行政院國家科學委員會專題研究計畫 成果報告

心房顫動引發心房組織 Annexin VI 之定量及其心房心肌細胞內分佈變化-心房電外收縮重構之可能機轉

研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 94-2314-B-040-038-
執 行 期 間 ：94 年 08 月 01 日至 95 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學系

計 畫 主 持 人：翁國昌
共 同 主 持 人：葉宏一
計畫參與人員：專科畢-專任助理：李淑珍
臨時工：陳威良

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 95 年 12 月 07 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
□期中進度報告

心房顫動引發心房組織 Annexin VI 之定量及其心房心肌細胞內分佈變化-心房電外收縮重構之可能機轉

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC－94－2314－B－040－038
執行期間： 94 年 8 月 1 日至 95 年 7 月 31 日

計畫主持人：翁國昌
共同主持人：葉宏一
計畫參與人員： 李淑珍、陳威良

成果報告類型(依經費核定清單規定繳交)：■精簡報告 □完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
■涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：中山醫學大學

中 華 民 國 95 年 7 月 31 日

報告內容

Introduction

Atrial fibrillation (AF) is associated with shortened action potential duration[1,2] and progressive atrial mechanical remodelling,[3, 4] which is implicated in the development of thromboembolic stroke. The mechanisms of atrial contractile dysfunction in AF remained unclear. Atrial dysfunction is attenuated by exposure to the Ca^{2+} antagonist verapamil and enhanced by calcium loading or the Ca^{2+} agonist Bay K8644,[5] suggesting that abnormalities in the Ca^{2+} handling play a central role in this process. The cytosolic Ca^{2+} homeostasis in cardiomyocytes involves the influx of extracellular Ca^{2+} through L-type Ca^{2+} channels, the release/uptake of Ca^{2+} by the sarcoplasmic reticulum, and efflux of intracellular Ca^{2+} though the cardiac Na^{+}/Ca^{2+} exchanger (NCX).[6] Previous studies had demonstrated that these calcium-regulating molecules are changed during chronic AF.[7-12] However, the question still remains regarding the mechanisms of failure in the Ca^{2+} cycling pathways, in particular, the upstream regulation of calcium handling proteins in AF.

Annexins are a superfamily of Ca^{2+}-dependent phospholipid binding proteins containing repeated domains of approximately 70 amino acids in length.[13] Annexin VI (Ax6) is a unique member of this family in that it contains eight conserved repeat domains instead of four. Ax6 is strongly expressed in the mammalian heart,[14] and has been shown in vitro to be a regulator of the L-type Ca^{2+} channel conductance,[15,16] the cardiac NCX,[17] and the cycling of Ca^{2+} release/uptake by the sarcoplasmic reticulum.[18-20] Overexpression of Ax6 has underlined physiological

alterations in contractile mechanics leading to dilated cardiomyopathy,[21] whereas knockout has been found to induce faster changes in Ca^{2+} transient and increased contractility,[22] suggesting a negative role of this protein on cardiomyocyte mechanics. However, whether atrial Ax6 is altered during AF remains unclear. To answer this question, we compared the expression and distribution of atrial Ax6 between patients with AF or sinus rhythm (SR).

Methods

Patients

All of the patients required coronary artery bypass grafting (CABG) for severe, multistenotic coronary artery disease. The clinical history was obtained before surgery. The presence and duration of AF were assessed according to previous serial electrocardiograms. Hemodynamic and echocardiographic data were obtained by reviewing data from preoperative cardiac catheterizations and echocardiograms. All patients were given informed consent. The study protocol was approved by the Institutional Review Board of the Chung Shan Medical University Hospital. The investigation conforms with the principles outlined in the Declaration of Helsinki. The left atrial (LA) and right atrial (RA) appendage tissues were obtained from 20 patients with chronic AF (defined as persistent AF$\geqq$3 months) at the time of bypass cannulation during CABG and/or aortic valve surgery. The AF patients were matched for age, sex, left ventricular function, and as far as possible for medication with 34 clinically stable patients in SR (without a history of AF) undergoing CABG. All 54 patients were euthyroid and in NYHA class I-III. In order to prevent potential confounding factors from mitral valve disease, we excluded the patients with significant mitral stenosis or regurgitation, as determined by preoperative echocardiography.

Ribonucleic Acid (RNA) Extraction and Reverse Transcription (RT)

Samples of atrial tissue were rapidly frozen in liquid nitrogen and stored until further analysis. Total RNA was prepared by applying the method of Chomczynski and Sacchi.[26] One μg of total RNA was transcribed into cDNA (complementary deoxyribonucleic acid) using Moloney murine leukemia virus reverse transcriptase (Promega).

Semiquantitative Polymerase Chain Reaction (PCR)

A 50-μL reaction mixture consisted of 5× reaction buffer; 10 mM dNTPs; 40 U RNase inhibitor; and 0.5 μM of the specific primers for Ax6 (5'-CAAATATAGGTGGCACTGAGGAG-3' and 5'-TAGCAGCAGTTCTCAAAGTGTG-3'). The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes was followed by 30 cycles with denaturation at 94°C for 60 seconds, annealing at 52°C for 60 seconds, and a final extension of 2 minutes at 72°C. The mRNA amount of the annexin genes was measured and normalized to the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Western blotting

Western blot analysis was performed as described previously.[23] In short, 25 μg of atrial homogenates were fractionated on SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond ECL, Amersham). The membrane was incubated with mouse monoclonal anti-Ax6 antibody (BD Transduction Laboratories; 1:500) and subsequently with goat anti-rabbit immunoglobulin (Promega; 1:1000) conjugated to a horseradish peroxidase (BD Biosciences;

1:2000) for 1 hour at 4°C. Signals were detected with the ECL detection system (Amersham Corp) on a standard x-ray system. The ß-actin data were used as an internal standard to normalize Ax6 data.

