Activation of the Calcineurin-Nuclear Factor of Activated T-Cell Signal Transduction Pathway in Atrial Fibrillation*

Chih-Chung Lin; Jiunn-Lee Lin, MD; Chich-Sheng Lin, PhD; Mei-Chuan Tsai; Ming-Jai Su, PhD; Ling-Ping Lai, MD, PhD; and Shoei K. Stephen Huang, MD

Study objectives: **The calcineurin-nuclear factor of activated T-cell (NFAT) signal transduction pathway regulates the expression of a plethora of genes in the myocardium. Cytosolic calcium overloading occurs in atrial fibrillation (AF), and this fulfills the condition needed for activation of this pathway. We therefore investigated the NFAT pathway in atrial tissue in a porcine model of AF.**

Methods and results: **AF was induced in eight adult pigs by rapid atrial pacing. Investigations on the calcineurin and NFAT pathway were performed on transmural left atrial tissue obtained 6 weeks after implantation of the pacemaker (pacing for 4 weeks, and AF without pacing for 2 weeks). In the AF group, the left atrial dimension increased significantly (26 ± 4 mm vs 31 ± 4 mm, respectively, $p < 0.05$ [mean ± SD]). Calcineurin enzyme activity increased significantly in pigs with AF ($n = 8$) when compared to control pigs ($n = 6$) [0.143 ± 0.034 vs 0.038 ± 0.063 mmol PO_4^- released, $p < 0.01$]. We found that both NFAT-c3 and NFAT-c4, the downstream effectors of calcineurin, increased significantly in the nuclei in AF tissue using immunoblotting. Translocation of NFAT-c3 and NFAT-c4 into the nuclei was also demonstrated in AF tissue microsections using immunohistochemistry. The electrophoresis mobility shift assay further demonstrated that nuclear extracts from AF tissue had a significantly larger binding capacity for NFAT-specific oligonucleotide probes.**

Conclusions: **Our results demonstrate that calcineurin activity was increased in AF with subsequent NFAT-c3 and NFAT-c4 translocation into the nucleus. Activation of this signal transduction pathway may play an important role in the pathogenesis of AF.**

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Key words: atrial fibrillation; calcineurin; calcium; nuclear factor of activated T cell

Abbreviations: AF = atrial fibrillation; EDTA = ethylenediaminetetraacetic acid; EMSA = electrophoresis mobility shift assay; NFAT = nuclear factor of activated T cell; PMSF = phenylmethylsulfonyl fluoride

Atrial fibrillation (AF) is the most common arrhythmia in humans. It causes palpitations, decreased cardiac output, heart failure, and systemic thromboembolism, and is a major issue in public health.[1,2] Current treatment modalities for AF are far from satisfactory. Despite aggressive treatment, the recurrence rate of AF is still high, and permanent AF refractory to any treatment including electrical cardioversion develops in many patients. These unsatisfactory outcomes are attributed, at least in part, to the lack of understanding about the pathophysiology of AF.

There is evidence showing that AF begets AF, and through this vicious cycle AF becomes incessant.[3,4] It has been reported that AF causes structural and functional changes in the atrial tissue, which, in turn, result in further AF. These changes include a short-

*From the Institute of Pharmacology (Mr. Lin, Ms. Tsai, and Drs. Su and Lai), National Taiwan University, Taipei; Department of Internal Medicine (Drs. J-L. Lin and Huang), National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei; and Department of Biological Science and Technology (Dr. C-S. Lin), National Chiao Tung University, Hsinchu, Taiwan.

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Correspondence to: Ling-Ping Lai, MD, PhD, No. 1, Jen-Ai Rd, Section 1, Institute of Pharmacology, School of Medicine, National Taiwan University, Taipei, Taiwan, 100; e-mail: lai@ha.mc.ntu.edu.tw

ening of atrial effective refractory period, a decrease of L-type calcium current, and changes in receptors, contractile proteins, and interstitial matrix.[5–7] However, the link between atrial rapid depolarization and these changes remains unknown.

