Targeting proteostasis in atrial fibrillation
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Inhibition of ER stress-induced autophagy preserves proteostasis and protects against cardiomyocyte dysfunction in experimental and human Atrial Fibrillation


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ABSTRACT

Backgrounds: Atrial fibrillation (AF) is characterized by a self-perpetuating nature, driven by structural remodeling of cardiomyocytes, which results in electrical disturbances, contractile dysfunction and increasing difficulty in maintaining sinus rhythm. Previous work suggests an important contribution of altered proteostasis to cardiomyocyte remodeling. Here, we explore the role of autophagy, an important player in proteostasis via protein and organelle degradation.

Methods and results: Tachypacing of HL-1 atrial cardiomyocytes caused a gradual and significant activation of autophagy, evidenced by increased autophagic flux, autolysosome formation, p62 degradation and processing of LC3B-I to LC3B-II, associated with loss of Ca\(^{2+}\) transients. Endoplasmic reticulum (ER) stress was responsible for autophagy-induction, as indicated by upregulation of ER-stress markers (ATF6, HSPA5, ATF4, ATG12 and CHOP) and autophagy-suppression by the ER-stress inhibitor 4-phenyl-butyric-acid and overexpression of the ER chaperone-protein HSPA5. Moreover, inhibition of both ER-stress and autophagy, by bafilomycin A1 or pepstatin A, prevented tachypacing-induced contractile dysfunction in cardiomyocytes and Drosophila pupae. Induction of ER stress and autophagy was also observed in atrial tissue of tachypaced dogs and permanent AF patients. In patients, atrial levels of autophagy markers correlated with cardiac troponins and \(\alpha\)-tubulin levels and correlated inversely with structural remodeling (myolysis).

Conclusions: Atrial-cardiomyocyte tachycardia and AF result in ER-stress and consequent activation of autophagy, contributing to structural remodeling and loss of contractile function. As Inhibitors of ER stress are currently in clinical development, notably the chemical chaperone 4-phenyl-butyric-acid, these findings may contribute to expanding therapeutic options in AF.
1. INTRODUCTION

Atrial fibrillation (AF) is the most common persistent clinical tachyarrhythmia, and contributes to increased cardiovascular morbidity and mortality.\(^1\) The self-perpetuating nature of AF is driven by structural remodeling, electric disturbances and contractile dysfunction.\(^2\) As cardiomyocyte remodeling ultimately limits the effectiveness of current drug therapy for sinus-rhythm restoration and maintenance, research is increasingly directed at uncovering the underlying mechanisms of AF progression.\(^1\)

Derailment of proteostasis, i.e. the homeostasis of protein production, breakdown and function, has been implicated in AF-substrate formation in various experimental models and in patients with AF.\(^3\)-\(^7\) Activation of proteases, including calpain, is an important contributor to proteostasis derailment. Studies in experimental and human AF have revealed calpain activation to induce breakdown of contractile and structural proteins (myolysis).\(^5,8\) In addition to protease activation, induction of auxiliary cellular protein degradation pathways, such as macroautophagy (hereafter autophagy)\(^9,10\), may play an important role in AF progression. Stress-induced stimulation of autophagy has already been associated with myolysis.\(^11\) Nevertheless, the contribution of autophagy to AF progression has not yet been investigated. Autophagy is an evolutionarily conserved cellular degradation pathway, which maintains cell proteostasis by removing damaged or expired proteins and organelles.\(^9,10\) This process is initiated by sequestration of proteins in cytoplasmic isolation vesicles, called autophagosomes, which are subsequently degraded in a lysosome-dependent manner.\(^9,10\) Selective autophagy adaptors, including p62, which recognize autophagic cargo, are important components. Autophagy adaptors mediate the formation of selective autophagosomes via binding to small ubiquitin-like modifiers, including LC3B and ATG5.\(^9\) Various markers of autophagy have been recognized, including p62 and LC3B. Since p62 is sequestered to autophagosomes during autophagy and degraded upon fusion with the lysosome, its levels are inversely proportional to activation of autophagy.\(^12,13\) LC3B-II is a protein produced from LC3B-I upon autophagy initiation and is also incorporated into autophagosomes. Consequently, LC3B-II levels are proportional to the number of autophagosomes.\(^12,13\)

Controlled autophagy during (mild) cardiac stress conditions, such as nutrient deprivation, brief hypoxia and oxidative stress, supports cardiomyocyte survival. In contrast, excessive activation of autophagy causes derailment of cell proteostasis by degradation of essential proteins and organelles and thereby triggers autophagic cell death, as found in mitral valve regurgitation\(^11,14\) and cardiac hypertrophy.\(^15\) As permanent AF features the degradation of contractile proteins and structural remodeling of atrial cardiomyocytes\(^5,8\), we investigated the activation of autophagy and upstream processes in...
AF. To address this subject, we utilized tachypaced HL-1 atrial cardiomyocytes, *Drosophila* and atrial-tissue samples from tachypaced dogs and AF patients.

