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Laminar shear stress alters endothelial KCa2.3 expression in H9c2 cells partially via regulating the PI3K/Akt/p300 axis

GUOJIAN LI[1*], QIONGHUI YANG[2*], YONG YANG[1], GUOKAI YANG[1], JIA WAN[1], ZHENHUAN MA[1], LINGJUAN DU[1], YI SUN[3] and GUIMIN ZHANG[3]

[1]Department of Vascular Surgery, The Second People's Hospital of Yunnan Province, Kunming Medical University; [2]Department of Pharmacy, The Third People's Hospital of Yunnan Province, Kunming, Yunnan 650200; [3]Department of Cardiovascular Surgery, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650021, P.R. China

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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 (KCa)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 KCa2.3 in cardiac myoblasts was examined. In cultured H9c2 cells, exposure to LSS (15 dyn/cm^2) for 12 h markedly increased KCa2.3 mRNA expression. Inhibiting PI3K attenuated the LSS-induced increases in the expression and channel activity of KCa2.3, 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 KCa2.3 gene. Therefore, the PI3K/Akt/p300 axis serves a crucial role in the LSS-dependent induction of KCa2.3 expression, by regulating cardiac myoblast function and adaptation to hemodynamic changes. The key novel insights gained from the present study are: i) KCa2.3 was upregulated in patients with atrial fibrillation (AF) and in patients with AF combined with mitral value disease; ii) LSS induced a profound upregulation of KCa2.3 mRNA and protein expression in H9c2 cells; iii) PI3K activation was associated with LSS-induced upregulation of the KCa2.3 channel; iv) PI3K activation was mediated by PI3K/Akt-dependent Akt activation; and v) LSS induction of KCa2.3 involved the binding of p300 to transcription factors in the promoter region of the KCa2.3 gene.

Correspondence to: Dr Yi Sun and Dr Guimin Zhang, Department of Cardiovascular Surgery, The First Affiliated Hospital of Kunming Medical University, 772 Xichang Road, Kunming, Yunnan 650021, P.R. China
E-mail: sunyi20082018@163.com
E-mail: zhangguimin2007@163.com

[*]Contributed equally

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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

species constitutively and predominantly express KCa2.3 and KCa3.1 (9). Previous studies have indicated that NO-mediated vasodilation is associated with KCa2.3 activation (10-12). Therefore, additional clarification of the effect of shear patterns on the expression of KCa.23 and the potential regulatory mechanisms in myocardial cells is required. In cardiac tissues, KCa2.3 channel blockers function to prevent atrial fibrillation (13,14).

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 ibrutinib 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 serves 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 cardiomyocytes 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% CO_2 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 as monolayers were exposed to static culture conditions (ST) or to a laminar shear stress condition (LS) using a computer-controlled shearing device at 15 dynes/cm^2 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

Table I. Primers sequence used in the present study.

Gene name	Primers	Product length, bp
PI3K	Forward: 5'-GATTGGTTCTTTCCTGTCTCTG-3' Reverse: 5'-CCACCCTATCAATTTACAACCA-3'	324
Akt	Forward: 5'-GCACAACGAGGGGAGTACAT-3' Reverse: 5'-CCTCACGTTGGTCCACATC-3'	113
p300	Forward: 5'-CAGTCCAGTAAATCAGCCTGCC-3' Reverse: 5'-AATCCTGTTTGTCCTCCCATCTG-3'	255
KCa2.3	Forward: 5'-CTTAATCACAGAACTCAATG-3' Reverse: 5'-TTAGCAACTGCTTGAACTTG-3'	178
β-actin	Forward: 5'-TTCCAGCCTTCCTTCTTG-3' Reverse: 5'-GGAGCCAGAGCAGTAATC-3'	153

PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; p300, histone acetyltransferase p300; KCa2.3, Ca^{2+}-activated K^+ channels.

