Cilostazol ameliorates atrial ionic remodeling in long-term rapid atrial pacing dogs

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ABSTRACT

Objective: Ionic remodeling has a close correlation with the occurrence of atrial fibrillation (AF). Atrial tachypacing remodeling is associated with characteristic ionic remodeling. The purpose of this study was to assess the efficacy of cilostazol, an oral phosphodiesterase 3 inhibitor, for preventing atrial ionic remodeling in long-term rapid atrial pacing (RAP) dogs.

Methods: We use the methods of patch-clamp and molecular biology to investigate the effect of cilostazol on ion channel and channel gene expression in long-term RAP dogs. Twenty-one dogs were randomly assigned to sham, control paced, and paced+cilostazol (5 mg/kg/d, cilo) groups, with 7 dogs in each group. The sham group was instrumented with a pacemaker but without pacing. RAP at 500 beats/min was maintained for 2 weeks in the paced and cilo groups. During the pacing, cilostazol was given orally in the cilo group. Whole-cell patch-clamp technique was used to record atrial L-type Ca\(^{2+}\) (ICaL) and fast sodium channel (INa) ionic currents. Western blot and RT-PCR were applied to estimate the gene expression of Cav1.2 and Nav1.5 subunits. Statistical analysis was performed using SPSS 13.0.

Results: The density of ICaL and INa currents (pA/pF) was significantly reduced in the paced group (ICaL: -6.55±1.42 vs. -4.46±0.59 pA/pF; INa: -48.24±10.54 vs. -30.48±5.20 pA/pF, p<0.01). The paced+cilo group could not increase the density of ICaL currents (ICaL: -4.37±1.25 pA/pF, p>0.05), while the INa currents were recovered (-44.54±12.65 pA/pF, p<0.01) compared with the paced group. The mRNA and protein expression levels of Cav1.2 and Nav1.5 were apparently down-regulated in the paced group (p<0.01), but after cilostazol treatment, both of these subunits were up-regulated significantly (p<0.01).

Conclusion: Cilostazol may have protective effects on RAP-induced atrial ionic remodeling. (Anatol J Cardiol 2015; 15: 963-9)

Key words: atrial fibrillation, cilostazol, ionic remodeling, channel gene expression

Introduction

Atrial fibrillation (AF) is the most frequently encountered arrhythmia in the cardiology department, and no single modality is effective for all patients (1). The pathogenesis underlying AF is multi-factorial. Accumulating evidence suggests that the substrate for AF typically results from the effects of both electrical and structural remodeling. Currently available therapeutic approaches have major limitations, including limited efficacy and potentially serious side effects, such as malignant ventricular arrhythmia induction (2). An improved understanding of the mechanisms underlying AF is needed for the development of novel therapeutic approaches (3). The investigations on non-antiarrhythmic drugs have demonstrated beneficial effects in preventing the episodes of AF in both animals and human (4-6).

Atrial remodeling is the construction of the arrhythmogenic substrate of atrial AF (7). Atrial electrical remodeling is characterized by altering cellular electrical properties. The electrical activities of the heart are orchestrated by the matrix of ion channels, and ion channel remodeling involves changes in current density or in ion channel mRNA or protein expression (8).

Cilostazol is a quinolinone derivative that selectively inhibits phosphodiesterase 3 (PDE3) and has been extensively investigated as the effects of vasodilation and anti-platelet aggregation (9-13). A previous study demonstrated that cilostazol increased heart rates and improved the symptoms in patients with bradyarrhythmia, tachycardia-bradycardia syndrome, and Wenckebach-type atrioventricular block (14). Cilostazol is currently of interest to many investigators for its antiarrhythmic effects.
effects in patients with Brugada syndrome. This was assumed to be related to an increase in cytoplasmic Ca (15, 16). Similarly, PDE3 inhibition may be pro-arrhythmic in long QT syndrome (16). Whether it will be a benefit to the tachyarrhythmia has not been reported; the purpose of this study was to assess the effects of cilostazol on atrial ionic remodeling in 2-week rapid atrial pacing (RAP) dogs. The electrophysiological and cardioprotective mechanisms of cilostazol would be investigated.

