Nkx2.5/CARP signaling pathway contributes to the regulation of ion channel remodeling induced by rapid pacing in rat atrial myocytes

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Abstract. Remodeling of atrial electrophysiology and structure is the primary feature of atrial fibrillation (AF). Evidence suggests that abnormalities in the expression levels of embryological cardiovascular development-associated transcription factors, including Nkx2.5, are crucial for the development of AF. Rat atrial myocardial cells (AMCs) in culture dishes were placed in an electric field and stimulated. Transmission electron microscopy was used to observe the ultrastucture prior to and following rapid pacing. The action potential durations and effective refractory periods were measured. RT-PCR and western blotting were performed to investigate the effect of rapid pacing on the expression levels of ion channel and nuclear proteins in AMCs. Nkx2.5 short interfering RNA (siRNA) transfection was performed. Through this *in vitro* rat AMC culture and rapid pacing model, it was demonstrated that rapid pacing shortened the action potential and downregulated the expression levels of L-type calcium and potassium channels. Expression levels of Nkx2.5 and cardiac ankyrin repeat protein (CARP) were significantly upregulated by rapid pacing at the mRNA and protein levels. siRNA-mediated Nkx2.5 silencing attenuated the rapid pacing-induced downreglation of ion channel levels. These results suggest that the Nkx2.5/CARP signaling pathway contributes to the early electrical remodeling process of AF.

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Introduction

Atrial fibrillation (AF) is one of the most common arrhythmias encountered in clinical practice (1). The prevalence of AF in the general population is ~1% and the risk increases with age. AF substantially increases cardiovascular morbidity and mortality. There is a 5-fold increase in the risk of stroke in patients with AF and 15-20% of strokes are caused by AF. AF is an independent risk factor for congestive heart failure and increases the mortality 2-fold. Genetic defects may be responsible for the pathogenesis of AF in a subset of patients. During AF, electrical and structural remodeling occurs continually (2-4). The electrophysiological and structural remodeling is crucial for the development, maintenance and recurrence of AF. Remodeling shortens the atrial wavelength of intraatrial reentry and leads to an increase in the potential number of electrical reentrant cycles, which is responsible for AF maintenance. Changes in the expression levels of ion channels, including L-type calcium (Ca) channel (LTCC) and potassium (K) channel, are important for early remodeling during AF (5). However, the pathogenic mechanisms underlying atrial structural remodeling remain to be elucidated.

Changes in atrial electrophysiology and structure, referred to as remodeling, constitute the primary features of AF occurrence, maintenance and recurrence (6,7). Changes in ion currents, including Ca^{2+} and K^+, form the basis for early electrical remodeling in AF (8,9). Calcium entry from the extracellular space through LTCCs and the resultant intracellular Ca^{2+} elevation (calcium overload) was demonstrated to be crucial in the regulation of atrial frequency-dependent action potential duration (APD) and effective refractory period (ERP) (10). The transient outward K^+ current mediated by the potassium channel Kv4.3 contributes to early repolarization (11).

Emerging evidence indicates that abnormalities in cardiovascular embryological development contribute to AF (12). Nkx2.5 is a critical transcription factor and its mutation is associated with AF development (13-16). As a member of the NK2 family, the expression and functions of Nkx2.5 overlap with those of the GATA family during cardiovascular development (17,18). However, whether Nkx2.5 affects ion channel proteins in the context of AF remains to be elucidated.

The aim of the present study was to investigate the effect of rapid pacing (rapid electrical stimulation used to simulate AF) on APD, ion channel proteins and the Nkx2.5/CARP (cardiac ankyrin repeat protein) signaling pathway.

