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ORIGINAL CONTRIBUTION

The effects of cilazapril and valsartan on the mRNA and protein expressions of atrial calpains and atrial structural remodeling in atrial fibrillation dogs

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Abstract Owing to relative inefficacy and side effects of currently available antiarrhythmic drugs, current interest has shifted to treatments that target atrial fibrillation (AF) substrate. It has been suggested that calpain-induced atrial structural remodelling is under the control of renin-angiotensin system during AF. The purpose of this study is to investigate the effects of cilazapril and valsartan on the mRNA and protein expression of atrial calpains and atrial structural remodelling in AF dogs induced by chronic rapid atrial pacing. Twenty-seven dogs were randomly divided into sham-operated group (n=6), control group (n=7), cilazapril group (n=7) and valsartan group (n=7). One thin silicon plaque containing 4 pairs of electrodes was sutured to each atrium. A pacemaker was implanted in a subcutaneous pocket and attached to a screw-in epicardial lead in the right atrial appendage. The dogs in control group, cilazapril group and valsartan group were paced at 400 beats per minutes for 6 weeks. The dogs in cilazapril and valsartan groups received cilazapril ($1 mg \cdot kg^{-1} \cdot d^{-1}$) or valsartan ($30 mg \cdot kg^{-1} \cdot d^{-1}$) 1 week before rapid atrial pacing until pacing stop respectively. Transthoracic and transoesophageal echocardiographic examinations were performed in order to detect the changes of left atrium volume and contractile function. The inducibility and duration of AF were measured in all the groups. The expressions of atrial calpain I and calpain II mRNA were semi-quantified by reverse transcription-polymerase chain reaction.The protein levels of calpain I and calpain II in atrial myocardium were measured by Western-blot method. Pathohistological and ultrastructural changes in atrial tissue were tested by light and electron microscopy. Compared with the sham-operated control group, dramatic smaller left atrium and left atrial appendage volumes and significant higher atrial contractile function were observed in the cilazapril and valsartan groups. After 6-week atrial tachy-pacing, the mRNA and protein expressions of calpain I increased dramatically in the control group than that in the sham group, tissue calpain protein expression in all groups significantly correlated with the myolysis ($r=0.89$, $P<0.01$). Cilazapril and valsartan could significantly inhibit the gene and protein expressions of calpain I. No differences were found in the expression of calpain II mRNA and protein between the groups. Compared with atrial myocytes obtained from sham dogs, atrial myocytes from the control group dogs showed a reduced number of sarcomeres, a significant higher myolytic area of atria (24.3% vs. 3.1%, $P<0.01$), increased vacuolization and dissolution. Cilazapril and valsartan could effectively prevent the pathohistological and ultrastructural changes induced by chronic rapid atrial pacing, dramatically decrease

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the area of myolysis ($P<0.05$) and significantly reduce the inducibility and duration of AF. The expression of calpain I mRNA and protein increased remarkably in AF dogs. Cilazapril and valsartan can inhibit calpain I up-regulation, suppress atrial structural remodeling, and prevent the induction and promotion of AF in chronic rapid atrial pacing dogs.

Key words calpains – angiotensin – structural remodelling – atrial fibrillation – dog

Introduction

The natural history of atrial fibrillation (AF) is characterized by a gradual worsening with time. Clinical and experimental studies have revealed that AF is associated with progressive structural changes of the atria including severe myolysis and atrial myocyte apoptosis, etc., which provides a possible explanation for the reduction of atrial contractility during AF and the prolonged atrial mechanical dysfunction after successful cardioversion of AF [10]. The depressed atrial contractility during AF can facilitate atrial dilatation, which may add to the persistence of AF. It has been confirmed that AF-induced changes at the structural level are of prime importance for the vulnerability to AF [3, 30]. Thus, conservation of the normal atrial architecture and size by preventing structural atrial remodeling due to AF seems critical in future AF management.

Calpains, a family of Ca^{2+}-dependent neutral cysteine proteases found in the cytosol of many cell types, have been demonstrated to play a harmful role in a variety of pathological states. Brundel et al. [6] have demonstrated that atrial myolysis might be owing to up-regulation of calpain I and contribute to the loss of atrial contractile force during AF. Calpains also could promote programmed cell death by triggering the intrinsic apoptotic pathway (down-regulating bcl-2 protein and activating caspase 3) [1, 28]. Recent studies have shown that AF is associated with the activation of the renin-angiotensin system (RAS), which is closely related to atrial structural remodeling in AF [9]. Sandmann et al. [25] found that ramipril and valsartan could effectively inhibit the up-regulation of cardiac calpains in myocardial infarction rat models, indicating that RAS has a close relationship with the transcriptional and translational control of the calpain system. In view of these findings, we speculate that inhibition of cardiac RAS may have therapeutic effects on atrial structural remodeling by inhibiting the calpain pathway in AF. Therefore, the present study was designed to observe the influence of pretreatment with the angiotensin-converting enzyme (ACE) inhibitor cilazapril and the angiotensin AT_1 receptor antagonist valsartan on the mRNA and protein expressions of atrial calpains in AF dogs induced by chronic rapid atrial pacing. Effects of the two drugs on calpain-mediated structural changes were also investigated by ultrasound and microscopy.

Methods

Animal preparation

For this study, 27 mongrel dogs of either sex, weighing between 15 and 25 kg, were randomly assigned to the sham-operated group (n=6), the control group (n=7), the cilazapril group (n=7) or the valsartan group (n=7). The dogs were anesthetized with pentobarbital sodium ($25\ mg \cdot kg^{-1}$). After intubation and mechanical ventilation, medial thoracotomy was performed. One thin silicon plaque containing four pairs of electrodes (electrode diameter, 1 mm; interelectrode distance, 1 mm; distance between electrode pairs, 10 mm) was sutured to each atrium (Fig. 1). The other ends of the electrode cables were tunneled subcutaneously and exposed at the back of the dogs where they were used for the pacing and electrophysiological measurements in the chronic phase. A pacemaker (made in Shanghai Fudan University, China) was implanted in a subcutaneous pocket and attached to a screw-in epicardial lead in the right atrial

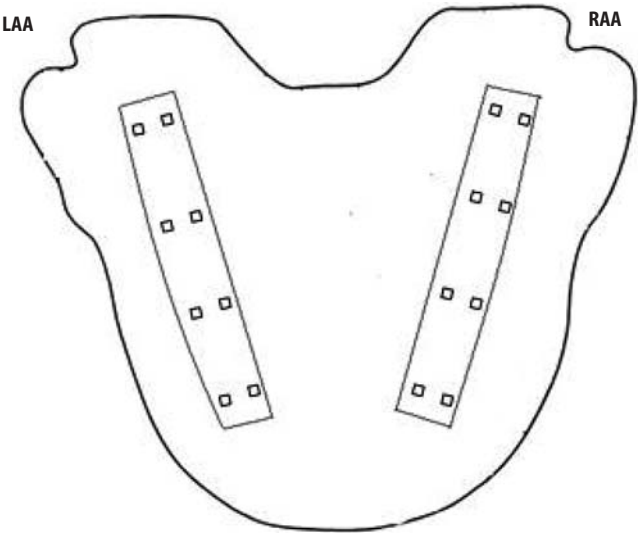

Fig. 1 Schematic of the positions of the atrial electrodes

appendage. After 1 week recovery, the dogs in the control group, cilazapril group and valsartan group were paced at 400 beats per minute for 6 weeks [18]. The dogs in the cilazapril and valsartan groups received cilazapril (1 $mg \cdot kg^{-1} \cdot d^{-1}$) or valsartan (30 $mg \cdot kg^{-1} \cdot d^{-1}$) 1 week before rapid atrial pacing until pacing stop respectively.