Here we show that tachypacing results in ER stress, which induces substantial autophagy in cultured atrial cardiomyocytes leading to contractile dysfunction. Moreover, inhibition of ER stress and autophagy prevented contractile dysfunction. In addition, we found that autophagy is activated in atrial tissue of tachypaced dog and AF patients. In patients, autophagy markers correlated significantly with the loss of contractile proteins and myolysis.

2. METHODS

2.1. *HL-1* atrial cardiomyocyte cell culture, transfections, constructs

*HL-1* atrial cardiomyocytes, derived from adult mouse atria, were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, USA).\(^{16}\) For detailed description of the cell culture and transfection methods, see Data supplement. Where indicated, *HL-1* cardiomyocytes were transiently transfected with the LC3B-GFP (kind gift Prof. T. Johansen)\(^{17}\), HSPA5 (kind gift Prof. H. Kampinga), or pcDNA3.1+ (empty) plasmid, by the use of Lipofectamin 2000 (Life Technologies, The Netherlands).

2.2. Tachypacing of *HL-1* cardiomyocytes and calcium transient measurements

*HL-1* cardiomyocytes were subjected to tachypacing as described before.\(^{3,8,18}\) In short, *HL-1* cardiomyocytes were subjected to 1-Hz (normal pacing) or 6-Hz (tachypacing) for a duration of up to 8 hours via the C-Pace100-culture pacer (IonOptix Corporation, The Netherlands). Detailed description of the calcium transient (CaT) measurements can be found in the Data supplement.

2.3. *Drosophila* stocks, tachypacing and heart wall contraction assays

Wild-type W1118 strains were obtained from Genetic Services Inc. All flies were maintained at 25°C on standard medium. After fertilization, adult flies were removed, and drugs (see Data supplement) were added to the medium containing fly embryos. After 2 days, prepupae were selected for tachypacing, as previously described.\(^3\) Groups of at least 5 pupae were subjected to tachypacing (5-Hz for 20 minutes) with a C-Pace100-culture pacer (IonOptix Corp). Before and after tachypacing, movies of whole pupae were recorded for 30 seconds. Heart wall contractions were analyzed with IonOptix software.

2.4. Antibodies and reagents

Description of all antibodies and reagents used can be found in the Data supplement.
2.5. Protein-extraction and Western blot analysis

Western blot analysis was performed as previously described. Equal amounts of total protein in SDS-PAGE sample buffer, were separated on SDS-PAGE 4-20% Precise™ Protein gels (Thermo Scientific, The Netherlands). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies and corresponding horseradish peroxidase-conjugated secondary antibodies. Signals were detected by the Western Lightning Ultra (PerkinElmer, Waltham, MA, USA) method and quantified by densitometry via the software Gene Gnome, Gene tools (Syngene, Cambridge, UK).

2.6. Quantitative RT-PCR

Total RNA was isolated from cardiomyocytes utilizing the nucleospin RNA isolation kit (Machery-nagel, The Netherlands). First-strand cDNA was generated by M-MLV reverse transcriptase (Invitrogen, The Netherlands) and random hexamer primers (Invitrogen, The Netherlands). Relative changes in transcription level were determined using the CFX384 Real-time system C1000 Thermocycler (Bio-Rad, The Netherlands) in combination with SYBR green supermix (Bio-rad, The Netherlands). Calculations were performed using the comparative CT method according to User Bulletin 2 (Applied Biosystems). Fold inductions were adjusted for GAPDH levels. For primer pairs, see Data supplement.

2.7. Immunofluorescent staining and confocal analysis

Forty eight hours after transient transfection of GFP-LC3B, or without transfection in case of endogenous LC3B, after normal- (NP) or tachypacing (TP), HL-1 cardiomyocytes were subjected to tachypacing, followed by fixation with 4% formaldehyde (Klinipath, The Netherlands) for 15 minutes at room temperature, washing three times with phosphate buffered saline (PBS) and permeabilization with 0.2% triton-X100 and blocking in 5% BSA (30 min room temperature). Nuclei were visualized by TOTO-3 (Molecular Probes, The Netherlands). Endogenous LC3B was visualized by an anti-LC3B antibody as described above and subsequent alexa488 labeled anti-rabbit antibody. Confocal images were obtained by confocal laser microscopy (Leica SP2 AOBS), captured at 125x magnification to demonstrate the formation of GFP-LC3 puncta, indicative of autophagosomes. The number of GFP puncta were counted manually from at least two independent experiments using imagePro. Mean values and SEM from each experimental condition were based on at least 20 cardiomyocytes.

