A calpain inhibitor elevated expressions of NF-κB in atrial fibrillation canine atria

Hong-Jie Xue¹, Zhi-Hua Gong¹*, Yue Li¹*, Yong-Tai Gong¹, Li Sheng¹, Guang-Zhong Liu¹, Wei-Min Li¹ and Song-Bin Fu²

¹Department of Cardiology, the First Affiliated Hospital, Harbin Medical University, Harbin, P. R. China.
²Department of Genetics, Harbin Medical University, Harbin, P. R. China.

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This aim of this study is to observe the alterations of calpain proteolytic system and effect of a calpain I inhibitor on NF-κB expressions in rapid pacing canine atrial fibrillation (AF) models. Twenty-one dogs were divided equally into three groups: a sham operation group, a control AF group and a calpain inhibitor group. Rapid right atrium pacing at 600 beats per min was applied in the control AF group and the calpain inhibitor group. N-Acetyl-Leu-Leu-Met (1.0 mg/kg/day) was administered in the calpain inhibitor group for three weeks. The protein expressions of NF-κB, calpain I, calpain II and calpastatin were assessed by immunohistochemistry and gene amplification of NF-κB and calpain I were detected by quantitative real time polymerase chain reaction. The highest density of calpain I was shown in the control AF group (p < 0.01, vs. calpain inhibitor group and sham operation group). Protein expression of calpain II and calpastatin had no significant difference among the three groups. Gene amplification results of calpain I unveiled the most active transcription was in the calpain inhibitor group (p < 0.01, vs. control AF group and sham operation group). Protein expression of calpain II and calpastatin had no significant difference among the three groups. Gene amplification results of calpain I unveiled the most active transcription was in the calpain inhibitor group (p < 0.01, vs. control AF group and sham operation group). The mRNA expression of NF-κB were elevated in the calpain inhibitor group, the cycle of threshold means were (20.1±5.1), while were (33.4±6.1) and (30.4±5.8) in the sham operation group and control AF group, respectively. NF-κB protein expressions in calpain inhibitor group were positive, while were negative in the other two groups. In canine heart muscle cells, atrial fibrillation elevated calpain I gene transcription, promoted protein expression and enhanced protease activity. The calpain I inhibitor N-Acetyl-Leu-Leu-Met promoted NF-κB gene transcription and protein expressions in AF canine models.

Key words: Calpain I inhibitor, atrial fibrillation, NF-κB.

INTRODUCTION

As a common arrhythmia, atrial fibrillation (AF) demands effective treatments. More and more researches documented that etiology of AF is mainly composed of three aspects, electrical remodeling, structural remodeling and cardiac neural remodeling (Ausma et al., 1997; Hobbs et al., 2000). Apoptosis plays an important role in cardiovascular structural remodeling. Abundant apoptosis cardiomyocytes were observed in atrial fibrillation models and patients (Li et al., 2009). According to Brundel et al. (2002) calpain activity was significantly increased in paroxysmal and persistent atrial fibrillation patients. Calpain was involved in apoptosis via complicated mechanisms (Li et al., 2009). Our previous findings demonstrated that calpain I played a pro-apoptotic role in AF canine models through activation of caspasas. Caspasas induced apoptosis directly as well as through their interactions with bcl-2 and ARC. N-Acetyl-Leu-Leu-Met, a calpain inhibitor inhibits the activity of calpain I and influences the expression of apoptosis-related proteins, playing an anti-apoptotic role in AF canine models. Recently new researches documented that NF-κB is involved in etiology of atrial fibrillation. Over expressions of NF-κB were recorded in cardiac tissues in

*Corresponding author. E-mail: gong_zhihua2@163.com. Tel: 86-451-85555672. Fax: 86-451-53675733.
patients with atrial fibrillation (Bukowska et al., 2008; Qu et al., 2009). We aimed to observe the alterations of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) expressions in atrial fibrillation dogs treated with calpain inhibitor, and try to provide another mechanistic link between calpain inhibitor and apoptosis in AF.

MATERIALS AND METHODS

The materials used include ALLM (N-Acetyl-Leu-Leu-Met, calbiochem, USA), DMSO (dimethyl sulfoxide, HuaShun Pharmaceuticals, Shanghai, China), embedding heart pacemaker (Electronic Engineering Department of Fudan University, Shanghai, China), polyclonal rabbit antibody to calpain I, calpain II, calpastatin (Boster Biotechnology, Inc), polyclonal rabbit antibody to NF-kB (p65) (Santa Cruz Biotechnology, Inc), peroxides conjugated goat anti-rabbit IgG (Santa Cruz, USA), TUNEL kit (Roche, USA), Trizol Reagent (Gibco BRL, USA), real time RT-PCR fluorescent quantitative detection kit (Dahui biotech Ltd., Guangzhou, China), polyclonal rabbit antibody to calpain I, calpain II, calpastatin (Boster Biotechnology, Inc, China), embedding heart pacemaker (Electronic Engineering Department of Fudan University, Shanghai, China), and healthy mongrel dogs (Animal Experimental Center of 111 affiliated hospital, Harbin medical university, Harbin, China).

