Calcium- and integrin-binding protein-1 and calcineurin are upregulated in the right atrial myocardium of patients with atrial fibrillation

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Aims
The aim of this study was to determine whether altered expression and distribution of calcium- and integrin-binding protein-1 (CIB1) is involved in the pathogenesis of different types of patients with atrial fibrillation (AF) associated with valvular heart disease (VHD).

Methods and results
Right atrial specimens obtained from 65 patients undergoing valve replacement surgery were divided into three groups: sinus rhythm group (n = 24), paroxysmal atrial fibrillation group (PaAF; n = 10), and persistent atrial fibrillation group (PeAF; AF lasting >6 month; n = 31). The expression levels of mRNA and protein content for CIB1, calcineurin B, calcineurin A, and Na\(^{+}\)–Ca\(^{2+}\) exchanger-1 (NCX1) were measured. We also measured the combination of CIB1 with calcineurin B, L-type Ca\(^{2+}\) channel, and NCX1 using immunoprecipitation. Expression of mRNA and protein content of CIB1, calcineurin B, calcineurin A, and NCX1 was increased in the AF group. Calcium- and integrin-binding protein-1 interacted with calcineurin B and L-type Ca\(^{2+}\) channel. Surprisingly, CIB1 also combined with NCX1.

Conclusions
The CIB1 and calcineurin expression was increased in AF atrial tissue and was related to the type of AF. This finding suggests that CIB1 may be involved in the pathogenesis of AF in VHD patients.

Keywords
Atrial fibrillation • CIB1 • Calcineurin • Atrial remodel • Human myocardium

Introduction
Atrial fibrillation (AF) is the most common cardiac arrhythmia in clinical practice. Valvular heart diseases (VHDs) such as pathological changes of the mitral and aortic valves or combination of both valves always accompany AF. The only way for patients with these diseases to avoid heart failure is to have a valvular operation.\(^1\)\(^,\)\(^,\)\(^,\)\(^2\) Atrial fibrillation is self-perpetuating, while tachyarrhythmia itself may produce electrophysiological and structural changes that exacerbate or maintain the problem.\(^3\) Structural remodelling is considered to play an important role in the development and maintenance of AF. As structural remodelling is difficult to reverse,\(^4\)\(^–\)\(^6\) the concept of AF-related structural remodelling helps to determine why paroxysmal AF tends to become persistent and why it is more difficult to treat.\(^7\)\(^,\)\(^8\) Therefore, interventions that prevent or reverse structural remodelling (upstream therapy) may be important for effective management of rhythm control. Unfortunately, the factors and mechanism that cause myocardial tissues to remodel remain unclear.

Recent studies suggest that Ca\(^{2+}\)/calcineurin-related mechanisms may also be crucial for the development of structural remodelling, and are emerging as a promising target in anti-AF therapy.\(^9\)\(^,\)\(^10\) Calcineurin is a heterodimer of the catalytic subunit calcineurin A and regulatory subunit calcineurin B. Calcium- and integrin-binding protein-1 (CIB1) has been identified as a protein that interacts with calcineurin B, and is required for the activation of calcineurin.\(^11\) This study was carried out to investigate the expression and distribution of CIB1 in patients with VHD with different types of AF, and to determine whether expression of CIB1 is increased in pathological atrial tissues and whether CIB1 interacts with proteins on the plasma membrane of human cardiac atrial myocytes.
Methods