Hemodynamic study

Right atrial pressure, mean pulmonary artery pressure and pulmonary capillary wedge pressure were monitored by a pulmonary arterial balloon catheter system (Swan-Ganz catheter) through a 6 F sheath placed in the femoral vein. Aortic systolic blood pressure and diastolic blood pressure were measured by a pigtail catheter through a 6 F sheath placed in the femoral artery.

Atrial structural and function evaluation

Left atrial (LA), left atrial appendage (LAA) structure and function were assessed by transthoracic and transesophageal echocardiographic examinations (GE VIVID5, USA). A 2.5 MHz transducer was used during the transthoracic echocardiographic study. By means of two-dimensional echocardiography, the LA up-down diameter (D_1) and left-right diameter (D_2) were measured in the apical four chamber view, while the LA anterior-posterior diameter (D_3) was measured at the level of the aortic valve in parasternal long-axis view. LA volume was measured using the following formula: $4\pi/3 \times D1/2 \times D2/2 \times D3/2$, where π is 3.14. Transesophageal echocardiographic examination was performed using a 5 MHz multiplane transducer. LAA area was measured by tracing a line starting from the top of the limbus of the left upper pulmonary vein along the appendage's endocardial border. The boundary of the base of the appendage was defined by a line drawn from the limbus of the left upper pulmonary vein to the exteriormost portion of the mitral annulus. LAA volume was calculated with plane length method. LA maximal volume (LAVmax) and LAA maximal volume (LAAVmax) were measured at the end of T wave of the simultaneously recorded electrocardiogram. LA minimal volume (LAVmin) and LAA minimal volume (LAAVmin) were recorded at the peak of R wave. LA ejection fraction (LAEF) was calculated as (LAVmax–LAVmin)/LAVmax. LAA ejection fraction (LAAEF) was calculated as (LAAVmax–LAAVmin)/LAAVmax. LAA maximal forward flow velocity (V-LAA^+) and LAA maximal backward flow velocity (V-LAA^-) were obtained with the pulsed Doppler sample volume placed immediately inside the appendage orifice.

AF inducibility and AF duration assessment

AF inducibility and duration were measured before and after 6 weeks rapid atrial pacing in the control, cilazapril and valsartan groups, respectively. Ten times the atrial burst pacing lasting for 10 s at a pacing cycle length of 100 ms was used to assess the inducibility and duration of AF. AF lasting more than 30 min was terminated by direct current electrical cardioversion and 30 min was allowed before the experiment continued.

RNA extraction and reverse transcription-polymerase chain reaction

At the end of the experiments, the dogs were anesthetized with pentobarbital sodium (25 $mg \cdot kg^{-1}$), sternotomies were performed. The hearts were quickly removed, and parts of the right atrial wall samples were rapidly frozen in liquid nitrogen and stored separately at –80 °C for further analysis. One aliquot of each tissue sample was used to investigate the mRNA expression of calpain I and calpain II, whereas the other part was used to determine the protein levels.

Total RNA was extracted from 100 mg atrial tissue using TRIZOL (Gibco, USA). Integrity of the RNA was confirmed by agarose gel electrophoresis, the quantity of RNA was assessed by obtaining the ratio of absorbance values 260 at 280 nm using a spectrophotometer (Ultrospec 3000, Pharmacia. Biotech, Sweden).

The reaction system was 50 μl: total RNA (2 μl), dNTP (2 μl), RNA PCR buffer (5 μl), sense and antisense primer (1 μl each), pfu Taq enzyme (1 μl), MgCl2 (10 μl), and RNase-free H_2O (up to 50 μl) according to the One-step RT-PCR kit (CLONTECH Laboratories, Inc., USA). β-actin was included as a control. Primers were obtained from Shanghai Genebase Gen-Tech Ltd., Shanghai, China. Details concerning the primers used in this study are given in Table 1. Reverse transcription at 50 °C for 1 h. Initial denaturation at 94 °C for 4 min was followed by 35 cycles with denaturation 45 s at 95 °C, annealing 45 s at 52 °C (calpain I), 45 s at 58 °C (calpain II), and elon-

Table 1 Primer sequences for amplification of calpain I, calpain II and β-actin

	Sequence	Product size?bp?
calpain I		
Sense	5′-GGAGTGGGTCGACGTGGTCATA-3′	279
Antisense	5′-CAGCCCAGCAAGGAGCCTCGTT-3′	
calpain II		
Sense	5′-CCTGGAGATCTGTAACCTGAC-3′	258
Antisense	5′-CCCATCTTCCTCTGCCGCCGT-3′	
β-actin		
Sense	5′-CAGAGCAAGAGGGGCATC-3′	392
Antisense	5′-AGGTAGTCGGTCAGGTCC-3′	

gation 60 s at 72 °C with a final extension at 72 °C for 10 min. 5 μl of product was analyzed by 1.52% agarose gel electrophoresis and all PCR products were purified using the plasmid DNA purification kit (Bocai Corp., Shanghai, China). Then, the resultant target gene was cloned into the pMD18-T vector and its sequence was identified by Shanghai Shenergy Biocolor BioScience & Technology Co. Ltd., Shanghai, China.

Protein extraction and Western blot analysis

Tissue samples were separately suspended in 5 ml of ice-cooled lysis buffer, and disrupted by a Wheaton-tissue homogenisator (neoLab, Hamburg, Germany). The particulate material was discarded by centrifugation at 100,000×g for 1 h at 4 °C in a Beckmann L8 ultracentrifuge. The clear supernatant of each tissue sample was collected and aliquots were frozen at –80 °C until use. The protein concentration was determined by the method of Lowry using bovine serum albumin as a standard. All preparations were carried out at 4 °C. For Western blotting, 50 μg of total protein solubilized for 5 min at 95 °C in one volume loading buffer (1% SDS, 30% glycerol, 0.8 M DTT, 1 mM Tris-HCl pH 6.8, 2% bromophenol blue) was loaded per lane onto a 10%/5% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a membrane and incubated overnight at 4 °C with the primary antibody (calpain I and calpain II, Santa Cruz, CA, USA; β-actin; Kangchen Bio-tech Co., Shanghai, Cina), then incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at 37 °C.

Morphological evaluation

Tissues from the right atrial wall were immediately fixed in 4% paraformaldehyde at 4 °C and embedded in paraffin. Light microscopy was performed using semi-thin sections (2 μm) stained with HE. To quantify the extent of myolysis in the cardiomyocytes, at least two sections per atrial site were examined and at least 200 cells per section were analyzed. The extent of cell change was evaluated only in cells in which the nucleus was present in the plane of the section. The myolytic area of the cardiomyocytes was measured with a digital imaging system (Motic Images Advanced, Richmond, BC, Canada). Cells were scored as mildly myolytic if myolysis involved 10% to 25% of the cytosol and as severely myolytic if >25% of the sarcomeres were absent.