Ca^{2+} is a major cation involved in many important physiologic responses in the heart, including excitation-contraction coupling, secretion, cell-signaling pathways, and transcription regulation.[8,9] Calcineurin-nuclear factor of activated T-cell (NFAT) pathway is a well-established calcium-dependent pathway in T cells. There is growing evidence showing the importance of this pathway in cardiac diseases such as ventricular hypertrophy.[10–12] It has also been reported that the NFAT plays an essential role in the regulation of many cardiac genes. Sustained elevation of cytosolic calcium occurs at early stage of AF, and it is the trigger for calcineurin-NFAT pathway activation.[13,14] Therefore, we tested the hypothesis that the calcineurin-NFAT pathway was activated in atrial tissue in AF.

Materials and Methods

Porcine Model of AF

The porcine model of AF has been reported in detail previously.[15] The investigation conforms to the National Institutes of Health guidelines for the care and use of laboratory animals. In brief, adult pigs of Yorkshire-Landrace strain weighing 50 to 80 kg were used. After IV anesthesia with ketamine, we implanted a high-speed pacemaker (Itrel III; Medtronic; Minneapolis, MN) to pace the atrium at a rate of 10 Hz (600 per minute). AFter pacing for 4 weeks, the pacemakers were turned off and the pigs were in persistent AF. The pigs were killed 2 weeks after turning off the pacemaker, and the total duration of rapid atrial depolarization was 6 weeks (rapid pacing for 4 weeks and AF without pacing for 2 weeks). In the sham (control) group, a pacemaker was implanted but remained off. The control pigs were killed 6 weeks after the implantation. The whole heart was removed from the chest cavity. Transmural left atrial free-wall tissue blocks were obtained and stored in liquid nitrogen for further use. There were eight pigs in the AF group and six pigs in the control group. Transthoracic echocardiography was performed at baseline and 6 weeks after implantation of the pacemaker. Left atrial dimension, left ventricular dimension (end-systolic and end-diastolic), and left ventricular ejection fraction were measured in two-dimension assisted M-mode in long-axis view.

Calcineurin Activity Assay

Calcineurin phosphatase activity was measured using a synthetic phosphopeptide substrate (R-II peptide) as described previously (AK-804 kit; BIOMOL; Plymouth Meeting, PA).[16] Tissue samples were homogenized in phosphatase lysis buffer containing 50 mmol/L Tris (pH 7.5), 0.1 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 0.1 mmol/L ethyleneglycol tetra-acetic acid, 1 μmol/L pepstatin A, and protease inhibitor cocktail tablets (Complete; Roche; Mannheim, Germany). Calcineurin enzymatic activity was measured in phosphatase buffer containing 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 6 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1 mmol/L dithiothreitol, 0.05% ethylphenyl-polyethylene glycol (NP-40). Phosphatase activity was determined as the dephosphorylation rate of the R-II peptide. The detection of free phosphate released from R-II peptide was based on the classic Malachite green assay.[17]

Preparation of Cytosolic Protein Extracts

The samples were homogenized in homogenization buffer containing 25 mmol/L Tris (pH 7.5), 0.5 mmol/L EDTA, 0.5 mmol/L ethyleneglycol tetra-acetic acid, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L dithiothreitol, 25 μg/mL leupeptin, 25 mmol/L NaF, and 1 mmol/L Na_3VO_4. The homogenates were centrifuged at 14,000*g* for 15 min, and the resulting supernatants were collected as cytosolic proteins for immunoblotting analysis. Protein concentrations were determined (BCA Protein Assay Reagent Kit; Pierce; Rockford, IL).