Immunoconfocal Microscopy

For immunofluorescence detection of the Ax6 proteins, in addition to the mouse monoclonal antibody mentioned above, goat polyclonal antibodies against human Ax6 (Santa Cruz) were used. For identification of other calcium handling proteins, a mouse monoclonal antibody against NCX (Affinity BioReagents), affinity purified rabbit polyclonal antibodies against L-type Ca^{2+} channel (Chemicon), and a mouse monoclonal antibody against ryanodine receptors (Affinity BioReagents) were used. For secondary antibodies, donkey anti-rabbit, anti-mouse, and anti-goat immunoglobulin conjugated to either CY3 or CY5 (Chemicon) were purchased. For single labeling, CY3-conjugated antibodies were used. For double labeling using two different primary antibodies, one CY3-conjugated antibody and one CY5-conjugated were used in combination. Background autofluorescence was detected in fluorescein isothiocynate (FITC) channel. For double labeling of Ax6 and downstream calcium-related handling proteins, cryosections of the samples from the last 16 patients (8 in AF, and 8 in SR) were fixed in -25 °C methanol (5 minutes), blocked in 0.5% BSA (15 minutes) and incubated with a mixture of goat anti-Ax6 (1:1000) plus anti-NCX (1:500), anti-L-type Ca^{2+} channel (1:250), or anti- ryanodine receptor (1:1000) at 37°C for 2 hours. The samples were then treated with a mixture of CY3-conjugated

(1:500) and CY5-conjugated secondary antibody (1:500) at room temperature for 1 hour. Finally the sections were mounted. Between each step, the slides were thoroughly washed with PBS.

Immunostained samples were examined by confocal laser scanning microscopy using a Leica TCS SP equipped with an argon/krypton laser with the appropriate filter spectra adjusted for the detection of FITC, CY3, and CY5 fluorescence. The images from sections of multiple labeling were taken using either simultaneous or sequential multiple channel scanning.

Statistical Analysis

Densitometric scanning and analysis were performed on immunoblots and agarose gels carrying RT-PCR products (stained with ethidium bromide) using Imagemaster (Amersham Pharmacia Biotech, New Jersey, USA). The data are expressed as mean value ± SD, unless otherwise stated. Differences between groups were compared using a Student's *t* test for continuous variables with normal distribution, and non-normal data were assessed by a Wilcoxon rank sum test. Categorical data were compared using a χ^2 test or a Fisher's exact test where appropriate. For determination of correlations between the mRNA and protein expression of Ax6 in each atrium the Spearman correlation test was used. Linear regression analysis was used for correlation between the expression levels of Ax6 protein and clinical parameters in all subjects. All tests were two-tailed, and a *P* value of <0.05 was considered to be statistically significant.

Results

Clinical characteristics

The clinical characteristics are shown in Table 1. There were 35 men and 19 women with a mean age of 67±12 years (range 40 to 85). Patients with AF did not differ from control subjects with SR with respect to clinical parameters that were evaluated except the diameter of left atrium, which was larger in patients with AF.

Ax6 gene expression in chronic AF

The difference in transcription of Ax6 between the two groups was determined by comparing the Ax6/GAPDH mRNA ratio. As shown in Figure 1, the expression level of Ax6 mRNA was significantly decreased in both RA and LA of AF patients compared to the corresponding atrium of SR patients (AF vs SR; RA: 0.21 ± 0.11 vs 0.61 ± 0.32; LA: 0.20 ± 0.10 vs 0.62 ± 0.32; both $p < 0.01$). In addition, in either SR or AF group, the gene expression levels were equivalent between the right and left atrium.

Ax6 protein expression in chronic AF

The difference in Ax6 protein expression between the two groups was determined by comparing

the Ax6/ß-actin ratio in Western blotting. As shown in Figure 2, the expression levels of Ax6 protein in chronically fibrillating atria were nearly half compared to SR (AF vs SR; RA: 0.18 ± 0.10 vs 0.34 ± 0.16; LA: 0.17 ± 0.09 vs 0.37 ± 0.17; both $p < 0.01$). In addition, similar to the transcript, the protein expression levels between RA and LA in either AF or SR group were comparable.

Immunohistochemistry

Single-labeling experiments showed that Ax6 protein is more or less evenly distributed in each sample. Consistent with the Western blotting results, a marked difference existed between the SR and AF groups. In general, the labels of Ax6 were abundant in the SR group, regardless of the RA or LA (Figure 3, A and B), though variations existed between samples (Figures 4B, 5B, and 6B). In contrast, in the chronically fibrillating atria the labels of Ax6 were less compared to the SR group (Figure 3, C and D). However, similar to the SR group, variations in expression also existed between samples of the AF group (Figures 4F, 5F, and 6F); in some sections of the fibrillating atria it was even difficult to identify the staining of Ax6 (Figure 3C).

Since the distribution of Ax6 appeared to surround the cell borders (Figure 3), we further studied the spatial relationship of Ax6 with NCX and L-type Ca^{2+} channel, both of which were known to exist in the sarcolemma, and ryanodine receptor, in the sarcoplasmic reticulum. For this purpose, randomly selected samples from the SR group and samples expressing Ax6 protein detectable in

the single labeling experiment from the AF group were used. In Figure 4, double immunolocalization of Ax6 (shown in red) with NCX (in green) and in Figure 5 of Ax6 (in red) with L-type Ca^{2+} channel (in green) demonstrated extensive overlapping (in yellow) of the Ax6 labels with those of the L-type Ca^{2+} channel and the NCX, respectively. In contrast, the Ax6 labels do not overlap with those of ryanodine receptor-marked sarcoplasmic reticulum in the cytoplasmic compartment (Figure 6).

Comparison of laboratory data and clinical parameters

A positive correlation was found between the mRNA ratio and the protein ratio of Ax6 in each atrium for all SR and AF patients (Figure 7, A and B). On the other hand, in the multivariate analysis, the protein level of Ax6 is not influenced by age, gender, history of diabetes, hypertension, severity of coronary artery disease, previous myocardial infarction, left ventricular ejection fraction, atrial filling pressure, and use of preoperative medication(s).

Discussion

The present study describes, for the first time, a parallel reduction in the expression of Ax6 at both the mRNA and protein levels in chronically fibrillating atria. In addition, we found that in both the SR and AF patients Ax6 was mainly distributed in close relationship to the sarcolemma and co-localized with the cardiac NCX and L-type Ca^{2+} channels in atrial myocytes.