For electron microscopy, ultrathin sections (50–100 nm) were cut from each sample, counterstained with uranium acetate and lead citrate, and examined with a transmission electron microscope (Philips 201, USA) by two professional staff.

Statistical analysis

Quantitative data were presented as mean ± SD. Comparisons between the quantitative data were made using the t test, whereas those for qualitative data were tested with the X^2 method. $P<0.05$ was considered statistically significant. The software SPSS 10.0 (SPSS, Chicago, IL) was used in the statistical analysis.

Results

Changes in properties of AF

As illustrated in Table 2, after 6-week rapid atrial pacing, AF became inducible in all the dogs in control group, cilazapril group and valsartan group, the inducibility and duration of AF increased dramatically compared with the baseline and the sham group ($P<0.01$), whereas the inducibility and duration of AF were found to be markedly lower in the cilazapril and valsartan group than those in the control group.

Hemodynamic changes

As displayed in Table 3, although there was no dramatic difference in aortic blood pressure between the sham, control, cilazapril and valsartan groups throughout the experiment, cilazapril and valsartan could prevent the increase of right atrial pressure, mean pulmonary artery pressure and pulmonary capillary wedge pressure induced by 6 weeks of atrial tachypacing.

Changes of atrial structure and function

From Table 4 we can see that in the control, cilazapril and valsartan groups, LAVmax, LAVmin, LAAVmax and LAAVmin increased significantly, while LAEF, LAAEF, V-LAA$^+$ and V-LAA$^-$ decreased dramatically after termination of the 6-week rapid atrial pacing. Compared with the control group dogs, the LA and LAA volume decreased significantly ($P<0.05$), whereas LAEF, LAAEF, V-LAA$^+$ and V-LAA$^-$ increased dramatically in the cilazapril and valsartan group dogs with 6 weeks of tachypacing, suggesting cilazapril and valsartan could prevent atrial structural remodeling and function reduction in chronic rapid atrial pacing dogs.

mRNA and protein expressions of calpains

Integrity of the RNA was confirmed by agarose gel electrophoresis, and the concentration was determined by densitometric measurement of UV absorption at 260

Table 2 Changes in inducibility and duration of AF before and after rapid atrial pacing in the sham, control, cilizapril and valsartan groups

	AF cases	AF times	AF inducibility[e] %[e]	mean AF duration[e] S[e]
Sham group[e] n=6[e]				
preoperation	2	6	10.0	41.1±13.6
7-week postoperation	2	8	13.3	46.8±16.0
Control group[e] n=7[e]				
baseline	2	9	12.8	39.4±10.2
6-week tachypacing	7[b, f]	67[b, f]	95.7[b, f]	1432.2±526.5[b, f]
Cilazapril group[e] n=7[e]				
baseline	3	11	15.7	43.6±11.1
6-week tachypacing	7[b, f]	46[b, c, f]	65.7[b, c, f]	531.5±301.2[b, d, f]
Valsartan group[e] n=7[e]				
baseline	2	8	13.8	38.5±12.9
6-week tachypacing	7[b, f]	48[b, c, f]	68.0[b, c, f]	556.8±323.4[b, d, f]

[a] $p<0.05$; [b] $p<0.01$, compared with baseline; [c] $p<0.05$; [d] $p<0.01$, compared with the control group; [e] $p<0.05$; [f] $p<0.01$, compared with sham group

Table 3 Hemodynamic parameters in the sham, control, cilazapril and valsartan groups (mmHg)

	SBP	DBP	RAP	MPAP	PCWP
Sham group[e] n=6[e]					
preoperation	109±13	70±9	6±2	15±5	7±3
7-week postoperation	110±15	70±8	5±1	16±5	8±4
Control group[e] n=7[e]					
baseline	111±15	71±9	5±1	16±4	8±2
6-week tachypacing	106±14	66±7	12±3[b, f]	23±5[a, e]	16±4[b, f]
Cilazapril group[e] n=7[e]					
baseline	107±15	68±9	4±1	14±3	8±3
6-week tachypacing	99±12	61±6	6±2[d]	17±4[c]	10±3[c]
Valsartan group[e] n=7[e]					
baseline	106±14	69±9	5±2	15±4	7±2
6-week tachypacing	101±11	64±7	7±3[c]	18±5[c]	9±4[c]

[a] $p<0.05$; [b] $p<0.01$, compared with baseline; [c] $p<0.05$; [d] $p<0.01$, compared with the control group; [e] $p<0.05$; [f] $p<0.01$, compared with sham group

nm. As shown in Fig. 2, compared with sham-operated dogs, calpain I mRNA abundance increased dramatically in control group dogs (P<0.01). The increase of mRNA expression of calpain I was significantly reduced by cilazapril and valsartan compared with control group dogs (P<0.01). No significant differences were found in the expression of calpain II mRNA among the four groups.

The data shown in Fig. 3 indicate that the amount of calpain I protein was significantly increased in control group dogs compared with sham-dogs (P<0.01). Cilazapril and valsartan could dramatically reduce calpain I protein up-regulation induced by rapid atrial pacing (P<0.01). Furthermore, tissue calpain protein expression in all groups significantly correlated with the myolysis (r=0.89, P<0.01, Fig. 4), whereas no dramatic differences were observed in the expression of calpain II protein level among the four groups.

Table 4 Changes of left atrium structure and function before and after rapid atrial pacing in the control, cilizapril and valsartan groups

	LAV_{max} (cm^3)	LAV_{min} (cm^3)	LAEF (%)	$LAAV_{max}$ (cm^3)	$LAAV_{min}$ (cm^3)	LAAEF (%)	V-LAA$^+$ (cm/s)	V-LAA$^-$ (cm/s)
Control group (n=7)								
baseline	13.4±2.7	6.5±1.7	51.4±10.5	1.9±0.6	0.9±0.3	52.6±10.5	40.2±8.5	37.4±7.9
6-week tachypacing	23.3±5.6[a]	15.6±3.2[b]	33.0±8.9[b]	2.9±1.1[a]	1.9±0.6[b]	34.4±8.9[b]	16.7±4.3[b]	15.9±3.5[b]
Cilazapril group (n=7)								
baseline	12.1±2.9	6.2±1.9	49.6±9.9	1.8±0.4	0.8±0.3	55.9±9.8	41.4±8.9	36.5±7.2
6-week tachypacing	17.1±3.9[a, c]	10.3±2.4[a, c]	40.5±9.0[a, c]	2.3±0.8[a, c]	1.3±0.4[a, c]	43.2±7.6[a, c]	29.2±6.2[a, d]	26.5±5.3[a, c]
Valsartan group (n=7)								
baseline	13.9±3.1	6.4±1.5	53.9±10.1	2.0±0.4	0.9±0.3	56.1±8.7	39.5±8.2	34.1±6.9
6-week tachypacing	18.0±3.8[a, c]	10.6±3.0[a, c]	41.6±8.9[a, c]	2.4±0.8[a, c]	1.3±0.4[a, c]	45.9±6.9[a, c]	27.1±5.4[a, c]	24.3±4.2[a, c]