Preparation of Nuclear Protein Extracts

The samples were homogenized in buffer A (10 mmol/L hydroxyethyl piperazine-ethanesulfonic acid [pH 7.9], 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L dithiothreitol, 25 μg/mL leupeptin, and 1 mmol/L PMSF). AFter a 10-min incubation on ice, the samples were centrifuged at 1,850*g* for 10 min at 4°C. The pellets were dissolved in buffer B (buffer A + 0.1% Triton X-100), incubated on ice for 10 min, and centrifuged as above. The crude nuclear pellets were washed once with buffer A and resuspended in buffer C (20 mmol/L hydroxyethyl piperazine-ethanesulfonic acid [pH 7.9], 25% glycerol (volume/volume), 0.42 M NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 1 mmol/L PMSF) for 30 min at 4°C. Nuclear proteins were recovered after centrifugation at 25,000*g* for 30 min. The resulting supernatants were the nuclear extracts.

Polyacrylamine Gel Electrophoresis and Immunoblotting

Proteins were separated by 8% sodium dodecylsulfate-polyacrylamine gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore; Bedford, MA). The membranes were blocked for 1 h at room temperature using nonfat dry milk dissolved in Tris-buffer with 0.1% Tween-20. The primary antibodies used in the present study included rabbit polyclonal anti–NFAT-c4 antibody, mouse monoclonal anti–NFAT-c3, and anticalcineurin antibody specific for calcineurin A-α (all from Santa Cruz Biotechnology; Santa Cruz, CA). Membranes were incubated with primary antibody in blocking buffer for 12 h at 4°C. Peroxidase-conjugated secondary antibodies were used for detection of primary antibody. Membranes were incubated in blocking buffer containing secondary antibody for 1 h at room temperature. Signals were detected with an enhanced chemiluminescence kit (Amersham Biosciences; Buckinghamshire, UK) and analyzed using image-editing software (Adobe Photoshop 6.0; Adobe Systems Incorporated; San Jose, CA; and Image Gauge V3.12; Fujifilm; Tokyo, Japan).

Immunohistochemistry

For immunohistochemistry, paraffin-embedded left atrial tissue was used. Deparaffinized and rehydrated sections were blocked at room temperature for 20 min with 5% nonimmune goat serum in Tris-buffered saline solution (pH 7.5) after microwave treatment (boiled 5 min in citrate buffer, pH 6) and quenching of endogenous peroxidase with 3% hydrogen peroxide/methanol for 15 min. Anti–NFAT-c3 (1:20 dilution) or

anti–NFAT-c4 (1:50 dilution) were used as primary antibody and incubated at room temperature for 1 h. Staining was performed (VECTASTAIN ABC; Vector Laboratories; Burlingame, CA) as described by the manufacturer, and the color was developed with diaminobenzidine. Hematoxylin was used for counterstaining.

Nonisotopic Electrophoretic Mobility Shift Assays

For nonisotopic electrophoresis mobility shift assay (EMSA), 10 μg of nuclear extracts were incubated with 10 ng of biotin-labeled double-strand oligonucleotide probe in 10-μL binding buffer containing 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 0.5 mmol/L EDTA, 5% glycerol, and 1 μg poly-d(I-C) (Panomics; Redwood City, CA). The oligonucleotide was the consensus NFAT binding site from the interleukin-2 promoter, and the base sequence was 5′-ACGCCCAAAGAG-GAAAATTTGTTTCATACA-3′. Competitive binding assays were conducted under the same condition with the addition of 50-fold molar excess of unlabeled NFAT or nonspecific (scrambled) probes. Anti-NFAT antibody from Santa Cruz Biotechnology was used for supershift assay. Complexes were resolved on 6% polyacrylamide gel at 4°C in 0.5 × Tris-borate-EDTA buffer and then transferred to a nylon membrane (positive charged, Roche, Mannheim, Germany). Following UV cross-linking (UV-Stratalinker-1800; Stratagene, La Jolla, CA), the membrane was incubated with strepavidin-horse radish peroxidase in blocking buffer (Gel-Shift Kit; Panomics). The membrane was washed and subsequently developed using an enhanced chemiluminescence kit (Amersham) and a chemiluminescence imaging system (Syngene; Cambridge, UK).