Materials and methods

Isolation and culture of rat atrial myocardial cells (AMCs). The present study and all experimental protocols involved were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University (Chongqing, China). A total of 20 female Wistar rats (2 weeks old) were purchased from the Experimental Animal Center of the Third Military Medical University. Rats were maintained under constant temperature and humidity conditions with a 12-h light/dark cycle and *ad libitum* access to standard chow and water. Prior to the experiment, rats were sacrificed by CO_2 inhalation and then fixed in a supine position. Following sterilization with 70% ethanol, an incision was made along the right edge of the sternum and the chest wall was removed. The heart was dissected out and washed in cold phosphate-buffered saline (PBS). The left and right atria were isolated and washed with serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with penicillin and streptomycin. Under aseptic conditions, the right atrial appendage was cut into small pieces with scissors, which were then digested with 0.08% trypsin at 37˚C for 5 min. The reaction was terminated by addition of DMEM containing 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA). This solution was maintained at room temperature and the supernatant was filtered through a 100-μm mesh filter. Digestion was performed twice. Finally, suspensions of single cells were prepared by treatment of the digested product with 0.1% collagenase at 37˚C for 15 min. The cells were seeded into flasks at a density of ~1x10^8/l, followed by incubation with DMEM containing 10% FBS at 37˚C with 5% CO_2. In the control group, following 72 h of routine culture, the medium was replaced with serum-free DMEM for 24 h, and then rapid pacing was performed.

Rapid pacing of AMCs. When the cell confluence reached ~80%, the culture dishes were placed in an electrical field and stimulated with 10 Hz, 1.5 V/cm using the BL-420E+ biological and functional experimental system (Chengdu Techman Software, Co., Ltd., Chengdu, China). The beating frequency of the cells was visually recorded. The survival rate of cells prior to and following the rapid pacing was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenytetrazolium bromide assay (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, and the APD at 50 and 90% repolarization was recorded with a patch clamp at the whole cell mode.

Transmission electron microscopy. AMCs prior to and following rapid pacing were collected, transferred into Eppendorf tubes, resuspended in cold PBS and centrifuged at 200 x g for 5 min at 4˚C. The supernatant was removed and cells were fixed in 2% glutaraldehyde for 2 h and post-fixed in 1% tetroxide osmium for 2 h. Following dehydration with an alcohol gradient, cells were embedded in epoxy resin 618 (Shanghai Kang Lang Biological Technology Co., Ltd., Shanghai, China). Ultrathin sections (100 nm) were prepared and contrast stained with uranyl acetate and lead citrate. Images were captured (magnification, x6,000) using a transmission electron microscope (H7500; Hitachi, Ltd., Tokyo, Japan).

RNA interference. The short interfering RNA (siRNA) for Nkx2.5 and negative control siRNA duplexes were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of siRNA duplexes were as follows: Sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3' and anti-sense, 5'-ACG UGA CAC GUU CGG AGA ATT-3' for the negative control; sense, 5'-CCC UCG GGC GGA UAA GAA ATT-3' and anti-sense, 5'-UUU CUU AUC CGC CCG AGG GTC-3' for Nkx2.5-310. In each group, 1x10^5 cells were seeded into 60-mm culture dishes without antibiotics. At 70% confluence, the siRNA duplexes for Nkx2.5 and negative control were added with Lipofectamine® RNAimax (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. mRNA expression levels were detected by RT-PCR. Total cellular RNA was extracted from the rat AMCs of each group using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was prepared using a Superscript II First-Strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. PCR was performed using Taq DNA Polymerase (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The primer sequences and annealing temperatures were as follows: Forward, 5'-ATG GAG GCT GGA GCC CAG ATT GA-3' and reverse, 5'-GAC ATT GAG GTC CGC ACC GAA GG -3' for α1c (annealing temperature, 61.3˚C); forward, 5'-GCA GCA ACC TGA AAT CTG AAA CT-3' and reverse, 5'-GAT AAG CAA TGA ACC CAT CTC CA-3' for Kv4.3 (annealing temperature, 56.1˚C); forward, 5'-TTG TTT CTG TCA CCA GTA AC-3' and reverse, 5'-GAT GAG GAA GGA AGA GAA GC-3' for connexin-43 (Cx43; annealing temperature, 56.3˚C); forward, 5'-GTA AGC GAC AGC GGC AGG AC-3' and reverse, 5'-CGA CGC CAA AGT TCA CGA AG-3' for Nkx2.5 (annealing temperature, 58.7˚C); forward, 5'-GGG GTA CCA GCC AAC ATG ATG-3' and reverse, 5'-CCC TCG AGG CCT CAG AAT GTA GC-3' for CARP (annealing temperature, 60.1˚C); and forward, 5'-TGA GAG GGA AAT CGT GCG TGA C-3' and reverse, 5'-ATC TGC TGG AAG GTG GAC AGT GAG -3' for β-actin (annealing temperature, 53.9˚C). The amplification process was performed for 35 cycles following an initial 45 sec denaturation at 94˚C, annealed for 30 sec at the above-indicated temperatures and extended for 5 min at 72˚C. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Band intensities were measured by densitometry and normalized to β-actin using ImageJ software version 1.5.0 (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. Rat AMCs (1.5x10^6) from each group were lysed with 0.5 ml radioimmunoprecipitation assay buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 5 μl phenylmethylsulfonyl fluoride. Cell lysates were centrifuged

