Abstract. In cardiac tissues, myoblast atrial myocytes continue to be exposed to mechanical forces including shear stress. However, little is known about the effects of shear stress on atrial myocytes, particularly on ion channel function, in association with disease. The present study demonstrated that the Ca\(^{2+}\)-activated K\(^+\) channel (K\(_{\text{ca}}\)2.3) serves a vital role in regulating arterial tone. As increased intracellular Ca\(^{2+}\) levels and activation of histone acetyltransferase p300 (p300) are early responses to laminar shear stress (LSS) that result in the transcriptional activation of genes, the role of p300 and the phosphoinositide3-kinase (PI3K)/protein kinase B (Akt) pathway, an intracellular pathway that promotes the growth and proliferation rather than the differentiation of adult cells, in the LSS-dependent regulation of K\(_{\text{ca}}\)2.3 in cardiac myoblasts was examined. In cultured H9c2 cells, exposure to LSS (15 dyn/cm\(^2\)) for 12 h markedly increased K\(_{\text{ca}}\)2.3 mRNA expression. Inhibiting PI3K attenuated the LSS-induced increases in the expression and channel activity of K\(_{\text{ca}}\)2.3, and decreased the phosphorylation levels of p300. The upregulation of these channels was abolished by the inhibition of Akt through decreasing p300 phosphorylation. ChIP assays indicated that p300 was recruited to the promoter region of the K\(_{\text{ca}}\)2.3 gene. Therefore, the PI3K/Akt/p300 axis serves a crucial role in the LSS-dependent induction of K\(_{\text{ca}}\)2.3 expression, by regulating cardiac myoblast function and adaptation to hemodynamic changes. The key novel insights gained from the present study are: i) K\(_{\text{ca}}\)2.3 was upregulated in patients with atrial fibrillation (AF) and in patients with AF combined with mitral valve disease; ii) LSS induced a profound upregulation of K\(_{\text{ca}}\)2.3 mRNA and protein expression in H9c2 cells; iii) PI3K activation was associated with LSS-induced upregulation of the K\(_{\text{ca}}\)2.3 channel; iv) PI3K activation was mediated by PI3K/Akt-dependent Akt activation; and v) LSS induction of K\(_{\text{ca}}\)2.3 involved the binding of p300 to transcription factors in the promoter region of the K\(_{\text{ca}}\)2.3 gene.

Introduction

A total of ~1% of the global population suffers from atrial fibrillation (AF), which is the most common clinically significant arrhythmia in adults (1). Hypertension, congestive heart failure, rheumatic and ischemic heart disease, and older age are the risk factors for AF. The 3 most important modes of treatment are rhythm control, rate control and anticoagulation therapy. Patients with AF generally suffer from increased morbidity and mortality rates due to events including ischemic stroke, heart failure and sudden cardiac death (2). Apart from increased risk of all-cause mortality (3), increased morbidity and cardiovascular mortality, AF also has a significant adverse effects on patient quality of life (4). In 2010, it was estimated that 5.2 million people in the United States of America suffered from AF, and a projected 12.1 million people are expected to suffer from AF by the year 2030 (5). Although atrial fibrosis has been associated with the AF process, little is known about the differing expression of the external cellular matrix (ECM) components collagen I, collagen III and fibronectin in the various forms of AF.

In endothelial cells (ECs), laminar shear stress (LSS) is a physiological regulator of a number of important homeostatic functions. It provokes a rapid elevation in the intracellular Ca\(^{2+}\) concentration in ECs that originates from intracellular stores and from Ca\(^{2+}\) influx (6). By controlling the production of major EC-derived relaxing factors including nitric oxide (NO) and prostacyclin, endothelial Ca\(^{2+}\)-activated K\(^+\) channels (KCa) serve an important role in regulating vasomotor tone and blood pressure (7). There are 5 KCa subtypes: KCa1.1, KCa2.1, KCa2.2, and KCa2.3, and KCa3.1 (8). In general, ECs in different regions of the vascular systems of several
previous studies have demonstrated that phosphoinositide 3-kinase (PI3K) is a cardioprotective protein that inhibits pathological signaling cascades downstream of G protein-coupled receptors (15); therefore, loss of PI3K may increase the likelihood of cardiotoxicity (16). In addition, previous data have also suggested that ibritinib increases the risk of AF, potentially by inhibiting cardiac PI3K-protein kinase B (Akt) signaling (17). The PI3K/Akt signaling pathway has certain potential advantages in this regard, due to intracellular dialysis of cardiomyocytes with phosphatidylinositol (3,4,5)-trisphosphate (PIP3) normalizes ion channels and eliminates arrhythmias. However, as the pathway regulates cell proliferation and survival by activating PIP3 downstream signals, transfusion of PIP3 at supraphysiological levels may result in abnormal cell growth that is detrimental to the cardiomyocytes. As the PI3K inhibitors used in the treatment of cancer have been identified to cause proarrhythmia, they represent a potential avenue for the study and evaluation of the potential effectiveness of a range of antiarrhythmic and anticancer drugs that are either presently in use or under development. PI3K may regulate cardiac ion channels when arrhythmias occur; however, limited information is available for PI3K/Akt signaling and arrhythmias. This demonstrates that there is a requirement to identify novel ways to improve the testing of antiarrhythmic drugs and increase understanding of PI3K/Akt signaling and arrhythmia (18).