Statistical Analysis

All data were expressed as mean ± SD. Parametric data were compared using Student *t* test. A p value < 0.05 was considered statistically significant.

Results

Porcine Model of AF

All eight pigs in the active pacing group showed AF at the end of the study, while all six pigs in the control group showed sinus rhythm. The two groups did not differ significantly regarding the left atrial dimension, left ventricular dimension, and left ventricular ejection fraction at the beginning. However, the left atrial dimension increased significantly in the AF group after 6 weeks of rapid atrial depolarization (Table 1), while there was no significant change of the left atrium size in the control group. The left ventricular dimension and left ventricular ejection fraction were not significantly altered in both groups.

Calcineurin Enzyme Activity and Protein Amount

We found that calcineurin (phosphatase 2B) enzyme activity was significantly higher in AF pigs than in control pigs (0.143 ± 0.034 vs 0.038 ± 0.063 nmol PO_4^- released, $p < 0.01$). The increase was greater than threefold (372 ± 87%) [Fig 1]. Calcineurin protein amount was also measured using antibody specific for calcineurin A-α for immunoblotting analysis. We found that the protein amount of calcineurin was not significantly different between AF and control pigs. These results indicated that calcineurin activity increased due to activation of the protein without significant changes in the protein amount.

Table 1—*Echocardiographic Measurements in AF and Control Groups

Variables	AF Group		Control Group	
	Baseline	6 wk	Baseline	6 wk
LAD, mm	26 ± 4	31 ± 4†‡	26 ± 3	27 ± 3
LVESD, mm	29 ± 4	31 ± 5	29 ± 5	30 ± 6
LVEDD, mm	48 ± 4	52 ± 6	49 ± 5	51 ± 6
LVEF, %	69 ± 10	67 ± 10	68 ± 9	67 ± 10

*Data are presented as mean ± SD.
†$p < 0.05$ when compared to LAD at baseline in AF group.
‡$p < 0.05$ when compared to LAD at 6 weeks in control group.
LAD = left atrial dimension; LVEDD = left ventricular end-diastolic dimension; LVEF = left ventricular ejection fraction; LVESD = left ventricular end-systolic dimension.

Translocation of NFAT-c3 and NFAT-c4

To investigate the translocation of NFAT-c from the cytosolic compartment to the nuclear compartment, we performed immunoblotting for NFAT-c3 and NFAT-c4 using cytosolic and nuclear fractions from atrial tissues (Fig 2, 3). These fractions were first confirmed using immunoblotting for β-actin and nucleolin to serve as cytosolic and nuclear markers, respectively. We demonstrated that there was little β-actin in the nuclear extract, while there was little nucleolin in the cytosolic extract (Fig 2). In the cytosolic fraction, NFAT-c4 decreased significantly in AF tissue, while NFAT-c3 also decreased but did not reach statistical significance ($p = 0.06$) [Fig 3, *top*, *A*]. In contrast, both NFAT-c3 and NFAT-c4 increased significantly in the nuclear fraction in pigs with AF (Fig 3, *bottom*, *B*).

Immunohistochemistry

Immunohistochemical studies were performed to investigate the distribution of NFAT-c3 and NFAT-c4 in atrial tissue. In microscopy, the brown signals indicate NFAT-c3 or NFAT-c4, and the nuclei appear blue with hematoxylin counterstain. On translocation of NFAT into the nuclei, darker signals were observed when brown and blue signals overlap. There was more NFAT-c3 and NFAT-c4 translocation into the nuclei in AF pigs than in control pigs. We also measured the percentage of nuclei showing overlapping signals. The ratio was significantly

