

Original Article

PDK4 regulates human cardiomyocyte viability, apoptosis, and inflammatory response via inhibiting TGF- β /Smad signaling pathway

Lizhong Song¹, Zhigang Zhang²

Departments of ¹Critical Care Medicine, ²Cardiology, Shanxi Cardiovascular Disease Hospital, Taiyuan, Shanxi Province, China

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Abstract: Objective: This study aimed to explore the impact of PDK4 on cardiomyocyte viability, apoptosis, and inflammatory response in patients with atrial fibrillation (AF) by targeting the TGF- β /Smad pathway. Methods: A total of 121 patients with AF and 32 controls with sinus rhythm (SR) were included in this study. Color sonography was used to detect the cardiac diameter of ventricles and atria. Left atrial appendages from patients were collected during the cardiac valvular replacement operation, and cardiomyocytes were separated and purified for subsequent experiments. The cardiomyocytes from AF patients were treated with PDK4 shRNA or TGF- β /Smad pathway inhibitor (LY2157299) and a negative control group (cardiomyocytes transfected with shRNA scrambled sequence) was set up. Masson staining was used to observe the degree of fibrosis in left atrial appendage. qRT-PCR and Western blot were performed to determine the expressions of related mRNA and protein. Cardiomyocyte viability and apoptosis were measured by CCK-8 assay and flow cytometry, respectively. Results: Compared with the SR group, the patients with AF had higher values of left ventricular end-diastolic diameter, left ventricular end-systolic dimension, and left atrial end-diastolic diameter. Moreover, PDK4 expression and TGF- β /Smad pathway were significantly activated in the myocardial tissues of patients with AF. After comparison with the negative control group, we found that PDK4 knockdown could inhibit TGF- β /Smad pathway, promote Cyclin D1 and Bcl-2 protein expressions and cell viability, down-regulate Bax and Caspase-3 protein expressions, and inhibit cell apoptosis. Conclusion: Silencing PDK4 can promote cardiomyocyte viability in AF patients and suppress cell apoptosis and the expression of inflammatory factors by inhibiting the TGF- β /Smad pathway.

Keywords: Pyruvate dehydrogenase kinase 4, TGF- β /Smad pathway, atrial fibrillation, cardiomyocyte, viability, apoptosis, inflammatory factor

Introduction

Atrial fibrillation (AF), the most common arrhythmia characterized by fast and irregular heart rate, is a serious life-threatening health issue, and its complications are important causes of high incidence and mortality of human heart disease [1, 2]. Although there have been many studies on AF, the pathogenesis and etiology of this disease remain unclear and require further investigation [3]. Some evidence indicates that the morbidity of AF may be related to factors such as ventricular remodeling, calcium homeostasis, oxidative stress, and inflammatory response [4, 5]. Previous studies have shown that in heart damage, a certain number of cardiomyocytes are required for maintaining

the normal function of the heart, and myocardial apoptosis plays an important role in the pathogenesis of AF [6]. Cardiomyocyte apoptosis not only means the loss of cells but also leads to cardiac hypertrophy and interstitial fibrosis for cellular compensation and repair, thereby causing ventricular remodeling and aggravation of AF [7].

Inflammation and apoptosis are believed to be involved in the occurrence of AF [8]. Some studies have indicated that the C-reactive protein (CRP) expression in plasma of AF patients is significantly up-regulated [9, 10]. Apoptosis can be influenced by many factors, and both gene expression and intracellular signaling pathway participate in the occurrence of apoptosis [11].

Pyruvate dehydrogenase kinase 4 (PDK4), a key enzyme in the process of glucose oxidation, can promote myocardium's glucose uptake in the process of myocardial ischemia-reperfusion, reduce myocardial injury, and improve cardiac function [12, 13]. Previous evidence has indicated that the overexpression of PDK4 may inhibit mitochondrial respiration in cardiomyocytes, thus causing damage to cardiomyocytes [14]. TGF β /Smad signaling pathway is one of the key signaling pathways in the pathogenesis of AF. Studies have confirmed that the process of myocardial fibrosis is often accompanied by the up-regulation of TGF- β 1, Smad2, and Smad3 [15]. Smad protein-dependent pathway is one of the classical signaling pathways of TGF- β 1, and its role in glycosaminoglycan elongation of cardiac non-myocyte is mainly achieved by phosphorylation of Smad2 [16]. Meanwhile, it has been found that in AF patients, the expressions of Smad2 and phosphorylated Smad2 can increase significantly [17]. However, whether the upstream of the signaling pathway is affected by PDK4 genes has not been fully elucidated. Based on all these findings, we propose that PDK4 might be an essential modulator in AF, possibly through the inhibition of the TGF- β /Smad signaling pathway.