at 12,000 x g at 4˚C for 30 min and the resulting supernatant (total tissue homogenate) was stored at -80˚C until further analysis. Protein (15 μg) from each group was separated by 15% SDS-PAGE (200 V, 45 min) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin (HyClone; GE Healthcare Life Sciences) in Tris-buffered saline containing Tween 20 (TBST) for 1 h at room temperature, and then were incubated with the following primary antibodies: Rabbit anti-rat α1c (1:2,000; catalog no. AB5156; EMD Millipore, Billerica, MA, USA), rabbit anti-rat Kv4.3 (1:1,000; catalog no. AB5194; EMD Millipore), rabbit anti-rat Cx43 (1:1,000; catalog no. sc-9059; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-rat Nkx2.5 (1:500; catalog no. sc-14033; Santa Cruz Biotechnology, Inc.), rabbit anti-CARP (1:500; catalog no. sc-30181; Santa Cruz Biotechnology, Inc.) and rabbit anti-β-actin (1:1,000; catalog no. ab8227; Abcam, Cambridge, MA, USA). Membranes were washed three times in TBST and incubated with a horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (1:500; catalog no. DGSP-H-KIT-4; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). The protein bands were visualized using SuperSensitive Enhanced Chemiluminescence solution (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and quantified using ImageJ software version 1.5.0. Band intensities were measured by densitometry and normalized to β-actin.

Statistical analysis. Statistical analysis was performed in SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Group comparisons were performed by one-way analyses of variance, and the Student-Newman-Keuls method was used as a *post-hoc* test. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Observation of cultured atrial myocardial cells (AMCs). AMCs cultured for three days were heterogeneous in shape, including rod, spindle, triangular and irregular (Fig. 1A). The number of spontaneously beating cells increased following 48 h in culture. AMCs exhibited clear ultrastructural features and regular arrangement of mitochondrial cristae (Fig. 1B). At 24 h following 3-h rapid pacing, AMCs presented with a more polygonal shape, irregular myofibril arrangement and sparse myofilaments (Fig. 1C and D). At 48 and 72 h following rapid pacing, vacuolar degeneration and expanded bubbles were observed (Fig. 1E and F). The beat frequency did not alter significantly following rapid pacing (Fig. 1G); however the survival rate decreased from 48 h following rapid pacing (Fig. 1H; P<0.001). Under normal conditions, cells would proliferate for one month. Therefore, the decrease in survival rate was as a result of rapid pacing.

Electrophysiological changes in AMCs. The APD measured at 50% repolarization (APD 50) was significantly decreased from 12 h following rapid pacing (12 h, P=0.0235; 24 h, P=0.0014; 48 h, P=0.0005; Fig. 2A and B). The APD 90 was significantly decreased at 24 (P=0.056) and 48 (P=0.0021) h following rapid pacing (Fig. 2A and B). No significant differences in ERP were observed following rapid pacing (6 h, P=0.6647; 12 h, P=0.3858; 24 h, P=0.3438; 48 h, P=0.3930; Fig. 2A and C).