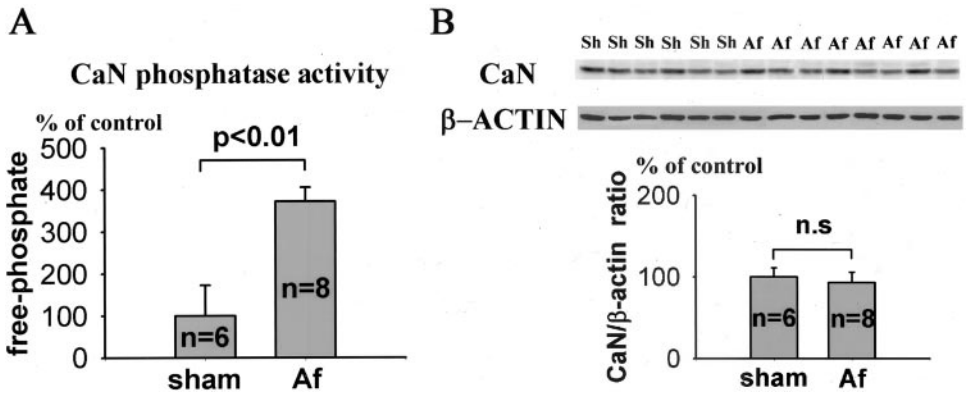

FIGURE 1. Enhancement of calcineurin enzyme activity but not protein amount in the atria of pigs with AF. The calcineurin activity was measured using a synthetic peptide R-II as the substrate. The calcineurin enzyme activity was significantly increased in pigs with AF than in control pigs. The elevation of calcineurin enzyme activity was greater than threefold (*left*, *A*). Immunoblotting of calcineurin revealed no significant change of calcineurin protein amount (*right*, *B*). CaN = calcineurin; n = No. of pigs; Sh = sham; n.s. = not significant. The error bars represent SEM.

higher in the AF group than in the control group (73.3 ± 27.8% vs 36.4 ± 21.9%, $p < 0.05$).

Nonisotopic EMSA for NFAT

To further investigate the NFAT-c activity in the nucleus, we performed EMSA using specific NFAT-c–binding oligonucleotides (Fig 4). We used an unlabeled NFAT probe and an unlabeled nonspecific probe for competition to confirm that the band showing the shift was NFAT specific. Furthermore, a supershift was observed when anti-NFAT antibody was added. In the nuclear extracts from AF pigs, the optical density of the band with the mobility shift was significantly larger than the control pigs. These results further indicated that the NFAT-c translocated to the nucleus had binding affinity with NFAT-c–responsive elements.

DISCUSSION

In the present study, we demonstrated the activation of calcineurin-NFAT signal transduction pathway in AF tissue after rapid atrial depolarization for 6 weeks. We showed that tissue calcineurin enzymatic activity was increased. We also showed that the downstream effectors of calcineurin, NFAT-c3 and NFAT-c4, were translocated into the nuclei. Binding activity to NFAT-c–specific probes was increased in nuclear extracts as demonstrated using EMSA.

