MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 2210-2217
DOI: 10.12659/MSM.906044

Received: 2017.07.04
Accepted: 2017.09.25
Published: 2018.04.13

Clinical Effects of "Selective Drug" Regulating Vagus Nerve Signal Pathway in Vagally-Mediated Atrial Fibrillation

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Source of support: Natural Science Foundation of China (NSFC81660071)

Background: The cardiac autonomic nervous system plays a crucial role in genesis and development of atrial fibrillation (AF) through the G protein signal transduction pathway. Therefore, intervening in the G protein signal transduction pathway may be a new "selective drug" method to regulate autonomic nerve activity to prevent vagally-mediated AF.

Material/Methods: Seventeen adult beagles were randomized into 3 groups: shame-operation control group (group A, n=5), empty vector gene control group (group B, n=6), and Gα_{i2}ctp gene experimental group (group C, n=6). Group A was injected with normal saline into the anterior atrial wall, and group B and group C animals were injected with recombinant adenovirus with empty vector or Gα_{i2}ctp vector in the same region. AF was induced by the method of rapid atrial pacing in groups B and C. To determine the clinical effect of vagal modulation, the effective refractory periods (ERP) and field action potential duration (FAPD) were evaluated by electrophysiological study. The expression levels of tyrosine hydroxylase (TH) and choline acetyl transferase (CHAT) in different parts were determined with immunohistochemistry.

Results: After successful Gα_{i2}ctp gene transfer, in group B, the ERP and FAPD significantly decreased ($P<0.05$), and TH and CHAT expression observably increased ($P<0.05$), while those differences were absent between groups A and C ($P>0.05$).

Conclusions: Recombinant adenovirus-mediated overexpression of Gα_{i2}ctp in canine myocardial cells can interfere with the activity of the vagus nerve, reverse the development and progression of electrical remodeling, and reduce the incidence of AF.

MeSH Keywords: Atrial Fibrillation • Atrial Remodeling • Autonomic Nervous System • GTP-Binding Protein Regulators

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/906044

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Background

Atrial fibrillation (AF) is a common tachyarrhythmia in clinical practice; it causes great harm to patients, and while stroke remains the most common complication, heart failure is the second [1–3]. Even more unfortunately, current clinical treatments such as drugs or catheter ablation achieve few demonstrable effects [4,5]. The cardiac autonomic nervous system is activated in AF, inducing autonomic nervous remodeling and electrical remodeling, which, in turn, aggravates AF [6,7]. Some studies proposed that low-level vagus nerve stimulation (LL-VNS) can inhibit the occurrence and persistence of AF through decreasing the cardiac autonomic nervous remodeling [8–11], and speculated that this anti-arrhythmia was associated with inhibitory vagus nerve and sympathetic activity. G protein features in autonomic nervous signal transduction pathway, and can regulate autonomic nervous activity [12]. Thus, the objective of this study was to find an optimal gene therapy that interferes in the G protein signal transduction pathway for AF prevention and treatment.

Material and Methods

Experimental animals

The study protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xinjiang Medical University, China (Permit Number: IACUC-20121122005), and conformed to guidelines developed by the Association for Assessment and Accreditation of Laboratory Care (AAALAC).

The 17 adult healthy beagle dogs were randomly divided into 3 groups: the sham-operation control group (group A, n=5), the empty vector gene control group (group B, n=6), and the $G\alpha_{i2}$ctp gene experimental group (group C, n=6). Our study was conducted with 17 healthy beagle dogs (mean body weight, 12.0±1.5 kg; aged 1–2 years) provided by the First Affiliated Hospital of Xinjiang Medical University Animal Center. The dogs were anesthetized with ketamine (20 mg/kg) and underwent thoracic surgery at the 4th intercostal rib on the left. Based on the methods described by Arora et al. [8], a 4-ml solution prepared from 500 µl of normal saline was injected into the anterior atrial wall following thoracotomy in the sham group (group A), while a 4-ml solution containing 500 µl of recombinant adenovirus with empty vector was administered to the group B dogs, and a 4-ml solution containing 500 µl of recombinant adenovirus with $G\alpha_{i2}$ctp vector was administered to the group C dogs. Injections were performed at multiple injection sites (6–8 equidistant sites that were 0.5–1 cm apart), with 0.5 ml injected per site.