Materials and methods

Subjects and clinical data

A total of 121 patients diagnosed with AF and received valve replacement under extracorporeal circulation at the Department of Thoracic Surgery in China-Japan Union Hospital of Jilin University from 2012 to 2017 were selected for the study. According to the joint proposal of the North American Society of Pacing and Electrophysiology and the European Society of Cardiovascular Diseases, inclusion requirements for patients were as follows: 1) patients with organic heart disease complicated by AF; 2) antiarrhythmic drug and drug for termination of atrial fibrillation were not effective to the patients; 3) patients who underwent thoracotomy for the first time; 4) patients without anemia or anemia had been corrected before surgery; 5) patients with no liver or kidney dysfunction; 6) patients who did not receive antiplatelet, aspirin, dipyridamole, warfarin, or any other anticoagulant drugs and hormone therapy within two weeks before surgery [18]. Exclusion cri-

teria were: 1) patients with low cardiac output syndrome after stopping the extracorporeal circulation machine; 2) patients who need re-thoracotomy within 24 hours after surgery [19]. There were 121 cases of patients included in the study (53 males and 68 females, age range: 26-74 years, weight range: 40-65 kg). Thirty-one patients underwent cardiac arrest valve replacement, whereas other 90 patients did not undergo this procedure. Meanwhile, 32 people with sinus rhythm (SR) were enrolled as control. All experiments were conducted according to the guidelines and principles of *Declaration of Helsinki*. The study was approved by the Institutional Review Board of China-Japan Union Hospital of Jilin University and written informed consent was obtained from all patients. Peripheral blood and left auricular tissue were collected at the time of surgery with the consent of the patients and their guardians.

Color doppler echocardiography

Cardiac ultrasound was performed in each group (model: ATL-5000; frequency: 2.5 Hz). The test items included left atrial diameter (LAD), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic dimension (LVESD), left atrial end-diastolic diameter (LAEDD), right atrial end-diastolic diameter (RAEDD), and left ventricular ejection fraction (LVEF).

Isolation, purification, and identification of cardiomyocyte

The left atrial appendage tissue in the AF group was cut into tissue blocks (1 mm \times 1 mm \times 1 mm) with surgical scissors, triturated with 0.1% trypsin and 0.1% collagenase repeatedly, and added into a complete medium to terminate the digestion. After centrifugation at 10,000 rpm for 8 min, the supernatant was discarded. The cells were resuspended with complete medium and filtered using 10 μ m mesh filter, followed by culturing at 37°C in 5% CO₂. The third generation cardiomyocytes were collected, and 0.1% trypsin was added for 1 min digestion. The digested cells were suspended by culture solution, and the suspension was centrifuged at 1,000 rpm for 8 min. After discarding the supernatant, the cells were collected and washed with PBS. Troponin I mouse anti-human (200 μ L, diluted to 1 μ g/mL, Abcam, Cambridge,

Table 1. qRT-PCR sequences

Gene	Sequence (5'-3')
GAPDH	Forward: GGAGCGAGATCCCTCCAAAT Reverse: GGCTGTTGCATACTTCTCATGG
PDK4	Forward: GGAGCATTCTCGCGCTACA Reverse: ACAGGCAATTCTGTGCGCAA
TGF-β1	Forward: GGCCAGATCCTGTCCAAGC Reverse: GTGGGTTCCACCATTAGCAC
Smad3	Forward: TGGACGCAGGTTCTCCAAC Reverse: CCGGCTCGCAGTAGGTAAC

UK) was added to the samples for overnight incubation at 4°C. Afterward, the sections were washed with PBS for three times and treated with Alexa Fluor® 488 conjugated goat-anti-rabbit secondary antibody (diluted to 1 µg/mL, Abcam, Cambridge, UK) at room temperature for 30 min. After rinsing with PBS three times, the purity of the cardiomyocytes was examined by flow cytometry.

Cell grouping

The third generation cardiomyocytes were randomized into five groups: control group (without any treatment), negative control (NC) group (transfected with blank plasmids), sh-PDK4 group (transfected with sh-PDK4), LY2157299 group treated with LY2157299 (galunisertib, a selective TGF-beta receptor type I (TGF-β) kinase inhibitor with an IC₅₀ of 56 nM), and sh-PDK4 + LY2157299 group (transfected with sh-PDK4 + LY2157299).

Cells were seeded in a 24-well plate and transfected with Lipofectamine™ 2000 (Invitrogen Inc., Carlsbad, CA, USA) when the cell confluence reached 50%-60%. The transfection process was carried out according to the manufacturer's instructions. During transfection, both Lipofectamine 2000 (1 µL) and PDK shRNA (20 pmol) were diluted in 50 µL serum-free medium, and their mixtures were incubated at room temperature for 20 min. Transfection for negative control sequence was based on the above steps. Subsequently, the mixtures were added into the cells under transfection and the cells were cultured at 37°C in 5% CO₂ for 24 h. The culture medium was replaced every 6-8 h.

LY2157299 (MCE, USA) was diluted with serum-free medium to 100 nM, and 2 mL dilution was added into the cells. The culture medium was replaced after 24 h incubation.