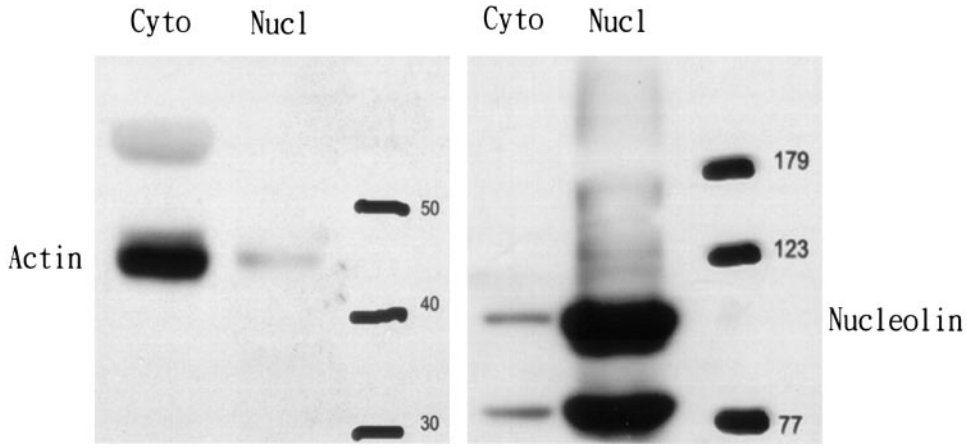

FIGURE 2. Confirmation of the purity of the cytosolic (Cyto) and nuclear (Nucl) protein extracts. The cytosolic and nuclear fractions were verified using immunoblotting for β-actin and nucleolin to serve as cytosolic and nuclear markers, respectively. There was little β-actin in the nuclear protein extract, while there was little nucleolin in the cytosolic protein extract.