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; Qiagen, Inc., Valencia, CA, 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 $2^{-\Delta\Delta Cq}$ method (20), where ΔΔCt 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 was resolved on 10% SDS-PAGE gels and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). The membrane were blocked in TBS-T containing 5% fat-free dry milk for 30 min at room temperature and then incubated with primary antibodies (1:2,000, sc-28621; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 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 (cat. no. PE0010; Beijing Solarbio Science & Technology). The primary antibodies used were directed against phosphorylated (p)-Akt (1:1,500, cat. no. 9271; Cell Signaling Technologies, Inc., Danvers, MA, USA), total Akt (1:2,000, cat. no. 4691; Cell Signaling Technologies, Inc.), p-p300 (1:500, cat. no. PA5-12652; Thermo Fisher Scientific, Inc.) and total p300 (1:2,000, cat. no. 86377; Cell Signaling Technologies, Inc.) and β-actin (1:1,500, sc-47778; Santa Cruz Biotechnology, Inc.). Representative immunoblots from ≥3 experiments are presented with the results of the densitometric analyses (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% TritonX-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 100 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.) or rabbit pre-immune IgG (1 μg/1 mg lysate) 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 cross-linking of protein-DNA complexes was reversed by incubation with 5 M NaCl at 65˚C for 4 h, and the DNA was digested with proteinase K for 1 h at 45˚C. The DNA was extracted with a 1:1 phenol-chloroform, and the purified DNA was precipitated with isopropanol at a concentration of 15%. Following washing, the DNA pellet was resuspended in H_2O and subjected to PCR amplification as aforementioned using the forward (5'-AGGACTGGGAACTGTAGTTTTTG-3') and reverse primers (5'-GAAAATGTCTACTTTTAA-3') (-688 to -203 bp). These primers were specifically designed based on the sequence of the KCa2.3 promoter region (supplied by Generay Biotech Co., Ltd., Shanghai, China). The PCR

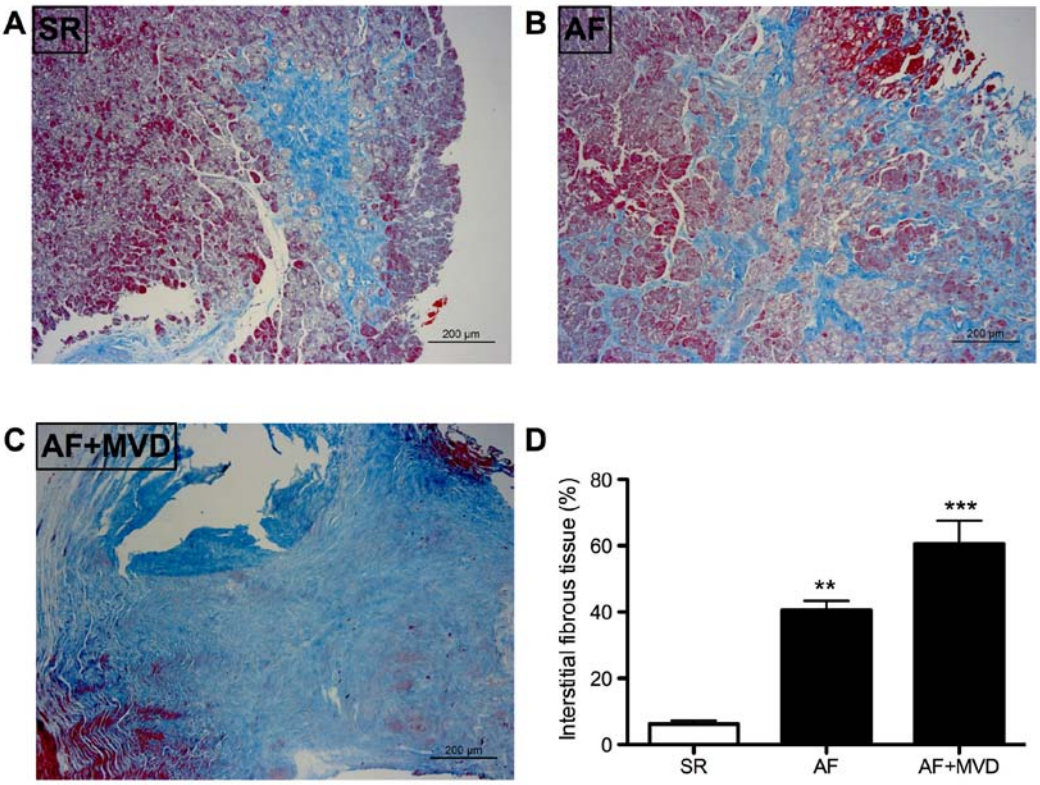

Figure 1. Histological analysis of atrial interstitial fibrosis. Representative Masson trichrome staining of left atrial myocardium in the (A) SR, (B) AF and (C) AF combined with MVD groups (magnification, x200; scale bar, 200 μm). (D) Percentage of areas of interstitial fibrous tissue in the three groups of patients; the bars indicate the standard deviation. **P<0.01 and ***P<0.001 vs. SR group. SR, sinus rhythm; AF, atrial fibrillation; MVD, mitral valve disease.