Down-regulation of Ax6 and calcium homeostasis in chronic AF

As mentioned before, previous works in human AF mainly attributed abnormalities in cardiomyocyte Ca^{2+} handling to the changes in expression of molecules regulating Ca^{2+} at both the sarcolemma and the sarcoplasmic reticulum.[9-12] The fact that Ax6 is the major cardiac annexins (up to 0.2% of total cardiac protein) suggests that this protein have important roles in heart function.[14] The finding of the present study suggests that Ax6, a major membrane-associated matrix protein of the heart, may also be involved in the Ca^{2+} handling abnormalities in AF. Although the functional roles of Ax6 in atrial myocytes have not yet been defined, the significant decrease in Ax6 expression described herein could have effects on the ability of fibrillating atria to regulate intracellular Ca^{2+}. This proposal is based on the reports that i) Ax6 increases conductance through modulation of the L-type Ca^{2+} channel;[15,16] ii) Ax6 rescues the function of cardiomyocyte NCX, which is inhibited after EDTA or EGTA treatment;[17,19] and iii) Ax6 was involved in the cycle of Ca^{2+} release/uptake of sarcoplasmic reticulum.[18-20] In addition, the

dynamic role of Ax6 in the modulation of Ca^{2+} homeostasis as well as contractility in cardiomyocytes has recently been elucidated. Recent work employing Ax6-knockout or transgenic models has provided new insights into the biological functions of Ax6 proteins. Studies with transgenic mice showed that Ax6 overexpression targeted to cardiomyocytes led to dilated cardiomyopathy and heart failure.[21] Cardiomyocytes isolated from these mice were found to have a reduced frequency-dependent percentage of shortening, decreased rates of contraction and relaxation, lower basal levels of intracellular free Ca^{2+} and a reduced rise in the peak free Ca^{2+} transient with concomitant changes in contractile properties. In contrast, the cardiomyocytes derived from Ax6 knockout mice demonstrate an increase in myocyte contractility and more rapid Ca^{2+} clearance from the cytoplasm during diastole.[22] These two studies suggested a negative inotropic role for Ax6 by acting as a regulator of cardiomyocyte membrane pumps and/or exchangers.

On the other hand, in the ventricular tissue, the expression of Ax6 is largely increased at the onset of heart failure,[23] whereas in end-stage heart failure its expression was largely down-regulated or remained unaltered,[24,25] suggesting that expression of ventricular Ax6 varies according to the severity and/or duration of heart pathology. These findings imply that our results may not be applied to patients with paroxysmal or short-term AF. In end-stage heart failure, in which AF is commonly seen, the expression of Ax6 in right atrial myocardium was reported to be unchanged.[25] In contrast, the present study shows that chronic AF was associated with a significant decrease in Ax6 in both atrial appendages. This discrepancy in atrial expression can be explained by the data of Li D et al., which indicate that heart failure, unlike AF, is not necessarily

associated with decreased action potential duration or effective refractory period in the atria.[27] Our results suggest that alternation in the expression levels of Ax6 may be one of the upstream defects related to the abnormalities of Ca^{2+} handling observed in human AF. However, the functional significance of the Ax6 change in chronic AF still remains unclear. Given that overexpression of Ax6 reduces the contractility of cardiomyocytes and that the opposite effect is observed in Ax6 null mutant mice, it is reasonable to speculate that down-regulation of Ax6 during AF is a form of molecular compensation that favors improved atrial contractile function. An increase in demand for cardiovascular work, as with a fibrillating atrium, is compensated for by a reduction in Ax6 which increases the Ca^{2+} cycling efficiency and atrial contraction–relaxation dynamics. However, the precise mechanism(s) and target site(s) of Ax6 on atrial Ca^{2+} cycling remain to be understood.

Location of Ax6 in atrial tissue

Clarification of the spatial relationship between Ax6 and cardiomyocyte is helpful to understand the function of Ax6 in the atria. In the present study, the findings from double immunolocalization strategy that in both SR and chronically fibrillating atria Ax6 is tightly associated with NCX and L-type Ca^{2+} channel in the sarcolemma suggest that spatially Ax6 directly acts on NCX and L-type Ca^{2+} channel. In contrast, the segregation of Ax6 with the cytoplasmic ryanodine receptor indicates that the effect of Ax6 on the cycle of Ca^{2+} release/uptake of sarcoplasmic reticulum is indirect. The findings of the present study further

supported the assumption that Ax6 maintains Ca^{2+} homeostasis through the regulation of NCX and/or L-type Ca^{2+} channels in cardiomyocytes.

Study limitations

All patients in the present study had severe coronary artery disease. Therefore, the confounding effects of ischemia on the atrial tissues from both the SR and AF patients should be considered. In addition, although we have speculated that the decrease of Ax6 may lead to changes in atrial Ca^{2+} cycling, we did not provide evidence that Ax6 is part of the functional unit accounting for atrial Na^{+}/Ca^{2+} exchange and/or Ca^{2+} inward current, and mechanical parameters or alterations in Ca^{2+} currents were not assessed in this study. Clearly, further studies are required to clarify the effect of ischemia on Ax6, to determine the temporal relationship between the decrease of Ax6 and the onset of AF, and to identify specific targets(s) of Ax6 responsible for the regulation of intracellular Ca^{2+} in atrial myocytes.

Conclusion

In the atria of patients with severe coronary artery disease, chronic AF is associated with a significant down-regulation of Ax6. This result together with the co-localization of Ax6 with cardiac NCX and L-type Ca^{2+} channels suggest that Ax6 may participate the regulation of calcium homeostasis by acting as an upstream regulator of atrial NCX and/or L-type Ca^{2+} channels at the site of sarcolemma, and down-regulation of Ax6 during AF may be a form of molecular compensation that favors improved atrial mechanical properties.

Figure legends

Figure 1. Down-regulation of Ax6 mRNA expression in chronic AF. A, representative polymerase chain reaction results of the transcripts encoding cardiac Ax6 from patients with chronic AF and sinus rhythm. B, relative amount of Ax6 gene expression in each atrium of the SR and AF group, after calibrated using GAPDH. SR, sinus rhythm; AF, atrial fibrillation; RA, right atrium; LA, left atrium; Ax6, annexin VI; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *P<0.01 compared with the same atrium of the SR group.

Figure 2. Down-regulation of Ax6 protein expression in chronic AF as determined by Western blotting. A, representative blots of Ax6 protein from patients with AF and SR. B, relative amount of Ax6 protein, in each atrium of both groups, after calibrated using ß-actin. Abbreviations are as in figure 1. *P<0.01 compared with the same atrium of the SR group.

Figure 3. Down-regulation of Ax6 protein expression in chronic AF as determined by immunoconfocal microscopy. A and B, Ax6 protein is abundantly expressed in both atria of the SR group, however, the expression is markedly reduced in each atrium of the AF group (C and D). Samples were immunostained with the mouse monoclonal antibody. Group and location of the samples are denoted at top left. Abbreviations are as figure 1. Bar, 50 μm.

Figure 4. Co-localization of Ax6 with NCX in human atrial appendage of both the SR and AF

groups. Note that in each row, the leftmost one is the superimposed image, in which the left half is split into the remaining 3 images according to the recording channels. In each row, the group and location of the samples are denoted at top left of the leftmost image. In the split image, the antibody used is marked at the top. See text for details. All images are of the same magnification. NCX, Na^+/Ca^{2+} exchanger. FITC, the autofluorescence background detected using the channel designed for fluorescein isothiocynate. Other abbreviations are as Figure 1. Bar, 20 μm.