2.8. Dog in vivo model for AF
Experiments with the dog in vivo model for AF were performed at the Montreal Heart Institute as described before\textsuperscript{18} and were according to the guidelines for animal-handling of the National Institutes of Health and approved by the Animal Research Ethics Committee of the Montreal Heart Institute. Ten mongrel dogs were included in the study: 5 non-paced control dogs and 5 atrial tachypaced dogs (ATP, 400 bpm) for 7 days. For more detailed description, see Data supplement.

2.9. Patient material

Prior to surgery, one investigator assessed patient characteristics (Table 1) as described before\textsuperscript{19} For detailed description, see Data supplement. The study conforms to the principles of the Declaration of Helsinki. The institutional review board approved the study and patient gave written informed consent.

2.10. Statistical analysis

Results are expressed as mean ± SEM of at least three independent experiments. Multiple-group comparisons were obtained by ANOVA, with one-way ANOVA for non-repeated measurements. Individual group-mean differences were evaluated with Student’s t-test with Bonferroni correction. Correlation was determined using the Spearman correlation test. All \( P \)-values were two-sided. A value of \( P<0.05 \) was considered statistically significant. SPSS version 20 was used for all statistical evaluations.

3. RESULTS

3.1. Tachypacing of HL-1 cardiomyocytes induces autophagy

Autophagy was examined by determining the levels of the autophagy markers p62, LC3B-I and LC3B-II and via visualization of autophagosomes by confocal microscopy.\textsuperscript{20} Tachypacing of HL-1 atrial cardiomyocytes induced a time-dependent decrease in p62 and increase in LC3B-II expression (Figure 1A–C), indicating the activation of autophagy. Consistent with Western Blot results, confocal analysis of tachypaced HL-1 cardiomyocytes revealed a time-dependent increase in LC3B levels in both LC3B-GFP transfected cardiomyocytes (Figure 1D) as well as in untransfected cardiomyocytes stained for endogenous LC3B (Figure 1E). Also, a clear redistribution of LC3B into discrete perinuclear puncta was observed (Figure 1F), indicative of autophagosome formation.

Next, we determined the autophagic flux to discern between the induction of autophagy and diminished degradation of autophagosomes (Figure 1G, H). Autophagic flux was measured as the difference in LC3B-II protein levels in the absence and presence of the lysosomal inhibitor bafilomycin A1 (BAF), which prevents the fusion of autophagosomes.
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to lysosomes and their subsequent clearance.\textsuperscript{21} BAF pretreatment further increased the levels of LC3B-II compared to those induced by tachypacing alone (Figure 1G, H), indicating that tachypacing induces the activation of autophagy rather than diminishing the degradation of autophagosomes. Together, these observations demonstrate that tachypacing induces the activation of autophagy in HL-1 atrial cardiomyocytes.

Table 1: Demographic and clinical characteristics of patients with paroxysmal AF (PAF), patients with permanent AF (PeAF), and control patients in sinus rhythm (SR).

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>PAF</th>
<th>PeAF</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>RAA (n)</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>LAA (n)</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56±8</td>
<td>51±7</td>
<td>61±10</td>
</tr>
<tr>
<td>Duration of AF</td>
<td>-</td>
<td>-</td>
<td>14.6 (8-56)</td>
</tr>
<tr>
<td>(median, range (months))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration SR before surgery (median, range (days))</td>
<td>-</td>
<td>1.5 (0-30)</td>
<td>-</td>
</tr>
<tr>
<td>Duration of last episode AF (median, range (h))</td>
<td>-</td>
<td>12 (0.2-24)</td>
<td>-</td>
</tr>
</tbody>
</table>

Underlying heart disease (n) and surgical procedure

| Coronary artery disease/CABG | 6   | 0* | 0* |
| Lone AF/Maze                | 0   | 7* | 7* |

New York Heart Association for exercise tolerance

<table>
<thead>
<tr>
<th>Class I</th>
<th>Class II</th>
<th>Class II</th>
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<tbody>
<tr>
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<td>5</td>
<td>3</td>
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<td>3</td>
<td>2</td>
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</table>