[a] $p<0.05$; [b] $p<0.01$, compared with baseline; [c] $p<0.05$; [d] $p<0.01$, compared with the control group; *LAV_{max}* LA maximal volume; *LAV_{min}* LA minimal volume; *LAEF* LA ejection fraction; *$LAAV_{max}$* LAA maximal volume; *$LAAV_{min}$* LAA minimal volume; *LAAEF* LAA ejection fraction; *V-LAA$^+$* LAA maximal forward flow velocity; *V-LAA$^-$* LAA maximal backward flow velocity

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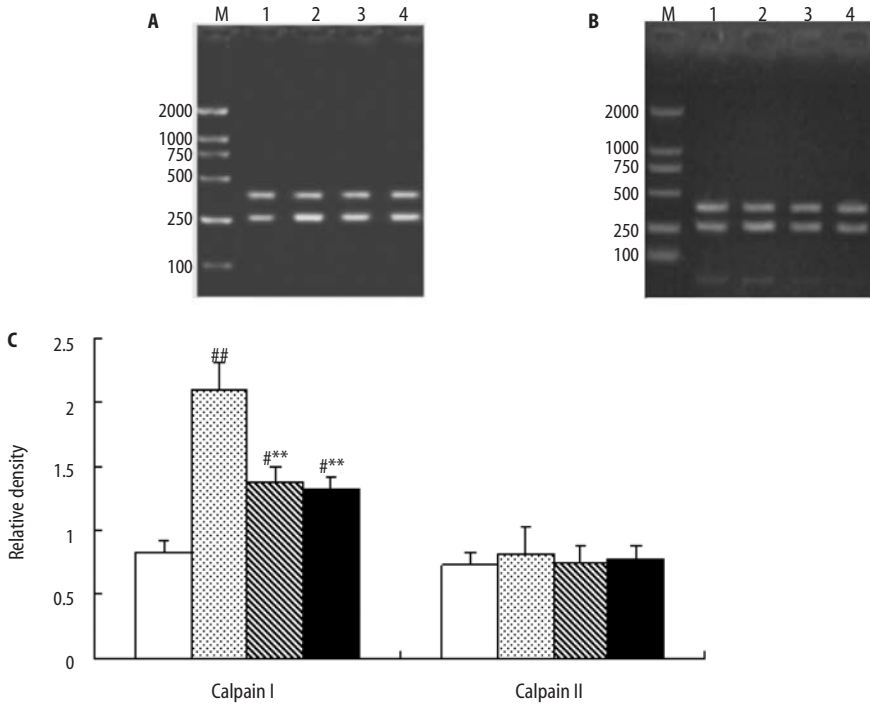

Fig. 2 Calpain I (**A**) and calpain II (**B**) mRNA expression in the sham-operated, control, cilizapril and valsartan groups. M=DL 2000 marker; 1 = Sham-operated group; 2 = Control group; 3 = Cilazapril group; 4 = Valsartan group. White bars = Sham-operated group; spotted bars = Control group; striped bars = Cilazapril group; solid bars = Valsartan group. # $p<0.05$; ## $p<0.01$, compared with the sham-operated group; ** $p<0.01$, compared with the control group (**C**)

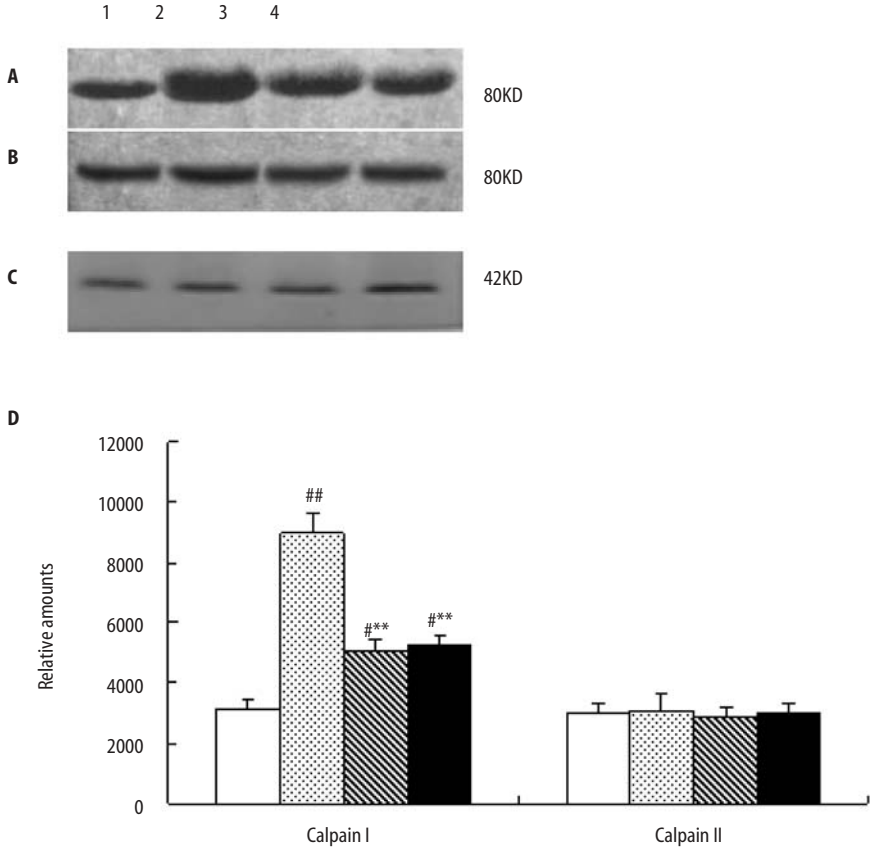

Fig. 3 Representative immunoblots (Western blots) showing calpain I (**A**), calpain II (**B**) and beta-actin (**C**) protein expression in atrial tissue. 1 = Sham-operated group; 2 = Control group; 3 = Cilazapril group; 4 = Valsartan group. Densitometric quantification of the Western blots (**D**). Amounts of protein are shown as relative densitometric absorption units. White bars = Sham-operated group; dotted bars = Control group; striped bars = Cilazapril group; solid bars = Valsartan group. # $p<0.05$; ## $p<0.01$, compared with the sham-operated group; ** $p<0.01$, compared with the control group

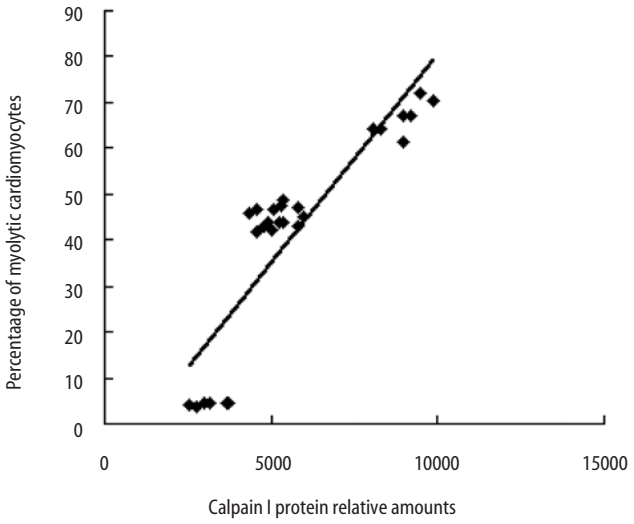

Fig. 4 The myolytic cardiomyocytes were the total cardiomyocytes with mild and severe myolysis. Tissue calpain protein amounts correlated with the myolysis in sham-operated group, Control group, Cilazapril group and Valsartan group ($r=0.89$, $P<0.01$)

