Syndecan-4 shedding is involved in the oxidative stress and inflammatory responses in left atrial tissue with valvular atrial fibrillation

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Abstract: Oxidative stress and inflammation play critical roles in the development and maintenance of atrial fibrillation (AF). In addition, syndecan-4 (Synd4) shedding induced by oxidative stress or inflammation plays a role in the migration of inflammatory cells. Therefore, we hypothesized that Synd4 shedding was also involved in the inflammatory response in atrial fibrillation patients with valvular heart disease. To confirm this suppose, left atrial appendages and clinical data were obtained from 65 patients with valvular disease undergoing valve surgery. Ten left atrial appendages obtained from healthy heart donors were used as controls. Analyses including histopathology, western blotting, and enzyme kinetics were performed to assess the oxidative injury, inflammation responses, and Synd4 shedding. The results showed that the inflammatory response and oxidative injury were increased significantly, whereas levels of the Synd4 ectodomain was decreased significantly in AF patients. Furthermore, Synd4 ectodomain levels were correlated with atrial oxidative and inflammatory markers. The results showed that Synd4 shedding is a molecular pathological alteration in the development and maintenance of inflammation-associated AF.

Keywords: Synd4 shedding, oxidative stress, inflammation, atrial fibrillation

Introduction

Atrial fibrillation (AF), the most prevalent arrhythmia disorder, is a major cause of morbidity and mortality. AF can occur secondary to valve heart disease (VHD), which impairs cardiac function and is associated with an increased risk of stroke [1]. Previously, a large number of studies on AF have focused on atrial remodeling, which is important in its pathogenesis. The structural changes that occur in the atrium are promoted by a variety of conditions, including inflammation and oxidative stress [2-6]. Although inflammation and oxidative stress have been studied heavily, many questions remain regarding the mechanisms underlying the progression of oxidative stress and inflammation.

Synd4, a member of the syndecan family, consists of an ectodomain carrying heparin sulfate- or chondroitin sulfate-rich glucosaminoglycan (GAG) chains, a transmembrane domain, and a short cytoplasmic tail. Synd4 can cooperate with many receptors, to subsequently play regulatory roles in processes including wound healing [7], inflammation [8], and angiogenesis [8]. Several lines of evidence have suggested that the ectodomain of syndecan could be shed from the membrane under many pathological conditions such as oxidative stress and inflammation [9, 10]. Recently, the cleavage and shedding of syndecan, which modulates the inflammatory response, was reported in lungs and heart [8, 11]. Because the oxidative shedding of syndecan is associated with inflammation, we hypothesized that Synd4 and its mediators may participate in AF-associated atrial inflammation and oxidative stress. Therefore, the aim of this study was to investigate the role of Synd4 in inflammation and oxidative injury during AF.

Methods

Patients

We recruited 65 patients with VHD who exhibited pathological changes in the mitral, aortic, or both valves, and were admitted to the Drum
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Tower Hospital of Nanjing University Medical College for valve replacement surgery. The patients were divided into three groups: sinus rhythm (SR; \( n = 20 \)), paroxysmal atrial fibrillation (PaAF; \( n = 15 \)), and persistent atrial fibrillation groups (PeAF, \( n = 30 \)). All patients underwent routine preoperative two-dimensional color echocardiography. Patients with familial paroxysmal AF, a history of disease (such as hyperthyroidism) that influences the AF-associated risk of pulmonary artery disease, cardiomyopathy, renal disease, or secondary thoracotomy were excluded from the study. Healthy heart donors were used as the control group (con, \( n = 10 \)).

Cardiac tissue collection and storage

LAA specimens were obtained prior to the establishment of extracorporeal circulation. One part of the tissue was fixed in 4% formalin for histological analysis, and the remaining tissue was frozen in liquid nitrogen and stored at -80°C for western blotting. Human tissue collection and analyses strictly abided by the principals outlined in the World Medical Association of Helsinki. All procedures involving human tissue were approved by the Drum Tower Hospital affiliated to Nanjing University Medical College Ethics committee. All patients recruited in the study gave written informed consent.

Histological staining

The specimens fixed in 4% formalin were embedded in paraffin and cut into slices ~4 μm thick. The slices were deparaffinized using dimethyl benzene followed by soaking in a series of solutions with decreasing ethanol concentrations 100-75%. Samples were then stained with hematoxylin-eosin (HE) according to routine procedures. Five different fields were observed from each stained tissue.