RT-qPCR

Total RNAs were extracted from the cardiomyocytes and the peripheral blood using a Trizol kit (Invitrogen Inc., Carlsbad, CA, USA). Optical density (OD) values of total RNA at 260 nm and 280 nm were detected with an ultraviolet spectrophotometer (Nano Drop 2000/2000c, Thermo Fisher, USA). The RNA sample with an OD 260/280 ratio between 1.8 and 2.0 was considered to have high purity. Reverse transcription was performed using the reverse transcription kit (K1622, Fermentas, MBI, USA) and the system was based on EasyScript First-Strand cDNA Synthesis SuperMix (AE301-02, Beijing Full Gold, Beijing, China). The reaction system consisted of 5 µL total RNA, 1 µL random primer, and 9 µL RNase-free H₂O, and the reaction conditions were: 70°C for 5 min, ice bath for 60 s, 37°C for 15 min, and 65°C for 1 h. The cDNAs were preserved at -80°C. Fluorescent quantitative PCR was performed according to the manufacturer's instructions of SYBR®PremixEx Taq™ II kit (Takara) with a Q-PCR instrument (ABI Company, Oyster Bay, NY, USA). The total volume of the PCR system was 20 µL, including 1.2 µL upstream and downstream primers, 2 µL cDNA templates, 10 µL SYBR Premix, and 6.8 µL RNase free H₂O. GAPDH was used as an internal control. The primer sequences are shown in **Table 1**. The running parameters of PCR were: pre-denaturation at 95°C for 30 s, 30 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 20 s, and extension at 72°C for 30 s. The expression of gene expression was measured using the ΔΔCt method.

Western blot

The left atrial appendage specimen and the cardiac muscle cells were placed in a labeled centrifuge tube and added with 1 mL protein lysis buffer (Beijing Suo Lai Bao Technology Co., Ltd., Beijing, China). The samples were shaken on ice for 30 min and centrifuged at 1,200 rpm for 30 min at 4°C. The BCA kit (Beijing Suo Lai Bao Technology Co., Ltd., Beijing, China) was applied to determine the protein concentration. We prepared 10% separating gel and 5% concentrated glue according to the manufacture's protocol of SDS-PAGE gel reagent (Beijing Suo Lai Bao Technology Co., Ltd., Beijing, China). The proteins were transferred onto a NC membrane (Millipore, Bedford, MA, USA). After blocking with 5% BSA for 1 h at room temperature,

the membrane was incubated with the antibodies overnight at 4°C, which were rabbit anti-PDK4 (1:1,000, Abcam Inc., Cambridge, MA, USA), anti-TGFβ1 (diluted to 1 µg/mL, Abcam Inc., Cambridge, MA, USA), anti-Smad3 (1:3,000, Abcam Inc., Cambridge, MA, USA), anti-p-Smad3 (1:2,000, Abcam Inc., Cambridge, MA, USA), anti-Cyclin D1 (1:10,000, Abcam Inc., Cambridge, MA, USA), anti-Caspase-3 (diluted to 1 µg/mL, Abcam Inc., Cambridge, MA, USA), anti-Bcl-2 (1:1,000, Abcam Inc., Cambridge, MA, USA), anti-Bax (1:1,000, Abcam Inc., Cambridge, MA, USA), anti-CRP (1:1,000, Abcam Inc., Cambridge, MA, USA), anti-TNF-α (1:1,000, Abcam Inc., Cambridge, MA, USA), and anti-IL-6 (1:500, Abcam Inc., Cambridge, MA, USA), followed by washing three times with PBST (10 min/wash). Subsequently, the membrane was added with secondary goat anti-rabbit antibody IgG (1:2,000, Abcam, Cambridge, MA, USA), slightly shaken for 2 h by a shaking table, and washed in PBS three times (10-15 min/wash). The electrochemiluminescence (ECL) solution (Pierce, Waltham, MA, USA) was added into the membrane, the protein expression was detected by Bio-Rad Gel imaging system (Beijing Tuo Morgan Biotechnology Co., Ltd., Beijing, China) and IPP7.0 software (Media Cybernetics, Singapore).

Masson staining

The left atrial appendages of each group were collected and made into paraffin sections. The sections were hydrated, washed by hematoxylin solution for 5-10 min, and then thoroughly washed by distilled water. After staining by Masson Fuchsin Acid for 5-10 min, the sections were sliced using glacial acetic acid. Phenylamine was utilized for staining the sections for 5 min after differentiation with 1% phosphomolybdate. The sections were then rehydrated in a gradient alcohol of 100%, 95%, 85%, and 80%, cleared in dimethylbenzene, and sealed with neutral gum. A computer image analysis system was applied to detect the collagen content. The paraffin section was from each subject. The collagen deposition area (areas stained in blue) was calculated, and the average value was deemed as collagen volume fraction (CVF).

Cell counting kit 8 (CCK-8) assay

Cells collected from each group were digested with 0.5% pancreatin and seeded into 96-well

plate with 1×10^4 in each well (five replicates for each group). The medium was changed after a 6 h incubation. At 24, 48, and 72 h after incubation, each well was added with 10 µL CCK8 solution (JK-021, Shanghai Jingke Chemical Technology Co., Ltd, Shanghai, China). An automatic quantitative plotting enzyme labeling instrument (MK3, Thermo, Pittsburgh, PA, USA) was then used to detect the OD value at 450 nm at 0, 24, 48 h.