Based on the aforementioned data, we hypothesized that the expression of major ECM proteins in left atrial tissue differs in patients with alterations in sinus rhythm (SR), AF alone and AF with underlying mitral valve disease (MVD). In addition, we hypothesized that KCa2.3 and the PI3K/Akt pathway may serve crucial roles in the AF process, and we hypothesized that the aberrant expression of KCa2.3 induced by LS is regulated by the PI3K/Akt signaling pathway.

Materials and methods

Patients. Patients with AF alone (n=8; 4 females and 4 males, age range, 48-72; left atrial diameter range, 43-57 mm) and patients with AF combined with MVD (n=6; 3 MVD- paroxysmal AF and 3 MVD-chronic AF, age range, 33-74; left atrial diameter range, 47-59 mm) were included. The control group consisted of patients with a normal SR (n=6; 3 females and 3 males; age range, 41-69; left atrial diameter range, 30-41 mm) and were matched to the AF groups according to age, left atrial size and left ventricular function. All patients provided written informed consent to participate in the present study. The Institutional Ethical Committee of the First Affiliated Hospital of Kunming Medical University approved the study, and the study was performed in concordance with the principles outlined in the Declaration of Helsinki. Atrial tissue from all patients was obtained from the left atrial free wall near the interatrial septum during cardiac surgery and was quickly placed in formaldehyde solution at room temperature, or frozen in liquid nitrogen and stored at -80°C until use.

Hematoxylin and eosin (H&E) staining, Masson staining and immunofluorescence. Left atrial tissue was fixed in 4% paraformaldehyde for 30 min at 4°C, embedded in paraffin, and transected into 4-µm thick sections along the center of the tissue. Under a light microscope (magnification, x20) formalin-fixed paraffin-embedded tissues were respectively stained with H&E and Masson's trichrome stain according to the manufacturer's protocols (cat. no. G134; Beijing Solarbio Science & Technology, Beijing, China). Following this, all collagen fibers were stained blue, and cardiomycocytes appeared red. Fibrous tissue areas were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) (19). The tissue expression of KCa2.3 was assessed by immunofluorescence. Briefly, glass covers were fixed on the tissue cross-section with 4% paraformaldehyde for 15 min at room temperature. Samples were then permeated by 0.3% Triton™ X-100 for 20 min at room temperature and blocked using PBS containing 5% donkey serum (ab7475; Abcam, Cambridge, MA, USA) and 1% BSA for 1 h at room temperature to reduce nonspecific reactions. Then, an antibody against KCa2.3 (1:500, ab28631; Abcam) overnight followed by incubation at 4°C with a fluorescein isothiocyanate-conjugated rabbit anti-human antibody (1:100; Anttene, Wuhan, China) for 2 h. DAPI at a final concentration of 0.5 μg/ml (Beyotime Institute of Biotechnology, Haimen, China) was used to stain the cell nuclei for 5 min at room temperature. Immunofluorescence was visualized using a confocal microscope (magnification, x20; Leica Microsystems, Inc., Buffalo Grove, IL, USA) to detect the expression of KCa2.3 in the left atrial tissue using ImageJ software [v.1.8; National Institutes of Health (NIH), Bethesda, MD, USA].

H9c2 cell culture. H9c2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in a standard humidified incubator at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (SH3008101, HyClone; GE Healthcare Life Sciences, Logan, UT, USA) for a 48 h starvation period prior to use in experiments to eliminate KCa induction by growth factors and to minimize background signaling. H9c2 cells at passages 2-4 were used in the experimental protocols.

Shear stress studies. H9c2 cells grown to 60% confluence were exposed to static culture conditions (ST) or to a laminar shear stress condition (LS) using a computer-controlled shearing device at 15 dynes/cm² for 12 h. In certain experiments, the cells were pretreated for 30 min with 20 nM dactolisib, a specific PI3K inhibitor, 10 nM GSK690693, a specific Akt inhibitor or 400 nM C646, a histone acetyltransferase p300 (p300) inhibitor binding to transcription factors, prior to exposure to LS.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using the RNASimple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to
the manufacturer's protocol. β-actin was used as a control. Genomic DNA was amplified by RT-qPCR using specific gene amplification primers. The forward and reverse PCR primers for measuring gene expression are summarized in Table I. The samples were amplified in an RT-qPCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with a SYBR®-Green detection system (cat. no. 204141; Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR parameters were denaturation at 95˚C for 5 min; followed by 40 cycles of (95˚C for 15 sec, 55˚C for 30 s and 72˚C for 15 sec) and another 60˚C for 5 min. To assess the amplification of specific transcripts, melting curve profiles were generated at the end of each PCR assay. All reactions were in triplicate, including controls without added template. The relative expression of gene of interest was calculated using the 2-ΔΔct method (20), where ΔΔcq is the difference between the Δcq of the two cDNA samples being compared.