Echocardiography

| Left atrial diameter (parasternal) | 42±3| 42±4| 48±4 |
| Left ventricular end-diastolic diameter (mm) | 50±4| 52±3| 52±3 |
| Left ventricular end-systolic diameter (mm) | 34±4| 38±3| 34±5 |

Medication (n)

<table>
<thead>
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<th>Verapamil</th>
<th>Beta-Blocker</th>
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<tr>
<td>1 (17)</td>
<td>2 (33)</td>
<td>4 (67)</td>
<td>5 (83)</td>
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<td>0 (0)</td>
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<td>1 (14)</td>
</tr>
<tr>
<td>3 (43)</td>
<td>3 (43)</td>
<td>3 (43)</td>
<td>2 (29)</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM or number of patients. CABG; Coronary Artery Bypass Grafting, MAZE; atrial arrhythmia surgery, MVD; mitral valve disease. *$P$≤0.05 vs SR.
Figure 1: Tachypacing induces autophagosome formation and activation of autophagy. A) Representative Western blot of tachypacing-induced autophagy markers p62, LC3B-I, and LC3B-II and loading control GAPDH. HL-1 cardiomyocytes were normal paced (NP) or tachypaced (TP) for the duration as indicated. B) Quantified data showing a significant reduction in p62 levels after 6 hours of TP. C) Quantified data showing a significant increase in LC3B-II levels after 2 hours of TP. D) Confocal images of tachypaced HL-1 cardiomyocytes, transfected with LC3B-GFP plasmid. E) Confocal images of tachypaced HL-1 cardiomyocytes for the period as indicated. Endogenous LC3B was visualized by immunostaining. Green puncta indicate autophagosomes. F) Quantified data showing accumulation of LC3B-GFP puncta/cardiomyocyte during TP. G) Representative Western blot of HL-1 cardiomyocytes NP versus TP for the duration as indicated, in the presence or absence of bafilomycin A1 (BAF). H) Quantification of the autophagic flux by determining the difference in LC3B-II levels in the presence versus absence of bafilomycin A1 (BAF). *P≤0.05, **P≤0.01, ***P≤0.001 versus NP.

3.2. Induction of autophagy is via ER stress

Activation of autophagy is regulated by various cellular signal transduction pathways. A key regulator is mammalian target of rapamycin (mTOR). mTOR assembles into two complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). Both complexes become activated by mTOR phosphorylation, although at different sites, after which they attenuate autophagy. To determine if tachypacing-induced autophagy results from the inhibition of mTOR signaling, we determined total mTOR, phosphorylation of mTOR at S2448 for mTORC1, S2481 for mTORC2 and their respective downstream targets ribosomal protein S6 (S6RP) and Akt (Figure 2). Tachypacing did not affect mTOR phosphorylation at S2448 or S2481 or phosphorylation of S6R at S235-236. However, tachypacing resulted in a significant increase in phosphorylation of Akt at S473 (Figure 2D). Since an increase in Akt S473 phosphorylation was previously observed during endoplasmic reticulum (ER) stress, and ER stress is an important regulator of
autophagy, the involvement of ER stress signaling in tachypacing-induced autophagy was examined. To this end, we determined levels of phosphorylated eIF2α, a downstream ER stress marker. Tachypacing resulted in a strong increase in eIF2α phosphorylation without affecting total eIF2α levels, indicating that tachypacing induces ER stress (Figure 3A,B). To substantiate the role of ER stress signaling in tachypacing-induced autophagy, levels of additional ER stress markers and the downstream autophagy gene ATG12, which is involved in autophagosome formation, were determined by qPCR (Figure 3C). Tachypacing induced the transcription of several different markers of ER stress, i.e. ATF4, ATF6, CHOP, HSPA5 and ATG12. Also, tachypacing gradually and significantly induced protein levels of HSPA5, an endogenous ER chaperone-protein (Supplemental Figure S1). Furthermore, treatment of the cardiomyocytes with the chemical chaperone and ER stress inhibitor 4PBA, prevented tachypacing-induced phosphorylation of eIF2α and activation of autophagy, as evidenced by attenuation of p62 breakdown and LC3B processing (Figure 3D, supplemental Figure S2). As expected, inhibition of autophagy by pepstatin A (lysosomal cathepsin D/E inhibitor) or BAF (lysosomal fusion inhibitor) also attenuated p62 degradation upon tachypacing, without affecting LC3B processing. Thus, these results show that induction of the ER stress signaling is upstream of tachypacing-induced autophagy in HL-1 atrial cardiomyocytes.