Patients
We recruited 65 patients with VHD such as pathologic changes of the mitral and aortic valves or both valves, who were admitted to the First Affiliated Hospital of Nanjing Medical University for valve replacement from November 2010 to April 2011. The patients were divided into three groups: 24 patients with sinus rhythm (SR), 31 patients with persistent atrial fibrillation (PaAF), and 10 patients with paroxysmal atrial fibrillation (PaAF). Patients with congenital heart disease (CHD) and SR who underwent heart surgery were included as a control group (n = 10). In order to avoid the influence of atrial stretch on these parameters, we selected patients with a right atrial diameter that was not significantly different prior to the operation. The following patients were excluded: (i) patients with renal dysfunction (serum creatinine >136 μmol/L) or type-II diabetes; (ii) patients who had undergone a coronary angiography and echocardiographic evaluation that indicated they needed coronary artery bypass grafting or other associated procedures; (iii) patients older than 70 years or with a history of diseases such as hyperthyroidism, which may have influenced the propensity to develop AF; and (iv) patients with severe post-operative complications that required special management. Pre-operative medications except warfarin and angiotensin-converting enzyme inhibitors were continued up to the morning of surgery. Prior to the operation, one investigator assessed the pre-operative clinical characteristics of the patients. Before discharge, another investigator recorded the detailed operative data. The Ethics Committee of Nanjing Medical University approved the study protocol, with all the patients providing written consent prior to enrollment in the study. The investigation was pre-incubated with 1.5 μL of oligo (dT)18 primer (10 μmol/L) and 1.5 μL of dNTP (10 mmol/L; Takara), 4 μL MgCl2 (25 mmol/L; Fermentas), 1.5 μL sense and anti-sense primers, 1 U Taq DNA polymerase (5 U/μL; Fermentas), and 2 μL cDNA in a total volume of 50 μL. Thirty cycles of PCR amplification were performed with initial incubation at 94°C for 5 min, and final extension at 72°C for 30 s. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The CIB1, calcineurin B, calcineurin A, and Na+–Ca2+ exchanger-1 (NCX1) genes were amplified using the following specific primers: CIB1 (sense: 5-GACGGGAACCTTGAA CAGAGAA-3, antisense: 5-ACGGAGAATGACAAACACG-3); calcineurin B (sense: 5-TGGAGTCAGCTGAGCCCGGA-3, antisense: 5-TGGACATGTGAGAGTGC-3); calcineurin A (sense: 5-GATCGGCTGAAGGGATTATA-3, antisense: 5-TTGA GGAAGCAGGAAGTGG-3); NCX1 (sense: 5-ACAAACATCGG CAGTTAGTC-3, antisense: 5-GGCCATGACCCGATC-3 ATG-3). The quantities of cDNA that produced an equal amount of β-actin PCR product were used for PCR using the primers for CIB1, calcineurin B, calcineurin A, and NCX1. Following RT-PCR, 5 μL samples of the amplified products were resolved by electrophoresis on 1% agarose gels (gene company, Chai Wan, Hong Kong) and stained with ethidium bromide. The level of each PCR product was determined semi-quantitatively using a digital camera and an image analysis system (Bio-Rad, Gel Doc XR, USA), followed by normalization against the expression of β-actin.