**Western blot analysis.** Western blot analysis was used to evaluate the expression of KCa2.3 protein. In brief, the cells were washed three times with ice-cold TBS and lysed in 200 µl lysis buffer (cat. no. N8031; Beijing Solarbio Science & Technology). Through a single round of freeze-thawing, the lysates were additionally homogenized. The protein concentration of each sample was quantified using an ND-1000 spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 10 µl proteins/lane from each sample remaining portion was divided into aliquots and incubated overnight at 4˚C followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1,000, SE134; Beijing Solarbio Science & Technology) at room temperature for 1 h. Protein expression was detected by chemiluminescence (ImageJ v.1.8; NIH).

**Chromatin immunoprecipitation (ChIP) assay.** To determine the binding of nuclear proteins to the KCa2.3 promoter, ChIP assays were performed as described previously (21). Briefly, 80% confluent H9c2 cells were treated with 4% paraformaldehyde for 20 min at room temperature and dissolved in ChIP buffer (50 mM Tris-HCl pH 7.9, 150 mM NaCl, 5 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM PMSF and protease inhibitor). These fixed cells were washed and lysed in SDS-lysis buffer (cat. no. 20-163; EMD Merck Millipore, Billerica, MA, USA) and sonicated with a frequency of 22.5KHz on ice until the DNA size was decreased to 200-800 bp. Following centrifugation at 10,000 x g for 2 min for 30 min at 4˚C with rotation, a portion of the pre-cleared solution was used as a DNA input control; the remaining portion was divided into aliquots and incubated with specific primary antibodies against p300 (1:2,000, cat. no. 86377; Cell Signaling Technologies, Inc.) and β-actin (1:1,500, sc-47778; Santa Cruz Biotechnology, Inc.) rabbit pre-immune IgG (1 µg/1 µl lysis buffer) at 4˚C for 8 h. The immunoprecipitated complexes of Ab-protein-DNA were collected and washed with low-salt buffer, high-salt buffer, LiCl buffer and Tris-EDTA, and then buffered with an elution buffer. The chromatin immunoprecipitation (ChIP) assay was performed using a PCR amplification as aforementioned using the 2-ΔΔcq method (20), where ΔΔcq is the difference between the Δcq of the two cDNA samples being compared.

### Table I. Primers sequence used in the present study.

| Gene name | Primers | Product length, bp |
|-----------|---------|--------------------|
| PI3K      | Forward: 5'-GATGTGGTCTTCTTCTGTC-3'  <br> Reverse: 5'-CCACCCCTATCAATTACACACA-3' | 324 |
| Akt       | Forward: 5'-GCACACACGAGGGAGTAC-3'  <br> Reverse: 5'-CCTCAGTGGTCCACATC-3' | 113 |
| p300      | Forward: 5'-AGTCCAGTAAATGCGTGC-3'  <br> Reverse: 5'-AATCCTTTGTCCTCCCATG-3' | 255 |
| KCa2.3    | Forward: 5'-CTTAACTACAGAATCAATG-3'  <br> Reverse: 5'-TTAGCAAATCTGATTCAGG-3' | 178 |
| β-actin   | Forward: 5'-TTCCAGCCTTCTTCTTG-3'  <br> Reverse: 5'-GGAGCCAGACAGCTATC-3' | 153 |

**PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; p300, histone acetyltransferase p300; KCa2.3, Ca2+-activated K+ channels.**
products were analyzed on agarose gels stained with ethidium bromide and analyzed with ImageJ (v.1.8; NIH).

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analysis was assessed by Student's t-test or one-way analysis of variance with a Dunnett's post hoc test and a nonparametric Kruskal-Wallis for data that are not distributed normally, where appropriate. All statistical procedures were performed using GraphPad Prism 5.0. (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Atrial structural remodeling. As presented in Fig. 1, according to the Masson trichrome staining, patients with AF exhibited significant interstitial fibrosis. The degree of atrial fibrosis in the AF and AF combined with MVD groups was significantly increased compared with that in the SR group. Specifically, the average degree of atrial fibrosis in patients with SR, AF and AF combined with MVD was 6.17±1.07, 40.6±2.8 and 60.63±6.93, respectively (P<0.05; Fig. 1).