**Figure 2:** Tachypacing-induced autophagy does not involve mTORC signaling. Top panels represent Western blots of proteins within the mTORC signaling and lower panels reveal quantified data of the ratio phosphorylated proteins normalized for basal protein levels. A) Phospho-mTOR 2448S (mTORC1). B) Phospho-mTOR 2481S (mTORC2), C) Phospho-S6RP 235-236S (downstream of mTORC1) and D) phospho-Akt 473S (downstream of mTORC2) in response to tachypacing for the duration as indicated compared to normal paced (NP). **P≤0.01, ***P≤0.001 versus NP.
Figure 3: Tachypacing augments mRNA levels of ER stress markers and autophagy gene ATG12. A) Representative Western blot of phospho-eIF2α 51S, an ER stress marker, and basal eIF2α and GAPDH levels in response to tachypacing (TP) for the indicated duration or normal pacing (NP). B) Quantitative real time PCR of ER stress markers, ATF4, ATF6, CHOP and HSPA5, and autophagy related genes, ATG12, in response to tachypacing for the indicated duration relative to normal pacing (NP). C) Representative Western blot of ER stress markers (eIF2α-P51S) and autophagy markers (LC3B and p62) in HL-1 cardiomyocytes pre-treated with 1% DMSO (control) or the autophagy modulators pepstatin A (PepA), bafilomycin A1 (BAF) or 4PBA. **P≤0.01, ***P≤0.001 versus NP.

3.3. Inhibition of ER stress induced autophagy protects against contractile dysfunction in tachypaced cardiomyocytes and Drosophila

To determine if autophagy contributes to tachypacing-induced contractile dysfunction, the effect of inhibition of autophagy on CaT was determined. Tachypacing of cardiomyocytes at 6-Hz for 8 h resulted in a nearly complete loss of CaT compared to normal paced cardiomyocytes (Fig. 4A, supplemental Figure S3 and movies S1-S12). To assess the role of autophagy, cardiomyocytes were preincubated with pepstatin A and BAF for 30 min prior to pacing. Both inhibitors completely attenuated tachypacing-induced loss of CaT (Figure 4A, B), demonstrating that inhibition of autophagy fully prevents against tachypacing-induced loss of CaT. Next, we determined whether a similar effect was accomplished by inhibition of ER stress. Indeed, pretreatment with 4PBA also completely attenuated loss of CaT (Figure 4A,B). Since 4PBA acts as a chemical chaperone to inhibit ER stress, we explored whether the endogenous ER chaperone-protein HSPA5 may serve a similar protection. Indeed, overexpression of HSPA5 also protected against tachypacing-
induced loss of CaT (Figure 4C, D, supplemental movies S13-S16). Finally, to exclude an indirect effect of the autophagy inhibitors through modulation of ER stress, HSPA5 expression was determined. None of the drugs at the concentrations applied influenced HSPA5 expression levels (Supplemental Figure S4). To extend these findings to a second experimental model, similar experiments were conducted in tachypaced *Drosophila.*

Tachypacing induced a marked reduction in contractility in vehicle treated *Drosophila* (Figure 4E, F). Similar to the findings in tachypaced HL-1 cardiomyocytes, inhibition of ER stress (4PBA) and autophagy (BAF) attenuated tachypacing-induced contractile dysfunction in *Drosophila* (Figure 4E, F; supplemental movies S17-S22). Pepstatin A was not protective at the concentrations applied. Thus, the inhibition of autophagy protects against tachypacing-induced loss of contractile function. Moreover, these data demonstrate that blockade of autophagy by the inhibition of upstream ER stress suffices to maintain proper cardiomyocyte function in tachypaced cardiomyocytes and *Drosophila.*

**Figure 4:** Inhibition of ER stress and autophagy protects against tachypacing (TP)-induced contractile dysfunction in HL-1 cardiomyocytes and *Drosophila melanogaster.* A) Representative CaT of HL-1 cardiomyocytes after normal pacing (NP) or tachypacing (TP). HL-1 cardiomyocytes were pre-treated with DMSO (Control) or the autophagy modulators pepstatin A (PepA), bafilomycin A1 (BAF) or 4PBA, followed by normal or tachypacing and measurement of CaT. B) Quantified CaT amplitude of NP and TP HL-1 cardiomyocytes, each from groups as indicated. C) Representative CaT of HL-1 cardiomyocytes transfected with empty plasmid (Control) or ER chaperone HSPA5, followed by NP or TP. D) Quantified CaT amplitude of NP and TP HL-1 cardiomyocytes transiently transfected with empty plasmid or HSPA5. 