Figure 5. Co-localization of Ax6 with L-type Ca^{2+} channel in human atrial appendage of both the SR and AF groups. See text for details. Assembly of the images, the abbreviations, and the scale are the same as in Figure 4.

Figure 6. Double labeling of Ax6 with ryanodine receptor in human atrial appendage shows different location of both proteins. See text for details. Assembly of the images, the abbreviations, and the scale are the same as in Figure 4.

Figure 7. Relationship between the mRNA and protein expression levels of Ax6 in right (A) and left (B) atrial appendages. (●) represents AF patients (n=20), (○) represents SR patients (n=34). Abbreviations are as Figure 1.

Table1. Baseline Characteristics of the Study Patients

Characteristic	AF (n= 20)	SR (n= 34)	p value
Age, years	67 ± 10	66 ± 11	0.792
Sex, male/female	13/7	22/12	>0.999
Duration of AF, months	21 ± 20	…	…
Diabetes mellitus	8 (40)	10 (29)	0.618
Hypertension	11 (55)	12 (35)	0.259
Vessel number of CAD	2.5 ± 0.7	2.6 ± 0.6	0.493
History of previous MI	6 (30)	10 (29)	>0.999
NYHA, functional class	2.2 ± 0.7	2.2 ± 0.6	0.987
Ejection fraction, %	50 ± 12	49 ± 11	0.725
RAP, mmHg	8.1 ± 2.7	7.4 ± 3.2	0.427
PCWP, mmHg	13.6 ± 3.1	12.0 ± 5.2	0.247
LAD, mm	48.6 ± 5.7	39.1 ±5.5	<0.0001
Therapy before surgery			
β-blocker	12 (60)	19 (56)	0.992
ACEI/AT1 antagonist	10 (50)	20 (59)	0.729
CCB	7 (35)	14 (41)	0.872
Diuretics	8 (40)	12 (35)	0.957
Digitalis	6 (30)	7 (21)	0.517
Type of heart surgery			
CABG	20 (100)	34 (100)	>0.999
AVR	2 (10)	1 (3)	0.548

Data are expressed as mean value ± SEM or number (%) of patients. AF = atrial fibrillation; SR = sinus rhythm; CAD = coronary artery disease; MI = myocardial infarction; NYHA = New York Heart Association; RAP = right atrial pressure; PCWP = pulmonary capillary wedge pressure; LAD = left atrial dimension; ACEI = angiotensin converting enzyme inhibitor; AT1 = angiotensin II type 1 receptor; CCB = calcium channel blockade; CABG = coronary artery bypass grafting; AVR = aortic valve replacement

Figure 1

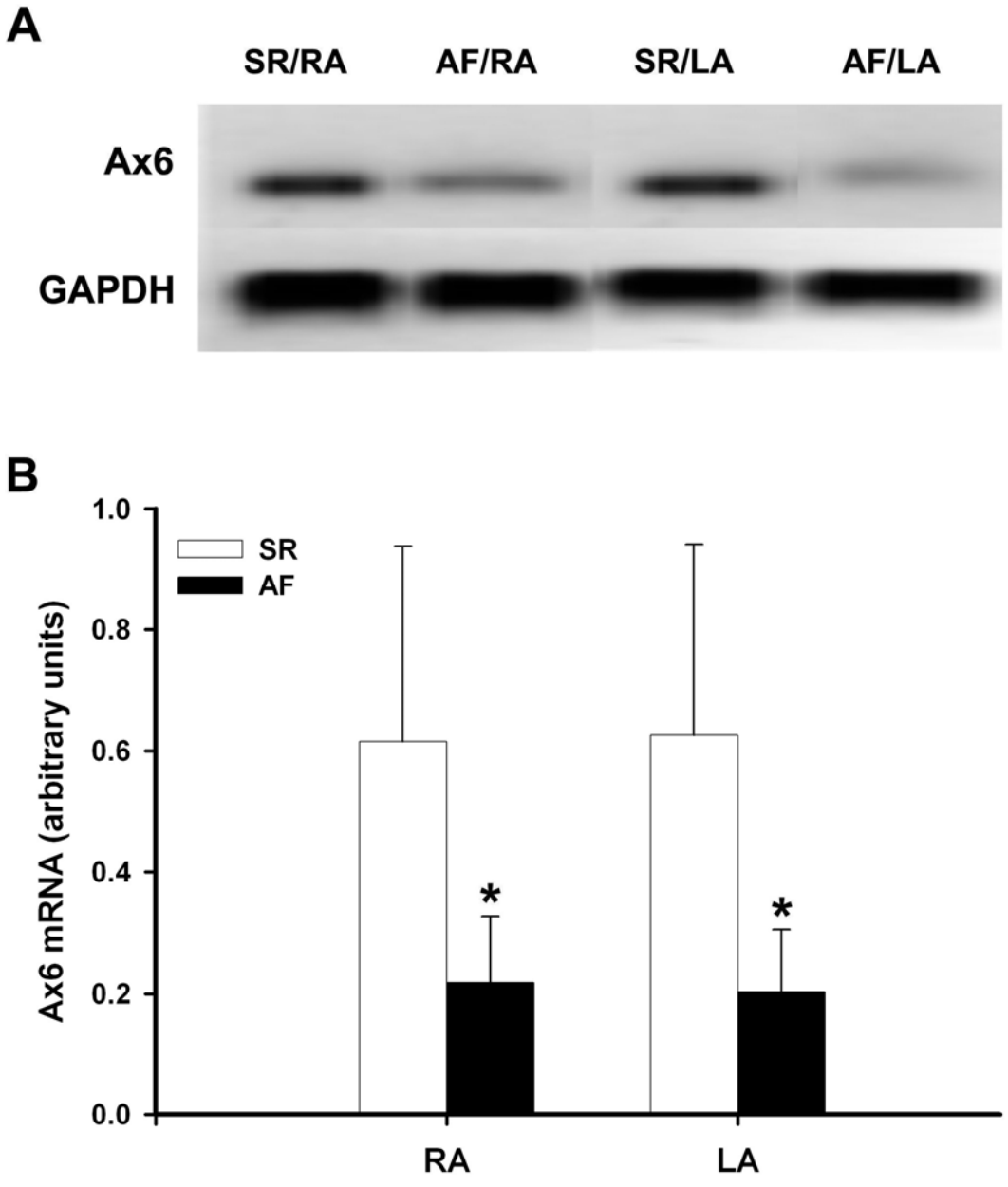

A
SR/RA
AF/RA
SR/LA
AF/LA
Ax6
GAPDH
B
Ax6 mRNA (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
SR
AF
*
*
RA
LA