KCa2.3 is highly expressed in patients with AF and AF combined with MVD. To investigate KCa2.3 gene expression, RNA was extracted from clinical tissues. KCa2.3 expression was initially investigated in samples from the three groups; significantly increased expression levels in the AF and AF combined with MVD groups compared with the SR group [a 2.66-fold increase (P=0.006) and a 3.1-fold increase (P=0.005), respectively] were observed (Fig. 2A). As the KCa2.3 protein has a specific function in regulating biological processes, KCa2.3 protein expression in the three groups was additionally investigated by western blot analysis. As indicated in Fig. 2B, the expression of KCa2.3 protein was significantly increased in the AF and AF combined with MVD groups compared with the control. Then, KCa2.3 protein expression was detected by immunofluorescence. As demonstrated in Fig. 2C, the expression of KCa2.3 was upregulated in the AF and AF combined with MVD groups compared with the control. In addition, to elucidate the molecular mechanisms underlying LS-induced atrial remodeling in patients with AF, the levels of the pivotal signal pathway initiator PI3K were investigated. Compared with the SR control, AF significantly altered the levels of PI3K protein expression. In the patients in the AF and AF combined with MVD groups, PI3K expression was significantly increased by 2.38- and 2.93-fold, respectively, compared with the control group (both P<0.05; Fig. 2D).

LS increases PI3K and KCa2.3 expression. The effect of shear stress on the expression of KCa2.3 in H9c2 cells was determined by RT-qPCR and western blot analysis. Exposure to LS (15 dynes/cm²) for 12 h markedly changed the morphology of the cells (Fig. 3A) and increased KCa2.3 mRNA and protein expression (1.16- and 0.91-fold, respectively; both P<0.01; Fig. 3B and C). The effect of LS on KCa2.3 expression was dose- and time-dependent; exposure to lower LS (5 dynes/cm²) for 12 h resulted in a moderate induction of KCa2.3, whereas exposure to LS at 15 dynes/cm² for 6 or 24 h decreased KCa2.3 expression (data not shown). To elucidate the potential
Figure 2. KCa2.3 gene expression analysis in clinical samples. (A) 2.66- and 3.1-fold increases in KCa2.3 expression levels were observed in the AF (n=8) and AF combined with MVD (n=6) groups compared to the SR groups (n=6; *P=0.006 and 0.005, respectively). (B) Increases in 85% and 63% in KCa2.3 expression was observed in the AF (n=8) and AF combined with MVD (n=6) groups compared to the SR group (n=6; **P<0.001 and ***P=0.004, respectively). (C) Expression levels of KCa2.3 in SR, AF and AF combined with MVD groups were examined by immunofluorescence. Fluorescence was observed by laser-scanning microscopy. Scale bars, 50 µm. (D) Western blot analysis was performed to detect the protein expression of PI3K in patients with SR, AF and AF combined with MVD; β-actin was used as an internal reference protein. Densitometric quantification of the western blot analysis results are presented; the bars represents the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. SR group. Kca, Ca2+-activated K+ channels; SR, sinus rhythm; AF, atrial fibrillation; MVD, mitral valve disease.

Figure 3. LS increases KCa2.3 partially via regulating PI3K expression. The effect of shear stress on the expression of KCa2.3 in H9c2 cells was determined by reverse transcription quantitative polymerase chain reaction and western blot analysis. (A) Exposure to LS (15 dynes/cm2) for 12 h markedly changed the morphology of the cells. (B) KCa2.3 mRNA levels were increased by 1.16-fold following LS. (C) KCa2.3 protein expression was increased by 0.91-fold following LS. (D) Exposure to LS (15 dynes/cm2) for 12 h upregulated the PI3K protein expression by 3.07-fold compared with the ST condition (P=0.0016). *P<0.01 vs. ST group. Kca, Ca2+-activated K+ channels; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; LS, laminar shear stress condition; ST, static culture conditions.
mechanisms by which the LS induced the KCa2.3 expression, based on the data obtained from the clinical samples, the PI3K expression between ST and LS exposure was also detected. As indicated in Fig. 3D, exposure to LS (15 dynes/cm²) for 12 h upregulated PI3K protein expression by 3.07-fold (P=0.0016) compared to the ST condition. Taken together, these data indicate that LS increases PI3K and KCa2.3 expression levels. Next, the subsequent studies were performed at the arterial level of LS (15 dynes/cm²) to determine the signaling pathway responsible for the physiological induction of these channels.