Western blotting

Tissue specimens were washed with PBS and then lysed using RIPA buffer containing a 1:100 dilution of protease inhibitor and phosphatase inhibitor (Sigma Aldrich). Protein concentrations were measured using a BCA protein assay (Pierce), and 30 μg protein samples were separated by SDS-PAGE. Proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore), and then incubated in Tris-buffered saline containing 0.1% Tween 20 (TBST) with 5% milk for 1 h at room temperature. Blots were then incubated with primary antibodies as follows: anti-rac1 (1:1000, Aabcam), anti-Synd4 (1:500, LifeSpan BioSciences), anti-HMGB1 (1:1000, Bioword), and anti-iNOS (1:1000, Bioword); anti-β-actin antibody (1:2000, Santa Cruz) was used as the internal control. After four washes in TBST, blots were incubated with horseradish peroxidase-conjugated secondary antibodies. The washes were repeated, and the membranes were then treated with SuperSignal Substrate Western Blotting Reagent (Millipore). The bands were quantified using BioRad Quantity One imaging software.

Malondialdehyde (MDA) levels

MDA levels were quantified using commercial assay kits according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

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**Table 1.** Baseline clinical characteristics of patients studied

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SR</th>
<th>PaAF</th>
<th>PeAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, M/F (n)</td>
<td>9/11</td>
<td>8/7</td>
<td>13/17</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.5±9.0</td>
<td>53.1±7.3</td>
<td>52.8±7.6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.5±3.1</td>
<td>23.2±2.4</td>
<td>22.8±2.7</td>
</tr>
<tr>
<td>NYHA class I/II/III/IV (n)</td>
<td>4/6/8/2</td>
<td>3/5/4/3</td>
<td>4/10/14/2</td>
</tr>
<tr>
<td>Echocardiographic parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>52.8±7.2</td>
<td>54.3±5.9</td>
<td>56.7±9.4</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>40.4±4.4</td>
<td>44.0±9.3</td>
<td>44.1±5.9</td>
</tr>
<tr>
<td>EF (%)</td>
<td>53.8±3.2</td>
<td>51.1±5.4</td>
<td>49.4±4.4</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>42.9±4.7</td>
<td>51.1±6.5</td>
<td>59.2±6.9*</td>
</tr>
<tr>
<td>Left atrial thrombus (n)</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cause of mitral valve disease (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatic/degenerative</td>
<td>15/5</td>
<td>12/3</td>
<td>23/7</td>
</tr>
<tr>
<td>Preoperative drugs (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digitalis</td>
<td>18</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>ACEI</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD or number of patients. ACEI, angiotensin converting enzyme inhibitor; EF, ejection fraction; LAD, left atrial diameter; LVDd, left ventricular end diastolic diameter; LVDs, left ventricular end systolic diameter; NYHA, New York Heart Association; PaAF, paroxysmal atrial fibrillation; PeAF, persistent atrial fibrillation groups; SR, sinus rhythm; *P<0.05, compared with the SR group; #P<0.05, compared with the PaAF group.
Statistical analysis

χ² test was used to compare categorical variables. One-way analysis of variance (ANOVA) or Kruskal-Wallis test was used when three or more experimental conditions were compared. Correlation analysis (Pearson) was used to assess the association between the different groups. Data for continuous variables were expressed as the mean ± SD. All statistical analyses were performed using SPSS 17.0. Statistical significance was assumed when P<0.05.

Results

Baseline characteristics of the patient population

In this study, we enrolled 65 valve disease patients. These patients were then divided into three groups depending on AF stage: 20 patients with SR, 15 with PaAF (AF lasting <7 days), and 30 with PeAF (AF lasting >7 days). Ten LAAs obtained from healthy heart donors were used as the control group. Table 1 shows the clinical characteristics of the study patients.
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The three groups did not differ significantly in age, body mass index, heart failure status, or left ventricular ejection fraction. Left atrial size and duration of valvular disease were significantly larger or longer, respectively, in the AF groups compared with the SR group. The three subgroups were also balanced in terms of drug use (including digitalis, calcium channel blockers, or angiotensin-converting enzyme inhibitors).

