Original Article

Endoplasmic reticulum stress associated apoptosis implicated in atrial fibrillation

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Abstract: Myocardial apoptosis is a key event in the initiation and progression of atrial fibrillation (AF). However, the trigger mechanism of apoptosis in AF has not been fully clarified. It has been reported that endoplasmic reticulum (ER) stress could mediate apoptosis and play a role in tissue remodeling after insult. This study explored the possible implication of ER stress and its association with apoptosis in AF patients. We analyzed the biopsies of left atrial appendage (LAA) from patients with paroxysmal AF (PaAF, n = 10), persistent AF (PeAF, n = 24) and sinus rhythm (SR, n = 20) undergoing isolated mitral valve surgery. Compared with patients in SR group, ER stress mediators, processed p50 activating transcription factor 6 (ATF6), spliced X-box binding protein 1 (XBP1), apoptosis-inductor C/EBP-homologous protein (CHOP) and Death Receptor 5 (DR5) were significantly elevated in PaAF and PeAF group. Caspase 3 cleavage and TUNEL positive cells were also increased in PaAF and PeAF group, paralleled with the expression of ER stress mediators. The results suggested that ER stress response was activated in AF, and prolonged ER stress was associated with myocardial apoptosis in AF patients.

Keywords: Endoplasmic reticulum stress, atrial fibrillation, apoptosis, ATF6, CHOP, XBP1

Introduction

Electrical and structural of atrial remodeling play a vital role in progression and maintenance of atrial fibrillation (AF) [1]. Above all, increased apoptosis has been described in patients with AF, resulting in atrial fibrosis which is considered a fundamental mechanism in the perpetuation of AF [2-4]. Though heavily studied, the cascade of apoptosis in AF has not been fully identified.

The endoplasmic reticulum (ER) is involved in protein folding, calcium homeostasis and apoptosis [5]. Conditions such as calcium depletion, ischemia, hypoxia, oxidative stress, aging, or elevated protein synthesis can potentially cause ER dysfunction, and result in ER stress [6, 7]. Three signaling pathways (ATF6, IRE1α and PERK) are induced by ER stress [6]. In response to ER stress, the ATF6 precursor is transported to the Golgi complex, and activated. The activated ATF6 behaves as a transcription factor to regulate the ER-associated degradation (ERAD) proteins to decrease the misfolded protein synthesis [8]. The second ER stress branch involves IRE1 and X-box-binding protein 1 (XBP1). Activated IRE1 promotes XBP1 to move in the nucleus and initiate genes encoding proteins involved in protein folding, transport, and degradation [8, 9]. The third ER stress branch is mediated by PERK, which can phosphorylate the eukaryotic translation initiation factor 2α (eIF2α). Phosphorylation of eIF2α subsequently attenuates translation of global mRNA and therefore the entry of new proteins into the ER [9, 10].

C/EBP homologous protein (CHOP) is a major downstream effector of PERK and ATF6. When severe ER stress is prolonged, CHOP can be induced and then build up Death Receptor 5 (DR5) transcription, a reliable marker coupling ER stress and apoptotic cell fate, leading to growth arrest and apoptosis [11]. ER stress-mediated apoptotic pathway was observed in
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Tachypacing-induced apoptosis of cultured atrial myocytes [12]. Until now, there is no report about ER stress in AF. Further evidence in vivo is needed to identify whether ER stress associated apoptosis occurred in AF.

In the present study, we explored the possible implication of ER stress and its association with apoptosis in atrial fibrillation patients.

**Materials and methods**

**Patients and tissue specimens**

The study has been performed according to the Declaration of Helsinki. All procedures involving human tissues were approved by the local ethics committee. All study subjects signed written informed consent. 54 patients with rheumatic mitral stenosis (MS) undergoing valve replacement surgery were recruited at the Drum Tower Hospital of Nanjing University Medical School and Huaian People’s Hospital. Patients with hyperthyreosis, chronic obstructive pulmonary disease, renal dysfunction, and detected rheumatic activity were excluded. Finally, the patients enrolled were divided into three groups: sinus rhythm group (SR, n = 20), paroxysmal atrial fibrillation group (PaAF, n = 10, self-terminating within 7 days) and persistent atrial fibrillation group (PeAF, n = 24, AF episode persisted > 7 days). Every patient had routine transthoracic echocardiographic examination.