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/cm^2) 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/cm^2) for 12 h resulted in a moderate induction of KCa2.3, whereas exposure to LS at 15 dynes/cm^2 for 6 or 24 h decreased KCa2.3 expression (data not shown). To elucidate the potential

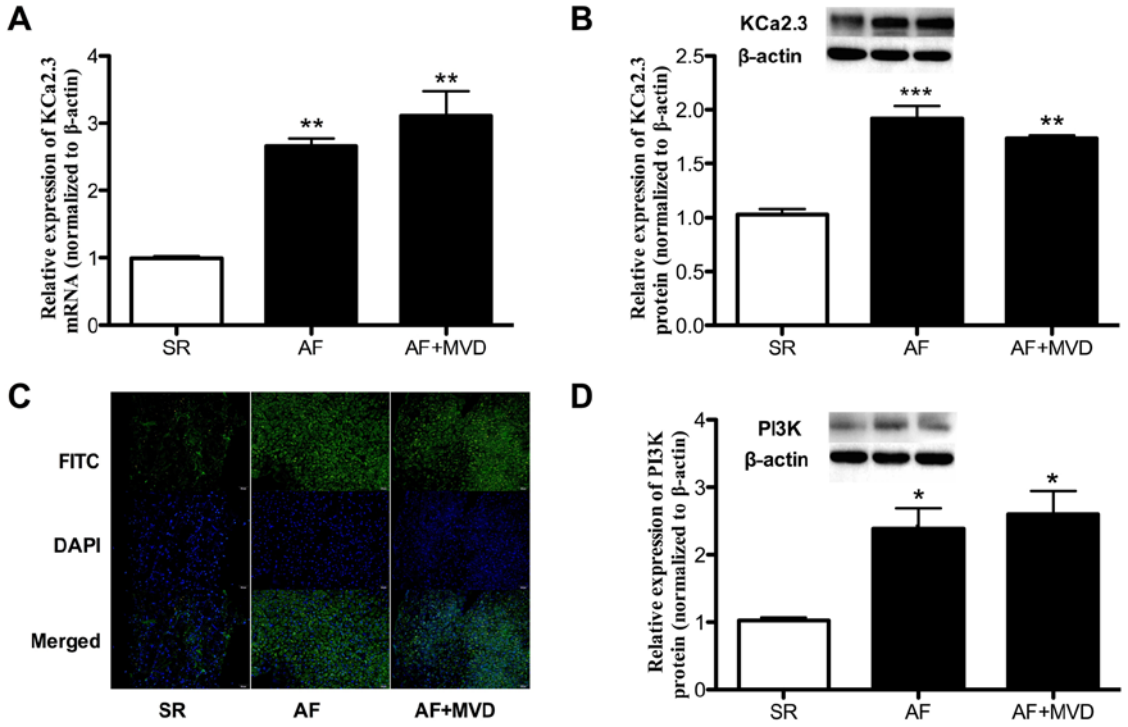

Figure 2. KCa2.3 gene expression analysis in clinical samples. (A) 2.66- and 3.1-fold increases in KCa2.3 expression levels were observed in the AF (n=8) and AF combined with MVD (n=6) groups compared to the SR groups (n=6; P=0.006 and 0.005, respectively). (B) Increases in 85% and 63% in KCa2.3 expression 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, Ca^{2+}-activated K^{+} channels; SR, sinus rhythm; AF, atrial fibrillation; MVD, mitral valve disease.

