Impaired cardiac autophagy in patients developing postoperative atrial fibrillation

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Objectives: Postoperative atrial fibrillation (POAF) is a common complication after on-pump heart surgery. Several histologic abnormalities, such as interstitial fibrosis and vacuolization, have been described in atrial samples from patients developing POAF. This ultrastructural remodeling has been associated with the establishment of a proarrhythmic substrate. We studied whether atrial autophagy is activated in patients who develop POAF.

Methods: A total of 170 patients in sinus rhythm who had undergone elective coronary artery bypass grafting were included. Systemic inflammatory markers were measured at baseline and 72 hours after surgery. During the procedure, samples of the right atrial appendages were obtained for evaluation of remodeling by light and electron microscopy. Protein ubiquitination and autophagy-related LC3B processing were assessed by Western blot.

Results: Of these patients, 22% developed POAF. The level of high-sensitivity C-reactive protein, fibrosis, inflammation, myxoid degeneration, and ubiquitin-aggregates in the atria did not differ between patients with and without POAF. Electron microphotographs of those with POAF showed a significant accumulation of autophagic vesicles and lipofuscin deposits. Total protein ubiquitination was similar in the patients with and without POAF, but LC3B processing was markedly reduced in those with POAF, suggesting a selective impairment in autophagic flow.

Conclusions: This study provides novel evidence that ultrastructural atrial remodeling characterized by an impaired cardiac autophagy is present in patients developing POAF after coronary artery bypass surgery. (J Thorac Cardiovasc Surg 2012;143:451-9)

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Postoperative atrial fibrillation (POAF) is a common complication after coronary artery bypass surgery (CABG), affecting approximately 30% of patients.¹ Although it is usually considered benign, POAF accounts for increased

short- and long-term mortality, postoperative hemodynamic instability, increased stroke risk, and overall increased health costs.^{2,3}

The underlying mechanisms for POAF remain largely unknown. Evidence has shown that inflammation and oxidative stress associated with surgical procedure serve as triggers in patients with a biologic substrate that renders them susceptible for arrhythmia development.^{4,5} However, the characterization of such a substrate has proved elusive. The histologic abnormalities described in the atria of patients developing POAF include interstitial fibrosis, vacuolization, and nuclear derangement of myocytes²; however, the association of such findings with POAF development is not conclusive.⁶ This suggests that patients with POAF have pre-existing ultrastructural atrial remodeling; however, simple histologic examination might not be sufficient to predict arrhythmia development.

One of the histologic findings most consistently reported in patients who develop POAF is cardiomyocyte vacuolization.² This morphologic feature has been associated with normal aging, as well as myocardial ischemia. Intriguingly, cardiomyocyte vacuolization is also the dominating morphologic feature related to autophagy.⁷ Autophagy is a dynamic and highly regulated cellular process in which components of the cytoplasm are self-digested by lysosomes, playing a role in the removal and recycling of

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Abbreviations and Acronyms

AF	= atrial fibrillation
CABG	= coronary artery bypass grafting
IP ₃ R	= IP ₃ receptor
POAF	= postoperative atrial fibrillation
RAA	= right atrial appendage

long-lived or damaged protein and organelles.⁸ In pathologic conditions, such as nutrient starvation and hypoxia, autophagy is upregulated and provides substrates for catabolic processes in a condition of metabolic stress. However, an excessive activation of autophagy can lead to cell death and has been associated with the early stages of pathologic cardiac remodeling.⁹ The protein LC3B (also called Atg8) is a key player in the autophagic process. LC3BI (its unprocessed form) is a constitutive cytoplasmic protein. In conditions of metabolic stress, LC3BI is processed by Atg4; the resulting protein (LC3BII) becomes enriched in the autophagosomes. The LC3BII/LC3BI ratio has been used as a measurement of autophagy.⁸ In the present study, we present biochemical and histologic evidence of impaired autophagy in the atria of patients who developed POAF, suggesting that ultrastructural atrial remodeling is associated with the establishment of a proarrhythmic substrate. The present study provides relevant information in the search for a better understanding of the mechanisms involved in POAF development.