**Methods**

**Animals and materials**

The animals we used in the study were approved by the Experimental Animal Administration Committee of Tianjin Medical University and Tianjin Municipal Commission for Experimental Animal Control. The approach we used was previously described to induce and maintain sustained AF in our experimental animals (17). For this study, 21 mongrel dogs of either sex, weighing between 12 and 17 kg, were randomly assigned to the sham group, paced group, or paced+cilostazol (5 mg/kg/d, cilo) group, with 7 dogs in each group. The dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg).

After intubation and mechanical ventilation, under sterile technique, a modified unipolar J pacing lead (St. Jude Medical, Saint Paul, MN, USA) was inserted through the right jugular vein, and the distal end of the lead was positioned in the right atrium. Initial atrial capture was verified with the use of an external stimulator (DF5A, Suzhou, China). The proximal end of the pacing lead was then connected to a programmable pacemaker (Fudan University, Shanghai, China), which was inserted into a subcutaneous pocket in the neck. The dogs in the paced group and paced+cilo group were paced at 500 bpm (120-ms cycle length) with the use of 0.2-ms square-wave pulses at twice-threshold current for 2 weeks. The electrocardiogram (ECG) was verified after 24 h in awake dogs and then every other day to ensure continuous 1:1 atrial capture. The dogs in the sham group were instrumented without pacing and were studied 2 weeks after pacing. The dogs in the paced+cilo group received cilostazol 5 mg/kg/d by gavage for 2 weeks during pacing. Systolic blood pressure was measured by carotid artery intubation during anesthesia at baseline and after 2 weeks of RAP. ECG and blood pressure were recorded by a multi-channel physiological recorder (Hongtong, TOP2001, Shanghai, China).

**Atrial cell isolation**

According to the previously described cell isolation methods (18, 19), a median sternotomy was performed, and the hearts were quickly excised. After cardiac excision, the hearts were immersed in Tyrode’s solution at 4°C. The solutions used for dissection and perfusion were equilibrated with 100% O2. The left circumflex artery was cannulated and connected to the Langendorff perfusion system filled with Tyrode’s solution free from CaCl2 and saturated with 100% O2. The heart was perfused at 25 ml/min, and the perfusion pressure was maintained at 80 mm Hg. The branches of the artery were ligated with silk thread to ensure adequate perfusion. The tissue was then perfused at 25 ml/min with nominally Ca2+-free Tyrode’s solution at 37°C for 20 min, followed by a 40-min perfusion with the same solution containing collagenase (150 U/ml, CLSII, Worthington Biochemical, USA), 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA), and 50 mM CaCl2. Softened tissue from a well-perfused region of the LA free wall was removed with forceps, and tissue pieces were washed by Krafibruhe (KB) solution and gently triturated. Cell were dispersed by a gentle pipette for 5 min in KB solution and then filtered. After the procedure above, the myocardial cells were kept at room temperature for 60 min.

The quiescent rod-shaped cells showing clear cross-stria- tions were chosen. A small aliquot of the solution containing the isolated cells was placed in a 1-ml chamber mounted on the stage of an inverted microscope (Olympus, Tokyo, Japan). Ten minutes was allowed for cells to adhere to the bottom of the chamber, and then the cells were superfused at 3 ml/min with Tyrode’s solution (6).