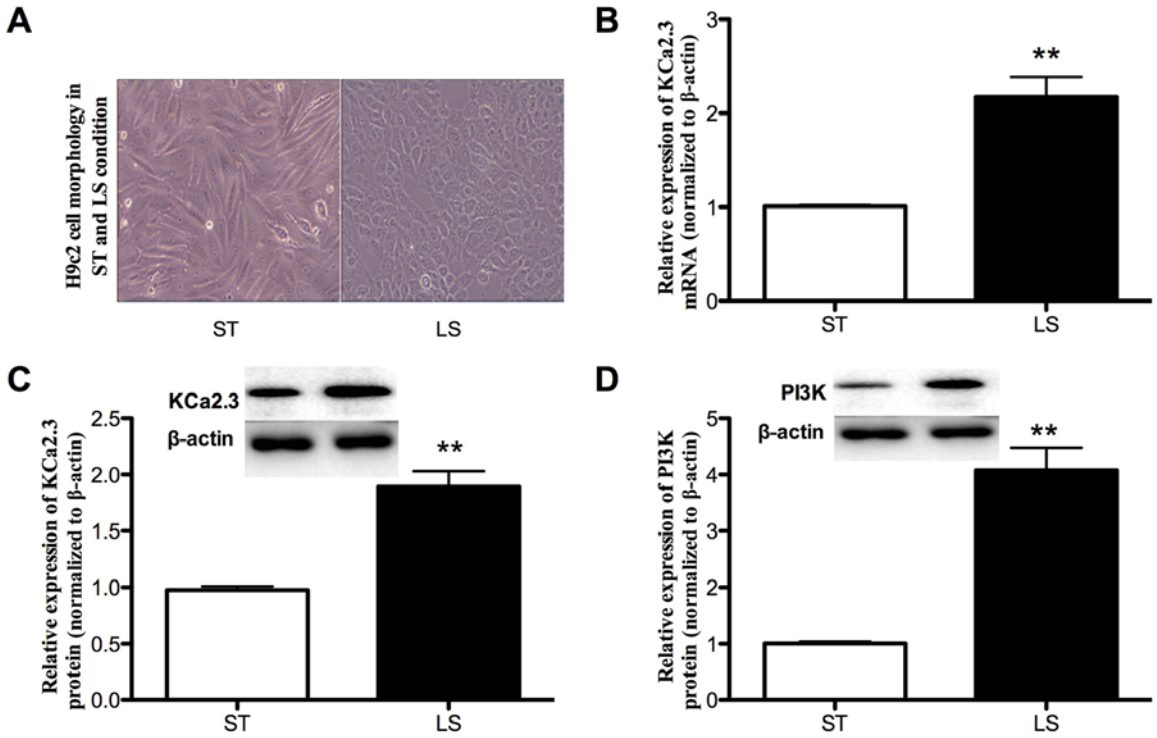

Figure 3. LS increases KCa2.3 partially via regulating PI3K expression. The effect of shear stress on the expression of KCa2.3 in H9c2 cells was determined by reverse transcription quantitative polymerase chain reaction and western blot analysis. (A) Exposure to LS (15 dynes/cm^2) for 12 h markedly changed 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/cm^2) 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, Ca^{2+}-activated K^{+} channels; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; LS, laminar shear stress condition; ST, static culture conditions.