METHODS**Subjects**

Patients in normal sinus rhythm who underwent elective CABG at Hospital Clinico de la Pontificia Universidad Católica de Chile from January 2008 to May 2010 were included. The baseline clinical characteristics, two-dimensional echocardiogram, and basic laboratory tests were obtained at admission. All patients underwent CABG using a roller pump and a membrane oxygenator with a standard cardiomyotomy suction setup. Hypothermia was used in all cases, cooling the patients down to a core temperature of 30°C. Myocardial protection was obtained with antegrade and retrograde crystalloid (St. Thomas cardioplegic solution) infused alternatively every 10 to 15 minutes. No aprotinin was used in any case at any time. Venous drainage was achieved with a single double-stage cannula introduced through right atrial appendage (RAA) amputation and directed toward the inferior vena cava. The resected RAA biopsies were obtained at right atrial cannulation, before the onset of cardiopulmonary bypass, immediately introduced in a portable nitrogen tank for transportation to the laboratory, and preserved in this condition for histologic examination and biochemical studies.

The patients were monitored continuously for 72 hours with a telemetry system with automated arrhythmia detection (IntelliVue MP70; Phillips Healthcare, Andover, Mass). In our previous work,¹ the peak for POAF was obtained after 11 ± 28 hours, and most POAF events occurred before day 3. In every patient with a suspected arrhythmic event, a standard 12-lead electrocardiogram was performed and reviewed by a trained cardiologist. Episodes of atrial fibrillation (AF) longer than 15 minutes were considered an episode of POAF for the purposes of the present study.

To exclude the effect of other proinflammatory factors from the analysis, the following exclusion criteria were applied: the need for urgent revascularization or pre-operative cardiogenic shock; a history of myocardial infarction within the previous 2 months; a need for associated heart valve surgery; a history of malignancy, rheumatologic disease, or any chronic inflammatory disease; chronic steroid treatment; thyroid dysfunction; and evidence of active infection. Our institutional ethics committee approved the present study, and all patients gave written informed consent.

Tissue Array

Tissue arrays were generated from paraffin stacks containing a sample of RAA. Each tissue cylinder was sectioned from the endocardium to epicardium using a precision instrument (Manual Tissue Microarrayer MTA-1; Beecher Instruments, Sun Prairie, Wis) and placed in a recipient paraffin block. Thin sections from this block (3 to 4 μm) were stained with hematoxylin-eosin and Masson trichrome; the images were reviewed by two independent pathologists who were unaware of POAF occurrence. In the case of differences between the evaluators, a third evaluator resolved the discrepancy.

Morphologic features, including atrial myocyte vacuolization, myxoid degeneration, and fibrosis and mononuclear infiltration, were assessed using a semiquantitative scale detailing the intensity of the characteristics evaluated from none to low, mild, and high.

Immunostaining

Paraffin sections of the tissue array were deparaffinized, and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 minutes. The slides were incubated in a moist chamber for 90 minutes with a primary rabbit polyclonal antibody against ubiquitin (Chemicon International, Temecula, Calif) and subsequently incubated with a bridge-antibody consisting of biotinylated anti-rabbit IgG (1:300 dilution; BioGenex, San Ramon, Calif). Between steps, the sections were washed with Tris-buffered saline. Diaminobenzidine and 0.04% hydrogen peroxide in distilled water was used as the chromogen, and the reaction was intensified by incubating in 0.1% osmium tetroxide for 3 minutes. All specimens were counterstained with hematoxylin-eosin.

Electron Microscopy

The tissue samples were fixed in 2% glutaraldehyde in 0.05 M (pH 7.3) cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were stained with aqueous uranyl acetate for 25 minutes and Reynolds lead citrate for 20 minutes and examined under a Philips Tecnai 12 BT electron microscope at 80 kV. Electron microscopy images were analyzed by two independent observers who were unaware of POAF occurrence using the NIH ImageJ software (Rasband WS, ImageJ, US National Institutes of Health, Bethesda, Md, available from: <http://rsb.info.nih.gov/ij/>, 1997–2009).

Total Protein Extracts

Atrial tissue was weighed and homogenized on ice in RIPA homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM sodium vanadate). The resulting homogenate was then centrifuged at 6,000g for 10 minutes. The protein concentration in the supernatant was determined by Lowry assay.