Apoptosis detection

After transfection for 48 h, the cells were digested with 0.25% pancreatin without EDTA and transferred to a flow cytometry tube. The supernatant was collected after centrifugation. Cell apoptosis was detected by Annexin V-FITC (fluorescein) assay (Biovision, USA) according to the manufacturer's instruction. Annexin V-FITC/PI dye was prepared by compounding the Annexin V-FITC, PI, and HEPES buffer solution at a ratio of 1:2:50. The cells (1×10^6) were resuspended with 100 µL dye and incubated at room temperature after shaking for 15 min and treated with 1 mL HEPES buffer solution. The fluorescence intensity of FITC and PI was detected within the scope of 488 nm and 515-620 nm of emission light to examine the apoptosis.

Statistical analysis

Statistical analysis was conducted using SPSS 18.0 software (IBM Corp., Armonk, NY, USA). Experiment data are presented as mean ± standard deviation. Kolmogorov-Smirnov test was performed to examine the normal distribution of the experimental data, and differences among multiple groups were compared by one-way analysis of variance followed by post hoc LSD t-test. Data noncompliant with normal distribution were examined by Wilcoxon and Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

Results

LVEDD, LVESD, and LAEDD were increased in AF patients

The results from the color doppler ultrasound of the heart are illustrated in **Table 2**. In comparison with the SR group, the values of LVEDD, LVESD, and LAEDD were higher in the AF group. No differences in the values of LAD, RAEDD,

Table 2. Color doppler ultrasound results in the two groups

	SR group (n=32)	AF group (n=121)	T	P
LAD	38.96±1.87	37.87±1.49	0.789	0.473
LVEDD	42.08±2.01	56.82±2.98	7.103	0.002
LAEDD	46.81±2.08	59.72±3.21	5.846	0.004
RAEDD	35.97±1.09	37.99±1.05	2.312	0.089
LVEF	57.13±1.89	56.22±2.06	0.563	0.603
LVESD	47.08±1.06	53.28±1.29	6.432	0.003

Note: SR, sinus rhythm; AF, atrial fibrillation; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic dimension; LAEDD, left atrial end-diastolic diameter; RAEDD, right atrial end-diastolic diameter; LVEF, left ventricular ejection fraction.

and LVEF, were found between the two groups (all $P > 0.05$).

PDK4 expression was up-regulated and TGF-β/Smad pathway was activated in AF patients

qRT-PCR and Western blot were applied to observe the activation of TGF-β/Smad pathway in the peripheral blood of the SR and AF groups. The results indicated that compared with the SR group, the mRNA and protein expression levels of PDK4 and TGF-β1 as well as the protein level of p-Smad3 were much higher in the AF group (all $P < 0.05$, **Figure 1**). No difference was observed in the protein level of Smad3 between these two groups ($P > 0.05$).

Since TGF-β/Smad pathway is closely related to myocardial fibrosis, Masson staining was performed to examine the fibrosis in the SR and AF groups. The results displayed that compared with the SR group, the AF group showed more disorderly arranged cardiomyocytes, higher CVF level, and less clear and complete blood vessels (all $P < 0.05$, **Figure 2**).

PDK4 silencing inhibits TGF-β/Smad pathway, promotes viability of cardiomyocytes, and inhibits cell apoptosis

The relationship between PDK4 and TGF-β/Smad pathway was investigated using Western blot. The results regarding the activation of the TGF-β/Smad pathway in each group are indicated in **Figure 3**. Compared with the control group, the protein levels of TGFβ1 and p-Smad3 significantly decreased in the groups with

PDK4 silencing (all $P < 0.05$). Meanwhile, there were no differences in levels of the markers related to TGF-β/Smad pathway between the sh-PDK4 and the LY2157299 groups and no differences in all the markers between the control and the negative control groups (all $P > 0.05$). These results indicate that PDK4 can regulate TGF-β/Smad pathway.

Western blot was conducted to detect the levels of proteins associated with cardiomyocyte proliferation and apoptosis in each group. The results showed that compared with the control group, the expression levels of Cyclin D1 and Bcl-2 in the sh-PDK4 group, the LY2157299 group, and the sh-PDK4 + LY2157299 group were higher, whereas the expression levels of Bax and Caspase-3 were lower (all $P < 0.05$). There were no significant differences in the level of these markers between the control group and the negative control group (all $P > 0.05$). Compared with the sh-PDK4 + LY2157299 group, the protein expression levels of Cyclin D1 and Bcl-2 were lower, whereas the Bax and Caspase-3 protein expression levels were higher in the sh-PDK4 group and the LY2157299 group (all $P < 0.05$). See **Figure 4**.