Reverse transcription polymerase chain reaction
Total RNA was isolated from the human atrial tissue samples and treated with RNase-free water in accordance with the Trizol method (Invitrogen, Carlsbad, CA, USA). Single-stranded cDNA was synthesized from total RNA as follows. Briefly, 2 μg of RNA was pre-incubated with 1.5 μL of oligo (dT)18 primer (10 μmol/L Genescript Corporation, Nanjing, China), and diethylpyrocarbonate (DEPC)-treated water (0.1%DEPC; Keygen, China) in a total volume of 10 μL. This solution was incubated at 70°C for 10 min and then chilled rapidly in ice. For the annealed primer/template, 2 μL of 10× RT (reverse transcriptase) buffer (Takara, Dalian, China), 1 μL of dNTP (10 mmol/L; Takara), 25 U of ribonuclease inhibitor (Takara), 200 U avian myeloblastosis virus RT (Takara), and DEPC-treated water were added to a final volume of 20 μL. The reaction was started by incubation at 42°C for 1 h in a gradient thermal cycler (Multigen Gradient,TC9600-G-230V, Edison, USA) and then stopped by deactivation at 70°C for 15 min, followed by immersion in ice. The resultant cDNA was used as a template for subsequent polymerase chain reaction (PCR). The PCR mixture contained 5 μL 10× Taq buffer (Fermentas, Vilnius, Lithuania), 1 μL dNTP (10 mmol/L; Takara), 4 μL MgCl2 (25 mmol/L; Fermentas), 1.5 μL sense and anti-sense primers, 1 U Taq DNA polymerase (5 U/μL; Fermentas), and 2 μL cDNA in a total volume of 50 μL. Thirty cycles of PCR amplification were performed with initial incubation at 94°C for 5 min, and final extension at 72°C for 30 s. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The CIB1, calcineurin B, calcineurin A, and Na+–Ca2+ exchanger-1 (NCX1) genes were amplified using the following specific primers: CIB1 (sense: 5-GACGGGAACCTTGAA CAGAGAA-3, antisense: 5-ACGGAGAATGACAAACACG-3); calcineurin B (sense: 5-TGGAGTCAGCTGAGCCCGGA-3, antisense: 5-TGGACATGTGAGAGTGC-3); calcineurin A (sense: 5-GATCGGCTGAAGGGATTATA-3, antisense: 5-TTGA GGAAGCAGGAAGTGG-3); NCX1 (sense: 5-ACAAACATCGG CAGTTAGTC-3, antisense: 5-GGCCATGACCCGATC-3 ATG-3). The quantities of cDNA that produced an equal amount of β-actin PCR product were used for PCR using the primers for CIB1, calcineurin B, calcineurin A, and NCX1. Following RT-PCR, 5 μL samples of the amplified products were resolved by electrophoresis on 1% agarose gels (gene company, Chai Wan, Hong Kong) and stained with ethidium bromide. The level of each PCR product was determined semi-quantitatively using a digital camera and an image analysis system (Bio-Rad, Gel Doc XR, USA), followed by normalization against the expression of β-actin.

Human cardiac tissue collection and storage
The same cardiac anaesthesiologist, perfusionist, and surgical team performed all the operations. All patients underwent cardiopulmonary bypass (CPB) with moderate hypothermia (33–34°C). Antegrade cardioplegic cardioplegic was used to arrest the heart, with local hypothermia being maintained with ice-slush. The cardioplegic solution was re-administered every 20–30 min. In each patient, ~250 mg of right atrial appendage (RAA) tissues were collected from the cannulation site before the start of extracorporeal circulation. The sampling sites in the patients were the same due to the similar surgical manoeuvres. A 50 mg portion of the RAAs was fixed in paraformaldehyde for histology and immunohistochemistry. The remaining tissue was snap-frozen immediately in liquid nitrogen for other analyses.

Western blot analysis
Atrial tissue samples were homogenized in lysis buffer containing 50 mmol Tris-HCl (pH = 7.4), 150 mmol NaCl, 1% Na2VO4, 1% Triton X-100, and 0.1% sodium dodecyl sulphate (SDS). Protein concentration was determined by the Lowry method with the absorbance read on a UV-2540 (Model Shimadzu, Japan). The denatured samples were subjected to western blot analysis. A 25 μg aliquot of the protein samples were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels for 1.5 h at 120 V and the proteins were then transferred to nitrocellulose membranes (Pall, Ann Arbor, USA). After blocking in 5% fat-free milk, the membrane was incubated overnight at 4°C with the primary antibody CIB1 (1:200; Boster, China), calcineurin B (1:200; Boster), calcineurin A (1:200; Boster), and NCX1 (1:200; Boster), followed by incubation for 2 h at 37°C in diluted secondary goat anti-rabbit IgG. The secondary antibody was diluted in phosphate-buffered saline containing 5% fat-free milk and 0.1%
Tween-20. The stained membranes were then visualized by an enhanced chemiluminescence reaction using the ECL Plus (GE Healthcare, Fairfield, CT, USA). The western blotting experiments were repeated at least three times on every sample with similar results.

**Immunoprecipitation**

For immunoprecipitation (IP) with anti-NCX1 (Boster, China), anti-CnB (Boster, China), anti-L-type Ca\(^{2+}\) channel subset alpha1C (Boster, China), and anti-CIB1 antibodies (Boster, China), the atrial tissues were collected in lysis buffer containing 50 mmol Tris-HCl (pH 7.4), 150 mmol NaCl, 0.5% Na\(_3\)VO\(_4\), and 1% NP-40. The immunocomplexes were captured with Protein A agarose beads (Beyotime, NanTong, China). After extensive washing, the proteins were resolved using SDS-PAGE electrophoresis and subjected to western blotting.