Western Blots

Aliquots of the extracted proteins (50 μg/lane) were separated according to the molecular weight on a monodimensional 15% and 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for LC3B and protein polyubiquitination. The resolved proteins were transferred to supported nitrocellulose sheets (BioRad, Hercules, Calif) at 400 mA for 105 minutes

and, after saturation of nonspecific-binding sites with 5% nonfat milk dissolved in Tris-buffered saline Tween-20 (20 mM Tris-HCl, 137 mM NaCl, 0.1% [vol/vol] Tween-20, pH 7.6), incubated overnight with specific polyclonal antibodies against LC3B (dilution 1:1,000, Cell Signaling Technology, Boston, Mass) and a monoclonal antibody against ubiquitin (dilution 1:100,000, Cell Signaling Technology). The blots were washed in Tris-buffered saline Tween-20 and incubated with the corresponding antibody conjugated with horseradish peroxidase (Calbiochem, Darmstadt, Germany), anti-rabbit antibody diluted 1:10,000, and anti-mouse diluted 1:5,000. After washing with Tris-buffered saline Tween 20, the protein bands were visualized using an enhanced chemiluminescence kit (EZ-ECL; Biological Industries, Kibbutz Beit Haemek, Israel) and revealed in radiographic films. The blots were scanned, and the digitized images were subjected to densitometry analysis using UN-SCAN-IT gel software, version 6.1 (Silk Scientific, Orem, Utah). The protein content was normalized using glyceraldehyde 3-phosphate dehydrogenase or β -actin as internal controls (both antibodies from Sigma-Aldrich, St. Louis, Mo).

Statistical Analysis

Continuous variables are expressed as the mean $\pm$ standard deviation; for categorical variables, n (%) was used. Continuous variables were tested for normality using the Kolmogorov-Smirnov test. For normally distributed variables, univariate comparisons between groups were done with the Student *t* test; otherwise, the Mann-Whitney *U* test was used. Categorical variables were analyzed with the chi-square test. Variables with significant differences between groups were included in a multivariate logistic regression analysis to evaluate their independence as predictors of POAF occurrence. Statistical analysis was performed using the Statistical Package for Social Sciences, version 16.0 (SPSS, Chicago, Ill). The authors had full access to the data and take full responsibility for its integrity.

RESULTS

Patient Clinical Characteristics

A total of 170 patients were included; 144 (84%) were men. Of the 170 patients, 38 (22%) developed POAF, and all returned to sinus rhythm within 8.2 ± 6.4 hours after POAF onset. The patients who developed POAF were significantly older (62.4 ± 10.5 vs 73.5 ± 8.3 years, $P < .01$), without significant differences in other comorbidities. In our cohort, no cases of postoperative atrial flutter developed. All the reported cases corresponded to POAF. The baseline therapy for the patients with POAF included angiotensin-converting enzyme inhibitors more often (79 vs 24 patients, $P = .01$). In contrast, β -blockers and statins were more commonly used in the group of patients who did not develop POAF (89 vs 16 patients, $P = .03$, and 100 vs 22 patients, $P = .01$, respectively). One patient from each group was receiving amiodarone at admission for ventricular arrhythmia. The EuroSCORE operative risk was greater for the group who develop POAF, mostly because of older age and comorbidities. The echocardiographic evaluation showed a moderate left atrial enlargement in the patients with POAF (39.4 ± 14.4 vs 32.5 ± 19.1 mm, $P = .04$) with no differences in systolic left ventricle function as assessed by the left ventricular ejection fraction. Age did not correlate with the use of β -blockers (57.9% in subjects older than 75 years vs 53.7% in those younger, chi-square test, $P = .75$) or statins (60% in subjects older than 75 years vs 58.5% in those younger, chi-square test, $P = .84$).

TABLE 1. Clinical, echocardiographic, and surgical characteristics of patients with and without postoperative atrial fibrillation