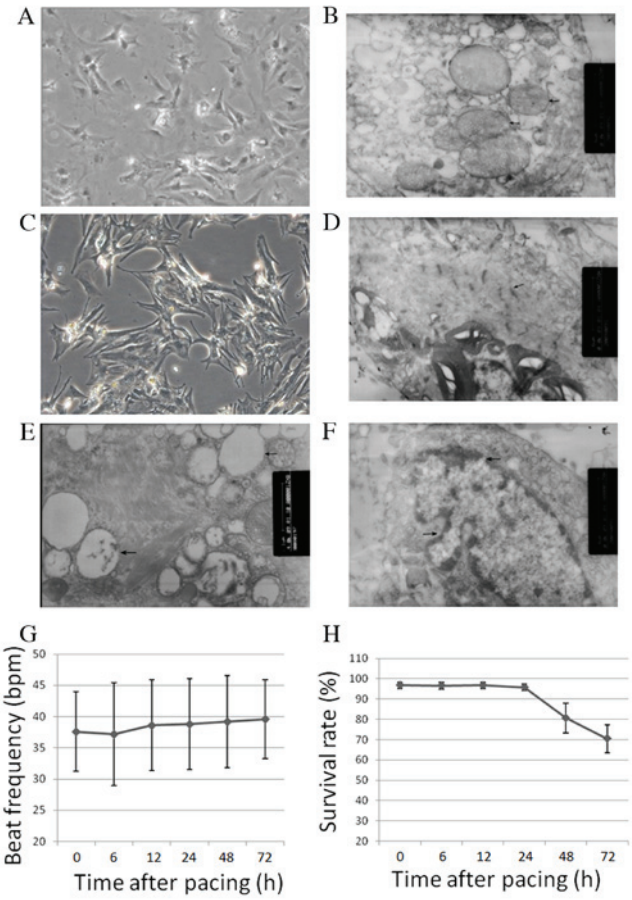

Figure 1. Characteristics of AMCs. AMCs were cultured for three days and visualized with (A) light microscopy and (B) transmission electron microscopy, in which mitochondrial cristaes were distinct. AMCs following rapid pacing were visualized with (C) light microscopy 24 h following rapid pacing, and transmission electron microscopy (D) 24 (E) 48 and (F) 72 h following rapid pacing. Light microscopy magnification, x400; transmission electron microscopy magnification, x14,000. Arrows in (E) indicate vacuolar degeneration of mitochondria at 48 h following rapid pacing. Arrows in (F) indicate vacuolization and swelling of organelles at 72 h following rapid pacing. Analysis of (G) the beat frequency and (H) survival rate of AMCs following rapid pacing revealed no change in beat frequency, although a decrease in survival was observed from 48 h following rapid pacing. Data are presented as the mean ± standard deviation (n=3). AMCs, atrial myocardial cells.

Effect of rapid pacing on the expression levels of ion channels and nuclear proteins in AMCs. mRNA expression levels of the LTCC protein α1c were significantly reduced at 6 h (P=0.023) following rapid pacing compared with prior to rapid pacing, and this difference increased at 12 (P=0.0053), 24 (P=0.0021) and 48 (P=0.0016) h (Fig. 3A). Western blotting correlated well with RT-PCR data, with α1c protein expression levels significantly reduced from 12 h following rapid pacing (P=0.0036; Fig. 3B). mRNA (P=0.0011) and protein (P=0.0085) expression levels of the potassium channel Kv4.3 were decreased from 12 h following rapid pacing (Fig. 3C and D). The mRNA and protein expression levels of the important gap junction protein Cx43 were not affected

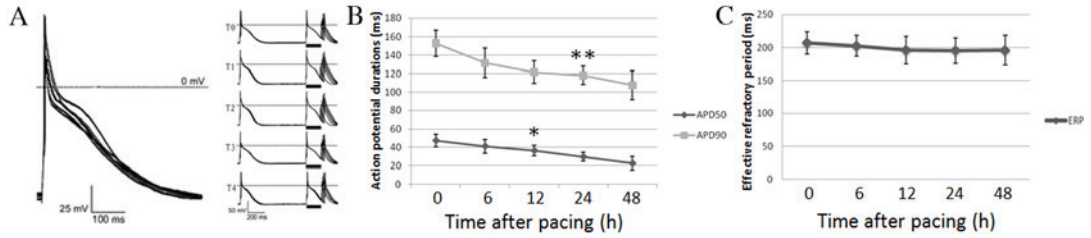

Figure 2. Electrophysiological alterations in atrial myocardial cells following rapid pacing. (A) Changes of APD and ERP at various time points following rapid pacing. (B) APD was measured at 50 and 90% repolarization. APD 50 and APD 90 decreased significantly following rapid pacing. (C) No significant differences were observed in ERP following rapid pacing. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. 0 h group. APD, action potential duration; ERP, effective refractory period.