**Solution dispensing**

Tyrode’s solution contained (mmol/L) NaCl 136, KCl 5.4, MgCl2 0.8, CaCl2 1.8, NaH2PO4 0.33, glucose 10, and HEPES 10, pH 7.4 (adjusted with NaOH). The KB solution contained (mmol/L) KCl 20, KH2PO4 10, glucose 10, L-glutamic acid 70, taurine 10, and EGTA 10, along with 0.2% bovine serum albumin, pH 7.4 (adjusted with KOH). The pipette solution that was used to record the L-type calcium channel (\(I_{\text{Ca,L}}\)) and fast sodium channel (\(I_{\text{Na}}\)) contained (mmol/L) CsCl 20, MgCl2 1, EGTA 10, L-glutamic acid 80, CsOH 80, Na2ATP 0.1, MgATP 5, HEPES 10, TEA-Cl 20, and Na2 phosphocreatine·4H2O 5, pH 7.25 (adjusted with CsOH). The extracellular solution recording \(I_{\text{ex}}\) contained (mmol/L) cholineCl 110, NaCl 10, CsCl 20, MgCl2 1, CaCl2 1, HEPES 10, glucose 10, pH 7.4 (adjusted with NaOH). The extracellular solution recording \(I_{\text{ex}}\) was the same as the Tyrode’s solution.

**Data acquisition**

For recording ionic currents, we used the whole-cell patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments). Borosilicate glass microelectrodes with a 1.5-mm outer diameter and 1.1-mm inner diameter were used, and tip resistances were kept at 2.5 to 5 MΩ. To control for cell size variability, currents were expressed as densities (pA/pF). Voltage command pulses were generated by a 12-bit digital-to-analog (D/A) converter (Digidata 1200, Axon Instruments), controlled by Clampfit 10.2 software. Recordings were sampled at 10 kHz and low-pass-filtered at 1 to 5 kHz.

**Western blotting**

We used the following antibodies: anti-Nav1.5a, anti-Cav1.2, and anti-GAPDH (Abcam Inc. Cambridge, UK). An equal amount of protein was loaded onto a 6% SDS denaturing polyacrylamide gel, separated by electrophoresis, transferred onto PVDF mem-

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brane (Merck Millipore, USA), and incubated with the specific primary antibody overnight at 4°C. Protein levels were expressed as the ratio to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The membranes were then washed and subsequently incubated with the secondary antibody conjugated to horseradish peroxidase (HRP). Protein was visualized using enhanced chemiluminescence. The resulting bands were quantified using Gene Tools software (Gene, Texas, USA).

RNA isolation and real-time RT-PCR
The LA tissue was used for molecular biological studies. Specimen were rapidly frozen in liquid nitrogen and stored separately at -80°C for further analysis. Specific oligonucleotide primer pairs for amplification of Na+ and Ca2+ channel genes were designed according to the sequences obtained from GenBank. The primers and GenBank sequence numbers specific for each channel are in Table 1. β-actin and GAPDH were included as internal controls.

A total 200 ng of total RNA underwent RT-PCR using a commercially available kit (Takara, Shiga-ken, Japan). The PCR consisted of 35 cycles of 94°C for 40 s; 51°C (Nav1.5a), 52°C (Cav1.2, β-actin), or 55°C (GAPDH) for 30 s; and 72°C for 30 s. Then, 5 μL of product was analyzed by 1% agarose gel electrophoresis.

Statistical analysis
Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., California, USA) Continuous variables are expressed as mean±SD. Kolmogorov-Smirnov test was used to test the distribution of numeric variables, and statistical comparisons among groups with normal distribution were performed by one-way analysis of variance (ANOVA). If significant effects were indicated by ANOVA, a t-test with Bonferroni correction or Dunnett’s test was used to evaluate the significance of differences between individual mean values. A two-tailed p<0.05 was considered significant.

Results
Hemodynamic parameters
As shown in Table 2, there was no significant difference in ventricular rate or systolic blood pressure between groups at baseline (p>0.05), and no difference was found during the pacing (p>0.05); the blood pressure and heart rate were not changed by rapid atrial pacing in each group after 2 weeks (p>0.05). All animals kept a good appetite and physiological conditions.