**PI3K/Akt/p300 axis activation mediates the LS-induced increase in KCa2.3.** The role of the PI3K/Akt/p300 signaling pathway in the LS-induced upregulation of KCa2.3 was examined. H9c2 cells were exposed to LS for 12 h in the absence or presence of 20 nM dactolisib, a PI3K inhibitor, 10 nM GSK690693, a specific Akt inhibitor or 400 nM C646, a p300 inhibitor. (A) Dactolisib decreased the ratio of PI3K. (B) Dactolisib decreased the ratios of p-Akt/Akt and p-p300/p300 and KCa2.3 expression levels by 27, 22 and 33%, respectively (all P<0.05). (C) GSK690693 decreased Akt expression. (D) GSK690693 had no effect on PI3K expression under LS conditions (P=0.063). However, GSK690693 decreased the ratio of p-p300/p300 ratio and KCa2.3 protein expression by 48 and 37%, respectively (both P<0.001). (E) Following the addition of C646, an inhibitor of p300, western blot analysis indicated that p300 protein levels decreased by 38% (P<0.001). (F) Following treatment with C646, no change in the PI3K expression or in the ratio of p-Akt/Akt was observed. **P<0.01 and ***P<0.001 vs. H9c2-LS group. PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; p300, histone acetyltransferase p300; p, phosphorylated; LS, laminar shear stress condition; KCa, Ca²⁺-activated K⁺ channels.

Recruitment of p300 to the KCa2.3 promoter is essential for LS-induced KCa2.3 upregulation. A previous study has suggested that phosphorylation of p300 is required for KCa2.3 transcription activation (8). In addition, activation of p300 may be regulated by Akt (22). Another study has demonstrated that p300 is required for the expression of certain genes (21). As demonstrated in Fig. 4F, pretreatment of H9c2 cells with C646 significantly inhibited LS-induced KCa2.3 expression in a...
time-dependent manner, suggesting that p300 may be involved in the regulation of the H9c2 promoter. Therefore, the aim of the present study was to investigate the hypothesis that recruitment of p300 is regulated by the PI3K/Akt cascade in LS-stimulated H9c2 cells. To investigate whether LS stimulated p300 recruitment to the Kca2.3 promoter region, recruitment of p300 was examined using a chIP-qPCR assay (Fig. 5A). A total of 31 cycles of qPCR amplifications were conducted using equal amounts of immunoprecipitated DNA in the presence of specific primer pairs encompassing the region between positions -688 and -203 bp of the rat Kca2.3 promoter (Fig. 5B-a). Following LS treatment, recruitment of p300 to the Kca2.3 promoter region was induced in a time-dependent manner; recruitment increased within 4 h and was sustained until at least 12 h (Fig. 5B-b). These results indicated that LS-induced Kca2.3 expression required the recruitment of p300 in H9c2 cells.

Discussion

There were 5 major novel insights gained from the present study. Firstly, KCa2.3 was upregulated in patients with AF and in patients with AF combined with MVD. Secondly, LS induced a marked upregulation of Kca2.3 mRNA and protein expression in H9c2 cells. Thirdly, PI3K activation was associated with LS-induced upregulation of the KCa2.3 channel. Fourthly, this upregulation was mediated by PI3K/Akt-dependent Akt activation. Finally, LS induction of Kca2.3 involved the binding of p300 to transcription factors in the promoter region of the Kca2.3 gene.

AF is the most common arrhythmia in humans. It affects 5% of the population >65 years of age, and its incidence is projected to increase as the mean population age increases (23). Experimental data from animal models of AF indicate that AF is associated with progressive structural and electrical remodeling of the atria. Atrial structural remodeling is characterized by atrial enlargement and interstitial fibrosis (24) and has been considered a major contributor to AF (25). Increased fibrosis has been observed in the atria of patients with AF (26). It is characterized by enhanced deposition of matrix collagen proteins; this leads to inhomogeneous atrial electrical conduction, and results in electrical reentry circuits that result in AF (27). Atrial fibrosis alters atrial electrical...
conduction and excitability and provides a substrate for AF maintenance. As a hallmark of atrial structural remodeling, atrial fibrosis serves a critical role in the maintenance of chronic AF. However, whether fibrosis is causally associated with AF or an epiphenomenon, and the precise mechanisms underlying atrial fibrosis, remain uncertain. The results of the present study suggest that the percentage of fibroblasts in patients with AF and AF combined with MVD is increased compared that in patients with SR (~10-fold), suggesting a complicated association between atrial fibrosis and AF, consistent with the results of previous studies (28).