**Decreased levels of Synd4 ectodomain in the LAA of patients with atrial fibrillation**

Western blotting revealed significantly decreased levels of Synd4 ectodomain in the PaAF and PeAF groups compared with con and SR. In addition, levels were lower in PeAF than PaAF, although this was not statistically significant (Figure 1A). Next, we analyzed the relationship between Synd4 ectodomain levels and other parameters including EF, valve number, and NYHA classification. There was no relationship between Synd4 expression and EF value in the SR, PaAF, and PeAF groups ($r=0.157$, $P=0.210$) (Figure 1B). In addition, there were no statistically significant differences between the three groups in terms of the number of valves involved in patients with valve disease (Figure 1C). Interestingly, as the New York Association (NYHA) classification increased, the levels of Synd4 ectodomain decreased gradually, although there was no relationship between Synd4 levels and EF value. Synd4 levels in the LAA of patients of NYHA class III and IV was lower than in NYHA class I, although there were no significant differences between class I and II groups. In the AF groups, Synd4 ectodomain levels exhibited a trend for being lower in class IV compared with class III (Figure 1D).

**Increased atrial myocardial oxidative stress and inflammation in patients with atrial fibrillation**

Increasing evidence has suggested that elevated oxidative stress plays a role in promoting and maintaining AF. Oxidative stress is a condition of elevated levels of reactive oxygen species (ROS), induced mostly by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family of enzymes. RAC1, an important subunit of NADPH oxidase, is a key factor in...
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many cardiovascular pathophysiological processes [12, 13]. The expression of malondialdehyde (MDA), another oxidative stress marker, is increased significantly in the serum of patients with AF [14]. In the current study, we measured RAC1 expression and MDA levels in the LAA to evaluate the levels of oxidative stress. As shown in Figure 2, the expression of RAC1 was increased significantly in the PaAF and PeAF groups compared with con and SR, although levels were similar between the PaAF and PeAF groups (Figure 2A). MDA levels confirmed the presence of oxidative stress in the AF groups; levels were highest in the PeAF

Figure 3. The inflammatory response in the left atrium of AF patients. A. Hematoxylin eosin (HE) staining showed more inflammatory cells had infiltrated the left atrial tissue in the AF groups. Arrows show inflammatory cells. B. Up-regulation of HMGB1 and iNOS in the AF groups. Con, n=10; SR, n=20; PaAF, n=15; PeAF, n=30; *P<0.05 between groups; Bar=100 μm; NS, no significant difference between groups.
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To assess the inflammatory response in AF, we used HE staining to estimate the number of infiltrating inflammatory cells in the left atrium. As shown in Figure 3A, HE staining revealed that there were more inflammatory cells in the AF groups compared with con and SR. To further confirm the state of inflammation in AF, western blotting was used to assess the expression of high mobility group box 1 protein (HMGB1) and inducible nitric oxide synthase (iNOS), which are inflammatory mediators. HMGB1, a novel proinflammatory cytokine, is released from active inflammatory cells; it then exacerbates the inflammation. Inducible nitric oxide synthase (iNOS) also plays an important role in inflammation. Compared with the con and SR groups, PaAF and PeAF exhibited significantly increased expression of iNOS and HMGB1 (Figure 3B). In the AF groups, the expression of HMGB1 was significantly higher in the PeAF group compared with PaAF. In contrast, there was no significant difference of expression of iNOS in the two AF groups compared with SR group. These data suggest that oxidative stress and inflammatory markers were upregulated in the AF groups.

Correlation between markers of inflammation and oxidative stress in AF

Although many studies have explored the role of inflammation and oxidative injury in AF, the relationship between these pathways remains unclear. Therefore, we assessed the relation-
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**Discussion**

In the present study, we found that decreased levels of the Synd4 ectodomain were followed by the upregulation of markers of inflammation and oxidative stress in the left atrial tissue of patients with AF. Importantly, our findings suggested that decreased Synd4 ectodomain levels were associated with markers of inflammation and oxidative stress.

Increasing evidence has supported the involvement of oxidative stress in AF [4, 15], and as such the application of antioxidants prevented atrial tachycardia remodeling-induced AF [16-18]. Therefore, many studies have assessed the potential role of the ROS-generating NADPH oxidase family of enzymes in the pathogenesis of AF [19]. Svetlana et al found that NADPH oxidase activity and NOX2 expression were increased significantly in the left atrium of goats with AF and in patients who developed postoperative AF [5]. Consistent with a recent study that reported higher MDA activity in the serum of AF patients compared with control [14], the present study demonstrated that MDA levels were higher in atrial tissue homogenates of AF compared with SR. We also revealed that the left atria of patients with AF are characterized by a marked upregulation of RAC1, which regulates NADPH oxidase activity; this is supported by the findings of previous studies [4, 5, 13]. These data suggest that oxidative stress is implicated in the pathogenesis of AF.