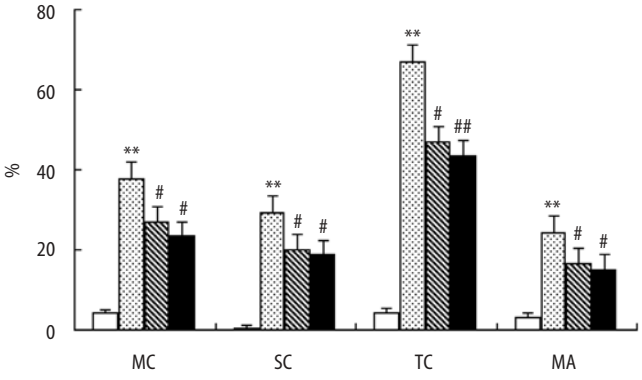

Fig. 6 Myolytic cardiomyocytes in atria of dogs. MC Mild changes, indicates myolytic changes between 10% and 25% of cell volume; SC Severe changes, myolytic changes > 25% of cell volume; TC Total changes, MC+SC; MA Myolytic area; White bars = Sham-operated group; dotted bars = Control group; striped bars = Cilazapril group; solid bars = Valsartan group. ** $p<0.01$, compared with the sham-operated group; # $p<0.05$; ## $p<0.01$, compared with the control group

Morphological changes

The results from the light microscopic analysis are shown in Figs. 5 and 6. The atrial myocytes from sham-operated dogs revealed a normal composition of sarcomeres distributed throughout the cell, and the intercellular space also appeared normal. After 6-week rapid atrial pacing, considerable numbers of atrial myocytes were typically affected by myolysis. With the treatment of cilazapril and valsartan, the number of both mildly and severely affected myocytes decreased dramatically, although they were still higher than that in the sham group. As an example, 4.3% of the myocytes from the right atria of sham-operated dogs showed some degree of myolysis, and 67.1% of the cardiomyocytes from dogs with 6 weeks of rapid atrial pacing showed mild or severe myolysis, whereas there were

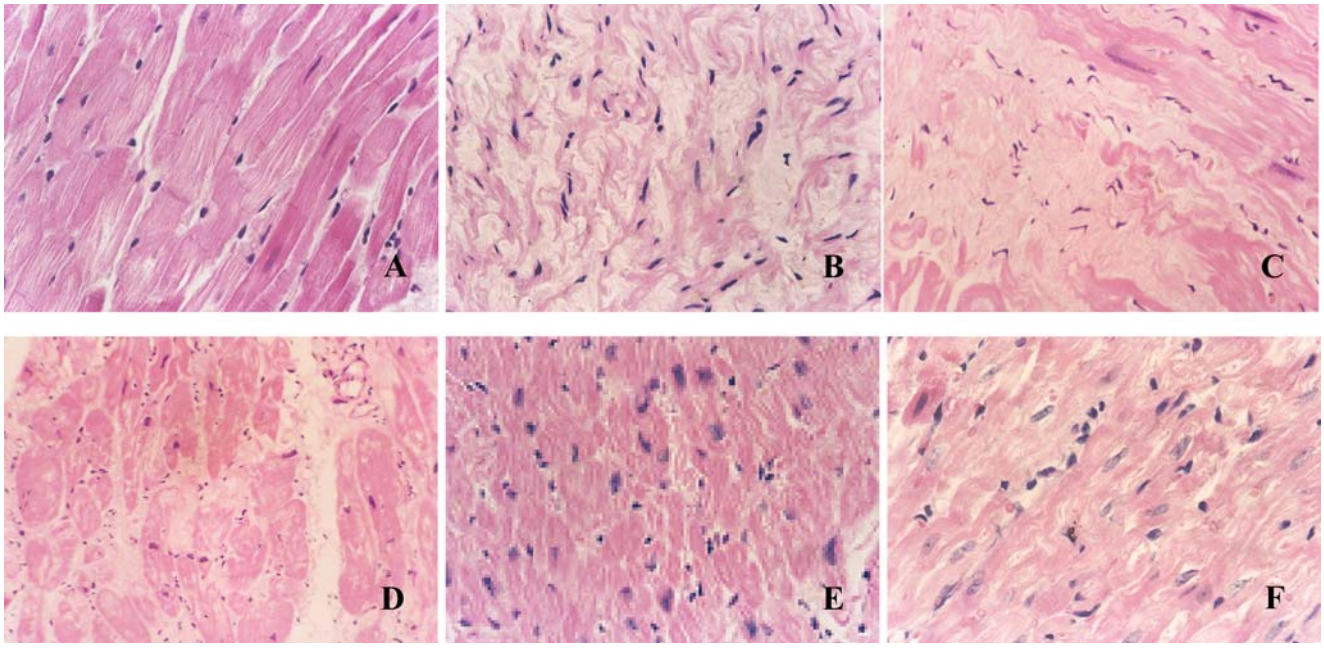

Fig. 5 Typical examples of structural changes in atrial myocytes. In the sham dogs, the composition of sarcomeres appeared normal (**A**). In the control dogs, severe myolysis (**B**), nucleus deformity (**C**), focal and early hypertrophy (**D**) were found. In cilazapril (**E**) and valsartan (**F**) treated dogs, these histopathological changes were primarily inhibited. Magnification: 1–400

only 48.8% and 45.6% of myocytes with myolysis in the cilazapril and valsartan group dogs, respectively. The myolytic area of atria was also significantly higher in chronic rapid atrial pacing dogs than that in sham dogs (24.3% vs. 3.1%, $P<0.01$). Cilazapril and valsartan could dramatically decrease the area of myolysis ($P<0.05$).

Atrial myocardial ultrastructure was examined by electron microscopy (Fig. 7). Atrial myocytes from dogs in the sham group showed a highly organized sarcomeric structure throughout the cytoplasm, with rows of normal-sized mitochondria between them and nuclei with clustered heterochromatin. Severe changes in the architecture of atrial myocytes were documented by electron microscopy after 6 weeks of atrial pacing. These changes were characterized by severe disintegration of myofilaments and mitochondrial swelling with a decrease in the density and organization of the cristae. Pyknotic nuclei with chromatin margination to nuclear membrane were also observed, indicating cell apoptosis. In contrast, the chronic tachypacing-induced ultrastructural changes were dramatically suppressed by treatment with cilazapril and valsartan.