### P≤0.001 vs NP control, **P≤0.01, *** P≤0.001 versus TP control. E) Representative heart wall contractions monitored before tachypacing (TP) and after TP with 2% DMSO (Control) pretreatment or pretreatment with pepstatin A (PepA), bafilomycin A1 (BAF) or 4PBA. F) Quantified data showing heart wall contraction rates each from groups as indicated. ***P≤0.001 vs control before TP; *P≤0.05, **P≤0.01 vs control TP; n=9 to 15 pupae for each group.
Figure 5: Patients with permanent AF show markers of ER stress and autophagy. A) Electron microscopic image of left atrial appendage of a patient with permanent atrial fibrillation (PeAF), arrows indicate the presence of autophagosomes and autolysosomes with a perinuclear (N) localization. B) Image of left atrial appendage of a patient with PeAF at a higher magnification, showing the presence of autophagosomes and autolysosomes. C) Image of left atrial appendage of a patient in SR, showing normal sarcomere structures and absence of perinuclear autophagosomes. D) Image of left atrial appendage of patient in SR, showing normal sarcomere structures and absence of perinuclear autophagosomes at a higher magnification. E) Representative western blot of the autophagy marker p62 and ER stress marker HSPA5 in right (RAA) and left atrial appendages (LAA) of patients in paroxysmal (PAF) and permanent AF (PeAF) versus SR. F) and G) Quantified data of autophagy marker p62 and ER stress marker HSPA5, in right (RAA) and left atrial appendages (LAA) of patients with paroxysmal AF (PAF), permanent (PeAF) and control patients in sinus rhythm (SR). *P≤0.05 versus SR.

3.4. Patients with permanent AF and dogs with AF reveal active autophagy

To extend our findings to human AF, right and left atrial appendages (RAA and LAA, respectively) of patients with paroxysmal (PAF) or permanent AF (PeAF) and of control patients in sinus rhythm (SR) were examined. Electron microscopic analysis of atrial tissue demonstrated that patients with PeAF show an accumulation of autolysosomes,
autophagosomes and the presence of myolysis (degradation of sarcomeres) in both RAA and LAA, which was absent in SR patients (Figure 5A-D). PeAF patients displayed a significant decrease in p62 levels in LAAs compared to RAAs (Figure 5E), indicating the presence of autophagy. In addition, the expression of HSPA5 was significantly reduced in LAA of PeAF compared to both their RAA and to control patients in SR or PAF (Figure 5F), indicating ER stress. No changes in LC3B-I and LC3B-II levels were observed (supplemental data Figure S5). To further substantiate the involvement of autophagy in structural remodeling and AF progression, correlations were made. We found that p62 expression correlated significantly with cardiac troponin cTnI, cTnT and alpha-tubulin expression in PeAF, PAF and SR patients (Figure 6A, B, C). The p62 levels also correlated inversely with the amount of myolysis (Figure 6D). In addition, the p62 levels correlated with HSPA5 levels (Figure 6E), suggesting that an ER stress response underlies autophagy and AF progression.

![Figure 6](image)

Figure 6: Significant correlations between levels of the autophagy marker p62 and markers of cardiomyocyte structural remodeling in patients with paroxysmal (PAF) and permanent (PeAF) and SR. A) Cardiac troponin T (cTnT), B) Cardiac troponin I (cTnI), C) α-tubulin (tub), D) Myolysis and E) HSPA5.
Figure 7: Dogs subjected to atrial tachypacing reveal induction of ER stress and autophagy in right and left atrial tissue. A) Representative western blot and quantified data of the autophagy marker p62 in right and left atrial tissue of dogs with and without atrial tachypacing (ATP). B) Representative Western blot and quantified data of ER stress marker eIF2α-P51S levels in RA and LA of dogs with and without ATP. C) Representative Western blot and quantified data of ER stress marker HSPA5 in RA and LA of dogs with and without ATP. D) Representative Western blot and quantified data of autophagy marker LC3B-I/II in RA and LA of dogs with and without ATP. *P<0.05 versus C. E) Proposed model for the role of autophagy and disease progression in AF. Starting from the top, AF triggers ER stress in cardiomyocytes through altered Ca²⁺ handling, derailment in redox homeostasis and reduction in ER chaperone HSPA5 levels. Subsequently, the ER stress response activates PERK by phosphorylation, leading to downstream phosphorylation of eIF2α. In turn, this results in the activation of the transcription factor ATF4, which regulates the expression of autophagy genes (e.g. ATGs) and LC3B, causing activation of autophagy by stimulating induction and elongation of autophagosomes. A second consequence of the ER stress response results in activation of ATF6, which upregulates the transcription of HSPA5 in an attempt to restore ER homeostasis. Initially, AF-induced activation of autophagy may preserve cardiomyocyte proteostasis, however, excessive stress-induced autophagy contributes to loss of contractile function and cardiac remodeling. ER stress-induced autophagy appears maladaptive, as inhibition of autophagy via 4PBA, HSPA5 overexpression, pepstatin A (PepA) and bafilomycin A1 (BAF) prevented AF-induced loss of calcium transients.
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Autophagy markers were also determined in left atrial (LA) and right atrial (RA) tissue of dogs subjected to tachypacing for 1 week and of non-paced control dogs. Similar to the findings in patients, a significant reduction in p62 and HSPA5 levels and induction of eIF2α phosphorylation was observed in LA of tachypaced dogs (Figure 7A, B, C). Furthermore, no differences in LC3B levels were found among the groups (Figure 7D).