	Without POAF (n = 142)	With POAF (n = 38)	P value
Clinical characteristics			
Age	62.4 ± 10.5	73.5 ± 8.3	$< .01$
Male	115 (81)	29 (76.3)	.50
Arterial hypertension	99 (69.7)	29 (76.3)	.55
Diabetes mellitus	45 (31.7)	11 (28.9)	.85
Dyslipidemia	97 (68.3)	21 (55.3)	.18
Stroke	6 (4.2)	0 (0)	.35
COPD	6 (4.2)	1 (2.6)	.99
Concurrent medication			
Beta blockers	89 (62.7)	16 (42.1)	.03
ACE inhibitors	79 (55.6)	24 (63.2)	.01
Statins	100 (70.4)	22 (57.9)	.01
Amiodarone	1 (0.7)	1 (2.6)	.37
Echocardiography			
Left atrial diameter (mm)	32.5 ± 19.1	39.4 ± 14.4	.04
Left ventricular ejection fraction (%)	39.2 ± 28.7	44.9 ± 27.7	.34
E wave (mm/s)	53.4 ± 35.9	63.8 ± 47.6	.19
A wave (mm/s)	55.2 ± 36.6	61 ± 39.8	.45
Surgery			
CPB time (min)	102.4 ± 43.7	105.4 ± 49.2	.72
Aortic clamp time (min)	65.7 ± 35.2	66.8 ± 44.2	.87
Postoperative balance (L)	2.1 ± 1.1	2.3 ± 1.2	.19
Euroscore	3.9 ± 1.9	5.8 ± 1.9	$< .01$
Water balance	2025 ± 1116	2772 ± 932	.03

Value expressed as mean $\pm$ SD or n (%). POAF, Postoperative atrial fibrillation; COPD, chronic obstructive pulmonary disease; ACE, angiotensin converting enzyme; CPB, cardiopulmonary bypass.

The left atrial diameter was slightly greater in the patients older than 75 years (39.7 ± 3.7 mm vs 38.6 ± 2.3 mm, $P = .83$). Accordingly, the left anterior descending artery seemed unrelated to age in our cohort (Pearson's $r = 0.177$, $P = .60$). No statistically significant difference was found in the use of amiodarone between patients with and without POAF ($P = .37$). The clinical and echocardiographic data are listed in Table 1.

Patient Laboratory Data

The baseline high-sensitivity C-reactive protein level was slightly increased in both groups (2.4 ± 2.3 vs 2.5 ± 2.7 mg/dL in patients with and without POAF, respectively). No significant differences were observed in the white blood cell count (6.9 ± 1.7 vs 7.4 ± 1.8 10^3 /mL in patients with and without POAF, respectively) or in the neutrophil count (4.5 ± 1.8 vs 4.7 ± 1.6 10^3 /mL in patients with and without POAF, respectively) or in the findings from other routine baseline laboratory tests, except for higher levels of blood urea nitrogen in patients with POAF (23.7 ± 6.7 vs 20.1 ± 8.2 , $P = .02$). After CABG, these inflammatory markers had increased in all patients, irrespective of POAF development.

FIGURE 1. Light microscopy histologic analysis of patients with postoperative atrial fibrillation (POAF) is not different from that of controls. A, Representative microphotograph of the right atrial appendage (RAA) of patients with and without POAF stained with ubiquitin antibody. Semiquantitative analysis of (B) fibrosis, (C) inflammation, (D) mixoid degeneration, and (E) ubiquitination. Results are presented as distribution of every score for each group of patients (without POAF, $n = 38$; with POAF, $n = 22$). The score details the intensity of the characteristic evaluated from none (N), low (L), mild (M), to high (H). NS, Not significant.

Atrial Histologic Findings

Thin sections of RAA from all patients with and without POAF were submitted for light microscopy analysis. Figure 1, A, shows representative images of ubiquitin-stained samples of patients without and with POAF. The atrial histologic features were qualitatively preserved in both groups, with no significant changes in the degree of fibrosis ($P = .08$), inflammation ($P = .34$), or myxoid degeneration ($P = .45$). Figure 1, C–E shows the distribution of the semiquantitative score for the aforementioned variables in patients with and without POAF. Although ubiquitin aggregates were more common in those with POAF (Figure 1, A), the difference did not reach statistical significance (Figure 1, E, $P = .06$).

Electron microphotographs of the atria samples from the patients with POAF showed marked myocyte vacuolization,

lipofuscin accumulation, and loss of sarcomere organization (Figure 2, A–D). Vacuoles were identified as autophagosomes using LC3 protein immunogold labeling in fixed human atrial samples (see Figure E1, A and B). Furthermore, patients with POAF had a significant increase in the number of early and late autophagic vacuoles (26 ± 15 vs 3 ± 1 per cell, $P = .03$, Figure 2, E), even when the relative area of the autophagosomes was similar ($15\% \pm 9\%$ vs $12\% \pm 4\%$ of total cell area, $P = .42$ for patients with and without POAF, respectively; Figure 2, F). Interestingly, the lipofuscin aggregates, a marker of undigested waste material, were larger in patients with POAF (relative area $4.1\% \pm 2.9\%$ vs $0.6\% \pm 0.6\%$, $P = .02$; Figure 2, G). Neither the number or autophagic vacuoles or lipofuscin aggregates area correlated with age ($r^2 = 0.04$, $P = .55$ and $r^2 = 0.02$, $P = .71$, respectively).