Establishing the acute AF model

At 7 days after revival, we opened the chest of each dog again. We sewed 10-bipolar electrodes into the left atrial appendage, left and right atria, and pulmonary veins to make pacing and record electrophysiological data. The blood oxygen saturation, rhythm and rate of heart, arterial blood pressure, and other vital signs were monitored in the experiment. In groups B and C, AF was induced by repetitive 1200-Hz burst pacing of the left atrial appendage at 2×threshold for 6 h, while group A was not stimulated. AF was induced by S1S1 method, which intermittently stimulated the vagus nerve (S1-S1=300 ms) by suprathreshold stimulation for 30 s. AF was considered to be induced successfully if an irregular 500 beats/min heart rate with an irregular atrioventricular conduction could be maintained for more than 5 s. Each part was repeated 5 times. All of the fast pacing and parameters recording were finished by a multi-conductive physiological recorder (Lead-7000 EP Control, Sichuan Jinjiang, China).

Recording of electrophysiological data

The ERP was defined as the longest interval with S1S2 method until failing to response (S1-S1=300 ms, S1: S2=8: 1, V=2× Threshold). The pacing was suspended at baseline, 1 h, 3 h, and 6 h, when measured.

Tissue samples (about 5–8 mm^2) were collected from the anterior wall of the atria, immersed in 4°C oxygenated Tyrode's solution, and placed onto an MEA electrode. After connecting the MEA electrode to the TCO2 thermal control and data collection system, electrocardiac signals were amplified 1200 times at 1060-AMP and recorded using Mc-Rack software. The field action potential duration (FAPD) was graphed based on the changes in peak amplitude, and the sequence of action potential was indicated by the difference in color and voltage in the diagram.

Immunohistochemistry studies

When the fast pacing finished, the fresh anterior right ganglionated plexi (ARGP) were routinely processed and fixed in 4% paraformaldehyde, stored in 70% ethanol, and paraffin-embedded. Then, the samples were cut into 5-um sections for histological evaluation. Rabbit polyclonal anti-TH (ab93291, Abcam, UK) and Cat polyclonal anti-CHAT (AB144P, Millipore, USA) were used to stain cardiac nerves. The positive staining area density, which was analyzed by Image-Pro Plus 6.0, was defined as the ratio of positive staining area to entire detection area (um^2/mm^2) to describe vagus nerve activity and sympathetic nerve activity. Three fields were selected for observation with a ×40 eyepiece in each section with highest levels of positive density. The mean density was defined as the positive area density of that section.

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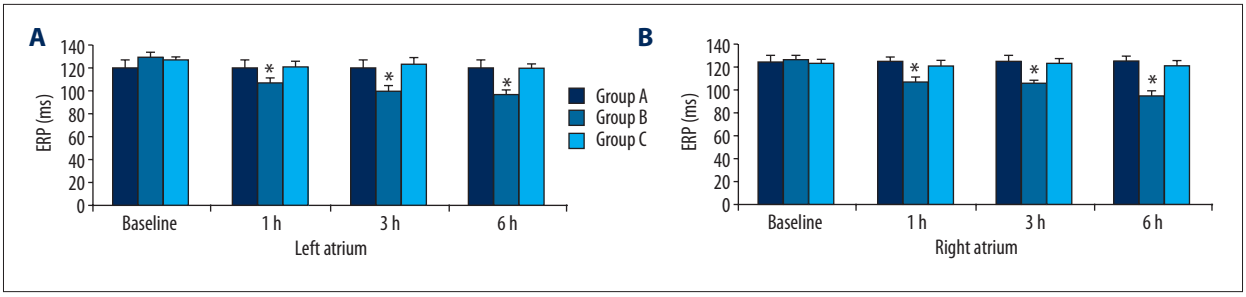

Figure 1. ERP at different time points in the 3 groups. * *P*<0.05 compared with baseline, ERP – effective refractory period.