CCK-8 assay was performed to determine the cardiomyocyte viability in each group. The results showed no difference in the cell viability at 0 h (all $P > 0.05$, **Figure 5**). Compared with the control group, the cell viability in the sh-PDK4 group, the LY2157299 group, and the sh-PDK4 + LY2157299 group was higher at 24 h and 48 h (all $P < 0.05$). No difference in the cell viability between the control group and the negative control group was observed ($P > 0.05$). Compared with the sh-PDK4 + LY2157299 group, the cell viability in the sh-PDK4 group and the LY2157299 group was lower (all $P < 0.05$). The results in **Figures 3-5** reveal that the knockdown of the PDK4 gene could promote the viability of cardiomyocyte in AF patients by regulating the TGF-β/Smad pathway. Meanwhile, the cell viability increased more significantly when cardiomyocyte was treated by both PDK4 silencing and LY2157299.

Annexin V-FITC assay was used to detect early and late apoptotic cardiomyocytes. The results showed that compared with the control group, the cardiomyocyte apoptosis rate in the sh-PDK4, LY2157299 and sh-PDK4 + LY2157299 groups was lower (**Figure 6**). Moreover, the

PDK4's role in atrial fibrillation

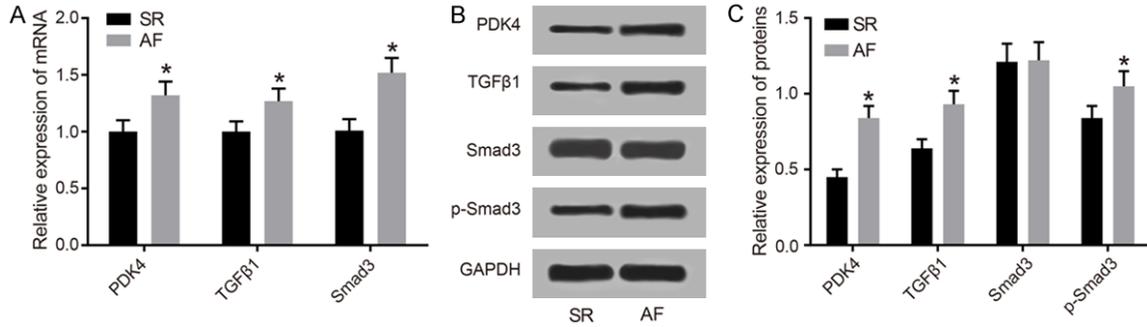


Figure 1. Expressions of PDK4, TGF-β1, and Smad3 in the left atrial appendage. A. The relative mRNA expressions of PDK4, TGF-β1, and Smad3; B. Expressions of PDK4, TGFβ1, and Smad3 determined by Western blot; C. Quantification of PDK4, TGFβ1, and Smad3 protein expression. SR, sinus rhythm; AF, atrial fibrillation. Compared with the SR group, *P<0.05.

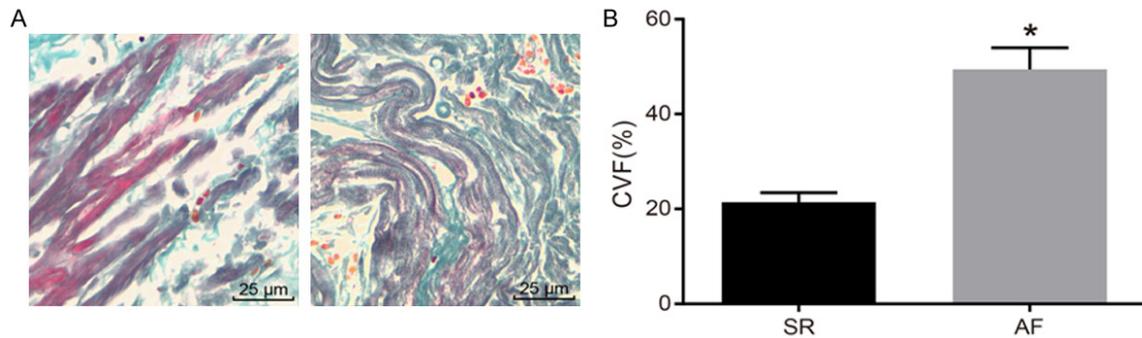


Figure 2. Masson staining of the left atrial appendage. A. Masson staining; B. Statistical results of CVF. CVF, collagen volume fraction. Compared with the SR group, *P<0.05. SR, sinus rhythm; AF, atrial fibrillation.