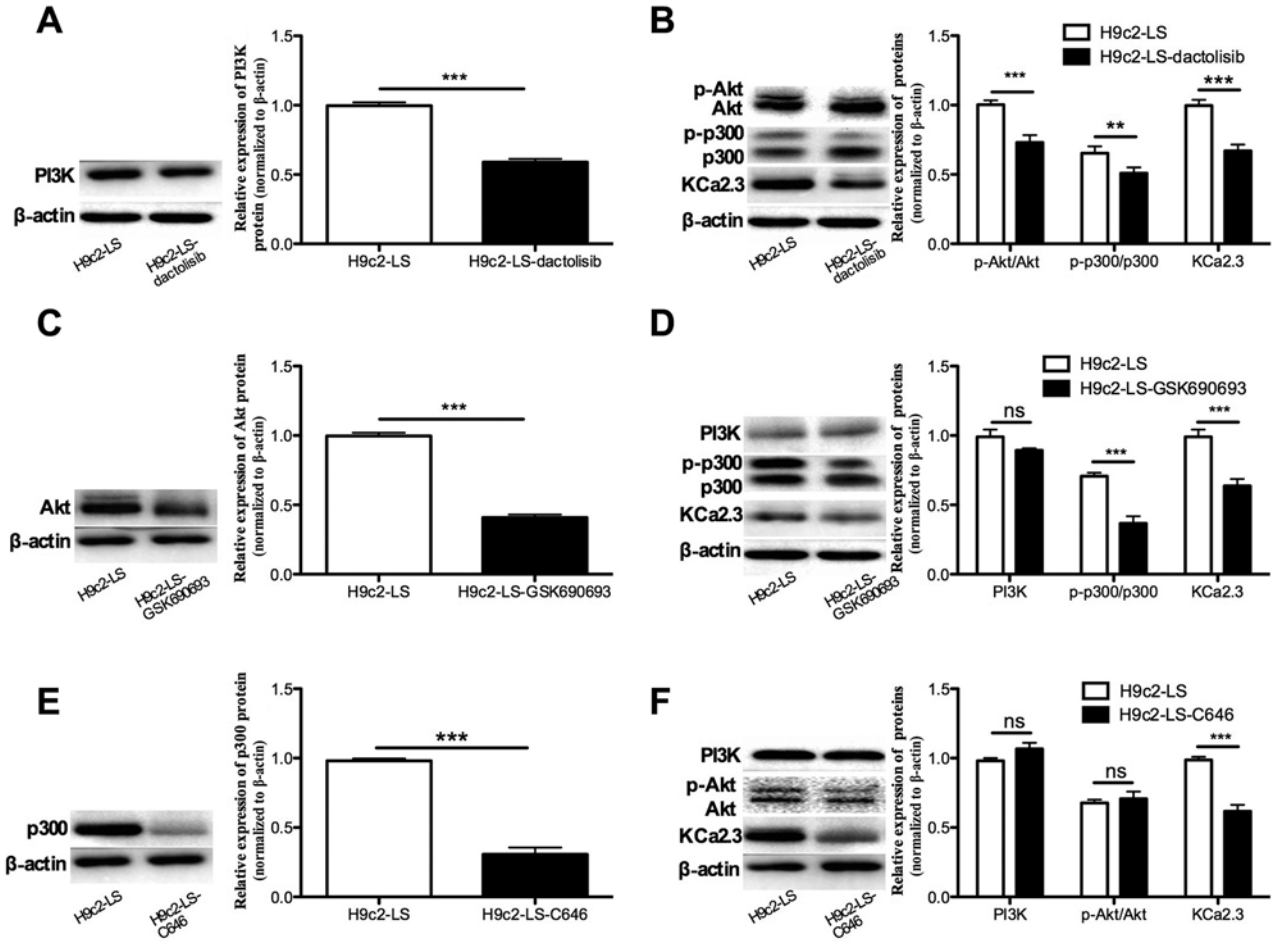

Figure 4. Activation of the PI3K/Akt/p300 axis mediates the LS-induced increase in KCa2.3. H9c2 cells were exposed to LS for 12 h in the absence or presence of 20 nM dactolisib, a PI3K inhibitor, 10 nM GSK690693, a specific Akt inhibitor or 400 nM C646, a p300 inhibitor. (A) Dactolisib decreased the 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 C464, 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, Ca^{2+}-activated K^+ channels.

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/cm^2) 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/cm^2) 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 specific inhibitor of PI3K, 10 nM GSK690693, a specific Akt inhibitor or 400 nM C646, an inhibitor of p300 transcription factor binding. As demonstrated in Fig. 4A, dactolisib markedly inhibited PI3K protein expression; it decreased the p-Akt/Akt and p-p300/p300 ratios and KCa2.3 expression levels by 27, 22 and 33%, respectively (all P<0.05; Fig. 4B). GSK690693 decreased Akt expression and had no effect on PI3K expression under LS conditions (P=0.063; Fig. 4C and D). However, GSK690693 attenuated the p-p300/p300 ratio and KCa2.3 protein expression by 48 and 37%, respectively (both P<0.01; Fig. 4D). By contrast, following the addition of C646, an inhibitor of p300, western blot analysis data suggested that the KCa2.3 protein level decreased by 38% (P<0.001) with no change in the expression of PI3K or in the ratio of p-Akt/Akt ratio (Fig. 4E and F). These results indicate that the PI3K/Akt/p300 signal axis serves an important role in mediating LS-induced upregulation of the KCa2.3 channel in H9c2 cells.