A sample of the left atrial appendage (LAA) tissues of each individual was obtained prior to the initiation of extracorporeal circulation. One part of the tissue was fixed in 4% paraformaldehyde, paraffin, and embedded in paraffin. The tissue was cut into 4 μm thick sections. Heat-induced epitope retrieval, non-specific-binding sites were blocked, and the sections were then incubated with anti-AFT6β and anti-CHOP overnight at 4°C. Following which they were visualized with HRP-conjugated goat IgG for 20 min.

**Western blot analysis**

Tissues were homogenized in RIPA solubilization buffer containing protease and phosphatase inhibitor (Sigma). Protein concentrations were determined by BCA protein assay (pierce, Rockford, IL, USA). 30 μg denatured samples were separated on 10% SDS-PAGE for 80 minutes at 200 mA, and then transferred to polyvinylidene difluoride membranes (Pall Corporation, Ann Arbor, MI, USA). The membranes were blocked with 5% non-fat dry milk and then incubated to primary antibodies, including XBP1s (619502, BioLegend, USA), XBP1u (sc-7160, Santa Cruz, USA), CHOP (2895P, CST, USA), ATF6β (sc-30597, Santa Cruz, USA), eIF2α (MAB3997, R&D, USA), p-eIF2α (MAB39971, R&D, USA), β-actin (AP0060, Bioworld, USA) was used as an internal control. Then the blots were performed with HRP-conjugated secondary antibodies. The signals were developed with ECL regents (Millipore) and quantified using Image-Pro Plus 6.0 Software. Protein content was normalized to β-actin.

**Immunohistochemistry**

The LAAs were fixed with 4% paraformaldehyde, subjected to alcoholic dehydration and embedded in paraffin. The tissue was cut into 4 μm thick sections. Heat-induced epitope retrieval, non-specific-binding sites were blocked, and the sections were then incubated with anti-AFT6β and anti-CHOP overnight at 4°C. Following which they were visualized with HRP-conjugated goat anti-IgG for 20 min.

**In situ apoptosis assay**

For the in situ detection of apoptosis in LAAs sections, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method was used by DeadEnd Fluorimetric TUNEL System (Promega).

**Statistical analyses**

Quantitative data are presented as mean ± SE. As for continuous variables, student’s t test (2 groups, normally distributed), Mann-Whitney (2 groups, non-normally distributed), one-way analysis of variance (3 groups, normally distrib-
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To analyze whether ER stress was activated in LAAs from PaAF and PeAF groups, we performed comparative western blot analysis by specific antibodies against the three distinct ER stress pathway sensor molecule respectively. Upon ER stress, the 90-kD ATF6 protein was processed to release a cytosolic fragment, ATF6β. ATF6β could translocate into nucleus to regulate the expression of genes of the ERAD pathway, further decrease the whole genome mRNA transcription [13]. In atrial tissue, ATF6β was increased in AF groups compared with SR group, while no significant difference was observed between PeAF and PaAF groups (Figure 1A, 1B).

Induction of ER stress in AF

ER stress could activate PERK. PERK activation resulted in eIF2α phosphorylation [14]. Here, we studied the levels of total eIF2α and phosphorylated eIF2α by western blot. No significant difference of eIF2α phosphorylation was found between AF groups and SR group (Figure 1A, 1D).

Next, we detected IRE1/XBP1 pathway. ER stress activated IRE1, cutting XBP1 mRNA into spliced XBP1 mRNA, and finally encoded transcriptionally active XBP1 protein [13]. In this study, we detected spliced XBP1 expression in LAAs to assess IRE1/XBP1 pathway activation. Spliced XBP1 (sXBP1) was robustly observed in LAAs from PeAF and PaAF group compared with SR group (Figure 1A, 1C).

Previous studies have demonstrated that activated ATF6 could induce the expression of CHOP and contribute to cell apoptosis [15]. We further analyzed CHOP expression by western blot. Similar to ATF6β, CHOP was up-regulated in PaAF and PeAF group (Figure 1A, 1D). To further confirm the up-regulation of ATF6β and CHOP in AF groups, we assessed their expression using immunohistochemical staining. In line with western blot, immunohistochemical

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<td>BMI (kg/m²)</td>
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<td>NYHA class I/II/III/IV (n)</td>
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<td>LAD (cm)</td>
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<td>PASP (mmHg)</td>
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<td><strong>Preoperative drugs (n)</strong></td>
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<td>ACEI or ARB (n)</td>
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<td>Beta blocker (n)</td>
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SR, sinus rhythm; PaAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation; NYHA, New York Association; LVDD, left ventricular diastolic diameter; LVDs, left ventricular end-systolic dimension; LAD, left atrial diameter; EF, ejection fraction; PASP, pulmonary artery systolic pressure; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker. MS, mitral stenosis. *P < 0.01 vs. SR group. **P < 0.05 vs. PaAF group.