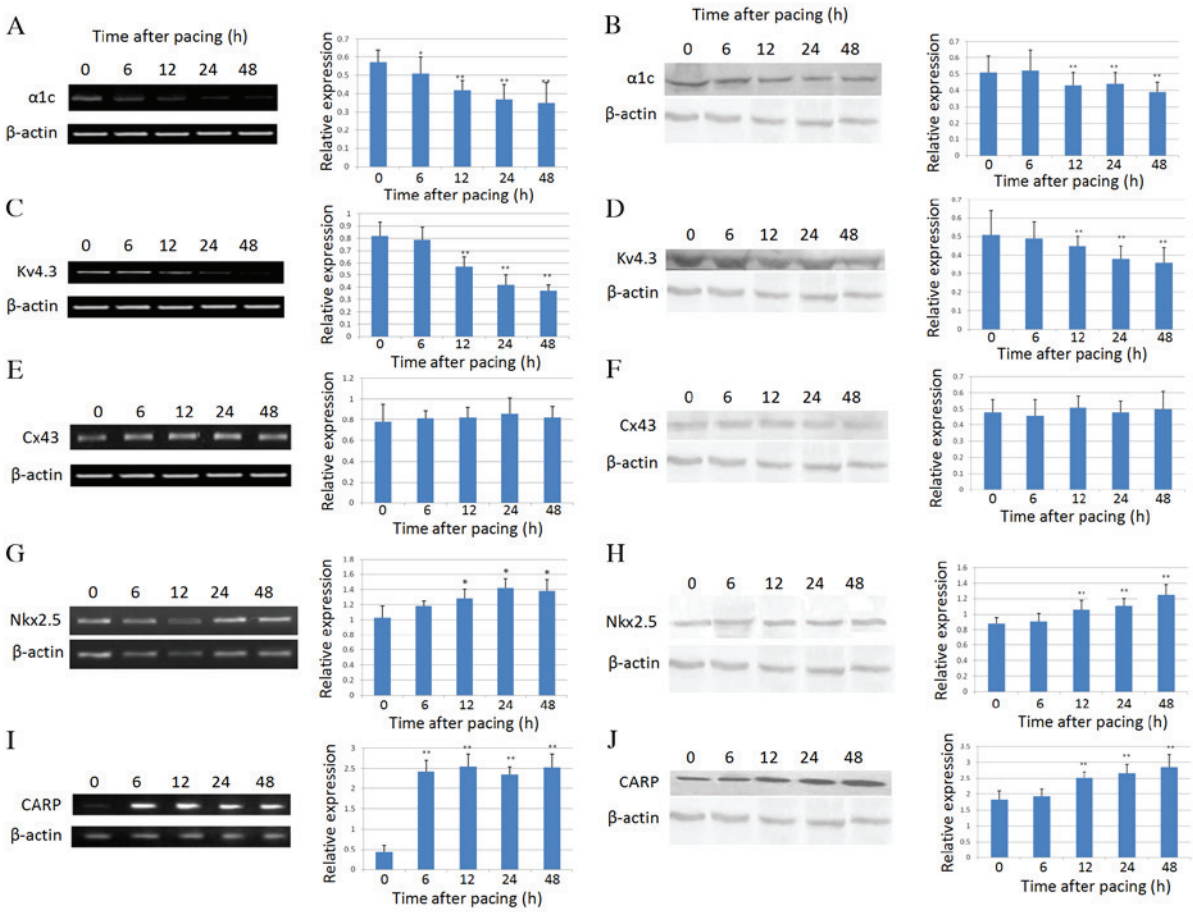

Figure 3. Effect of rapid pacing on the mRNA and protein expression levels of ion channel and nuclear proteins in atrial myocardial cells, determined by reverse transcription-polymerase chain reaction and western blotting, respectively. α1c (A) mRNA and (B) protein expression levels were reduced following rapid pacing. Kv4.3 (C) mRNA and (D) protein expression levels were decreased following rapid pacing. Cx43 (E) mRNA and (F) protein expression levels were not affected by rapid pacing. Nkx2.5 (G) mRNA and (H) protein expression levels were increased following rapid pacing, as were CARP (I) mRNA and (J) protein expression levels. Cx43, connexin-43; CARP, cardiac ankyrin repeat protein. *P<0.05 and **P<0.01 vs. 0 h group.

by rapid pacing (Fig. 3E and F). The mRNA (P=0.022) and protein (P=0.0073) expression levels of Nkx2.5, a critical cardiac transcription factor, were upregulated from 12 h following rapid pacing (Fig. 3G and H). CARP, a downstream molecule in the Nkx2.5 homeobox gene signaling pathway, exhibited a similar pattern to Nkx2.5 (mRNA, P=0.0005 and protein, P=0.0032 at 12 h; Fig. 3I and J).