**Histology and immunohistochemistry**

The RAAs were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 24 h. After alcoholic dehydration, the tissues were embedded in paraffin, followed by preparation of 2 \(\mu\)m serial sections that were then stained with Van-Gieson solution for microscopic examination. For detection of CIB1 (Boster), immunoreactivity was performed on 4 \(\mu\)m-thick sections from each paraffin-embedded tissue. Brown staining in the cells or on the cell membrane was considered positive.

**Statistical analysis**

All the values are expressed as means ± s.e.m. Differences between three or more groups were analysed using the Kruskal-Wallis test. The \(\chi^2\) test or Fisher’s exact test was used for analysis of percentage differences between the patient groups. \(P\) values <0.05 were considered significant.

**Results**

**Clinical characteristics and haemodynamic data**

The left atrial diameters measured by echocardiography in patients with PeAF were significantly larger than in patients with SR. There was no significant difference in left ventricular end systolic dimension (LVDs), left ventricular end diastolic dimension (LVDd), and left ventricular function between the three groups. The pre-operative haemodynamic and echocardiographic data in the three groups are shown in Table 1.

**Calcium- and integrin-binding protein-1 interacted with calcineurin B, L-type Ca\(^{2+}\) channel, and Na\(^+\)–Ca\(^{2+}\) exchanger-1**

The IP experiments showed that CIB1 combined with calcineurin B and L-type Ca\(^{2+}\) channel in atrial tissues of patients with AF. Surprisingly, we found that CIB1 also combined with NCX1 (Figure 1).

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**Table 1** Analysis of clinical data

<table>
<thead>
<tr>
<th>Variables</th>
<th>SR + CHD</th>
<th>SR + VHD</th>
<th>PaAF + VHD</th>
<th>PeAF + VHD</th>
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<tr>
<td><strong>Basic data</strong></td>
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<td>Patient number</td>
<td>10</td>
<td>24</td>
<td>10</td>
<td>31</td>
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<tr>
<td>Sex, M/F (n)</td>
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<td>14/10</td>
<td>4/6</td>
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<td>Age (years)</td>
<td>15.20 ± 3.41</td>
<td>45.75 ± 2.68</td>
<td>53.6 ± 9.83</td>
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<td><strong>Pre-operative data</strong></td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>103.4 ± 4.51</td>
<td>77 ± 9.00</td>
<td>80 ± 10.0</td>
<td>79 ± 9.20</td>
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<td>NYHA class I/II/III/IV</td>
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<td>0/4/6/0</td>
<td>0/13/14/4</td>
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<td><strong>Echocardiographic parameters</strong></td>
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<td>LVDd</td>
<td>34 ± 2.30</td>
<td>55.54 ± 1.70</td>
<td>53.66 ± 19.13</td>
<td>51.97 ± 1.36</td>
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<tr>
<td>LVDs</td>
<td>24 ± 3.11</td>
<td>35.38 ± 1.26</td>
<td>39 ± 20.22</td>
<td>34.13 ± 1.03</td>
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<td>EF (%)</td>
<td>64 ± 4.12</td>
<td>63.01 ± 4.80</td>
<td>55.07 ± 5.32</td>
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<td>LAD (mm)</td>
<td>23 ± 6.73</td>
<td>46.15 ± 5.19</td>
<td>50.33 ± 6.80</td>
<td>58.13 ± 5.92</td>
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<td>RAD (mm)</td>
<td>27 ± 8.71</td>
<td>39.4 ± 5.20</td>
<td>39 ± 5.64</td>
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<tr>
<td>Pre-operative length of stay</td>
<td>10.4 ± 9.36</td>
<td>17.6 ± 11.80</td>
<td>17.3 ± 11.53</td>
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<td><strong>Operative data</strong></td>
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<td>Surgical procedure</td>
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<tr>
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<td>5/2/3</td>
<td>21/9/1</td>
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<td>CPB duration</td>
<td>80 ± 12.44</td>
<td>133 ± 13.42</td>
<td>145 ± 23.44</td>
<td>142 ± 14.23</td>
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<td>Aortic clamp time</td>
<td>48.32 ± 33.32</td>
<td>83 ± 36.41</td>
<td>90 ± 32.25</td>
<td>95 ± 43.34</td>
</tr>
</tbody>
</table>