In the present study, it was demonstrated that PI3K was upregulated in patients with AF and in patients with AF combined with MVD, indicating that PI3K may be involved in the increased KCa2.3 expression observed in these patients. An overexpression of the KCa2.3 channel may affect the vascular structure of the heart during development (29). In cardiac muscle, blockers of KCa2.3 channels have been demonstrated to prevent atrial fibrillation (13,14), but at present it is unknown whether specific openers of KCa2.3 channels will incur pro-arrhythmic effects. It was noted that previous studies conducted by Pretorius et al (30), revealed that PI3K activity was decreased in atrial samples from patients with acute or chronic AF compared with patients without AF, which is markedly different from the results from the present study. The disparity between the data from Pretorius et al (30) and the present study were examined, and the potential explanations include, but are not limited to: i) A small sample size in the present study and that of Pretorius et al (30). Only 4 patients with AF and 6 patients with SR were included in the study by Pretorius et al (30), and 8 patients with AF and 6 patients with SR were included in the present study. Statistically, larger sample sizes would yield more accurate results, and studies with small sample sizes may provide more deviation; ii) the disparate sex distribution of the study samples may have contributed to the differences in the results. In the present study, the ratios of females to males in the AF and SR groups were 1:1, whereas in the study of Pretorius et al (30), the sex distribution was markedly disparate (3 females and 1 male in the AF group, and 1 female and 5 males in the SR group). This may lead to markedly different results, as sex is an independent contributory factor in AF (30-32); iii) race and ethnicity differences. The present study was performed in a Chinese population, whilst the study by Pretorius et al (30), was undertaken in an Australian population; this may have resulted in different findings as race may also contribute to the AF process (33). Other studies have demonstrated that mice with decreased cardiac PI3K-Akt activity were highly susceptible to AF and concluded that ibrutinib increased the risk of AF partially via inhibition of the cardiac PI3K-Akt pathway (17,30), was also considered. We hypothesized that additional data should be collected to achieve an improved understanding of the potential association between AF and PI3K as the specific electrophysiological properties of cardiac cells may lead to major functional differences between human and nonhuman hearts (34,35).

In fact, although cyclic stretch and hydrostatic pressure affect the function of ECs, Ohashi et al (36) concluded that physiological shear stress is dominant to physiological hydrostatic pressure up to 100 mmHg, suggesting the relative contribution of physiological hydrostatic pressure and fluid shear stress to endothelial monolayer integrity. Concomitantly, the aim of the present study was to reveal the potential role of shear stress in inducing AF; therefore, all experiments were focused on shear stress only. Previous studies have indicated that shear stress may induce mechanotransduction in vascular endothelial cells (37) and three-way activation of mechanically sensitive ion channels, including direct physical effects mediated by shear stress, changes in the mechanical tension of the cytoskeleton and the changes in cell membrane fluidity induced by shear stress (38). Akt is a major regulator of vital cellular processes including cell cycle progression, growth and survival, and activation of extracellular signal-regulated PI3K/Akt signaling pathways has been suggested to be critical for increased KCa2.3 channels expression. As calcium-activated, voltage-gated SK3 potassium channels, KCa2.3 channels function as major regulators of Ca2+ transport through the cell membrane (39,40). As demonstrated in the present study, treating H9c2 cells with an Akt inhibitor decreased Akt protein expression and subsequently decreased LS-induced KCa2.3 expression in H9c2 cells. These results are consistent with those of other previous studies, in which it was demonstrated that Akt is a key regulator for expression of KCa2.3 (39,40).

Although novel therapeutics for the treatment of AF are undergoing experimental and clinical evaluation, small molecule signal transduction inhibitors are the most promising class of agents. However, the involvement of PI3K/Akt/p300 in LS-induced KCa2.3 expression in H9c2 cells remains unknown. To the best of our knowledge, the results of the present study demonstrated for the first time that in H9c2 cells, LS-induced KCa2.3 expression was mediated through the activation of PI3K/Akt and downstream transcriptional co-factor p300, as revealed by the pharmacological inhibitors dactolisib, GSK690693 and C646. These data not only confirm the results of previous studies indicating that LS may induce KCa2.3 expression (8,13), but also reveal the potential underlying mechanisms by which LS induces its expression. Dai et al (41) suggested that the PI3K-dependent pathway participates in the regulation of nuclear factor erythroid 2-related factor 2 nuclear translocation and binding to cofactors in the LS-induced condition, which provides insight into the potential roles of LS in inducing transcription factor translocation or recruitment. Therefore, the present study utilized ChIP assays to determine whether LS-induced KCa2.3 expression is regulated via p300, one of the cofactors in KCa2.3 transcription. The results suggested that, under the condition of 15 dynes/cm2, the amount of PCR product increased gradually in a time-dependent manner, suggesting that p300 is the key regulator of KCa2.3 gene expression. Based on the data from the present study, useful clinical trials aiming to assess the efficacy of PI3K/Akt/p300 inhibitors in patients with AF should be performed, and we propose that these data will provide substantial information regarding the importance of this novel pathway in patients with AF and AF combined with MVD.

In conclusion, the present study revealed that atrial fibrosis in patients with AF and AF combined with MVD is more serious than that in patients with SR, and that KCa2.3 was upregulated in patients with AF and in those with AF combined with MVD. In addition, based on the data obtained from clinical samples, the significant upregulation of KCa2.3 mRNA...
and protein expression in H9c2 cells was identified. Subsequent experiments revealed that this upregulation was mediated by PI3K/Akt-dependent Akt activation, and that LS induction of KCa2.3 involves the binding of p300 to transcription factors in the promoter region of KCa2.3 gene. Taken together, these data suggest that a PI3K/Akt/p300 cascade mediates LS induction of KCa2.3. Through understanding this potentially crucial pathway involved in AF and AF combined with MVD, rational drug designs may be proposed for these molecules, which are critical to cell signaling. Based on the specific expression of molecular targets, clinicians will then be able to appropriately individualize treatment for patients with AF.