Effect of Nkx2.5 inhibition on the expression levels of ion channel proteins in AMCs. As presented in Fig. 4A, transfection with Nkx2.5 siRNA inhibited the rapid pacing-induced increase in Nkx2.5 expression at the mRNA level (P=0.0089). In addition, the increase in mRNA expression levels of CARP induced by rapid pacing was inhibited by Nkx2.5 siRNA (P=0.0068; Fig. 4B and C). Protein expression levels of Nkx2.5 (P=0.046) and CARP (P=0.031) followed the same pattern (Fig. 4D and E).

Furthermore, treatment with Nkx2.5 siRNA attenuated the decrease in α1c and Kv4.3 mRNA (α1c, P=0.028; Kv4.3, P=0.043) and protein (α1c, P=0.017; Kv4.3, P=0.019) expression levels induced by rapid pacing (Fig. 5).

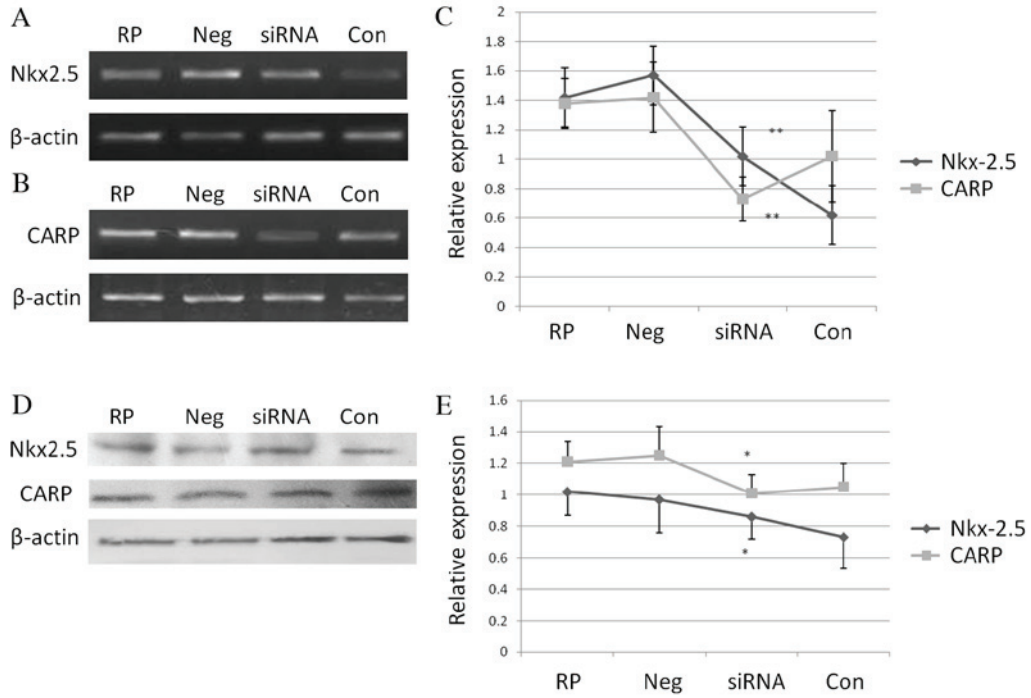

Figure 4. Effect of Nkx2.5 inhibition on the mRNA and protein expression levels of Nkx2.5 and CARP in atrial myocardial cells, determined by reverse transcription-polymerase chain reaction and western blotting, respectively. mRNA expression levels of (A) Nkx2.5 and (B) CARP were reduced following transfection with Nkx2.5, but not negative control, siRNA. (C) Relative mRNA expression of Nkx2.5 and CARP. (D) Protein expression levels of Nkx2.5 and CARP were reduced following transfection with Nkx2.5, but not negative control, siRNA. (E) Relative protein expression of Nkx2.5 and CARP. Data were normalized to β-actin. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. RP and negative groups. CARP, cardiac ankyrin repeat protein; RP, rapid pacing; Neg, negative group; siRNA, Nkx2.5 siRNA transfection group; Con, control group without pacing.