Results

Patient clinical characteristics

Demographic and clinical data regarding patients with MS were shown in Table 1. There was no statistical difference among the three groups with regard to basic data, history of hypertension or diabetes, and preoperative drugs. In UCG, left atrial diameter (LAD) before surgery was increased in AF group compared with SR group (P < 0.01). Similarly, increased tendency of LAD was observed between PeAF and PaAF group, but not reached significant (P < 0.05).

Other parameters, such as left ventricular dimensions, or left ventricular ejection fraction were similar among the three groups.

| Parameters                             | SR   | PaAF | PeAF | P value |
|-----------------------------------------------|
| Basic data                             |      |      |      |         |
| Sex, M/F (n)                           | 8/12 | 4/6  | 8/16 | 0.881 |
| Age (years)                           | 49.7±9.8 | 53.0±9.2 | 54.3±9.4 | 0.244 |
| BMI (kg/m²)                           | 22.6±2.7 | 21.9±2.2 | 21.3±3.2 | 0.746 |
| NYHA class I/II/III/IV (n)               | 9/11/0 | 4/6/0 | 13/10/1 | 0.769 |
| Echocardiographic parameters            |      |      |      |         |
| LVDD (cm)                             | 4.7±0.9 | 4.9±0.8 | 5.1±1.1 | 0.684 |
| LVDs (cm)                             | 4.0±0.8 | 4.1±0.7 | 4.2±0.8 | 0.502 |
| EF (%)                                | 61.1±10.2 | 58.8±5.3 | 57.2±7.8 | 0.107 |
| LAD (cm)                              | 3.8±0.4 | 5.1±1.1 | 3.8±0.8 | <0.01 |
| PASP (mmHg)                           | 49.8±12.3 | 56.4±14.6 | 60.6±16.2 | 0.146 |
| Preoperative drugs (n)                 |      |      |      |         |
| ACEI or ARB (n)                       | 0    | 2    | 2    | 0.392 |
| Beta blocker (n)                      | 1    | 2    | 3    | 0.556 |
| CCB (n)                               | 0    | 1    | 2    | 0.400 |
| Digoxin (n)                           | 14   | 7    | 18   | 0.926 |

SR, sinus rhythm; PaAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation; NYHA, New York Association; LVDD, left ventricular diastolic diameter; LVDs, left ventricular end-systolic dimension; LAD, left atrial diameter; EF, ejection fraction; PASP, pulmonary artery systolic pressure; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker. MS, mitral stenosis. *P < 0.01 vs. SR group. **P < 0.05 vs. PaAF group.

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staining showed that the expressions of ATF6β and CHOP were higher in PeAF and PaAF group than SR group (Figure 1F).

Taken together, AF was associated with activation of the ER stress via ATF6 and IRE1/XBP1 pathway.

**ER stress was associated with apoptosis rate in AF**

Persistent CHOP depended apoptosis has been reported in many diseases. In this study, we assessed the apoptosis rate in atrial tissue by detecting the expression of DRS, cleaved-caspase 3 and TUNEL assay.

DR5, a sensitive gene responding to the ER stress and mediating cell apoptosis [9], was increased in AF groups compared with SR group (Figure 2A). Similarly, as shown in Figure 2B, the expression of cleaved-caspase 3 by western blot was also up-regulated in AF groups compared with SR group. Meanwhile, cleaved-caspase 3 in PeAF group was significantly increased than that in PaAF group. Additionally,
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using TUNEL assay, we observed prominently enhanced apoptosis rate in AF groups compared with SR group, especially in PeAF group (Figure 2C). Thus, the level of apoptosis rate in AF groups was higher than that in SR group.

Next, to ascertain the correlation of ER stress mediators and apoptosis, we conducted correlation analysis. As illustrated in Figure 3A-C, it revealed a strong positive correlation between ATF6β, CHOP and cleaved caspase 3 expression, further indicating that ER stress was associated with apoptosis in AF group.

Correlation between ER stress mediators and NYHA heart function classification

As reported recently, ER stress was enhanced in failing myocardium [16]. Therefore, we detected whether the ER stress mediators in LAAs were associated with cardiac function. To address this issue, we evaluated the ER stress proteins in different NYHA classes. In AF group, the expressions of ATF6β and CHOP from severe heart failure patients (NYHA class III) were higher than that from moderate HF patients (NYHA class II). However, no significant difference was observed in SR group (Table 2).