ICaL changes and gene expression
In the present study, any contaminating effects of ICaL rundown were minimized by beginning all studies 5 min after membrane rupture. Depolarizing 200-millisecond pulses from -40 mV to voltages ranging from -50 mV to +50 mV elicited typical ICaL. Figure 1A shows the I-V curves of ICaL obtained from the dogs of each group. Figure 1B shows the mean±SEM of peak ICaL current densities; RAP was associated with a decrease in ICaL density. ICaL density was reduced significantly by RAP . Maximum peak ICaL density averaged to -4.46±0.59 pA/pF in the paced group (n=10 cells) compared with -6.55±1.42 pA/pF in sham dogs (n=9 cells) (p<0.01). After 2 weeks of RAP , cilostazol (5 mg/kg/d, cilo) (-4.37±1.25 pA/pF, n=8 cells) did not change the maximum peak density of ICaL compared with the paced group (p>0.05). The protein levels of Cav1.2 decreased in the paced group compared with the sham group (Cav1.2: paced group 0.31±0.03 vs. sham group 1.0, p<0.01).

Table 1. Primers for RT-PCR

| Gene   | Sequence numbers Primer (5’→3’) | Size (BP) |
|--------|---------------------------------|-----------|
| Nav1.5α| NM_001002994 F: TGAATGTCCTCCTGTGCTG  | 424       |
|        | R: TGTTGGTTGAAGTTGTCG            |           |
| Cav1.2 | AB262537.1 F: CCGTTCCTGTGGACCTTCA | 288       |
|        | R: CACCTTCCGTGCGCTGTG             |           |
| β-actin| AF021873.2 F: CAGAGCAAGCAGAGGATC | 392       |
|        | R: AGGTAGTCATGTCAGGT              |           |
| GAPDH  | NM0001003142.1 F: ACCACAGTCATGCCATCAC | 261       |
|        | R: CACACACTTCTTGTAGTCATC          |           |

Table 2. Hemodynamic parameters before and after pacing in each group

| Group | Heart rate, bpm | Systolic blood pressure, mm Hg |
|-------|-----------------|-------------------------------|
|       | Before pacing   | P                              | Before pacing   | P                              |                             |
|       | 197±12          | >0.05                          | 136.4±7.5       | >0.05                          |                             |
| Sham  |                 |                                | 138.3±10.1      | >0.05                          |                             |
| Paced | 198±9           | >0.05                          | 132.4±7.5       | >0.05                          |                             |
| Cilo  | 196±14          | >0.05                          | 136.2±7.8       | >0.05                          |                             |
| F     | 0.185           | 0.029                          | 0.122           | 0.389                          |                             |
| P     | >0.05           | >0.05                          | >0.05           | >0.05                          |                             |

Data are presented as mean±SD; *Bonferroni t-test
decreased in the paced group compared with the sham group (p<0.01). The paced+cilo group increased the mRNA levels of Cav1.2 compared with the paced group (p<0.01). Figure 1E shows representative western blots of Cav1.2. The 288-kDa immunoreactive band. Figure 1F illustrates quantitative Cav1.2 immunoreactivity of the western blot hybridization. Data are presented as relative gene expression (n=6). GAPDH was used as a loading control. The protein levels of Cav1.2 were upregulated in the paced+cilo group (p<0.01).

INa changes and gene expression

The I-V curves relation for INa in each group are revealed in Figure 2A; peak INa density was shown as a function of test potential (TP). Any contaminating effects of INa rundown were also minimized by beginning all studies 5 min after membrane rupture. Depolarizing 200-millisecond pulses from -90 mv to voltages ranging from -80 mv to +60 mv elicited a typical INa. Figure 2B illustrates that RAP was associated with a decrease in INa density. INa density was reduced highly significantly by RAP. Maximum peak INa density averaged to -30.48±5.20 pA/pF in the paced group (n=6 cells) compared with -48.24±10.54 pA/pF in sham dogs (n=10 cells) (p<0.05). After 2 weeks of RAP, in the cilostazol group (-44.54±12.65 pA/pF, n=9 cells), the density of INa (-58.62±16.17 pA/pF, n=8 cells) was significantly higher than that in the paced and sham group (p<0.01).