Acknowledgements

Not applicable.

Funding

The present study was funded by the National Natural Science Foundation of China (grant no. 31360227) and the Yunnan Province-Kuming Medical University Joint Foundation for Applied Basic Research (grant no. 2017NS015).

Availability of data and materials

The datasets used in the present study are available from the corresponding author on reasonable request.

Authors’ contributions

GL, QY and YY contributed to the design of the study and drafted the manuscript. GY and LD performed the experiments. JW, ZM, YS and GZ made substantial contributions to the analysis of data, revised and amended the manuscript. All authors have read and approved this manuscript.

Ethics approval and consent to participate

All patients provided written informed consent to participate in the present study. The Institutional Ethical Committee of the First Affiliated Hospital of Kunming Medical University approved the study, and the study is in concordance with the principles outlined in the Declaration of Helsinki.

Patient consent for publication

All patients provided written informed consent to participate in the present study.

Competing interests

The authors declare that they have no competing interests.

References

1. Go AS, Hylek EM, Phillips KA, Chang Y, Renault LE, Selby JV and Singer DE: Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study. JAMA 285: 2370-2375, 2001.

2. Chugh SS, Havmoeller R, Narayanam K, Singh D, Rienstra M, Benjamin EJ, Gillum RF, Kim YH, MacAnulty JH Jr, Zheng ZJ, et al: Worldwide epidemiology of atrial fibrillation: A Global Burden of Disease 2010 study. Circulation 129: 837-847, 2014.

3. Benjamin EJ, Wolf PA, D’Agostino RB, Silbershatz H, Kannel WB and Levy D: Impact of atrial fibrillation on the risk of death: The Framingham Heart Study. Circulation 98: 946-952, 1998.

4. Ynsaurriaga FA, Peinado RP and Ormaetxe Merodio JM: Atrial fibrillation and quality of life related to disease and treatment: Focus on anticoagulation. Future Cardiol 10: 381-393, 2014.

5. Colilla S, Crow A, Petkun W, Singer DE, Simon T and Liu X: Estimates of current and future incidence and prevalence of atrial fibrillation in the U.S. adult population. Am J Cardiol 112: 1144-1147, 2013.

6. Kwan HY, Leung PC, Huang Y and Yao X: Depletion of intracellular Ca²⁺ stores sensitizes the flow-induced Ca²⁺ influx in rat endothelial cells. Circ Res 92: 286-292, 2003.

7. Grgic I, Kaistha BP, Hoyer J and Köhler R: Endothelial Ca²⁺-activated K⁺ channels in normal and impaired EDHF-dilator responses—relevance to cardiovascular pathologies and drug discovery. Br J Pharmacol 157: 509-526, 2009.

8. Takai J, Santu A, Zheng H, Koh SD, Otta M, Filinbman LM, Lemaitre V, Teraoka R, Jo H and Miura H: Laminar shear stress upregulates endothelial Ca²⁺-activated K⁺ channels KCa2.3 and KCa3.1 via a Ca²⁺-calmodulin-dependent protein kinase kinase/Calcium/Akt/p300 cascade. Am J Physiol Heart Circ Physiol 305: H484-H493, 2013.

9. Ledoux J, Werner ME, Brayden JE and Nelson MT: Calcium-activated potassium channels and the regulation of vascular tone. Physiology (Bethesda) 21: 69-78, 2006.

10. Bährler S, Kaistha A, Schmidt VJ, Wölflse SE, Busch C, Kaistha BP, Kacik M, Hasenau AL, Grgic I, Si H, et al: Genetic deficit of SK3 and IK1 channels disrupts the endothelium-derived hyperpolarizing factor vasodilator pathway and causes hypertension. Circulation 119: 2323-2332, 2009.

11. Milkau M, Köhler R and de Wit C: Crucial importance of the endothelial K⁺ channel SK3 and connexin40 in arteriolar dilations during skeletal muscle contraction. FASEB J 24: 3572-3579, 2010.

12. Si H, Heyken WT, Wölflse SE, Tysiak M, Schubert R, Grgic I, Vilianovich L, Giebing G, Maier T, Gross V, et al: Impaired endothelium-derived hyperpolarizing factor-mediated dilations and increased blood pressure in mice deficient of the intermediate-conductance Ca²⁺-activated K⁺ channel. Circ Res 99: 537-544, 2006.