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

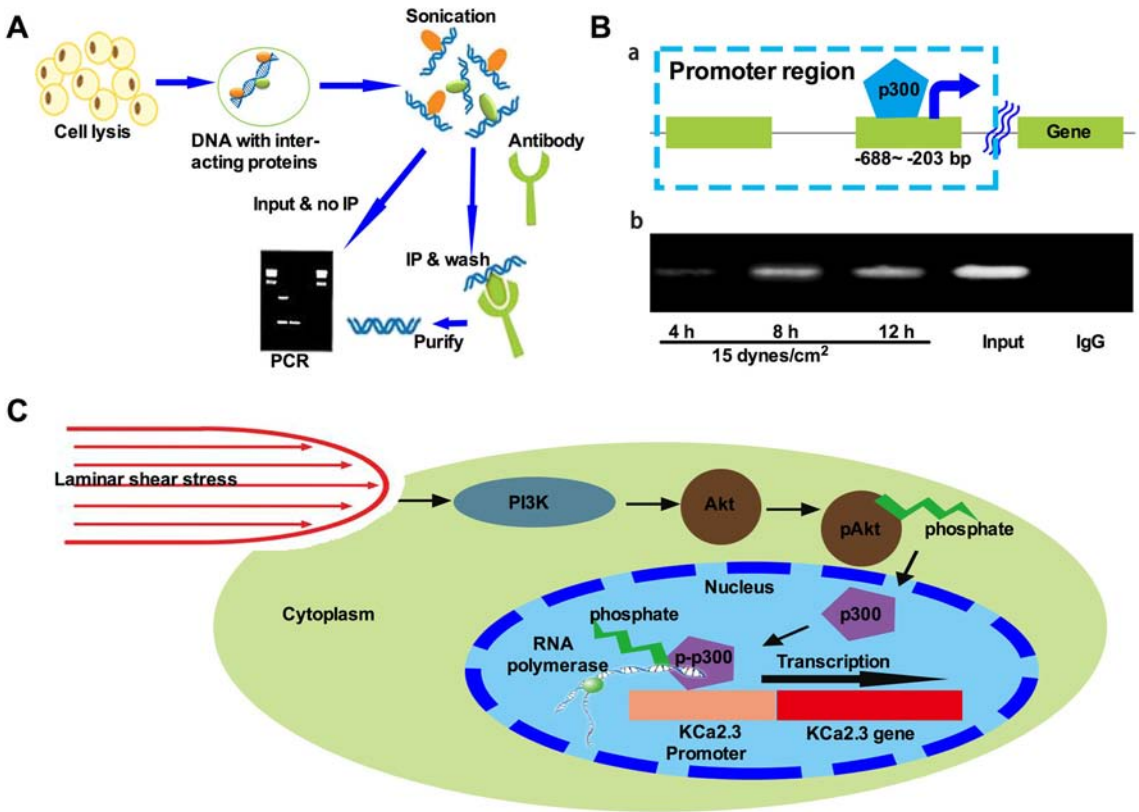

Figure 5. Transcriptional cofactor p300 is required for LS-induced expression of KCa2.3. (A) An overview of the ChIP procedure. Generally, cells are initially treated with a cross-linking agent that covalently links DNA-interacting proteins to the DNA. The genomic DNA is then isolated and sheared by sonication to yield a suitable fragment size distribution. An antibody that specifically recognizes the protein of interest is then added, and immunoprecipitation is used to isolate specific protein-DNA complexes. The cross-links are then reversed, and the DNA fragments are purified and subjected to PCR analysis. (B-a) Schematic of the KCa2.3 genomic DNA region. The square boxes indicate the DNA region amplified by PCR in the ChIP assay. (B-b) Time dependence of LS-stimulated recruitment of p300 to the KCa2.3 promoter as measured in ChIP assays. The cells were treated with LS for the indicated times. The precipitated KCa2.3 promoter region (-688 to -203 bp) was amplified by PCR. The figure represents one of at least three individual experiments. The proximal KCa2.3 promoter region (-688 to -203 bp) was amplified by PCR following ChIP. Mouse IgG represents negative control. The ChIP experiments were repeated three times. (C) Proposed model of LS-induced KCa2.3 activation in H9c2 cells. Under normal conditions, KCa2.3 is expressed at a basal level. When cells are subjected to LS, p300 is recruited to the KCa2.3 promoter, where it activates KCa2.3 transcription. LS results in activation of the PI3K/Akt cascade and recruitment of p300 to the promoter of the KCa2.3 promoter. KCa2.3 transcription is regulated in a p300-dependent manner by a PI3K/Akt cascade. This signaling pathway may contribute to the sustained expression of KCa2.3 in H9c2 cells. p300, histone acetyltransferase p300; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; LS, laminar shear stress condition; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; KCa, Ca^{2+}-activated K^+ channels.

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, recruitment of p300 was examined using a ChIP-PCR assay (Fig. 5A). A total of 31 cycles of PCR amplifications were conducted using equal amounts of immunoprecipitated DNA in the presence of specific primer pairs encompassing the region between positions -688 and -203 region 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 Ca^{2+} 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/cm^2, 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.

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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.

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