Discussion

AF has a self-perpetuating character, with paroxysmal AF often progressing to sustained AF. This is illustrated by the fact that about 30% of patients with paroxysmal AF eventually develop persistent or even permanent AF [14]. It has been demonstrated that sustained AF causes changes in atrial electrophysiological function, a process referred to as atrial electrical remodeling, and leads to atrial morphological alterations including myolysis, apoptosis and atrial enlargement, called structural remodeling, which in turn promote the occurrence and persistence of AF [12, 34]. Atrial electrical remodeling has been shown to be completely reversible within a few days after cardioversion of AF [35]. In contrast, the recovery of atrial structural remodeling and transport function may take several months [22], some structural

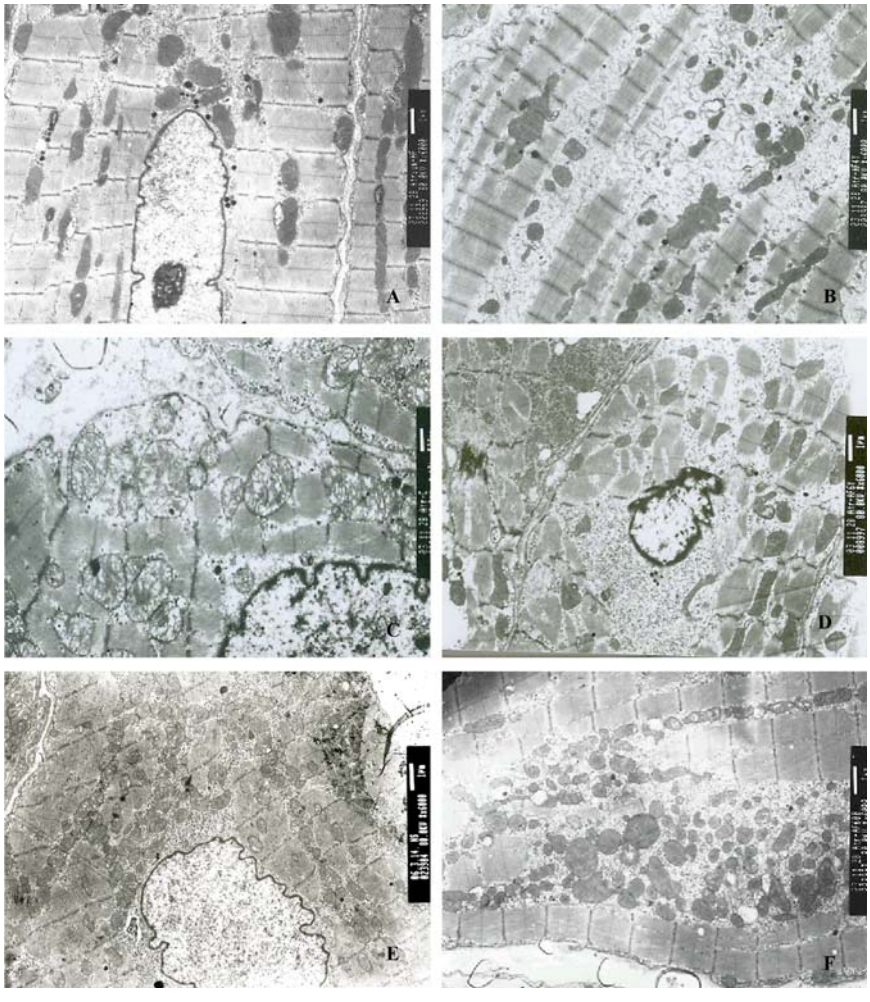

Fig. 7 Representative transmission electron micrographs of atrial myocardium. Atrial myocytes from sham-operated dogs had regular sarcomere organization, uniformly sized mitochondria. Nuclei show normal clumping of chromatin at the nuclear membrane (**A**). Samples of atrial tissue taken from control group dogs showed abnormal ultrastructure: severe disintegration of myofilaments (**B**), mitochondrial swelling with a decrease in the density and organization of the cristae (**C**), karyopyknosis with chromatin margination to nuclear membrane indicating cell apoptosis (**D**). In contrast, these chronic tachypacing-induced ultrastructural changes were markedly attenuated by the treatment of cilazapril (**E**) and valsartan (**F**). (Magnification A, B, D, E and F 6000x, C 10000x)

changes may be even irreversible [4]. Considering previous observations, the arrhythmogenic atrial structural abnormalities might play a critical role in the initiation and maintenance of chronic AF; therefore, after decades of focus on the electrical aspects of AF with unsatisfactory outcomes, recent research is focusing increasingly on atrial structural remodeling therapy.

Atrial structural and function changes in AF

Ausma et al. [5] found that atrial myolysis can be seen in a considerable percentage of atrial myocytes (up to 92%) in AF goats. Compared with sinus rate patients, the contractility of atrial muscle bundles isolated from AF patients was markedly reduced and correlated positively with the extent of atrial myolysis [26]. Atrial myolysis and replacement of sarcomeres by glycogen contribute to the delayed recovery of atrial function after conversion to sinus rhythm [26]. Schotten et al. [27] have clearly demonstrated that there is a strong correlation between the maximum force of contraction and sarcomere content in atrial muscle, loss of atrial contractility gives rise to progressive dilatation of fibrillating atria. According to multiple wavelet theory, a determinant of AF is the presence of at least 4 to 6 wandering reentrant atrial wavelets in the atria. Atrial dilatation caused by depression of atrial contraction may lead to a greater number of wave fronts circulating through the atria. Atrial size has been proved to correlate positively with the stability of AF in heart failure dogs [29]. Ak et al. [2] verified that the degree of myolysis in right atrial myocardium is a significant predictor for the development of postoperative AF in coronary artery bypass grafting patients. Our present experimental study demonstrates that compared with sham myocytes, atrial myocytes from control group dogs showed a severely reduced number of sarcomeres, dissolution and vacuolization.

Besides severe myolysis, consistent with the results reported by Aime-Sempe et al. [1], pyknotic nuclei with chromatin margination to nuclear membrane were also observed in the atria of chronic atrial tachypacing dogs indicating programmed cell death, which may lead to further contractile dysfunction. The current data show that after 6 weeks of rapid atrial pacing, the volume of LA and LAA increased significantly with a dramatic depression of atrial function, and sustained AF became inducible in all the control group dogs.

The calpain system and atrial structural remodeling in AF

The calpain system is an intracellular, strongly Ca^{2+}-dependent, neutral cysteine protease system existing in all mammalian and in some non-mammalian cells. The best characterized calpains are the widely distributed isoenzymes, calpain I, or μ-calpain, which requires micromolar intracellular calcium concentrations for half-maximum activity, which could be activated easily during AF; however, calpain II, or m-calpain, requires millimolar concentrations for activation, so stimulation of this enzyme is not expected during chronic forms of arrhythmia. Although the precise intracellular functions of the calpains have not been fully defined, a number of studies indicated their potential importance in regulated proteolysis of structural proteins and key enzymes. Brundel et al. [7] established that calpain activation is indeed a key molecular switch in AF-related atrial myocyte remodeling, and inhibition of calpain activity may be superior to verapamil treatment to prevent the myocyte remodeling processes. Consistent with the results reported by Goette et al. [15], the present research demonstrates that the gene and protein expression of calpain I increased dramatically in AF dogs.

There is evidence that calpain I is involved in cardiac remodeling in the late phase and calpain II only contributes to cardiac remodeling in the early phase [25]. The current findings imply that in contrast to calpain I, there were no dramatic differences in the expression of calpain II mRNA and protein between the sham-operated dogs and dogs with 6 weeks of atrial tachypacing.