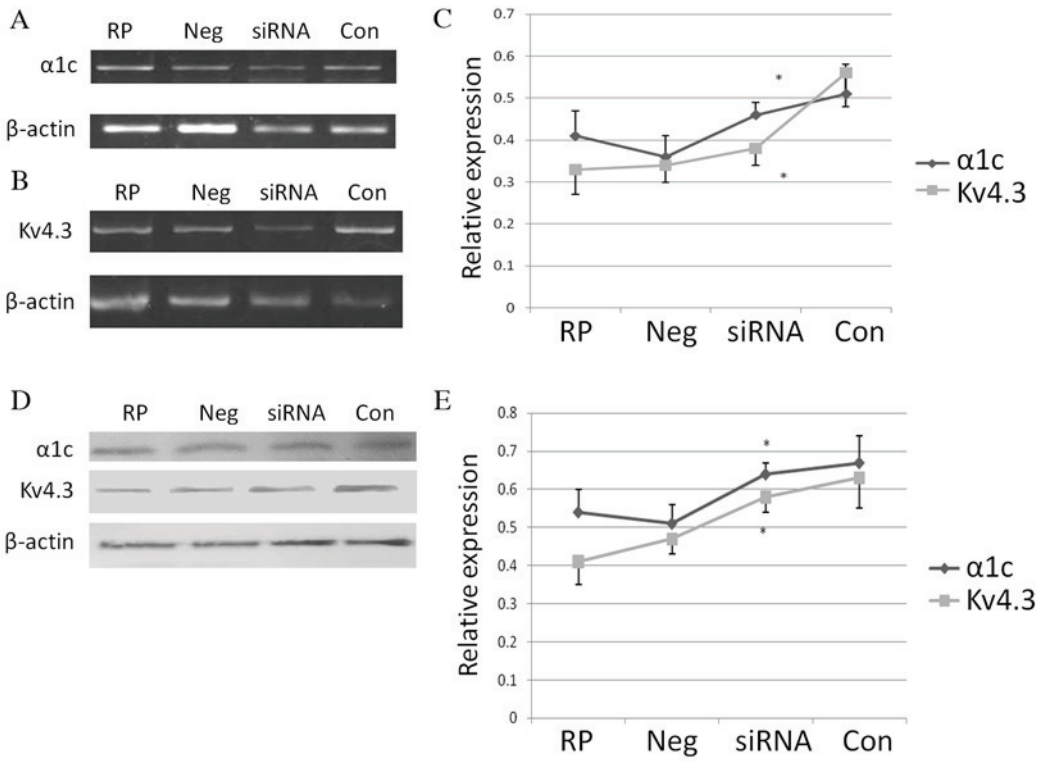

Figure 5. Effect of Nkx2.5 inhibition on the mRNA and protein expression levels of the ion channel proteins α1c and Kv4.3 in atrial myocardial cells, determined by reverse transcription-polymerase chain reaction and western blotting, respectively. mRNA expression levels of (A) α1c and (B) Kv4.3 were increased following transfection with Nkx2.5, but not negative control, siRNA. (C) Relative mRNA expression of Nkx2.5 and CARP. (D) Protein expression levels of α1c and Kv4.3 were increased following transfection with Nkx2.5, but not negative control, siRNA. (E) Relative protein expression of Nkx2.5 and CARP. Data were normalized to β-actin. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. RP and negative groups. RP, rapid pacing; Neg, negative group; siRNA, Nkx2.5 siRNA transfection group; Con, control group without pacing.

Discussion

AF, the most common form of sustained cardiac arrhythmia, is characterized by uncoordinated atrial activation and chaotic electrical activity, with consequent deterioration of atrial mechanical function (19). Using the *in vitro* rat AMC culture and rapid pacing model, the present study demonstrated that rapid pacing shortened the APD and downregulated the expression levels of LTCC and potassium channels. Expression of Nkx2.5 and CARP were significantly upregulated by rapid pacing at the mRNA and protein levels. siRNA-mediated Nkx2.5 silencing rescued the rapid pacing-induced downreglation of ion channel expression levels, suggesting that the Nkx2.5/CARP signaling pathway contributes to the early electrical remodeling process of AF.