EF, ejection fraction; MVR, mitral valve replacement; DVR, double valve replacement; AVR, aortic valve replacement.
Figure 1 (A) Calcium- and integrin-binding protein-1-specific antibody, calcineurin B-specific antibody, and control IgG was used for immunoprecipitation. Immunoblots show calcineurin B coimmunoprecipitated with calcium- and integrin-binding protein-1. (B) Calcium- and integrin-binding protein-1-specific antibody, L-type Ca\textsuperscript{2+} channel specific antibody, and control IgG was used for immunoprecipitation. Immunoblots show L-type Ca\textsuperscript{2+} channel coimmunoprecipitated with calcium- and integrin-binding protein-1. (C) Calcium- and integrin-binding protein-1-specific antibody, Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger-1-specific antibody and control IgG was used for immunoprecipitation. Immunoblots show Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger-1 coimmunoprecipitated with calcium- and integrin-binding protein-1.

Figure 2 Immunohistochemistry for CIB1 (stained brown) in sections obtained from CHD + SR, VHD + SR, VHD + PaAF, and VHD + PeAF group. (A) CHD + SR; (B) VHD + SR; (C) VHD + PaAF; (D) VHD + PeAF. Nuclei are in blue. Elevated levels of CIB1 in right atrial tissues were identified in VHD + PeAF and VHD + PaAF groups, compared with VHD + SR and CHD + SR groups. CIB1, calcium- and integrin-binding protein-1; CHD, congenital heart disease; SR, sinus rhythm; VHD, valvular heart disease; PaAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation.
Figure 3 (A) Calcium- and integrin-binding protein-1 protein expression in different groups. (B) Calcium- and integrin-binding protein-1 mRNA expression in different groups. *P < 0.05. (D) Expression of levels of calcium- and integrin-binding protein-1 mRNA in different groups. *P < 0.05. (E) Calcineurin B protein expression in different groups. (F) Calcineurin B mRNA expression in different groups. (G) Protein expression of calcineurin B in different groups. *P < 0.05. (H) Expression of levels of calcineurin B mRNA in different groups. *P < 0.05. (I) Na⁺–Ca²⁺ exchanger-1 protein expression in different groups. (J) Na⁺–Ca²⁺ exchanger-1 mRNA expression in different groups. (K) Protein expression of Na⁺–Ca²⁺ exchanger-1 in different groups. *P < 0.05. (L) Expression of levels of Na⁺–Ca²⁺ exchanger-1 mRNA in different groups. *P < 0.05. (M) Calcineurin A protein expression in different groups. (N) Calcineurin A mRNA expression in different groups. (O) Protein expression of calcineurin A in different groups. *P < 0.05. (P) Expression of levels of calcineurin A mRNA in different groups. *P < 0.05.
CIB1 and calcineurin expression in patients with atrial fibrillation

Figure 3 Continued.
The protein and gene expression of calcium- and integrin-binding protein-1, calcineurin B, calcineurin A and Na\(^{+}\)-Ca\(^{2+}\) exchanger-1 was increased in atrial fibrillation

Immunohistochemistry showed enhanced expression of CIB1 in atrial tissues from patients with AF compared with patients with SR (Figure 2). Western blot analysis demonstrated that the protein levels of CIB1 in samples from patients with either PeAF + VHD or PaAF + VHD were higher than in patients with either SR + VHD or SR + CHD. In addition, protein levels of calcineurin B and calcineurin A in samples from patients with PeAF + VHD or PaAF + VHD were higher than those from patients with SR + VHD or SR + CHD. The expression levels of mRNA for CIB1, calcineurin A, and calcineurin B confirmed the protein expression results. Western blot analysis revealed that the protein levels of NCX1 in samples from PeAF + VHD patients were higher than those from SR + VHD and SR + CHD patients. Similarly, RT-PCR analysis showed that mRNA levels of NCX1 from PeAF + VHD patients were higher than in patients with SR + VHD or SR + CHD (Figure 3, Table 2).