Increasing evidence has revealed that inflammation is also a key player in the development, recurrence, and perpetuation of AF. Several studies have demonstrated that several inflammatory markers were involved in the mainte-
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nance or development of AF, such as interleu-
kin (IL)-6 [20], C-creative protein (CRP) [20-22],
IL-8, TNF-α [23], and CCL2 (MCP-1) [24]. To
assess inflammation associated in AF, we mea-
sured the expression of the inflammatory medi-
ators HMGB1 and iNOS. HMGB, a novel inflam-
matory cytokine, is released by necrotic
macrophages and monocytes [25]. Similarly,
iNOS plays a role in the induction of pro-inflam-
matory cytokines and oxidative stress. A recent
study reported that the serum concentrations
of HMGB1 were higher in patients with AF than
control [14]. As expected, our data revealed
that both HMGB1 and iNOS were upregulated
in the LAA of patients with AF compared with
the SR group. More importantly, the expres-
sion of HMGB1 was significantly higher in PeAF
patients compared with PaAF. However, the dif-
ference in iNOS expression was not signifi-
cantly different. These data suggest that the inflam-
matory markers HMGB1 and iNOS may play an
important role in the pathogenesis of AF by
inducing the inflammatory response.

In the current study, correlation analysis re-
vealed a strong correlation between oxidative
stress and inflammation, consistent with previ-
ous studies [14]. This suggests that oxidative
stress may be related to inflammation in AF. A
recent investigation reported that antioxidant
administration attenuated inflammation during
postoperative AF [18]. However, the endoge-
 nous mechanisms of inflammation and oxida-
tive stress in AF remain poorly understood.

Syndecans are major heparin sulfate proteogly-
cans that are found at the surface of most
mammal cells. They play important roles in
defense mechanisms including inflammation,
angiogenesis, and tissue remodeling. Syndecan
ectodomains are constitutively shed and
replaced under physiological conditions to
maintain balance; however, in response to cer-
tain stimuli, syndecan shedding is increased
dramatically [11, 26]. In AF, Synd4 shedding
was revealed by the reduced levels of Synd4
ectodomain in the AF groups. Moreover, Synd4
protein was correlated with markers of oxida-
tive stress and inflammation, suggesting that
these pathways may contribute to Synd4 shed-
ing. Consistent with this, several studies have
reported that MMPs regulate Synd4 shedding
under pathological conditions [27, 28]. During
the pathological progression of AF, there is
increased expression and activity of MMPs, as
well as markers of oxidative stress and inflam-
mation [29]. This suggests that oxidative stress
or inflammation may be responsible for Synd4
shedding via MMPs.

In addition to inflammation-induced syndecan
shedding, accumulating evidence suggests that
ectodomain shedding might in turn act as a
key inflammatory mediator (Figure 5) [11,
30]. As described above, we observed that
Synd4 was reduced in high grade NYHA and AF
groups as well as enhanced inflammation cells
migration. These data are consistent with previ-
uous studies, which demonstrated that Synd4
was sheded in ventricular and atrial tissue of
failure hearts [11, 31]. It is clear that HSPGs
play an important role in many aspects of
inflammation. First, HSPGs can bind to chemo-
kines to protect them from proteolysis, increas-
ing the concentration of active chemokines at
inflammatory sites [32]. Second, HSPGs can
immobilize chemokines to establish chemokine
gradients and promote inflammation [30, 33].
Third, shed HSPGs, such as syndecan-1, can
form stable chemokine gradients that may
facilitate leukocyte migration [30]. A recent
study highlighted this by demonstrating that
inflammation-induced shed Synd4 could pro-
mote the transmigration of inflammatory cells
in a pressure-overloaded heart [11]. Therefore,
the shed Synd4 in the current study is likely to
enhance the inflammatory response in atrial
tissue, because we found a strong correlation
between the expression of Synd4, HMGB1, and
iNOS. Thus, we propose that shedding Synd4
might be an important mediator of the oxidative
stress and inflammatory responses in AF.

In summary, we demonstrated that the levels
of Synd4 ectodomain decreased in the atrial tis-
sue of AF. It was also inversely correlated with
MDA levels and the expression of inflammatory
markers. Therefore, these findings reveal a
potential role of the Synd4 ectodomain in oxida-
tive stress and inflammation-associated AF.

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Disclosure of conflict of interest
None.

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