13. Dines JG, Skihsbeylje L, Jespersen T, Bartels ED, Sørensen US, Hansen RS and Grunnet M: Effects on atrial fibrillation in aged hypertensive rats by Ca²⁺-activated K⁺ channel inhibition. Hypertension 57: 1129-1135, 2011.

14. Qi XY, Dines JG, Brandel BJ, Zhou XB, Naud P, Wu CT, Huang H, Harada M, Allak M, Dobrev D, et al: Role of small-conductance calcium-activated potassium channels in atrial electrophysiology and atrial fibrillation in the dog. Circulation 129: 830-840, 2014.

15. McMullen JR, Amriahmadi F, Woodcock EA, Schinke-Braun M, Bouwman RD, Hewitt KA, Mollica JP, Zhang L, Zhang Y, Shiio T, et al: Protective effects of exercise and phosphoinositide 3-kinase(p110alpha) signaling in dilated and hypertrophic cardiomyopathy. Proc Natl Acad Sci USA 104: 612-617, 2007.

16. Rossello X, Riquelme JA, He Z, Taferner S, Vanhaesebroeck B, Davidson SM and Yellon DM: The role of PI3Kα in isofrom protection in cardioprotection. Basic Res Cardiol 112: 66, 2017.

17. McMullen JR, Boey EJ, Ooi JY, Seymour JF, Keating MJ and Tam CS: Ibrutinib increases the risk of atrial fibrillation, potentially through inhibition of cardiac PI3K-Akt signaling. Blood 124: 3829-3830, 2014.

18. Ezeani M and Elom S: Necessity to evaluate PI3K/Akt signalling pathway in proarrhythmia. Open Heart 4: e000596, 2017.

19. Seyed Jafari SM and Hunger RE: IHα Optical Density Score: A new practical method for quantitative immunohistochemistry image analysis. Appl Immunohistochem Mol Morphol 25: e12-e13, 2017.

20. Seyed Jafari SM and Hunger RE: IHα Optical Density Score: A new practical method for quantitative immunohistochemistry image analysis. Appl Immunohistochem Mol Morphol 25: e12-e13, 2017.

21. Seyed Jafari SM and Hunger RE: IHα Optical Density Score: A new practical method for quantitative immunohistochemistry image analysis. Appl Immunohistochem Mol Morphol 25: e12-e13, 2017.
22. Huang WC and Chen CC: Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. Mol Cell Biol 25: 6592-6602, 2005.

23. Zoni-Berisso M, Lercari F, Carazza T and Domenicucci S: Epidemiology of atrial fibrillation: European perspective. Clin Epidemiol 6: 213-220, 2014.

24. Nattel S and Harada M: Atrial remodeling and atrial fibrillation: Recent advances and translational perspectives. J Am Coll Cardiol 63: 2335-2345, 2014.

25. Iwasaki YK, Nishida K, Kato T and Nattel S: Atrial fibrillation pathophysiology: Implications for management. Circulation 124: 2264-2274, 2011.

26. Frustaci A, Chimenti C, Bellucci F, Morgante E, Russo MA and Maseri A: Histological substrate of atrial biopsies in patients with lone atrial fibrillation. Circulation 96:1180-1184, 1997.

27. Friederichs K, Baldus S and Klinke A: Fibrosis in atrial fibrillation - role of reactive species and MPO. Front Physiol 3: 214, 2012.

28. Tan AY and Zimetbaum P: Atrial fibrillation and atrial fibrosis. J Cardiovasc Pharmacol 57: 625-629, 2011.

29. Wandall-Frostholm C, Skaarup LM, Sadda V, Nielsen G, Hedegaard ER, Mogensen S, Köhler R and Simonsen U: Pulmonary hypertension in wild type mice and animals with genetic deficit in KCa2.3 and KCa3.1 channels. PLoS One 9: e97687, 2014.

30. Pretorius L, du XJ, Woodcock EA, Kiriazis H, Lin RC, Marasco S, Medcalf RL, Ming Z, Head GA, Tan JW, et al: Reduced phosphoinositide 3-kinase (p110alpha) activation increases the susceptibility to atrial fibrillation. Am J Pathol 175: 998-1009, 2009.

31. Fang MC, Singer DE, Chang Y, Hylek EM, Renault LE, Jensvold NG and Go AS: Gender differences in the risk of ischemic stroke and peripheral embolism in atrial fibrillation: The AnTicoagulation and Risk Factors In Atrial Fibrillation (ATRIA) study. Circulation 112: 1687-1691, 2005.

32. Dagres N, Nieuwlaat R, Vardas PE, Andresen D, Lévy S, Cobbe S, Kremastinos DT, Breithardt G, Cokkinos DV and Crijs HJ: Gender-related differences in presentation, treatment, and outcome of patients with atrial fibrillation in Europe: A report from the Euro Heart Survey on Atrial Fibrillation. J Am Coll Cardiol 49: 572-577, 2007.