Establishment of chronic AF canine models

This investigation conformed to the guiding principals of the Declaration of Helsinki and in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Twenty-one dogs (weight 15 ± 5 kg) were categorized into three groups: A sham operation group, a control AF group and a calpain inhibitor group. After anesthetized with pentobarbital 30 mg/kg intravenous injection, spiral mono-polar pacing leads were positioned in the right auricle (RA) then connected to pacemakers. After recovery, atrial pacing was set at 600 beats /min in AOO mode. The RA leads in the sham operation group were nonfunctional. In the calpain inhibitor group, dogs received intravenous injection of ALLM (1.0 mg/kg/day) dissolved in DMSO for 3 weeks. In the control AF group and sham operation group, dogs were injected intravenously with DMSO (1.0 mg/kg/day) for 3 weeks. ALLM is a non-specific cysteineyl protease inhibitor. A significantly higher affinity to calpain I made ALLM a good choice for calpain I inhibiting. The tissues from the left atrial appendage were removed after general euthanasia.

Table 1. Primers and conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NF-kB</th>
<th>Calpain I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>TTCGAGGGCCTGTATCCAGT</td>
<td>CACGGAGCTGCTCTCAAACC</td>
</tr>
<tr>
<td>Backward primer</td>
<td>TCTACTTCAGCCCGCTGTC</td>
<td>GCAGCCAGAAAGCCACAGTC</td>
</tr>
<tr>
<td>Program</td>
<td>1 cycle 50°C 1 min, 93°C 10 min, 40 cycles 93°C 15 s, 55°C 20 s, 1 cycle 72°C 30 s</td>
<td>1 cycle 93°C 2 min, 40 cycles 93°C 1 min, 55°C 1 min</td>
</tr>
</tbody>
</table>

Ribonucleic acid isolation and quantitative real time PCR

Total RNA was isolated from tissues lysate following Trizol reagent instructions. RNA quantity and quality were evaluated using absorbance measurements at 260 and 280 nm.

Reverse transcription was performed in a total volume of 20 μl, containing 5 μl RNA, 4 μl first-strand buffer (5x), 0.2 μl dNTPs, 0.4 μl oligo(dT), 1 μl M-MLV reverse transcriptase, and 9.4 μl DEPC purified water. This mixture was incubated at 37°C for an hour and 95°C for 3 min, and then subsequently stored at -20°C.

The real-time polymerase chain reaction was performed in a 50 μl total volume, containing the following mixture: 5 μl aforementioned cDNA, 10 μl PCR buffer (5x, contains fluorescent probe), 0.5 μl forward primer (Table 1), 0.5 μl reverse primer, 0.5 μl dNTPs, 2 μl 7q DNA polymerase, 31.5 DEPC purified water. Different dilutions of positive controls (10², 10⁶, 10⁷ and 10⁸, respectively) generated standard curve. This curve is linear for double stranded DNA concentrations.

Immunohistochemistry

5 μm paraffin sections were incubated with polyclonal rabbit antibody to calpain I, calpain II, calpastatin (Boster Biotechnology, Inc), polyclonal rabbit antibody to NF-kB (p65) (Santa Cruz Biotechnology Inc, USA) at 1:200 dilution, overnight at 4°C. Subsequently, sections were rinsed with phosphate buffered saline for 5 min, three times. The sections were incubated with peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc, USA) at 1:1000 dilution, overnight at 4°C for 20 min. Sections were visualized with a DAB-based colorimetric method. The percentage of positively stained cells in the total cell population was regarded as immunohistochemical index.

Statistical analysis

Results were expressed as mean ± SD. Statistical significance of the differences between means was assessed with ANOVA. Statistical analysis was performed with the Kruskal-Wallis test, for the post-hoc comparison (using SPSS 11.0). Statistical significance was set as P < 0.05.