The mRNA levels of Nav1.5α were also evaluated. Figure 2C shows a gel obtained by semiquantitative RT-PCR for the Nav1.5α subunit. The left band in each lane corresponds to the Nav1.5α mRNA product, and the right band is the internal standard. Figure 2D shows the mean±SEM Nav1.5α mRNA concentration in hearts (1 independent determination per heart) from each group of dogs. Data are presented as relative gene expression (n=6). The mRNA levels of Nav1.5α decreased in the paced group compared with the sham group (p<0.01). The paced+cilo group increased the mRNA levels of Nav1.5α compared with the paced group (p<0.01). The protein expression of Nav1.5α was down-regulated by RAP. Cilostazol could induce the up-regulation of protein expression of Nav1.5α (Fig. 2E, 2F).

Discussion

The major finding of this study is that cilostazol may have beneficial effects on atrial ionic remodeling in long-term RAP dogs.

Atrial electrophysiology change is one of the main characteristics of AF, which is called electrical remodeling. Wijffels et al. (7) reported that atrial electrical remodeling played a key role
in the development, maintenance, and recurrence of AF. Research (20-22) has shown that the changes in reduced ion current density, including $I_{CaL}$, $I_{Na}$, etc., are an important basis for early electrical remodeling in AF and result in a functional substrate that supports the maintenance of AF.

In the experiment, we used the AOO model of a buried implanted pacemaker, with the right external jugular vein approach. The incision was small, had a low rate of infection, avoided the operation method of thoracotomy, and improved the operation efficiency and success rate. During the experiment, we used the method of monitoring the limb lead ECG to detect the effectiveness of high-speed continuous atrial pacing and evaluate the successful rate of the model. After 2 weeks, a median sternotomy was performed; we checked the location of the electrode at the same time, and the hearts were quickly excised, and next, the electrophysiological experiments were performed. The electrode position on the right atrial and right or appendage was regarded as a successful model. The application of the model was safe and reliable, and the AF-induced rate was more than 80%. In the present study, the ventricular rate was not controlled by atrioventricular (AV) node block, and it was a pity that we did not assess ventricular function with echocardiography. But, we found that the ventricular rate and blood pressure were not significantly changed by RAP in each group. Thus, the ventricular tachycardia-induced left ventricular dysfunction might not have been caused by the pacing or might not have contributed to the development of atrial remodeling in the RAP dog model.

Yue et al. (18) showed that L-type calcium channel ($I_{CaL}$) plays a significant role in maintaining the plateau in canine atrial myocytes. Grunnet et al. (23) indicated that $I_{CaL}$ plays a crucial part in the regulation of human atrial frequency-dependent action potential duration (APD) and endocardial return percentage (ERP). Nav1.5 is the principal Na⁺ channel isoform expressed in cardiomyocytes. Nav1.5 has also been observed in the endoplasmic reticulum of dog myocytes (24). Abriel et al. (25) reported that Nav1.5 associates with partner proteins, which may be adaptor proteins, enzymes which interact with and modify the channel, and proteins modulating the biophysical properties of Nav1.5 upon binding. Nav1.5 is essential for the conduction of electrical impulse and has a close correlation with AF (26-28). Lu et al. (29) indicated that RAP could induce acute stages of atrial electrical remodeling. Atrial tachycardia is a sufficient stimulus to induce the changes typical of AF-induced remodeling (17).