FIGURE 2. Histologic alterations in patients with postoperative atrial fibrillation (POAF) observed by electron microscopy. A and B, Representative electron microscopic images of the right atrial appendage (RAA) of patients without POAF showing well-preserved mitochondria and filaments, with few autophagosomes visible. C and D, Images of patients who developed POAF, showing mitochondria with focal disorganization of the cristae, lipid droplets, autophagosomes with cellular debris, and lipofuscin granules. Arrows denote autophagic vesicles, and arrowheads mark lipofuscin aggregates. E, Number of cells with 0 to 5 or more than 5 autophagosomes in controls (n = 9) and patients with POAF (n = 11). F and G, Relative area of autophagosome vesicles and lipofuscin aggregates in the electron microscope images from patients with and without POAF. Asterisk indicates significant difference. NS, Not significant.

LC3B Processing

Figure 3, A shows the determination of the two forms of LC3B, namely I (unprocessed) and II (processed) by Western blotting. LC3BII was significantly reduced in the patients with POAF compared with those without POAF (Figure 3, B). The LC3BII/LC3BI ratio was also significantly decreased in patients who developed POAF in relation to those who maintained sinus rhythm after CABG

(0.65 ± 0.02 vs 0.56 ± 0.03 , $P = .02$; Figure 3, C). Regarding autophagy markers, only lipofuscin aggregates were increased in the older patients. The LC3BII/LC3BI ratio, total autophagosome area, or autophagosome count did not appear to be related to age in our cohort. Although our study was not powered to assess the effect of concurrent medication, the use of statins was related to a decreased number of autophagosomes (3.2 ± 1.9 vs 7.9

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FIGURE 3. LC3B processing is impaired in patients with postoperative atrial fibrillation (POAF). A, Western blot of LC3B in right atrial appendage (RAA) samples from patients with and without POAF. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level is shown as load control. B, Quantification of the LC3BII/ β -tubulin ratio ($n = 16$). C, Quantification of the LC3BII/LC3BI ratio ($n = 16$). * $P < .01$ versus patients without POAF.

± 2.9 , $P = .03$) without changes in lipofuscin deposits or total autophagosome area. Previous β -blocker use was not associated with any changes in the autophagosome count or lipofuscin deposits.

Protein Ubiquitination

As mentioned, light microscopy did not reveal any significant differences in the number of ubiquitin aggregates between patients with and without POAF (Figure 1, A and E). To further assess proteasome-dependent degradation, we evaluated the polyubiquitination pattern of total protein samples by Western blotting. As shown in Figure 4, A, no changes were found in total protein polyubiquitination normalized to total β -actin ($P = .05$; Figure 4, B).

DISCUSSION

To our knowledge, this is the first report of impaired autophagy in patients who develop POAF, contributing additional evidence of a relationship between POAF and pre-existing histopathologic changes in the right atrium such as interstitial fibrosis, vacuolization, and nuclear derangement of myocytes, as has been previously reported.¹⁰ These changes contribute to the development of a susceptible substrate for arrhythmia development. After CABG, several different mechanisms might trigger the arrhythmia, including pericarditis, altered fluid mobilization, increased sympathetic activity, and autonomic imbalance during the postoperative period.^{2,3} This evidence suggests a “2-hit”

mechanism for POAF development, in which atrial morphologic and metabolic remodeling serve as favorable substrate and surgical-related variables as triggers for arrhythmia. These mechanistic considerations might also explain the limited utility of our current prophylactic strategies and the challenges in identifying subjects more likely to benefit from interventions aimed to prevent POAF development.