A previous study reported that in fibrillating and dilated atria, apoptosis of myocytes contributes to cellular remodeling, which may not be entirely reversible [1]. The atrial apoptosis could be the consequence of AF, but the mechanisms underlying the cellular alterations seen in diseased atria are still poorly understood. Aime-Sempe et al. [1] found stronger caspase 3 expression and down-regulation of the antiapoptotic bcl-2 protein in fibrillating and dilated human right atria. Calpain is known to activate the downstream protease caspase 3 whose activity has been reported to be increased in chronic AF patients [1, 32]. Gil-Parrado et al. [13] also reported that ionomycin-induced calpain activation could cause a decrease of bcl-2 proteins. Thus, the up-regulation of calpain I may play a key role in promoting the execution of apoptotic cascades during AF. Further studies are still needed to clarify the detailed mechanisms.

Effects of RAS inhibitor on calpains expression and atrial structural remodeling

RAS over-activation in AF is a well-documented fact. The expression of ACE was increased three-fold in patients with chronic AF compared to patients who had no history of AF [9]. Willems et al. [33] established that the development of AF by atrial tachypacing was associated with an increase in plasma level of angiotensin II in a sheep

model. Pedersen et al. [24] observed that trandolapril could reduce the incidence of AF in patients with left ventricular dysfunction after acute myocardial infarction by 55%. ACE inhibitor therapy could inhibit the progression from paroxysmal AF to chronic AF [17]. The combination of amiodarone plus losartan could significantly decrease the rate of AF recurrences in hypertensive patients with a history of recent paroxysmal AF [11]. Sandmann et al. [25] reported that angiotensin II infusion could induce significant up-regulation of cardiac calpains in myocardial infarction rat models, which could be effectively reduced by ramipril and valsartan. Our study indicates that cilazapril and valsartan could significantly decrease gene expression and the protein level of calpain I, and diminish the area of myolysis in AF dogs.

Contard et al. [8] also found that the elevated left ventricular diastolic and/or systolic pressure during heart failure which determines the inner ventricular wall stress might be responsible for transcriptional and translational up-regulation of calpain I. Nakashima et al. [23] demonstrated that angiotensin II could significantly increase atrial pressure, leading to increased atrial wall stretch, which may be partly due to the positive inotropic effect of angiotensin II on the atrial myocardium, whereas atrial pressure did not change during rapid atrial pacing after coadministration with captopril or candesartan. It is conceivable that the inhibition effects of cilazapril and valsartan on the up-regulation of calpain I may be explained to some extent by preventing the elevation of atrial pressure [20].

Angiotensin II also could aggravate cytosolic Ca^{2+} overload by increasing the intake of extracellular calcium and promoting Ca^{2+} release from sarcoplasmic reticulum in myocytes through the activation of membrane L-type Ca^{2+} channel or phosphatidylinositol-phospholipase C pathways, then promote the activation of calpains. Touyz et al. [31] found that angiotensin II increases the intracellular Ca^{2+} concentration significantly more in atrial myocytes than in ventricular myocytes, because the density of the angiotensin II receptor in atria is generally higher than that in ventricles. The rise of intracellular calcium in myocytes has been considered to be a pivotal event in the activation of calpains leading to cardiac cell death and structural damage of myocardium during myocardial ischemia [1]. Brundel et al. [6] found that calpain I activity was significantly increased in AF patients and closely correlated with degree of structural changes. It is imaginable that treatment with an ACE inhibitor or AT_1 receptor antagonist might inhibit the activation of calpain I by preventing calcium overload. Our present data show that cilazapril and valsartan could effectively attenuate the pathohistological and ultrastructural changes, maybe partially depend on preventing intracellular calcium overload caused by RAS activation in chronic rapid atrial paced dogs.

Recent studies pointed out that the RAS plays a pivotal role in cardiac apoptosis; early treatment with quinaprilat could attenuate cardiomyocyte apoptosis in an isolated hemoperfused working porcine heart model of acute ischemia, followed by reperfusion [19]. Losartan could significantly prevent apoptosis in surfactant-depleted lungs in rats [21]. The current results of electron microscopy strongly suggest that ACE inhibitor and AT_1 receptor antagonist may provide beneficial effects on preventing atrial cell apoptosis in AF dogs which may be partly due to the inhibition of calpain I up-regulation. It has been confirmed that angiotensin II may activate p53 to mediate apoptosis [16], ACE inhibitor can attenuate programmed cardiomyocyte death by suppressing bax and p53 protein elevation and reducing caspase 3 gene expression [19]. The concrete anti-apoptosis mechanisms of RAS inhibitors in the fibrillating atria still need to be clarified.

After 6-week rapid atrial pacing, compared with the sham group dogs, the LA and LAA volume decreased significantly, the LAEF, LAAEF, V-LAA^+ and V-LAA^- increased dramatically in the dogs with the treatment of cilazapril or valsartan. These results imply that RAS inhibitor could prevent atrial dilation and function reduction in the canine pacing model. The inducibility and duration of AF were found to be markedly lower in the cilazapril and valsartan groups than that in the control group.

Potential clinical relevance

Our study for the first time demonstrated that cilazapril and valsartan could effectively prevent atrial myolysis and programmed atrial myocyte death by inhibiting calpain I up-regulation, suppress atrial structural remodeling and mechanical atrial dysfunction, and significantly decrease the inducibility and duration of AF in chronic rapid atrial pacing dogs. Hence, interference with the RAS may represent an important tool to prevent atrial structural remodeling, and perhaps a future strategy for patients with persistent AF.

Limitations of the study

Ideally, the activity of calpain I should have been determined in the present study, because only after being activated, the calpain could play the role of proteolysis in AF related atrial structural remodeling. Calpain I requires only micromolar intracellular calcium concentrations for half-maximum activity, which is expectable during AF, and in fact the activation of calpain I in animal models of AF has been demonstrated by many investigators. In this study, the causal relationship between calpain protein expression and the myolysis suggests,

but does not prove, that the activity of calpain I increased due to the rapid atrial pacing.