RESULTS

Establishment of sustained AF canine models, measurement of maximal diameters of left atria, protocol of TUNEL assay and assessment of calpain I activity
Table 2. Heart weight/ body weight ratio in different groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of dogs</th>
<th>Body weight at baseline (kg)</th>
<th>Body weight before death (kg)</th>
<th>Heart weight (g)</th>
<th>Heart weight/body weight (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation group</td>
<td>7</td>
<td>14.72 ± 5.16</td>
<td>14.66 ± 4.89</td>
<td>130.50 ± 29.41</td>
<td>9.14 ± 1.05</td>
</tr>
<tr>
<td>AF control group</td>
<td>7</td>
<td>15.46 ± 4.54</td>
<td>11.18 ± 3.92</td>
<td>132.6 ± 13.47</td>
<td>12.60 ± 2.92*</td>
</tr>
<tr>
<td>Calpain inhibitor group</td>
<td>7</td>
<td>13.96 ± 3.81</td>
<td>12.32 ± 3.29</td>
<td>119.4 ± 20.32</td>
<td>9.90 ± 1.32*#</td>
</tr>
</tbody>
</table>

*, p < 0.05, compared with sham operation group; #, p > 0.05, compared with sham operation group; Δ, p > 0.05, compared with AF control group.

were reported in our previous work (Li et al., 2009) (baseline information of the dogs were shown in Table 2).

**Expression of calpain I, calpain II and calpastatin localized by immunohistochemistry**

The calpain proteolytic system was composed of the calpain proteases, the small regulatory subunit CAPNS1, and the endogenous calpain-specific inhibitor, calpastatin. Two main isoforms of the calpain proteases, dubbed μ-calpain and m-calpain (also known as calpain I and II), that differed primarily in their calcium requirements in vitro. While indeed which one should be responsible for the biological effect of apoptosis in AF cardiomyocytes is unclear. Semi-quantitative immunohistochemical analysis of calpain I demonstrated that calpain I mainly locates in the nuclei, intercalated disk and plasma, and the highest density of calpain I was shown in the control AF group (p < 0.01, vs. control AF group and sham operation group) (Figure 1). Calpain II mainly locates in the intercalated disk and plasma (Figure 2). The expressions of calpain II had no significant difference among the three groups. Calpastatin locates in the plasma, no expression difference among the three groups neither (Figures 3 and 4).

**Expression of calpain I mRNA was detected by real-time PCR**

The threshold cycle value, Ct for short, is the cycle number at which the fluorescence generated crosses the fluorescence threshold within a reaction, a fluorescent signal significantly above the background fluorescence. At the threshold cycle, a certain amount of amplification product has been generated. The threshold cycle is inversely proportional to the original relative expression level of the gene of interest. The comparison of Ct among the three groups demonstrate that gene transcript was the most active in the inhibitor group (p < 0.01, vs. control AF group and sham operation group, Figure 5, Table 3).

**Expression of NF-κB mRNA was detected by real-time PCR**

NF-κB/Rel is a family of transcription factors which plays...
Figure 2. Calpain II mainly located in the intercalated disk and plasma. No significant difference of calpain II protein expression among the three groups.

Figure 3. Calpastatin located in the plasma; no expression difference among the three groups neither.
Figure 4. Semi quantitative immunohistochemical analysis demonstrated that in control AF group, the protein expressions of calpain were significantly elevated (*, p < 0.01, vs. sham operation group; #, p < 0.01, vs. control AF group). Protein expressions of calpain II and calpastatin had no significant difference among the three groups (△, p > 0.05, vs. sham operation group; ▲, p > 0.05, vs. control AF group).

Figure 5. The calpain I amplification in each group. The red straight line indicates threshold. The dark blue curve represents amplification of calpain I gene in calpain inhibitor group; the light blue curve, green curve stands for AF control group and sham operation group respectively. Intersections with the red line are read as threshold cycle value (Ct). The cycle of threshold were (28.6 ± 6.3) in the sham operation group, (27.1 ± 5.5) in the control AF group and (22.7 ± 4.6) in the inhibitor group (p < 0.01 vs. control AF group and sham operation group).
Table 3. mRNA expressions of calpain 1 and NF-κB were detected by real-time PCR.

<table>
<thead>
<tr>
<th>Group</th>
<th>Calpain1 (Ct)</th>
<th>NF-κB (Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation group</td>
<td>28.6 ± 6.3</td>
<td>33.4 ± 6.1</td>
</tr>
<tr>
<td>Control AF group</td>
<td>27.1 ± 5.5</td>
<td>30.4 ± 5.8</td>
</tr>
<tr>
<td>Calpain inhibitor group</td>
<td>22.7 ± 4.6*#</td>
<td>20.1 ± 5.1*#</td>
</tr>
</tbody>
</table>

mRNA expressions of calpain I and NF-κB were detected by real-time PCR in each group, and expressed as the threshold cycle value (Ct). The threshold cycle value is inversely proportional to the gene expression level. In the sham operation group and control AF group, Ct had no difference (p > 0.05). A significant amplification was recorded in the calpain inhibitor group (p < 0.01, vs. control AF group and sham operation group). Gene expression of NF-κB followed the same trend, (*, p < 0.01, vs. sham operation group, *#p < 0.01, vs. control AF group).