Our study implies a potential link between the metabolic and morphologic changes previously described in patients who develop POAF¹¹ by providing evidence for an increase in autophagic vesicles compared with the controls. Autophagy is involved in the recycling of long-lived proteins and organelles, especially mitochondria, and contributes to the maintenance of cellular energy homeostasis in the heart, particularly in response to an increased energy demand or decreased nutrient availability.¹² Several studies have implicated autophagy in the pathophysiology of heart failure⁹ by increasing protein degradation and decreasing the cardiac mass. Increased autophagic activity in hypertrophied hearts is thought to be involved in the transition from stable cardiac hypertrophy to decompensated heart failure.^{9,12} The high levels of autophagy observed during heart failure support the theory that an excess in autophagic flux is responsible for autophagic cell death and cardiomyocyte loss. Furthermore, dead and dying cardiomyocytes showing the characteristics of excessive autophagic activity have been reported in heart failure due to dilated

FIGURE 4. Total protein ubiquitination is not modified in patients with postoperative atrial fibrillation (POAF). A, Western blot of polyubiquitination pattern of patients with and without POAF. B, Quantification of polyubiquitinated proteins relative to the amount of β -actin ($n = 9$).

cardiomyopathy,¹³ chronic ischemia, and stunned or hibernating myocardium but not in normal hearts.¹³ However, it remains unclear whether autophagy is a sign of failed cardiomyocyte repair or a suicide pathway for the failing cardiomyocytes.¹² Autophagy is also related to the aging process, characterized by progressive collagen deposition, atrophy, vacuolar degeneration of myocytes, and accumulation of lipofuscin and other structures damaged by oxidation that are useless and often harmful. This myofibrillar derangement has been linked to increased atrial heterogeneity, which might serve as a structural substrate for age-associated AF.¹⁴

Alterations in the frequency and/or amplitude of a Ca^{2+} signal can trigger apoptosis or autophagy in several models.

Høyer-Hansen and colleagues¹⁵ were the first to report an association between Ca^{2+} and autophagy, showing that stimulation of Ca^{2+} mobilization from the sarcoplasmic reticulum by different pharmacologic agents induces autophagy. New evidence has also shown that the activation of the inositol triphosphate–inositol trisphosphate receptor (IP_3R) signaling pathway inhibits autophagy.¹⁶ Atrial cardiomyocytes express greater levels of IP_3R than ventricular cardiomyocytes, and the increase in IP_3R activity can render these cells more susceptible to arrhythmia development.¹⁷ In agreement with this finding, the expression of IP_3R is increased in RAA samples from patients with chronic AF.¹⁸ However, a direct relationship between autophagy and IP_3R in the context of POAF has not yet been studied. Alterations in Ca^{2+} signaling act as a causal factor for the generation and maintenance of AF.^{17,19} Atrial cardiomyocytes differ from ventricular cardiomyocytes in structure and intracellular Ca^{2+} distribution mechanisms. Although the latter has an extensive network of T tubules across the intracellular space, atrial cardiomyocytes have considerably less or no T tubular formation. In atrial cells, the sarcoplasmic reticulum forms an intracellular network called Z-tubules, which is enriched in ryanodine receptors and coordinates the rhythmic release of Ca^{2+} and contraction.¹⁹ Impaired autophagy might disrupt this delicate sarcoplasmic reticulum structuration, leading to defective Ca^{2+} conduction and the establishment of a proarrhythmic substrate.

Connexins are permeable to second messengers such as inositol triphosphate and Ca^{2+} . Current evidence suggests that heterogeneity of connexin distribution might play an important role in the susceptibility to atrial fibrillation.²⁰ Autophagy has been linked to connexin degradation in the failing heart and might contribute to the electrically isolated area within the atria and the development of AF.²⁰ In summary, impaired autophagic activity could affect both intracellular and extracellular Ca^{2+} handling, making these hearts more prone to arrhythmia development.

Dysregulated autophagy might provide an appealing link between the aforementioned morphologic changes observed in the present study preceding POAF and the metabolic changes that occur before the onset of the arrhythmia (decreased glucose and phosphocreatine content), which in turn resemble those observed in the early stages of AF.¹⁰ Even when after a few days of sustained arrhythmia, the phosphocreatine pool is replenished, myofibrillar energetics is persistently impaired and ultimately leads to atrial contractile dysfunction.¹²

To date, the demonstration of autophagic vacuoles by conventional electron microscopy is accepted as the reference standard for assessing autophagy, in both tissues and cultured cells.²¹ In our study, we observed a significant increase in the number of autophagic vacuoles in the atrial samples of patients who developed POAF. At first, this result does not seem to agree with the observed reduction in