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References

1. Aime-Sempe C, Folliguet T, Rucker-Martin C, Krajewska M, Krajewska S, Heimburger M, Aubier M, Mercadier JJ, Reed JC, Hatem SN (1999) Myocardial cell death in fibrillating and dilated human right atria. J Am Coll Cardiol 34: 1577–1586
2. Ak K, Akgun S, Tecimer T, Isbir CS, Civelek A, Tekeli A, Arsan S, Cobanoglu A (2005) Determination of histopathologic risk factors for postoperative atrial fibrillation in cardiac surgery. Ann Thorac Surg 79:1970–1975
3. Allessie M, Ausma J, Schotten U (2002) Electrical, contractile and structural remodeling during atrial fibrillation. Cardiovasc Res 54:230–246
4. Ausma J, Van der Velden H, Lenders MH, Duimel H, Borgers M, Allessie MA (2001) Partial recovery from structural atrial remodeling after prolonged atrial fibrillation. Circulation 104 (Suppl II): II/77
5. Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, Borgers M (1997) Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. Circulation 96:3157–3163
6. Brundel BJ, Ausma J, van Gelder IC, Van der Want JJ, van Gilst WH, Crijns HJ, Henning RH (2002) Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. Cardiovasc Res 54:315–324
7. Brundel BJ, Kampinga HH, Henning RH (2004) Calpain inhibition prevents pacing-induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation. Cardiovasc Res 62:521–528
8. Contard F, Koteliansky V, Marotte F, Dubus I, Rappaport L, Samuel JL (1991) Specific alterations in the distribution of extracellular matrix components within rat myocardium during the development of pressure overload. Lab Invest 64:65–75
9. Dai Y, Wang X, Cao L,Wu T (2004) Expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. J Huazhong Univ Sci Technolog Med Sci 24:32–36
10. Everett TH 4th, Li H, Mangrum JM, McRury ID, Mitchell MA, Redick JA, Haines DE (2000) Electrical, morphological, and ultrastructural remodeling and reverse remodeling in a canine model of chronic atrial fibrillation. Circulation 102:1454–1460
11. Fogari R, Mugellini A, Destro M, Corradi L, Zoppi A, Fogari E, Rinaldi A (2006) Losartan and prevention of atrial fibrillation recurrence in hypertensive patients. J Cardiovasc Pharmacol 47:46–50
12. Frustaci A, Chimenti C, Bellocci F, Morgante E, Russo MA, Maseri A (1997) Histological substrate of atrial biopsies in patients with lone atrial fibrillation. Circulation 96:1180–1184
13. Gil-Parrado S, Fernandez-Montalvan A, Assfalg-Machleidt I, Popp O, Bestvater F, Holloschi A, Knoch TA, Auerswald EA, Welsh K, Reed JC, Fritz H, Fuentes-Prior P, Spiess E, Salvesen GS, Machleidt W (2002) Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. J Biol Chem 227: 27217–27226
14. Godtfredsen J (1975) Atrial fibrillation Etiology, Course and Prognosis. A Follow-up Study of 1212 Cases. Copenhagen: University of Copenhagen
15. Goette A, Arndt M, Rocken C, Staack T, Bechtloff R, Reinhold D, Huth C, Ansorge S, Klein HU, Lendeckel U (2002) Calpains and cytokines in fibrillating human atria. Am J Physiol Heart Circ Physiol 283:H264–H272
16. Grishko V, Pastukh V, Solodushko V, Gillespie M, Azuma J, Schaffer S (2003) Apoptotic cascade initiated by angiotensin II in neonatal cardiomyocytes: role of DNA damage. Am J Physiol Heart Circ Physiol 285: H2364–H2372
17. Hirayama Y, Atarashi H, Kobayashi Y, Horie T, Iwasaki Y, Maruyama M, Miyauchi Y, Ohara T, Yashima M, Takano T (2005) Angiotensin-converting enzyme inhibitor therapy inhibits the progression from paroxysmal atrial fibrillation to chronic atrial fibrillation. Circ J 69:671–676
18. Kinebuchi O, Mitamura H, Shiroshita-Takeshita A, Kurita Y, Ieda M, Ohashi N, Fukuda Y, Sato T, Miyoshi S, Hara M, Takatsuki S, Nagumo M, Ogawa S (2004) Oral verapamil attenuates the progression of pacing-induced electrical and mechanical remodeling of the atrium. Circ J 68:494–500
19. Kossmehl P, Kurth E, Faramarzi S, Habighorst B, Shakibaei M, Wehland M, Kreutz R, Infanger M, J Danser AH, Grosse J, Paul M, Grimm D (2006) Mechanisms of apoptosis after ischemia and reperfusion: role of the renin-angiotensin system. Apoptosis 11:347–358
20. Li Y, Li WM, Xue JY, Han W, Yang SS, Gu HY (2004) An experimental study on the effects of cilazapril on atrial remodeling in atrial fibrillation dogs. Zhonghua Xin Xue Guan Bing Za Zhi 32:1033
21. Lukkarinen HP, Laine J, Aho H, Zagariya A, Vidyasagar D, Kaapa PO (2005) Angiotensin II receptor inhibition prevents pneumocyte apoptosis in surfactant-depleted rat lungs. Pediatr Pulmonol 39:349–358
22. Manning WJ, Silverman DI, Katz SE, Riley MF, Doherty RM, Munson JT, Douglas PS (1995) Temporal dependence of the return of atrial mechanical function on the mode of cardioversion of atrial fibrillation to sinus rhythm. Am J Cardiol 75:624–626
23. Nakashima H, Kumagai K, Urata H, Gondo N, Ideishi M, Arakawa K (2000) Angiotensin II antagonist prevents electrical remodeling in atrial fibrillation. Circulation 101:2612–2617
24. Pedersen OD, Bagger H, Kober L, Torp-Pedersen C (1999) Trandolapril reduces the incidence of atrial fibrillation after acute myocardial infarction in patients with left ventricular dysfunction. Circulation 100:376–380
25. Sandmann S, Yu M, Unger T (2001) Transcriptional and translational regulation of calpain in the rat heart after myocardial infarction – effects of AT(1) and AT(2) receptor antagonists and ACE inhibitor. Br J Pharmacol 132: 767–777

26. Schotten U, Ausma J, Stellbrink C, Sabatschus I, Vogel M, Frechen D, Schoendube F, Hanrath P, Allessie MA (2001) Cellular mechanisms of depressed atrial contractility in patients with chronic atrial fibrillation. Circulation 103:691–698
27. Schotten U, de Haan S, Neuberger HR, Eijsbouts S, Blaauw Y, Tieleman R, Allessie M (2004) Loss of atrial contractility is primary cause of atrial dilatation during first days of atrial fibrillation. Am J Physiol Heart Circ Physiol 5: H2324–H2331
28. Sharma AK, Rohrer B (2004) Calcium-induced calpain mediates apoptosis via caspase-3 in a mouse photoreceptor cell line. J Biol Chem 279:35564–35572
29. Shi Y, Ducharme A, Li D, Gaspo R, Nattel S, Tardif JC (2001) Remodeling of atrial dimensions and emptying function in canine models of atrial fibrillation. Cardiovasc Res 52:217–225
30. Thijssen VL, Ausma J, Liu GS, Allessie MA, van Eys GJ, Borgers M (2000) Structural changes of atrial myocardium during chronic atrial fibrillation. Cardiovasc Pathol 9:17–28
31. Touyz RM, Sventek P, Lariviere R, Thibault G, Fareh J, Reudelhuber T, Schiffrin EL (1996) Cytosolic calcium changes induced by angiotensin II in neonatal rat atrial and ventricular cardiomyocytes are mediated via angiotensin II subtype 1 receptors. Hypertension 27:1090–1096
32. Wang KK (2000) Calpain and caspase: can you tell the difference? Trends Neurosci 23:20–26
33. Willems R, Sipido KR, Holemans P, Ector H, Van de Werf F, Heidbuchel H (2001) Different patterns of angiotensin II and atrial natriuretic peptide secretion in a sheep model of atrial fibrillation. J Cardiovasc Electrophysiol 12:1387–1392
34. Yue L, Feng J, Gaspo R, Li GR, Wang Z, Nattel S (1997) Ionic remodeling underlying action potential changes in a canine model of atrial fibrillation. Circ Res 81:512–525
35. Yu WC, Lee SH, Tai CT, Tsai CF, Hsieh MH, Chen CC, Ding YA, Chang MS, Chen SA (1999) Reversal of atrial electrical remodeling following cardioversion of long-standing atrial fibrillation in man. Cardiovasc Res 42:470–476