and-effect relationship for the development of AF but is an important factor that can contribute to the progression of AF substrate. Further studies utilizing interventions that can improve mitochondrial function and reduce oxidative stress can help to clarify the cause-and-effect relationship and potential therapeutic target for preventing progression and complications of AF.

In summary, our study in a well-matched group of patients with well-preserved LV function undergoing open-heart surgery provides evidence of a selective downregulation of mitochondrial ETC functional activity predominantly affecting complexes I and II with associated increase in oxidative stress in atrial tissue from patients with AF. This provides cues for further investigation to narrow down targets that can be modulated to prevent progression of the substrate that promotes AF, a common arrhythmia whose increasing prevalence and associated complications are a major public health concern.

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COMPLEX I IS DOWNREGULATED IN HUMAN ATRIAL FIBRILLATION

GRANTS
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AUTHOR CONTRIBUTIONS

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