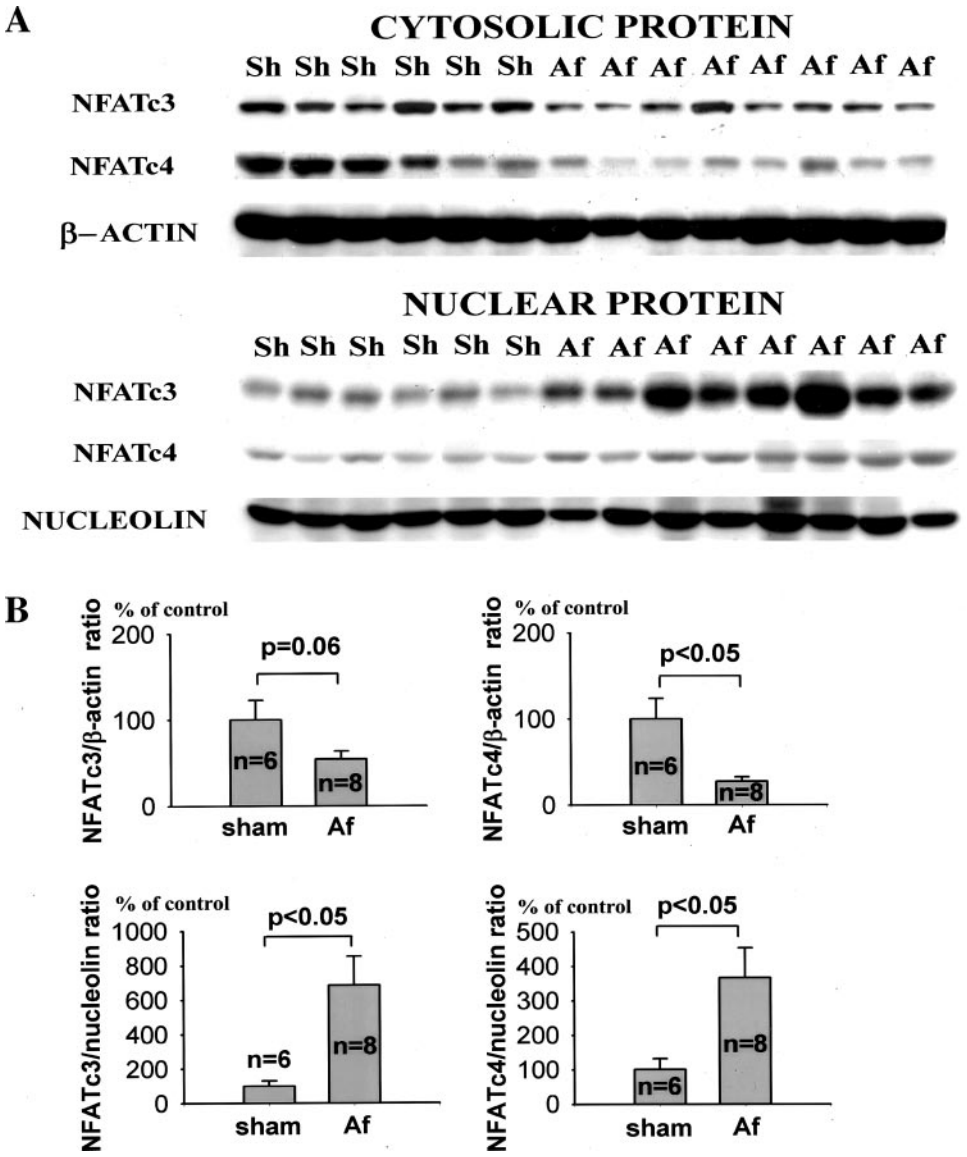

FIGURE 3. Translocation of NFAT-c3 and NFAT-c4 from cytosolic fraction to nuclear fraction in atrial tissue in pigs with AF. Immunoblotting studies on both cytosolic and nuclear fractions using anti–NFAT-c3 and anti–NFAT-c4 antibodies were performed respectively (*panel A*). *Panel B* shows the summary data. In the cytosolic fraction, NFAT-c4 decreased significantly in pigs with AF, while NFAT-c3 also showed a decrease, although not statistically significant ($p = 0.06$). In the nuclear fraction, both NFAT-c3 and NFAT-c4 increased significantly in pigs with AF. Error bars represent SEM. See Figure 1 legend for expansion of abbreviations.

NFAT-c and the Heart

NFAT-c has been extensively studied in the immune system. It was named NFAT because of its essential roles in T-cell activation.[18,19] The association between NFAT-c and the heart was found by a yeast two-hybrid study showing binding affinity between NFAT-c and the heart-specific GATA4 transcription factor.[10] It is therefore hypothesized that NFAT-c plays important roles in the regulation of gene expression in cardiac tissue. Later studies[10–12] on NFAT-c in the heart focused on ventricular hypertrophy. Both a transgenic animal study[20] and a pharmacologic study[12] using cyclosporine A and FK506 indicate that calcineurin-NFAT pathway is involved in cardiac hypertrophy.

Past studies on the calcineurin-NFAT pathway in atrial tissue are few. To the best of our knowledge, we showed for the first time that the calcineurin-NFAT pathway was activated in AF. In AF, the cytosolic calcium level undergoes characteristic changes. During rapid atrial depolarization, the diastolic period shortens, which results in a decrease of