Figure 2

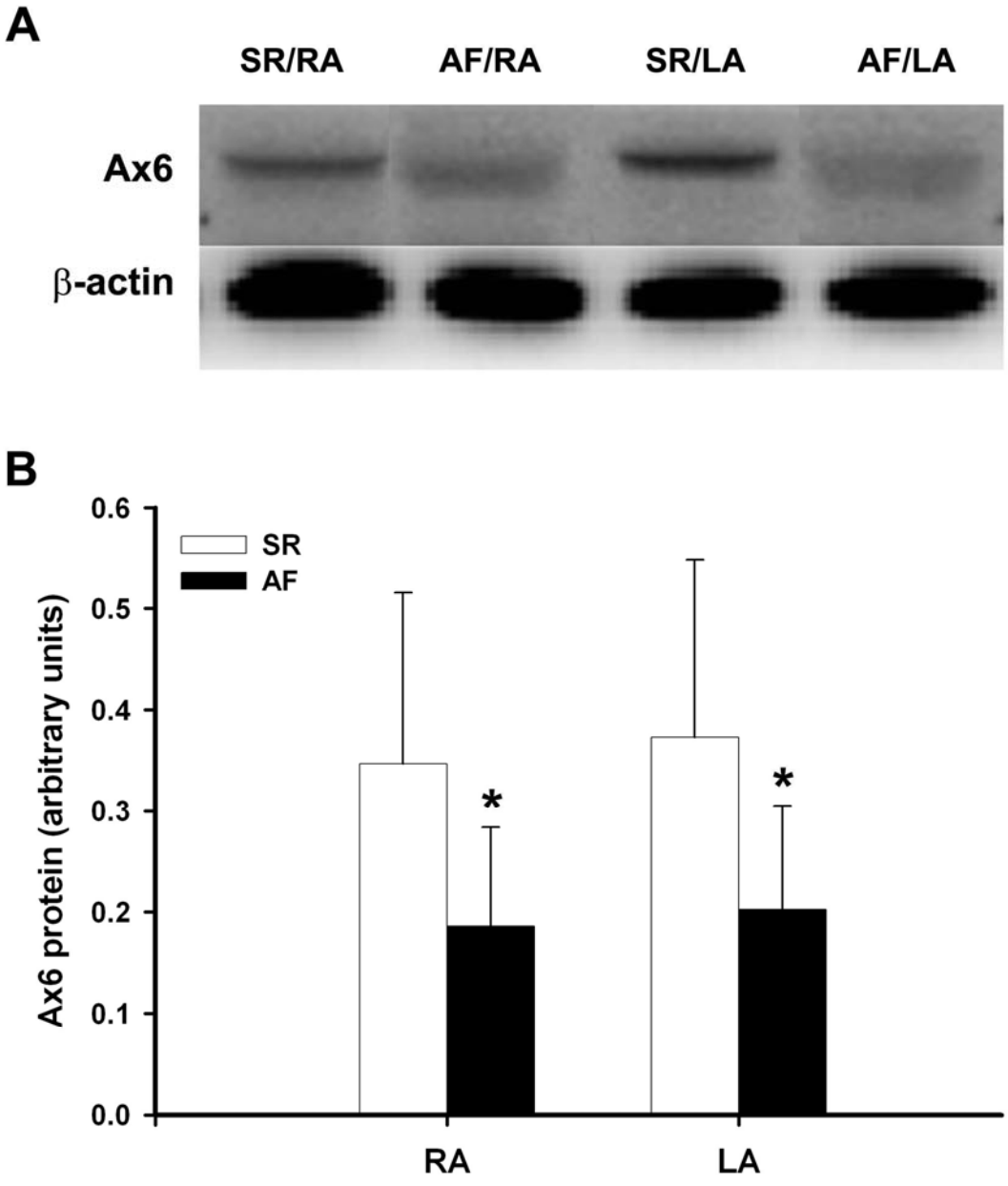

A
SR/RA
AF/RA
SR/LA
AF/LA
Ax6
β-actin
B
SR
AF
Ax6 protein (arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
RA
LA