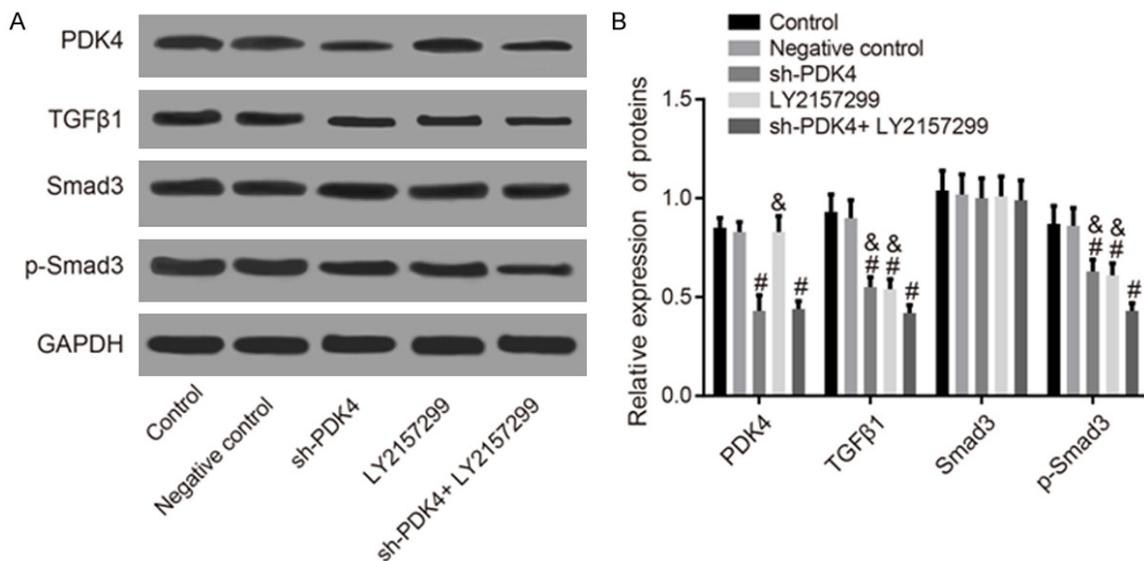


Figure 3. Expressions of the proteins associated with TGF-β/Smad pathway in cardiomyocytes of each group. A. TGFβ1, p-Smad3, and Smad3 protein expressions determined by Western blot; B. Quantification of TGF-β1, p-Smad3, and Smad3 protein expressions. Compared with the control group, #P<0.05; compared with the sh-PDK4 + LY2157299 group, &P<0.05.

PDK4's role in atrial fibrillation

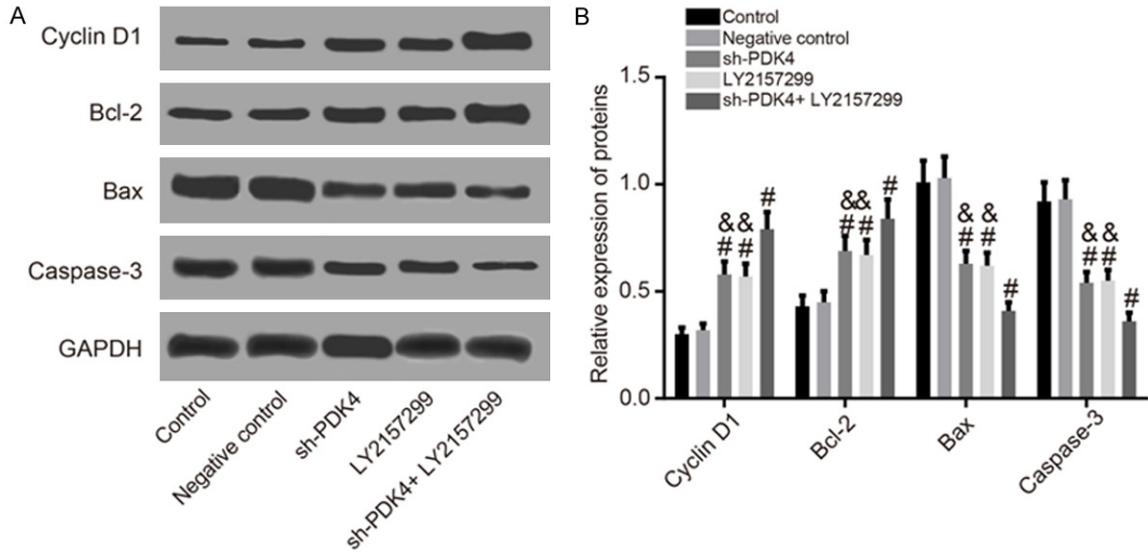


Figure 4. Expressions of proteins associated with cardiomyocyte proliferation and apoptosis in each group. A. Cyclin D1, Bcl-2, Bax, and Caspase-3 protein expressions determined by Western blot; B. Quantification of Cyclin D1, Bcl-2, Bax, and Caspase-3 protein expressions. Compared with the control group, # $P<0.05$; compared with the sh-PDK4 + LY2157299 group, & $P<0.05$.