Together, these results demonstrate the activation of autophagy both in patients with permanent AF and in the dog model for AF. Most likely, autophagy is caused by ER stress in response to the increased activation rate of the atrial cardiomyocytes. A clinically relevant contribution of autophagy is suggested by the correlation of autophagy with the degradation of contractile proteins and the amount of structural remodeling.

4. DISCUSSION

In the present study, we reveal tachypacing of cardiomyocytes to activate autophagy. Apparently, activation of autophagy plays a major role in cardiomyocyte remodeling, as inhibition of autophagy by pepstatin A and bafilomycin A1 fully protected against tachypacing-induced contractile dysfunction. Furthermore, we show that activation of autophagy is caused by upstream activation of the ER stress response, as autophagy was attenuated by the blocker of ER stress, 4PBA and overexpression of HSPA5. In accord, tachypacing induced phosphorylation of the ER stress marker eIF2α and thereby stimulated ATF6, HSPA5, ATF4, CHOP, and ATG12 expression. Importantly, 4PBA blocked the phosphorylation of eIF2α, and the subsequent activation of autophagy and loss of contractile function. In addition, the activation of autophagy was observed in patients with permanent AF and in the dog model for AF. In permanent AF patients, autophagy was demonstrated by perinuclearly localized autophagosomes, and reduced levels of the autophagy marker p62. In these patients, a reduction in the ER chaperone HSPA5 was found, suggesting a role for the ER stress response as activator of autophagy in human AF. Moreover, the correlation of p62 levels with levels of contractile proteins and myolysis suggests that activation of autophagy also contribute to cardiomyocyte structural damage and dysfunction and thereby disease progression in human AF. Together, our data indicate that activation of autophagy is involved in the derailment of cardiomyocyte proteostasis, contributing to loss of function and AF progression.

4.1. Autophagy via ER stress

The first finding of our study is that tachypacing of cardiomyocytes activates autophagy, a known key modulator of cell proteostasis. Activation of autophagy is evidenced by the accumulation of autophagosomes, p62 degradation, and LC3B processing. Moreover, two inhibitors of autophagy, bafilomycin A1 and pepstatin A, prevented
tachypacing-induced loss of contractile function. In addition, patients with permanent AF revealed active autophagy, which correlates with structural remodeling. The second finding is that tachypacing-induced autophagy is activated via upstream ER stress signaling, and this also seems to apply for patients with permanent AF and tachypaced dogs. Consequently, inhibition of ER stress, via 4PBA or overexpression of HSPA5, protects HL-1 cardiomyocytes and Drosophila against tachypacing-induced contractile dysfunction. Previous studies revealed that the inhibition of basal autophagy, by bafilomycin A1 and lysosomal enzyme inhibitors (such as pepstatin A) induces ER stress and increases expression of the cardioprotective HSPA5. While such effect may result in protection from loss of CaT in tachypaced cardiomyocytes, no evidence of increased HSPA5 by the compounds used was found in the current study (Supplemental Figure S4). Thus, here we identify a prominent role for ER stress in the induction of autophagy and derailment of cardiomyocyte proteostasis which underlies AF progression.