Figure 3

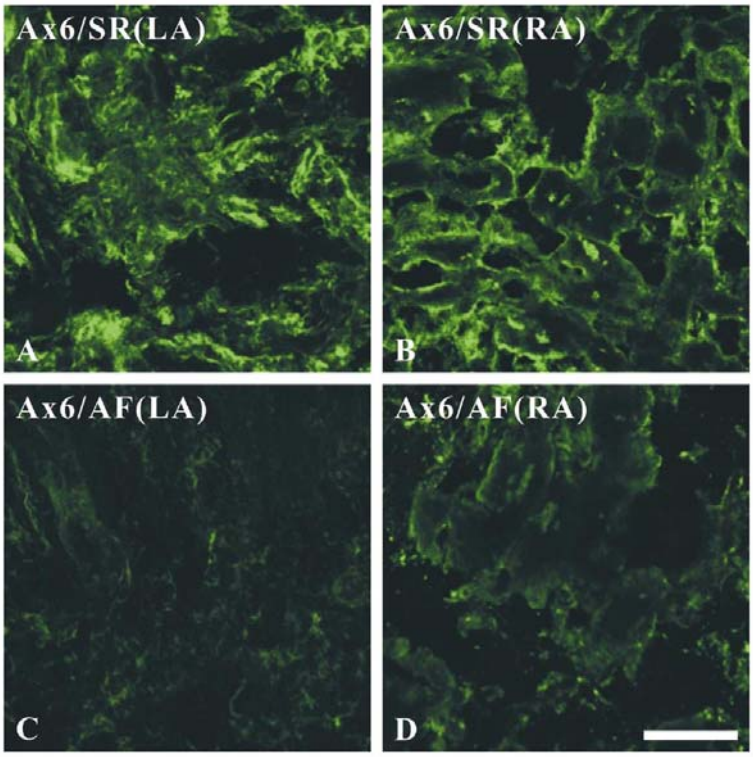


Figure 4

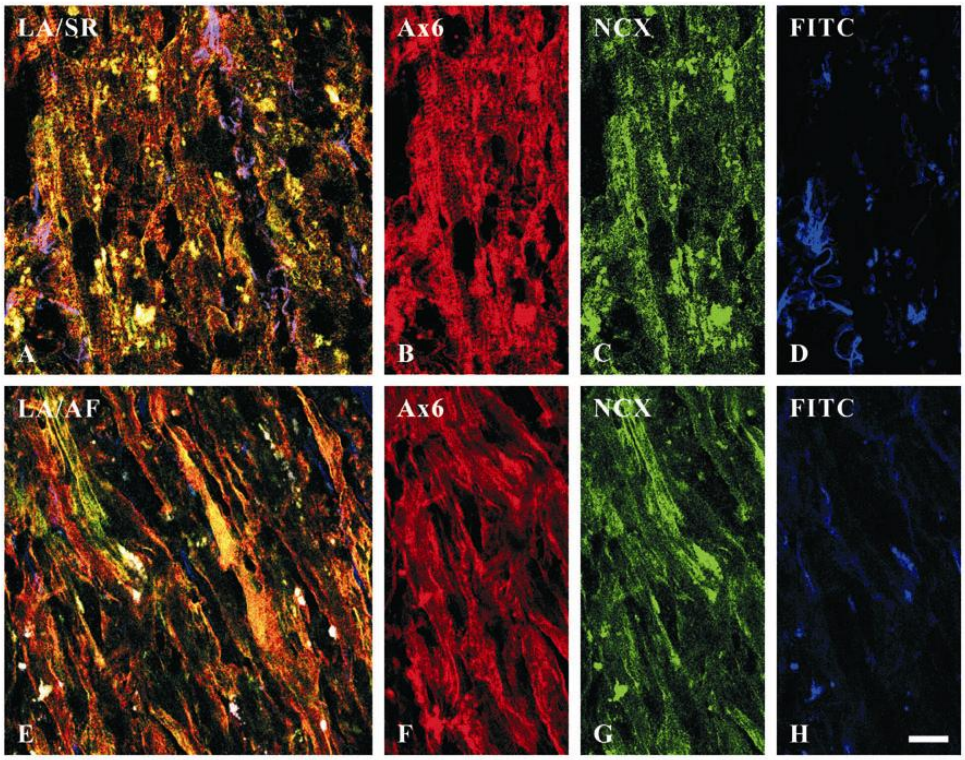


Figure 5

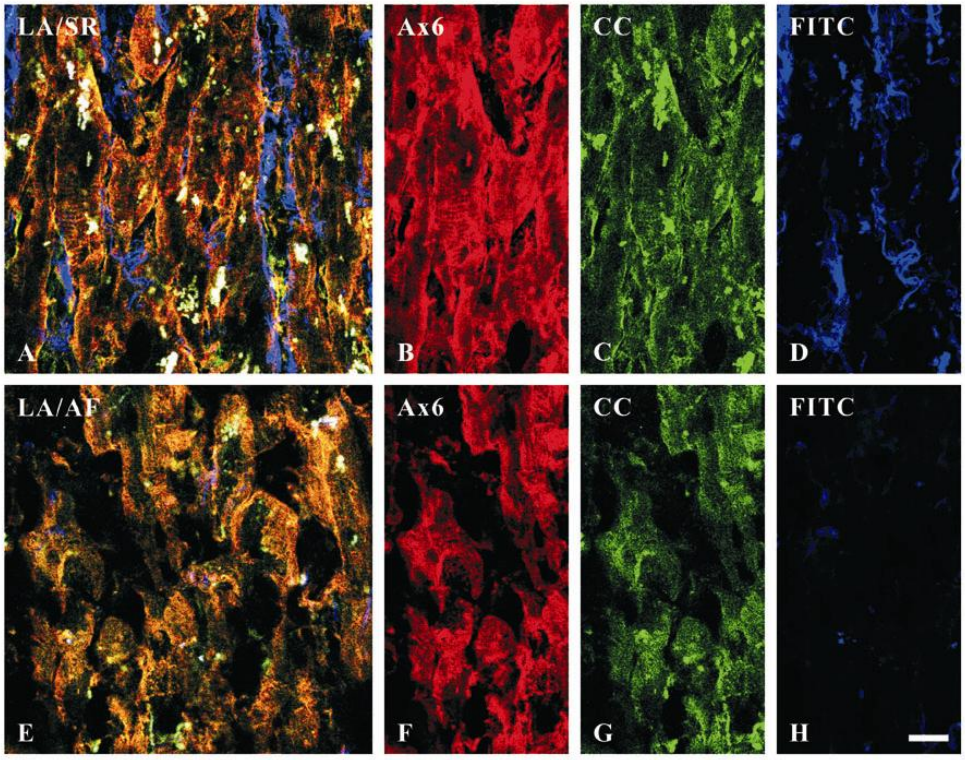


Figure 6

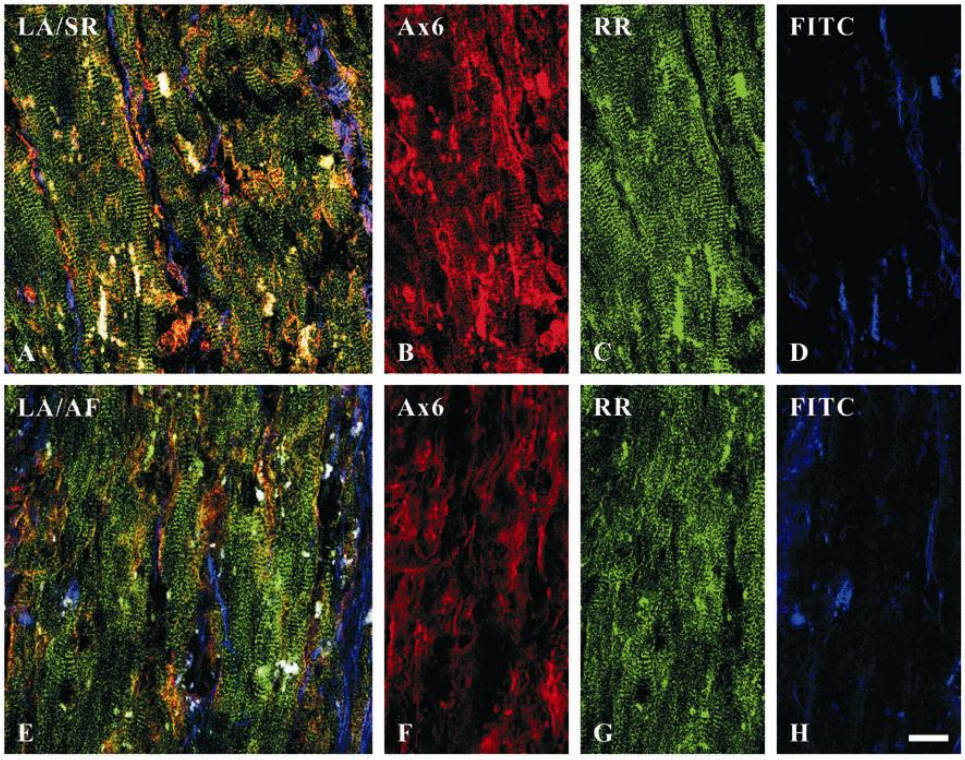
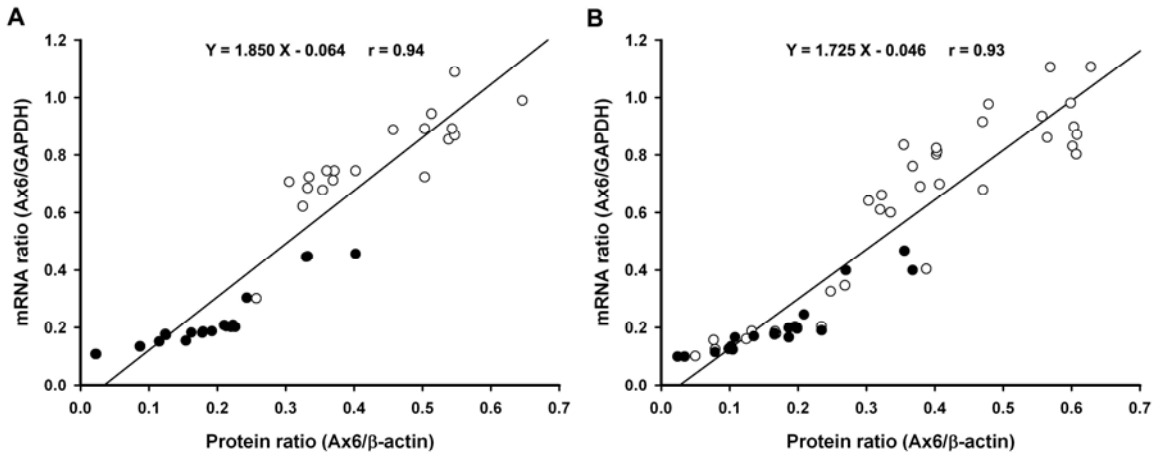


Figure 7


A
Y = 1.850 X - 0.064 r = 0.94
mRNA ratio (Ax6/GAPDH)
Protein ratio (Ax6/β-actin)
B
Y = 1.725 X - 0.046 r = 0.93
mRNA ratio (Ax6/GAPDH)
Protein ratio (Ax6/β-actin)

參考文獻

1. Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. Circulation. 1995; 92:1954-1968.
2. Goette A, Honeycutt C, Langberg JJ. Electrical remodeling in atrial fibrillation. Time course and mechanisms. Circulation. 1996;94:2968-2974.
3. Sun H, Gaspo R, Leblanc N, Nattel S. Cellular mechanisms of atrial contractile dysfunction caused by sustained atrial tachycardia. Circulation. 1998;98:719-727.
4. Schotten U, Ausma J, Stellbrink C, Sabatschus I, Vogel M, Frechen D, Schoendube F, Hanrath P, Allessie MA. Cellular mechanisms of depressed atrial contractility in patients with chronic atrial fibrillation. Circulation 2001;103:691-698.
5. Leistad E, Aksnes G, Verburg E, Christensen G. Atrial contractile dysfunction after short-term atrial fibrillation is reduced by verapamil but increased by BAY K8644. Circulation. 1996;93:1747-1754.
6. Eisner DA, Choi HS, Díaz ME, O'Neill SC, Trafford AW. Integrative analysis of calcium cycling in cardiac muscle. Circ Res. 2000;87:1087-1094.
7. Skasa M, Jungling E, Picht E, Schondube F, Luckhoff A. L-type calcium currents in atrial myocytes from patients with persistent and non-persistent atrial fibrillation. Basic Res Cardiol. 2001;96:151-159.
8. Hove-Madsen L, Llach A, Bayes-Genis A, Roura S, Rodriguez Font E, Aris A, Cinca J. Atrial fibrillation is associated with increased spontaneous calcium release from the sarcoplasmic reticulum in human atrial myocytes. Circulation. 2004;110:1358-1363.