In the current study, the APD of rat AMCs was significantly reduced 12 h subsequent to rapid pacing, while no effect on the ERP was observed at any time point. In AF patients, shortened APD results in decreased wavelength of reentry circuits and atrial electrical remodeling, thus facilitating the maintenance of AF and inhibiting the natural termination of AF (20,21). The lack of an effect on ERP may be due to differences in pacing rate, electric field strength and varying cell sources. The causes of APD shortening include: i) Increased outward K^+ currents; ii) decreased inward Ca^{2+} current; and iii) a combination of the above two factors (22). L-type Ca^{2+} current is activated by membrane depolarization and contributes to the formation of the action potential plateau phase (23). As in the ventricular muscle cells, the transient outward K^+ current (I_{to}) is the basis of early rapid repolarization of atrial action potential, while Kv4.3 is the major pore-forming subunit of I_{to} channels (24). Atrial rapid pacing, as occurs in atrial fibrillation, may lead to a decrease in the density of functional ion channels (Na^+, Ca^{2+} and K^+) (25); however, no effect was observed on the intrinsic properties of single ion channels.

A calcium channel current (I_{Ca}) is essential for action potential and excitation-contraction coupling of myocardial cells (26). The voltage-dependent calcium channels are typically divided into L- and T-type channels, and Ca^{2+} influx mediated by LTCCs is an important factor regulating human atrial frequency-dependent APD. The present study revealed that the expression level of α1c at 6 h subsequent to rapid pacing was significantly reduced compared with prior to pacing, becoming stable at 24 h. The reduction in LTCC currents is critical for the shortening of the action potential cycle, and decreased calcium influx is harmful to atrial mechanical contraction. The expression level of Kv4.3 was significantly reduced from 12 h subsequent to rapid pacing. These results are largely in accordance with other experimental models of atrial fibrillation, and may reflect an attempt to prevent the shortening of APD and ERP; however, the underlying mechanism requires further study.

Gap junctions between cardiac cells provide connections and a low-resistance pathway interconnecting cardiomyocytes (27). These coordinate myocardial action potential and synchronous contraction. The gap junction Cx proteins present in heart cells include Cx40 and Cx43 (28). A change in the structure and density of Cx may result in changes in conductivity anisotropy and conduction velocity of atrial myocytes, ideal conditions for reentrant arrhythmia (29). In the present study, no significant changes in Cx43 were observed, which may be due to various factors: The pacing duration may not have been long enough; changes in Cx43 may be the result of long-term AF; or changes in the distribution of Cx43 may be of greater importance than its expression levels. Further investigations are required to elucidate the role of gap junction proteins in atrial electrical remodeling.

Anomalies in embryological cardiovascular development contribute to the initiation of AF (30,31). Various transcription factors, including Nkx2.5, were identified as essential in cardiovascular genesis (32,33). Gutierrez-Roelens *et al* (34) first identified an Nkx2.5 mutation suggested to be associated with the atrial fibrillation phenotype. Homeobox gene Nkx2.5, also referred to as cardiac-specific homeobox gene, belongs to the NK-2 homeobox family. Nkx2.5 is crucial for myocardial cell differentiation and heart tube formation, and is involved in the atrioventricular separation and conduction system (35). As a downstream mediator of Nkx2.5, CARP contributes to the maintenance of complete sarcomere structure and function, and is involved in the regulation of intracellular calcium (36). The present study demonstrated that the expression levels of Nkx2.5 and CARP were significantly elevated during the early phase following fast pacing, indicating that Nkx2.5 is important in undifferentiated cells and in differentiated cardiomyocytes. As the Nkx2.5/CARP signaling pathway is a critical regulator of cell development, cell communication and intracellular calcium, it was hypothesized that the Nkx2.5/CARP signaling pathway may be critical for ion channel remodeling in the early stages of atrial fibrillation.

In the present study, although Nkx2.5-siRNA transfected AMCs inhibited the downregulation of α1c and Kv4.3 expression levels induced by rapid pacing, this downregulation was not completely reversed, suggesting that ion channel remodeling is regulated by multiple factors.

In conclusion, the results of the present study demonstrated that rapid pacing may shorten APD and induce the downregulation of the LTCC protein, α1c and potassium channel, Kv4.3, resembling the electrophysiological properties of atrial fibrillation. The Nkx2.5/CARP signaling pathway was upregulated by rapid pacing, while Nkx2.5 siRNA-mediated gene silencing inhibited the rapid pacing-induced ion channel downregulation. These results indicate that the Nkx2.5/CARP signaling pathway may be involved in the early channel remodeling process during rapid pacing. These findings may have implications for the early detection of AF, and suggest potential targets for prophylaxis.

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