Expression of NF-κB was localized by immunohistochemistry

Classic NF-κB is composed of a p50 and p65 subunit. We investigated the expression and localization of NF-κB in atrial myocytes. In the sham operation group and control...
AF group, cardiomyocyte nuclei were negative. Only in the calpain inhibitor group, NF-κB positive cells were observed (Figure 7).

**DISCUSSION**

Our research demonstrated alteration of NF-κB expression in AF. NF-κB is an antiapoptosis factor, which is activated by proteolysis of IκB. Calpain was involved in proteolysis of IκB. These studies provided a mechanistic link between the calpain inhibitor and the NF-κB in AF.

In clinical, for most patients suffering from AF, they always had enlarged atria. The relationship between the diameter of atria and AF was investigated by a lot of researchers. Zaca et al. (2007) documented that extraordinary length of atria diameter would be due to onset and persistence of atrial fibrillation. Our previous findings unveiled that rapid atrial pacing lead to increase of atria diameter, and apoptosis index was positively correlated to atria diameter. Apoptosis is much potent on the structure remodeling in atrial fibrillation (Li et al., 2009).

Calpain is involved in apoptosis via complicated mechanisms like caspase family, bax/bcl-2, ARC etc. (Li et al., 2009). We dedicated to reveal the relationship between calpain and NF-κB in AF canine models. NF-κB/Rel is a family of transcription factors, which migrate into nucleus and bind to decameric NF-κB DNA binding sites in activate form, regulates oxidative stress responses, proliferation, and apoptosis. Upon stimulation, such as cytokines, growth factors, chemical stress, UV or ionizing radiation, bacterial lipopolysaccharide or tetradecanoyl phorbol acetate, induce release of activate NF-κB/Rel dimmers. NF-κB/Rel family is antiapoptotic, down-regulation of NF-κB/Rel activity induced apoptosis of murine B lymphocytes and ectopic expression of c-Rel led to survival of aforementioned cells (Bellas et al., 1997). Mice lacking the rel A gene, which encodes the p65 subunit of NF-κB, exhibit embryonic lethality at 15th to 16th day of gestation, accompanied by severe liver apoptosis (Beg et al., 1995). These documents proved that NF-κB is essential for cell survival. In our research, NF-κB expression was hardly detected in normal heart muscle cells, or in the AF group cells. Gene transcription of NF-κB in these two groups had no significant difference [Ct was (33.4 ± 6.1) and (30.4 ± 5.8) in sham operation group and control group respectively, p > 0.05]. These results incline that normally NF-κB up-regulation was not independent in the atrial fibrillation, while artificial interference with calpain I inhibitor leads to gene and protein expression. Protein expression of NF-κB was detected only in the calpain inhibitor group. Ct value in the calpain inhibitor group was remarkably smaller than that in sham operation group and control group (both p < 0.01). Elevated expression of NF-κB was believed to protect cells from apoptosis.
The activation of NF-κB/Rel is phosphorylation-dependent, which is controlled by associated inhibitor protein, IκB. The IκBα protein inactivates the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins, keeping them sequestered in the cytoplasm, in an inactive state. IκB degeneration is an essential event in signal pathways leading to NF-κB/Rel activation. In HepG2 liver cells, IκB proteolysis is mediated by calpain (Han et al., 1999) and rapid IκB proteolysis can be inhibited by some calpain inhibitors in WEHI231 immature B cells (Miyamoto et al., 1998). In former documents, calpain contributes to IκBα degradation and NF-κB activation, calpain inhibitor inhibited rapid IκBα proteolysis. Conditions that prevented degradation of IκBα inhibit nuclear NF-κB activity. In this theory, calpain inhibitor would reduce NF-κB expression, object to our results. We have possible explanations for this phenomenon: one, ALLM had different biological effect on IκBα degradation, or the mechanism differs in different cells, like in WEHI231 immature B cells calpain inhibitors reduced NF-κB expression while in atria fibrillation canine heart muscle cells, a controversial effect take place; third, although as a calpain inhibitor, ALLM, promoted concentration of IκBα, this reduction of NF-κB is limited compared with elevated amplifications of NF-κB.

**Conclusion**

Calpain inhibitor ALLM promotes the transcription of NF-κB, leads to gene and protein over-expressions of NF-κB p65 in atrial fibrillation canine atria.

**Limitation of the study**

As apoptosis of atrial myocyte is more prominent in complex of AF and heart failure (Goette and Lendeckel, 2006), to prevent dogs from heart failure, the AF canine models should be designed to control ventricular rate. We did not create an atrioventricular block by radiofrequency-catheter ablation in this model, because the rapid atrial pacing model with AV-block is a pure model, however, our model mimics actual clinical AF (Sato et al., 2006; Niwano et al., 2007), and thus the results are considered to reflect the clinical phenomenon.

**ACKNOWLEDGEMENTS**

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