9. Brundel BJ, van Gelder IC, Henning RH, Tuinenburg AE, Deelman LE, Tieleman RG, Grandjean JG, van Gilst WH, Crijns HJ. Gene expression of proteins influencing the calcium homeostasis in patients with persistent and paroxysmal atrial fibrillation. Cardiovasc Res. 1999;42:443-454.

10. Lai LP, Su MJ, Lin JL, Lin FY, Tsai CH, Chen YS, Huang SK, Tseng YZ, Lien WP. Downregulation of L-type calcium channel and sarcoplasmic reticular Ca^{2+} -Atlases mRNA in human atrial fibrillation without significant change in the mRNA of ryanodine receptor, calsequestrin and phospholamban: an insight into the mechanism of atrial electrical remodeling. J Am Coll Cardiol 1999;33:1231–1237.

11. Ohkusa T, Ueyama T, Yamada J, Yano M, Fujumura Y, Esato K, Matsuzaki M. Alterations in cardiac sarcoplasmic reticulum Ca^{2+} regulatory proteins in the atrial tissue of patients with chronic atrial fibrillation. J Am Coll Cardiol. 1999;34:255-263.

12. Schotten U, Greiser M, Benke D, Buerkel K, Ehrenteidt B, Stellbrink C, Vazquez-Jimenez JF, Schoendube F, Hanrath P, Allessie M. Atrial fibrillation-induced atrial contractile dysfunction: a tachycardiomyopathy of a different sort. Cardiovasc Res. 2002;53:192–201.

13. Crompton MR, Moss SE, Crumpton MJ. Diversity in the lipocortin/calpactin family. Cell. 1988;55:1-3.

14. Doubell AF, Lazure C, Charbonneau C, Thibault G. Identification and immunolocalization of annexins V and VI, the major cardiac annexins, in rat heart. Cardiovasc Res. 1993;27:1359-1367.

15. Pollard HB, Guy HR, Arispe N, de la Fuente M, Lee G, Rojas EM, Pollard JR, Srivastava M,

Zhang-Keck ZY, Merezhinskaya N. Calcium channel and membrane fusion activity of synexin and other members of the annexin gene family. Biophys J. 1992;62:15-18.

16. Naciff JM, Behbehani MM, Kaetzel MA, Dedman JR. Annexin VI modulates Ca^{2+} and K^{+} conductances of spinal cord and dorsal root ganglion neurons. *Am J Physiol Cell Physiol.*1996;271:2004-2015.