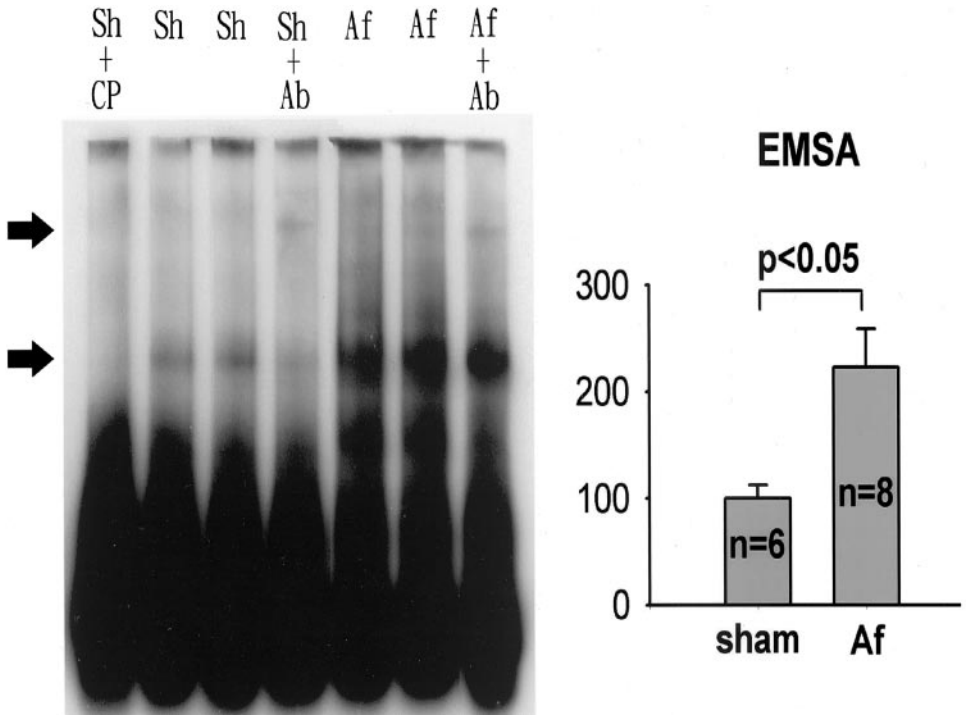

FIGURE 4. Increased NFAT-specific probe bind capacity in nuclear extracts from pigs with AF. Biotin-labeled NFAT-c–specific probes used EMSA for nuclear extracts from atrial tissue. The lower arrow indicates the mobility shift due to binding of the probe with NFAT-c, while the upper arrow indicated a supershift after adding anti–NFAT-c antibody. Ab = antibody against NFAT-c; CP = cold probe in 50 × excess. Error bars represent SEM.

calcium re-uptake into the sarcoplasmic reticulum. It has been demonstrated that the diastolic cytosolic calcium level increases and the calcium transient decreases in AF.[13,14] This exactly fulfills the condition needed for activation of calcineurin-NFAT pathway, which depends on a sustained calcium elevation instead of a transient increase of cytosolic calcium.

The gene expression regulatory effects of NFAT-c are promiscuous. It has been shown that NFAT-c regulated the expression of myosin heavy chain,[22] inflammatory cytokines such as interleukins, tumor necrosis factors, and inducible cyclooxygenase 2[23–25]; ion channels such as calcium-activated potassium channel[26]; and apoptosis-related genes such as Fas legend and tumor necrosis factor-related apoptosis-inducing ligand genes.[27,28] Brain-type natriuretic peptide, endothelin-1, and myocyte-enriched calcineurin interacting protein 1 are also under the regulation of NFAT-c.[29,30] It has also been reported that overexpression of calcineurin resulted in a decrease of Ito potassium channel.[31] Another group of researchers[32] reported that expression of Kv4.2 potassium channel was regulated by GATA4 transcription factor, which is also called NFAT-n, and binds with NFAT-c. The remodeling processes of atrial tissue in AF are manifold and include structural remodeling, electrical remodeling, and contractile remodeling. The activation of calcineurin-NFAT activation may contribute to these changes by altering the expression of a plethora of genes.

NFAT-c Subtypes

There are five subtypes of NFAT-c identified in mammalian tissues.[18] In adult cardiac tissue, NFAT-c3 and NFAT-c4 are the most important ones.[18,29] There have been reports showing that these two subtypes are redundant. The DNA-binding sequence was 100% homologous between NFAT-c3 and NFAT-c4.[33] They might regulate the transcription of the same genes, and both can compensate for the loss of each other. At the ventricular level, it has been reported that NFAT-c3 is more important than NFAT-c4 in causing cardiac hypertrophy.[34] In the present study, we showed an increase of both NFAT-c3 and NFAT-c4 in the nuclei.

Limitations

Although we showed that calcineurin-NFAT pathway is activated in AF and a lot of genes are under the regulation of NFAT-c, a direct link between atrial tissue remodeling and calcineurin-NFAT path-

way activation is lacking. Pharmacologic blockade of the calcineurin-NFAT pathway using cyclosporine A or FK506 was not performed in the present study.

The study was performed after rapid atrial depolarization for 6 weeks. The changes therefore can only represent the change at 6 weeks. A time course study was not performed. We cannot answer how quick the pathway was activated.

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