Discussion

ER stress mediated apoptosis emerged as a crucial contributor to the pathophysiology of a variety of human diseases, such as heart failure, diabetes and renal fibrosis [16-18]. In this study, we firstly reported up-regulation of ATF6β, enhanced expression of CHOP, and exclusive activation XBP1 pathway in AF patients compared to SR group, indicating a chronic ER stress response occurred in the LAAs of AF patients.

It has been known that ER stress induced apoptosis is mainly mediated by CHOP, which is

Figure 2. Apoptosis rate in LAAs. A. DR5 mRNA expression in LAA normalized to GAPDH. B. Cleaved caspase 3 and β-actin immunoblotting in LAAs were shown in SR, PaAF and PeAF groups, and quantification of optical density normalized to β-actin. C. Enhanced red nuclear fluorescence as labeled by arrows reflected the increase of endonucleolytic DNA degradation and apoptosis. Bar = 50 um. SR, sinus rhythm; PaAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation; LAAs, left atrial appendages. Measurements of individual samples were done in duplicate. SR group, n = 20; PaAF group, n = 10; PeAF group, n = 24. *P < 0.05; **P < 0.01; ns = not significant.
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Downstream of the PERK (PERK-eIF2α pathway) and ATF6 pathway [15], CHOP levels are increased when ER stress is severe and prolonged. CHOP inhibits protective anti-apoptotic factors like Bcl-2 and promotes apoptotic caspase activity [9]. In our study, we observed ATF6β, in parallel with the induction of CHOP, has strong correlations with apoptosis rate, indicating CHOP associated apoptosis may occur in AF patients. In addition, recently, Lu et al. confirmed that DR5 played a central role in ER stress induced apoptosis pathway: It was speculated that DR5 transcription was resulted by CHOP activity, and may act synergistic action with CHOP, to define cells to survival or apoptosis [11, 19]. Totally consistent with the tendency of CHOP, in the present study, DR5 expression was increased in AF group, further indicating ER stress associated apoptosis pathway was involved in AF.

As indicated in our study, even in the relative early clinical stage (NYHA II), an increasing trend of ER stress mediators was observed in AF group than in SR group. ER stress might be an early event in AF. What could be the underly-

**Table 2. Analysis of protein expression of ATF6-CHOP axis in different heart function classifications**

<table>
<thead>
<tr>
<th>Grade</th>
<th>ATF6β</th>
<th>CHOP</th>
<th>ATF6β</th>
<th>CHOP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SR group</td>
<td>AF group</td>
<td>SR group</td>
<td>AF group</td>
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<tr>
<td>NYHA II</td>
<td>0.95±0.14b</td>
<td>0.94±0.09b</td>
<td>1.75±0.08a,b</td>
<td>1.34±0.07a,b</td>
</tr>
<tr>
<td>NYHA III</td>
<td>1.06±0.16b</td>
<td>1.03±0.15b</td>
<td>2.18±0.14a,b</td>
<td>1.58±0.10a,b</td>
</tr>
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*P < 0.05; NYHAIII VS NYHA II; *P < 0.05; AF group VS SR group.

**Figure 3.** Correlation between ATFβ/CHOP and other parameters. A. Relationship between protein expression of ATF6β and CHOP. r = 0.67, P < 0.01, n = 54. B. Relationship between protein expression of ATF6β and cleaved caspase 3. r = 0.66, P < 0.01, n = 54. C. Relationship between protein expression of CHOP and cleaved caspase 3. r = 0.69, P < 0.01, n = 54. D. Relationship between mRNA expression of DR5 and cleaved caspase 3. r = 0.60, P < 0.01, n = 54.
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Several limitations should still be acknowledged in our study. First, though patients with detected rheumatic activity were excluded in our study, we couldn’t exclude the possibility that chronic rheumatic activity may induce the ER stress response. More studies are necessary to evaluate the impact of rheumatic activity on ER stress. Next, as a systematic defect of pathophysiology study using heart tissues of patients, whether the up-regulation of the ER stress sensors represents a cause-effect relationship remains uncertain. Further animal studies are required to solve the puzzle.

In conclusion, our report provided evidence that ER stress occurred in the process of AF. Severe ER stress may contribute to myocytes apoptosis in AF.

Acknowledgements

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Disclosure of conflict of interest

None.

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