On the mechanistic level, the current study reveals tachypacing to induce ER stress via phosphorylation of eIF2α. It is well known that the ER is involved in protein synthesis, folding and maturation. Also, the ER constitutes a source of the autophagic isolation membrane, also called phagophore, which is necessary for the inclusion of autophagy-prone proteins (Figure 8E). Upon ER stress, eIF2α gets phosphorylated at S51, as observed in our study, which then initiates a cascade of events aiming to mitigate ER stress. These events include a general inhibition of protein translation as well as the selective translation of stress-responsive transcripts including ATF4 and ATF6 (Figure 7E). As a consequence, ATF4 and ATF6 signaling induces the expression of CHOP, ATG12, LC3B and HSPA5 (also named BiP, Grp78, or ER chaperone), respectively. During prolonged ER stress, the phosphorylated eIF2α replenishes cellular supplies of ATG12, CHOP and LC3B allowing for sustained and excessive autophagy flux. Consistent with the upstream role of ER stress, we found the chemical chaperone 4PBA to attenuate eIF2α phosphorylation at S51 and subsequent activation of autophagy, resulting in conservation of contractile function after tachypacing. Thus, our data identifies ER stress and downstream activation of autophagy as a prominent pathway to underlie AF progression.

4.2. The role of autophagy in clinical AF

Our results obtained in atrial tissue samples from patients and dog further substantiate the role of the activation of autophagy in AF. Patients with permanent AF displayed perinuclearly localized autophagosomes, and reduced levels of p62, the most prominent marker for autophagy. In addition, a correlation between p62 and contractile proteins cTnT, cTnI and alpha-tubulin was observed, together with an inverse correlation between p62 and the amount of myolysis. Since reduced levels of HSPA5 were observed in patients with
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permanent AF and in the dog model for AF, ER stress also likely represents the main pathway to induce autophagy in AF in vivo (Figure 7E). The reduction in p62 and HSPA5 levels was observed only in left atrial tissue of patients and dog, suggesting that ER-stress-induced autophagy is more pronounced in the left atrium. This finding is in line with previous studies that found marked changes in ion channel and alpha-tubulin expression in patients and dog to be confined to the left atrium.\textsuperscript{3,35,36} Thus, our data indicate that activation of autophagy, likely caused by ER stress, plays a prominent role in the derailment of cardiomyocyte proteostasis, structural remodeling and progression of clinical AF. In line with our findings, also an association between autophagy and the presence of myolysis was found in patients with mitral valve regurgitation.\textsuperscript{11} Furthermore, an accumulation of autophagosomes was observed in patients who developed post-surgery AF.\textsuperscript{37} These studies further substantiate a role for autophagy in structural remodeling and AF progression.

Our results in tachypaced HL-1 cardiomyocytes demonstrate that autophagy promotes the progression of cardiomyocyte remodeling. However, it should be kept in mind that activation of autophagy is not as such a detrimental response, but should be appreciated in the context of its time frame and level of activation. In tachypacing, the benefit of autophagy is that it initially attenuates ER stress by activation of ER chaperone-proteins.\textsuperscript{9} However, the excessive and prolonged activation of autophagy, as reflected by eIF2α phosphorylation and increased expression of ATF4, autophagy genes and LC3B, conveys substantial cellular damage. Such view explains why excessive autophagy is present mainly in permanent AF, thus contributing to myolysis and structural remodeling of the cardiomyocytes, and its presence in various chronic conditions, including other cardiac diseases.\textsuperscript{11,26}

4.3. Inhibition of autophagy and ER stress as a therapeutic intervention strategy in AF

Pharmacological approaches preventing or limiting AF progression and substrate formation are extensively being studied, with the aim of identifying novel effective therapeutic agents for AF treatment.\textsuperscript{1} Given our results, pharmacological intervention to inhibit autophagy may constitute a promising therapeutic strategy in clinical AF. At present, autophagy can be modulated by a number of small molecules.\textsuperscript{32,38,39} Since basal autophagy is crucial for normal cell physiology, chronic treatment with autophagy inhibitors, such as in permanent AF, may be detrimental to the cardiomyocyte.\textsuperscript{14,40-42} Such view is corroborated by the high toxicity of bafilomycins, which precludes its use in the clinical setting.\textsuperscript{43} As the clinical options for autophagy inhibition are currently limited, inhibitors of ER stress may represent a suitable alternative, as identified in the current study. From the available compounds, the chemical chaperone 4PBA seems the most
promising, as this compound not only inhibits ER-stress induced autophagy, but also has been approved for clinical use. More importantly, 4PBA was reported to have minor side effects and is considered safe in patients.\textsuperscript{44} Therefore, our findings suggest 4PBA as a therapeutic agent with great potential in clinical AF.

In summary, the present study demonstrates AF to activate autophagy, caused by upstream stimulation of the ER stress signaling, contributing to structural remodeling and contractile dysfunction. Inhibition of autophagy protected against loss of contractile function. These findings imply that inhibition of autophagy, preferably via inhibition of ER stress, form a novel therapeutic target for intervention.

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