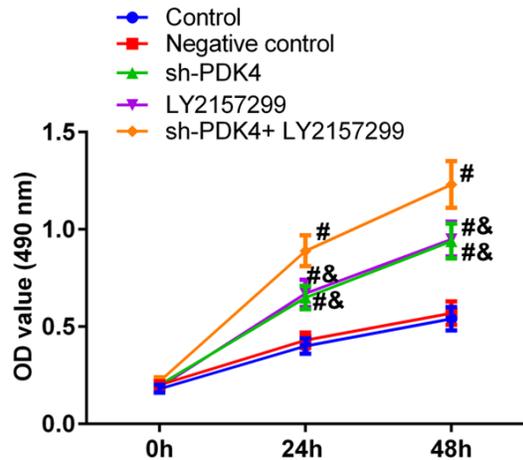


Figure 5. Cardiomyocyte viability in each group detected by CCK-8 assay. CCK-8, Cell Counting Kit-8; OD, optical density. Compared with the control group, # $P<0.05$; compared with the sh-PDK4 + LY2157299 group, & $P<0.05$.

apoptosis rate was lower in the sh-PDK4 + LY2157299 group than in the sh-PDK4 and LY2157299 groups (all $P<0.05$). There was no significant difference in the cell apoptosis between the control and the negative control groups ($P>0.05$). The results in **Figures 3, 4** and **6** indicate that the inhibition of PDK4 could impede the activation of TGF- β /Smad pathway, thereby reducing the apoptosis of cardiomyo-

cytes. The treatment using the combination of PDK4 shRNA and LY2157299 markedly suppressed the cell apoptosis ($P<0.05$).

PDK4 silencing down-regulates the expression of inflammatory cytokines in cardiomyocyte through inhibiting TGF- β /Smad pathway

Western blot was used to determine the protein expressions of CRP, TNF- α , and IL-6. The results showed that there were no significant differences in the levels of these proteins between the control group and the negative control group (all $P>0.05$, **Figure 7**). Compared with the control group, the protein expression levels of CRP, TNF- α , and IL-6 in the other groups were lower (all $P<0.05$). Compared with the sh-PDK4 + LY2157299 group, the protein expression levels of CRP, TNF- α , and IL-6 in the sh-PDK4 group and LY2157299 group were higher (all $P<0.05$).

Discussion

AF is a common type of arrhythmia, and its prevalence has increased substantially in recent years [20]. Studies report that the morbidity of AF is closely associated with many diseases such as coronary heart disease and heart failure [21, 22]. Thus, it is of great importance to investigate the pathogenesis for the

PDK4's role in atrial fibrillation

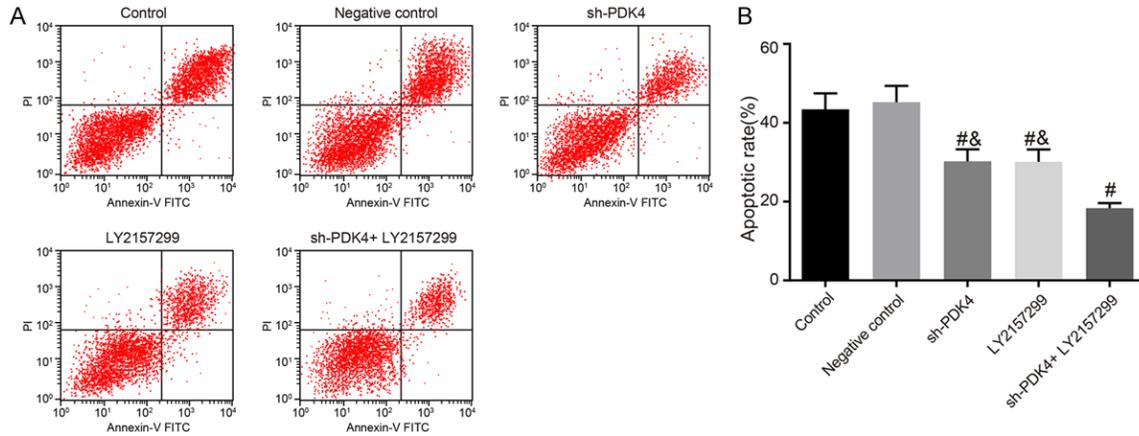


Figure 6. Cardiomyocyte apoptosis in each group detected by Annexin V-FITC assay. A. Results of Annexin V-FITC detection; B. Apoptosis rate in each group. Compared with the control group, [#]*P*<0.05; compared with the sh-PDK4 + LY2157299 group, [&]*P*<0.05.