17. Sobota A, Cusinato F, Luciani S. Identification and purification of calpactins from cardiac muscle and their effect on Na^{+}/Ca^{2+} exchange activity. Biochem Biophys Res Commun. 1990;172:1067-1072.

18. Diaz-Muñoz M, Hamilton SL, Kaetzel MA, Hazarika P, Dedman JR. Modulation of Ca^{2+} release channel activity from sarcoplasmic reticulum by annexin VI (67-kDa calcimedin). J Biol Chem. 1990;265:15894-15899.

19. Hazarika P, Sheldon A, Kaetzel MA, Diaz-Muñoz M, Hamilton SL, Dedman JR. Regulation of the sarcoplasmic reticulum Ca^{2+}-release channel requires intact annexin VI. J Cell Biochem. 1991;46:86-93.

20. Hazarika P, Kaetzel MA, Sheldon A, Karin NJ, Fleischer S, Nelson TE, Dedman JR. Annexin VI is associated with calcium-sequestering organelles. J Cell Biochem. 1991;46:78-85.

21. Gunteski-Hamblin A, Song G, Walsh RA, Frenzke M, Boivin GP, Dorn GW 2nd, Kaetzel MA, Horseman ND, Dedman JR. Annexin VI overexpression targeted to heart alters cardiomyocyte function in transgenic mice. Am J Physiol 1996;270:1091-1100.

22. Song G, Harding SE, Duchen MR, Tunwell R, O'Gara P, Hawkins TE, Moss SE. Altered mechanical properties and intracellular calcium signaling in cardiomyocytes from annexin 6

null-mutant mice. FASEB J. 2002;16:622-624.

23. Trouvé P, Legot S, Bélikova I, Marotte F, Benevolensky D, Russo-Marie F, Samuel JL, Charlemagne D. Localization and quantitation of cardiac annexins II, V, and VI in hypertensive guinea pigs. Am J Physiol. 1999;276:1159-1166.

24. Song G, Campos B, Wagoner LE, Dedman JR, Walsh RA. Altered cardiac annexin mRNA and protein levels in the left ventricle of patients with end-stage heart failure. J Mol Cell Cardiol. 1998;30:443-451.

25. Matteo RG, Moravec CS. Immunolocalization of annexins IV, V and VI in the failing and non-failing human heart. Cardiovasc Res. 2000;45:961-970.

26. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156-159.

27. Li D, Fareh S, Leung TK, Nattel S. Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort. Circulation 1999;100:87-95.

自評

本研究在人類首次發現，Annexin VI蛋白質及mRNA在慢性顫動之心房組織，有明顯之下降，此外吾等也發現，無論在正常心律或者心房顫動，Annexin VI主要分佈於心房心肌細胞膜，同時與心臟鈉鈣交換蛋白質及L型鈣離子通道息息相關。

一、 Annexin VI蛋白質的降低與鈣離子調控之關係

過去的研究顯示，心房顫動會導致心房細胞膜及內質網上鈣離子調控分子之表現的變化，本研究發現 Annexin VI 蛋白質可能參與在心房顫動中鈣離子調節異常之機轉，Annexin VI 佔所有心臟蛋白質總量可高達 0.2%，雖然 Annexin VI 蛋白質在心房心肌細胞所扮演之功能角色並未完全清楚，Annexin VI 表現之降低在心房顫動之心房中，可能在心房細胞內鈣離子調控扮演重要的角色。這樣的假設是基於過去報告顯示。

（1）Annexin VI 會增加 L 型鈣離子通道之傳導特性

（2）Annexin VI 可以改善心肌細胞鈉鈣交換蛋白質之功能

（3）Annexin VI 參與內質網鈣離子之釋放及回收機轉。

此外，Annexin VI 近來在心肌細胞鈣離子調控及收縮也扮演重要之角色，已經被明顯闡釋，在過度表現 Annexin VI 的老鼠研究中發現，Annexin VI 之過度表現，會降低細胞內鈣離子濃度降低去極化後鈣離子流，最後導致細胞收縮之功能降低，有趣的是在 Annexin VI 完全去除之老鼠心室心肌細胞，發現會增加細胞質內鈣離子之移除，也影響到收縮功能。

此兩個研究顯示正常的 Annexin VI 表現對於心肌細胞細胞內鈣離子調控扮演非常重要的角色。

相反的，在心室組織中在心臟衰竭初期 Annexin VI 表現則明顯降低，然而在末期心臟衰竭，Annexin VI 的表現則維持不變或者降低，顯示心室 Annexin VI 的表現跟心臟病理發現之嚴重程度有關，然而，在末期心臟衰竭，Annexin VI 在右心房的表現則沒有變化，相反的，本研究顯示慢性心房顫動會導致 Annexin VI 在左、右心房心耳組織中 Annexin VI 之表現大大降低。這樣的差異可以由 Li 等人的研究來做解釋，在此研究中作者發現心臟衰竭不像心房顫動並不一定合併心房之有效不反應期或者動作定位之降低，我們的研究顯示，心房組織中 Annexin VI 之降低可能是在人類之心房顫動鈣離子調控異常之上游缺陷，然而 Annexin VI 在鈣離子調控之真正機轉及作用點則仍有待進一步釐清。

二、 Annexin VI 在心房組織中之分佈

Annexin VI 與心肌細胞相關蛋白質之相關空間關係的釐清，將有助於了解 Annexin VI 在心房所扮演的角色，本研究藉由雙染免疫定位研究發現，在心房組織中 Annexin VI 主要分佈於心房心肌細胞膜上，與鈉鈣交換蛋白質及 L 型鈣離子通道有完全重疊之現象，相反的，Annexin VI 的分佈與細胞質內之 ryanodine 接受體則完全沒有重疊現象，顯示 Annexin VI 在心房心肌細胞鈣離子調控，可能主要經由鈉鈣交換蛋白質及 L 型鈣離子通道，對於肌漿網之鈣離子釋放，及回收作用可能是間接的。

三、 本研究限制

本研究中所有的病人皆具有嚴重之冠狀動脈病變，因此心房組織之缺氧可能造成某些影響，此外，我們雖然假設 Annexin VI 之降低可能導致心房鈣離子調控之異常，但我們並未提供直接證據顯示，Annexin VI 之降低到底是心房顫動之因或者是果，因此必須進一步研究來釐清心肌缺氧對 Annexin VI 表現之影響並決定心房顫動之時程與心房 Annexins VI 降低之關係，並確定 Annexin VI 調控鈣離子之真正作用點。

本研究已經撰寫成論文正在投稿當中。