LC3BII processing seen by Western blotting. LC3B processing is an important step in the formation of autophagosomes, and its determination is widely used as a marker to study autophagy in cells.⁸ However, this apparent contradiction is resolved if we remember the dynamic nature of autophagy. The accumulation of autophagic vacuoles can result from either increased formation or decreased degradation and removal.²¹ If autophagic flow is impaired, a reduction in early autophagic processing (with a decrease in the LC3BII/I ratio) and an accumulation of “intermediate” and “late” autophagic vacuoles can be expected, such as was seen in the patients with POAF in our study. Although the mechanisms underlying this proposed alteration in autophagic flux in patients with POAF are far beyond the scope of the present study, the significant increase in the amount of lipofuscin aggregates observed in those with POAF suggest an insufficient capacity for autophagy on the lysosomal compartment, leading to accumulation of undigested waste material.²² Other degradative pathways such as the ubiquitin/proteasome mechanism seem to be unhindered, as evidenced by the lack of ubiquitin aggregates or increased total ubiquitination in sodium dodecyl sulfate-polyacrylamide gel electrophoresis assays. Although granular cytoplasmic-ubiquitin inclusions by immunohistochemistry have been used by other groups as a method to detect autophagic cell death in cardiomyocytes, it is important to remember that ubiquitin aggregates can be a consequence of changes impairing protein degradation, as well as autophagic activity, and its appearance should be interpreted with caution.¹⁴

As the heart ages, it shows signs of progressive collagen deposition, atrophy, vacuolar degeneration of cardiomyocytes, and accumulation of structures damaged by oxidation. Lipofuscin is a heterogeneous polymer composed of fragments of proteins linked by aldehyde bridges deriving from lipid-derived peroxidation products. Autophagy is also related to the aging process, because it has been suggested that mitochondria undergoing autophagy (mitophagy) acts as major source of lipofuscin precursors.²³ The accumulation of waste material inversely correlates with the life span, and cardiomyocytes become loaded with lipofuscin at different rates, compromising the lysosomal degradative pathway.²⁴ The myofibrillar derangement associated with age has been linked to increased atrial heterogeneity, which might serve as a structural substrate for age-associated AF.^{14,25} In this scenario, impaired autophagic activity might play an important role in the establishment of the cellular substrate for POAF development.

The limitations of the present study included the lack of left atrial samples. The RAA biopsies were obtained at right atria cannulation before beginning elective CABG in patients in normal sinus rhythm. This is the normal procedure, and samples from the left atria could not be obtained because of ethical considerations. The left atrium is more

susceptible to the development of POAF than the right atria. Second, although we identified the structures shown in Figure 3, A as autophagosomes (Figure E1) and we observed a reduction in LC3IIB levels, we acknowledge that our data interpretation could be controversial. It is not possible to adequately address the flux issue in human samples, and this new concept that impaired autophagy could play a role in atrial fibrillation in humans warrants additional investigation.

In conclusion, these findings support a role for impaired autophagy in the establishment of a susceptible substrate for POAF development. Additional research is needed to elucidate whether this same substrate is present in patients who develop paroxysmal or permanent AF.

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FIGURE E1. LC3B protein is localized in autophagosomes. Tissue samples of atrial appendage were prepared for immunogold labeling of LC3B followed by a secondary antibody coupled to gold. A and B, Electron microscopy images of patients with postoperative atrial fibrillation; *arrows* denote LC3B protein in the autophagosomes. C, Negative control (without primary anti-LC3 antibody). Tissue samples were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3), postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were placed on nickel grids, allowed to dry for 2–24 hours at 20°–35°C, incubated with saturated sodium metaperiodate for 90 minutes, incubated with 10% hydrogen peroxide for 10 minutes, and washed with Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, 0.1%, pH 7.6). Aldehyde groups were blocked with Tris buffer (0.1 M glycine, pH 7.2) for 30 minutes at room temperature and incubated 90 minutes with rabbit polyclonal anti-LC3B antibody (dilution 1:300, catalog number NB 600-1384; Novus Biological LLC, Littleton, Colo), washed in Tris-buffered saline, incubated 90 minutes with gold-labeled secondary antibody (dilution 1:100, catalog number: P-9785; Sigma Aldrich, St. Louis, Mo), and washed with Tris-buffered saline and distilled water. Samples were contrasted with uranyl acetate and lead citrate.