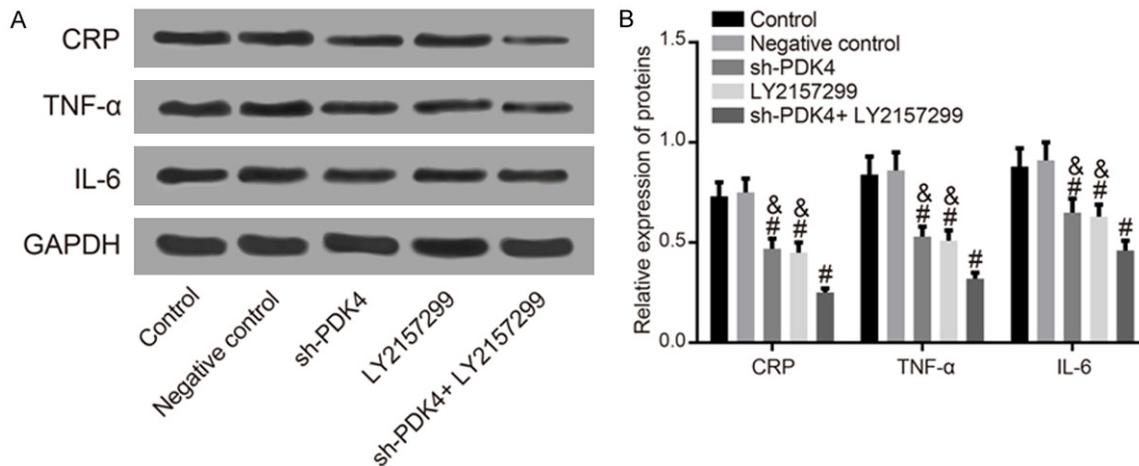


Figure 7. Expressions of proteins associated with cardiomyocyte proliferation and inflammation in each group. A. CRP, TNF- α , and IL-6 protein expressions determined by Western blot; B. Quantification of CRP, TNF- α , and IL-6 protein expressions. Compared with the control group, [#]*P*<0.05; compared with the sh-PDK4 + LY2157299 group, [&]*P*<0.05.

prevention and treatment of this disease. Apoptosis and inflammation are two important factors in the pathogenesis of AF [23]. In the present study, we explored the impacts of PDK4 and its interaction with TGF- β /Smad signaling pathway on the biological behavior and inflammatory response of cardiomyocytes in patients with AF.

The results of cardiac ultrasound showed that the levels of LVEDD, LVESD, and LAEDD were higher in the AF group than in the SR group, indicating that AF can affect the levels of these markers. Rohm et al. reported that in diabetic mice treated with glibenclamide, the blood glu-

cose level and cardiac metabolism and function were significantly improved and the PDK4 expression in plasma was down-regulated [24]. A study by Raman reported that the PDK4 expression was much higher in the plasma of patients with AF than in people with SR, thus PDK4 may be used as a biomarker to identify the sinus rhythm of AF patients [25]. In the present study, we compared the expressions of PDK4 gene and protein in the left atrial appendage between the AF group and SR group and found that the expression of PDK4 in the left atrial appendage was higher and the TGF β /Smad signaling pathway was markedly activated in AF patients. TGF β /Smad pathway is a key

pathway in the process of atrial remodeling and myocardial fibrosis, and its activation can aggravate the progression of AF [26]. In the present study, qRT-PCR, Western blot, and Masson staining were performed to confirm that there were marked upregulation of PDK4 expression in left atrial appendage, noticeable myocardial fibrosis, and activation of TGF β /Smad pathway in patients with AF.

It has been reported that cardiomyocyte apoptosis are factors to the onset of AF, and the increase of cardiomyocyte apoptosis can promote myocardial interstitial fibrosis and atrial structural enlargement [27-29]. Moreover, cardiomyocyte apoptosis is an irreversible process, which is also an essential reason why it is difficult to restore the sinus heart rate in patients with persistent AF [30]. Cyclin D1, as a typical cyclin, can shorten the G1 phase of cells when overexpressed, thus speeding up cell cycle progression and promoting cell viability [31]. In the present study, the expressions of proteins associated with cardiomyocyte proliferation and apoptosis were examined. The results showed that the expressions of anti-apoptotic proteins Cyclin D1 and Bcl-2 increased, whereas the pro-apoptotic factors Bax and Caspase-3 were down-regulated in cardiomyocytes treated with PDK4 silencing, and the sh-PDK4 + LY2157299 group exhibited the most significant change of all the groups. CCK8 and Annexin V-FITC (Fluorescein) assay further identified that PDK4 silencing and TGF β /Smad signaling pathway inhibition could promote cardiomyocyte viability and inhibit apoptosis. Besides, reports have demonstrated that inflammatory reaction is another factor in the morbidity of cardiovascular diseases [32]. The elevation of plasma CRP and inflammatory factors such as TNF- α and IL-6 have become risk predictors of acute coronary syndrome and peripheral arterial diseases [33]. It has been documented that inflammatory reaction is involved in the occurrence and development of AF, while the incidence of AF can, in turn, aggravate the inflammatory response [34]. A study by Aguilar confirmed that the inflammatory mediators can promote cardiomyocyte apoptosis and induce atrial remodeling by activating fibroblast fibrosis, which can further lead to AF [35]. Thus, we speculate that the reason PDK4 silencing can regulate AF through inhibiting TGF- β /Smad signaling pathway may be that it can affect cardiomyocyte inflammatory factors.

In summary, our study presents a rationale for the hypothesis that PDK4 silencing can inhibit the activation of TGF- β /Smad signaling pathway, thereby suppressing the cardiomyocyte apoptosis and expression of inflammatory factors and promoting the cardiomyocyte viability. PDK4 may become a potential target in the treatment of AF. However, more studies are required in the future to further verify this method for the clinical application.

Disclosure of conflict of interest

None.

Address correspondence to: Lizhong Song, Department of Critical Care Medicine, Shanxi Cardiovascular Disease Hospital, No. 18 Yifen Street, Taiyuan 030024, Shanxi Province, China. Tel: +86-13835126875; E-mail: songlizhong58y@163.com

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