Biochemical analysis

Fresh myocardial tissues (200 mg) were collected and homogenized in RIPA buffer. The tissue homogenates were centrifuged, and the supernatants (20 μl) were collected. Protein samples were mixed with 5× loading buffer, separated by SDS-PAGE for 45 min, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with Western blocking buffer (5% skim milk powder in 100 ml PBST) for 60 min. Each membrane was incubated overnight with 10 ml of primary antibodies directed against $G\alpha_{i2}ctp$ (1: 4000), M_2R (1: 2000), or β-actin (1: 5000) at 4°C. After washing primary antibodies twice for about 20 min at room temperature, membranes were incubated with 10 ml of alkaline phosphatase-labeled anti-rabbit, anti-mouse, and anti-goat secondary antibodies for 2 h at room temperature. Substrate solution was added to the membranes for protein visualization, and when purple bands were clearly visible, the reaction was stopped. Proteins were quantified using Image-ProPlus (version 4.1) software.

Statistical analysis

The mean ERP and FAPD values at different time points were analyzed using analysis of variance for repeated measurements. Comparisons of TH and CHAT protein levels were performed by one-way ANOVA, while pair-wise comparisons between multiple groups were analyzed using the LSD method. P-values <0.05 were considered statistically significant.

Results

In the process of the experiment, the blood pressure of the animals remained stable and the oxyhemoglobin saturation remained above 90%. No signs of heart failure due to rapid pacing were observed.

The effect of $G\alpha_{i2}ctp$ gene overexpression on basic electrophysiological study

The ERP were measured at baseline, 1 h, 3 h, and 6 h. In group B compared to groups A and C, the ERP was clearly shortened (P<0.05), but these differences were absent between groups A and C (P>0.05). In group B, the ERP values gradually decreased over time and reached a minimum at 6 h (P<0.05), but these changes disappeared in groups A and C (P>0.05) (Figure 1).

In the empty vector gene control group, the field action potential duration (FAPD) of atrial tissues at different time was observed in the microelectrode array (MEA) detection map, which indicated the transmission sequence of electric shock from bottom left to upper right (Figure 2A). The frequency of excitation was significantly increased and disordered, with inconsistent rhythm and rate. During signal transduction, significant changes in voltage were observed as indicated by disappearance of the step-wise voltage changes and the presence of signals with inconsistent amplitude and direction (Figure 2B). Further analysis of the electrocardiac signal revealed disorganized shapes of different colors and shades (Figure 2C). The FAPD of left atrial tissues in the 3 groups were 76.84±4.25 ms, 62.35±3.18 ms, and 78.13±7.18 ms. The FAPD of right atrial tissues in the 3 groups were 82.35±6.23 ms, 59.75±3.54 ms, and 81.36±5.32 ms. Compared to groups A and C, the FAPD of group B was significantly decreased (P<0.05), while that difference was absent between groups A and C (P>0.05) (Figure 2D).

Histology

Ganglionated plexi, which are an important element of the epicardial neural network, feature in occurrence and persistence of AF [13]. In this experiment, TH and CHAT protein expression levels in ARGP were measured by immuohistochemical staining. The niger-brown-staining areas were positive ganglion cells and unpigmented areas were negative ganglion cells, while the blue-staining areas were cores (Figure 3A–3C).

The CHAT expression levels of ARGP in groups A, B and C were (39.35±6.18) ×1000 um^2/mm^2, (87.35±7.63) ×1000 um^2/mm^2, (42.50±7.64) ×1000um^2/mm^2.

The TH expression levels of ARGP in groups A, B and C were (41.25±7.63) ×1000 um^2/mm^2, (81.24±6.72) ×1000 um^2/mm^2, (39.45±6.57) ×1000um^2/mm^2.