The major finding of this study is that cilostazol may have beneficial effects on atrial ionic remodeling in long-term RAP dogs. Our data indicate that the $I_{CaL}$ current was not changed in the cilostazol-treated group but increased Cav1.2 gene expression in our animal model of RAP. The reasons for the increase of Cav1.2 gene expression found in the paced+cilostazol group in our study are not apparent and deserve further investigation.
The I-V curve indicates that $I_{CaL}$ was not changed in the cilostazol-treated group. As a result, we investigated the active curve of $I_{CaL}$ and found that there were significant differences in the sham group and paced+cilo group in the curve; although the entire current range had no significant change, cilostazol still restored the active status of $I_{CaL}$ (Fig. 3A) probably because the drug dose and treatment time were not enough.

The present study showed that cilostazol could up-regulate $I_{Na}$ current and its channel gene expression in the paced+cilostazol group compared to in paced group. The human cardiac Na⁺ channel, voltage-gated Nav1.5α (encoded by the SCN5A gene), is responsible for the fast depolarization upstroke of the cardiac action potential and serves as a molecular target for developing antiarrhythmic drugs (30, 31). The mutations in SCN5A may predispose patients with or without underlying heart diseases to AF; expanding the clinical spectrum of disorders of the cardiac Na⁺ channel to AF (31, 32).

Cilostazol could up-regulate $I_{Na}$ current in the paced+cilostazol group compared to the paced group. However, there were no obvious changes of the $I_{Na}$ active curve between the paced and paced+cilo groups (Fig. 3B). These results indicated that cilostazol may have the function to restore the $I_{Na}$ current by not affecting its active status and might act by other ways to regulate the current.

Increased evidence has demonstrated that cilostazol could have many potential benefits on the heart, and cilostazol may have dual inhibitory effects in the heart (33). Cilostazol has presented different effects from other PDE3 inhibitors, especially adenosine uptake inhibition. Therefore, cilostazol may serve as an effective cardioprotective drug, with its beneficial effects in preventing arrhythmia. Investigations on the effects of cilostazol on heart rate variability (HRV) have shown that cilostazol significantly improves a slow heart rate (34, 35). However, it is important to note that the evidence regarding cilostazol and tachyarrhythmia is still limited and unclear. Currently, as we know, there are fewer studies that directly have investigated the effect of cilostazol in AF. In a recent study, Alizade et al. (36) indicated that cilostazol could decrease total atrial conduction time duration in patients with peripheral artery disease, which may also prevent the development and/or recurrence of AF.

### Study limitations

In the present study, we did not assess the overall electrophysiological inter-atrial conduction time (IACt), atrial effective refractory period (AERP), and vulnerability to AF. We also did not evaluate the cardiac function with echocardiography. We also did not evaluate other ion currents in our study. These limitations should be solved in future studies with different designs.

### Conclusion

This study, for the first time, demonstrated that in the atrial tissue of chronic rapid atrial pacing dogs, cilostazol 5 mg/kg/d could effectively suppress the down-regulation of $I_{CaL}$ currents and up-regulation of Cav1.2 and Nav1.5α gene expression. In conclusion, we found for the first time that cilostazol ameliorated atrial ionic remodeling in a canine model of RAP. Our study provides further evidence for the role of cilostazol in regulating tachyarrhythmia. Cilostazol 5 mg/kg/d did not affect the current densities of $I_{CaL}$ in paced dogs. Cilostazol may prevent atrial ionic remodeling and may serve as a novel therapeutic approach to the prevention of AF.

### Conflict of interest

None declared.

### Peer-review

Externally peer-reviewed.

### Authorship contributions

Concept - G.L.; Design - G.L., T.L., E.L.; Supervision - Z.Z., L.C.; Resource - G.L.; Materials - J.L., X.W., W.Y.; Data collection &/or processing - Z.Z., W.L., L.C.; Analysis &/or interpretation - Y.C., L.C., W.L., X.W.; Literature search - T.L., E.L., X.W., J.L.; Writing - Z.Z.; Critical review - T.L., E.L., W.Y., Y.C.; Other - W.L., Z.Z., W.Y., J.L., Y.C.
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