Discussion

Numerous studies have demonstrated that structural remodelling may primarily determine the generation of AF. A number of factors associated with atrial structural remodelling have already been identified. Of these factors, hypertrophy is considered an important factor in atrial structural remodelling.\(^{12}\)

Researchers have found that single myocytes in patients with AF are wider and longer than in patients with SR.\(^{13}\) It is therefore clear that atrial cells may become hypertrophic. Atrial hypertrophy hinders conduction in the atrium and leads to ectopic activity and diffuse conduction, further initiating and sustaining AF. Many reports have demonstrated the role of Ca\(^{2+}\)/calcineurin-related mechanisms in pathological hypertrophy heart tissues in mice.\(^{11}\) However, a CIB1 and Ca\(^{2+}\)/calcineurin-related mechanism has not been researched extensively in human AF tissues. Calcineurin is a heterodimer of a catalytic subunit, calcineurin A and regulatory subunit, calcineurin B.\(^{14}\) Calcineurin B is the exclusive and obligate binding partner of the calcineurin A catalytic subunit, with neither protein being very stable without expression of the other.\(^{15}\) We demonstrated that catalytic subunit (calcineurin A) and regulatory subunit (calcineurin B) were overexpressed in atrial tissues of patients with AF. From those data, we concluded that the activity of the catalytic subunit was probably increased. As calcineurin is activated by Ca\(^{2+}\), possibly in response to Ca\(^{2+}\) flux through the L-type channel itself, it has been proposed that a feed-forward loop may activate the calcineurin pool.\(^{14}\) A Ca\(^{2+}\)-calcineurin-nuclear factor of activated T cell (NFAT) signalling cascade has been identified as a crucial node where inputs from several signalling pathways are integrated to promote pathological hypertrophy.\(^{16}\) Studies have also suggested that the intracellular calcineurin-NFAT signalling pathway may be a potential mediator of matrix metalloprotease alterations that result in...
atrial fibrosis. Indeed, in agreement with the findings of Heineke, the IP test in our study demonstrated that CIB1 interacts with calcineurin B and 1αc subunit of the L-type Ca^{2+} channel. Our study also showed that protein and mRNA expression of CIB1 was greater in the AF groups than in the SR groups. Over-expression of CIB1 may be a primary mechanism for recruiting calcineurin that causes atrial myocytes to hypertrophy associated with AF.

We were surprised to find that CIB1 combined with NCX1. Increased activity of the NCX will produce a transient inward current that underlies delayed afterdepolarization and potentially triggers arrhythmogenic activity. Our study demonstrated that the gene expression and protein content of NCX1 were increased in atrial tissues of patients with AF. As a result, critical prolongation of the AP plateau phase due to expression of NCX1 can give rise to a delayed afterdepolarization and provide an extra depolarizing current. Unstable membrane potentials either at the AP plateau or resting level can serve as triggers for ectopic activity. Generation of an inward depolarizing NCX current may produce triggered activity that causes focal activity, thereby contributing to maintenance of AF. Therefore, we suggest that greater interaction between CIB1 and NCX1 may induce delayed afterdepolarizations that provide favourable circumstances for the development of AF.

Conclusion

We have presented data that showed over-expression of CIB1, calcineurin B, calcineurin A, and NCX1 in atrial tissues from patients with AF. In addition, we showed a combination between CIB1 and NCX1, calcineurin B, and L-type calcium channels. These results support the hypothesis that CIB1 and calcineurin are possibly involved in the pathogenesis of AF in VHD patients. However, as there are many risk factors for AF, the mechanism of CIB1 and calcineurin in atrial structural and electrical remodelling needs to be investigated further.

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