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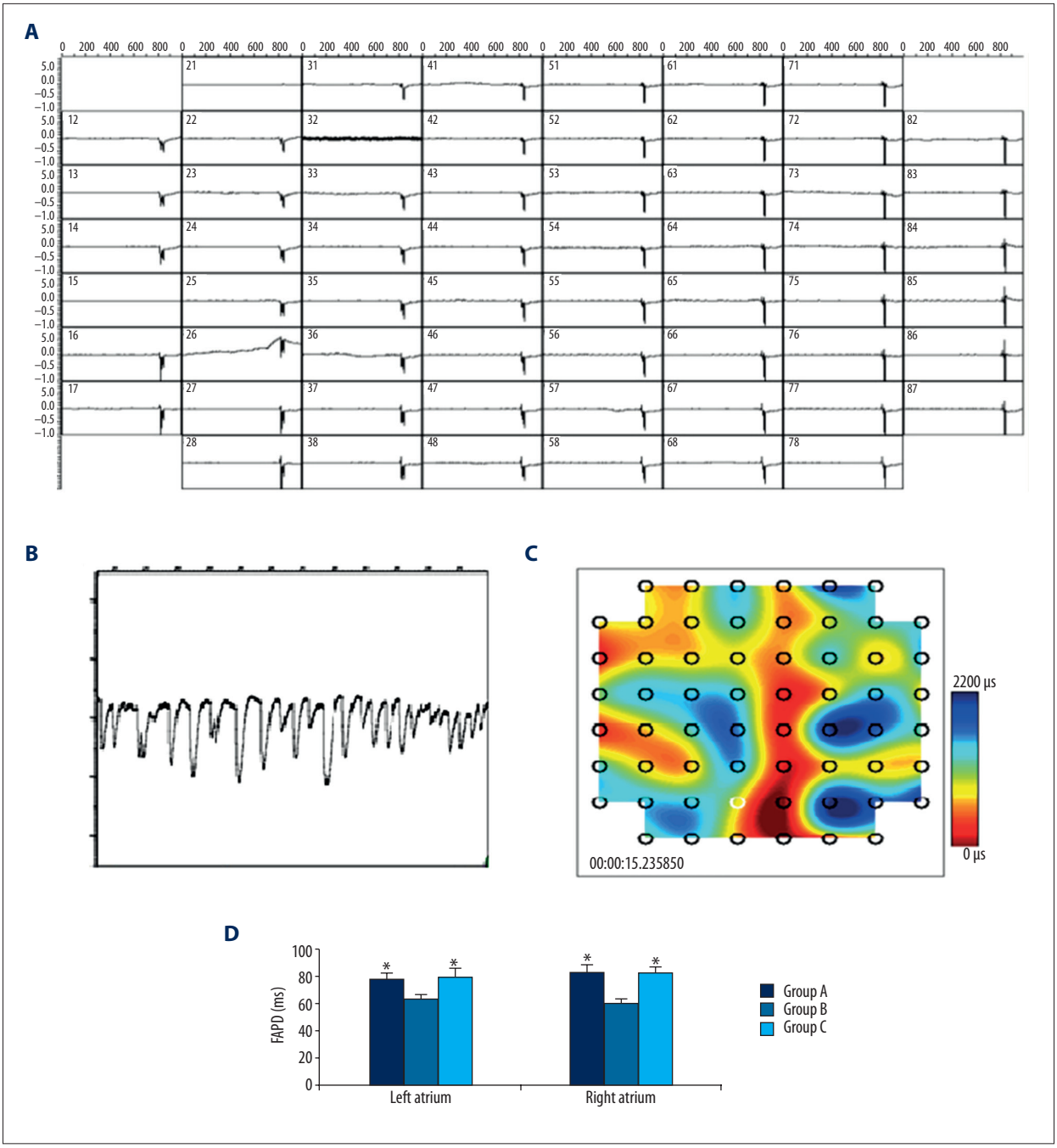

Figure 2. MEA detection in atrium. (**A**) Left atrial tissues MEA detection in group B; (**B**) recorded single-window graph of MEA detection for left atrial tissues in group B; (**C**) electrocardiac signal analysis of left atrial tissues; (**D**) FAPD comparison for right or left atrial tissue MEA detection potential. MEA – microelectrode array; FAPD – field action potential duration. * *P*<0.05 compared with group B.

Compared to groups A and C, the expression levels of TH and CHAT observably increased in group B (P<0.05), but that difference was absent between groups A and C (P>0.05) (Figure 3D).

Biochemical analysis

As shown in Figure 4, protein expression of $G\alpha_{i2}$ctp was observed in all parts of the atrium, and $G\alpha_{i2}$ctp protein expression compared to β-actin expression could be calculated.

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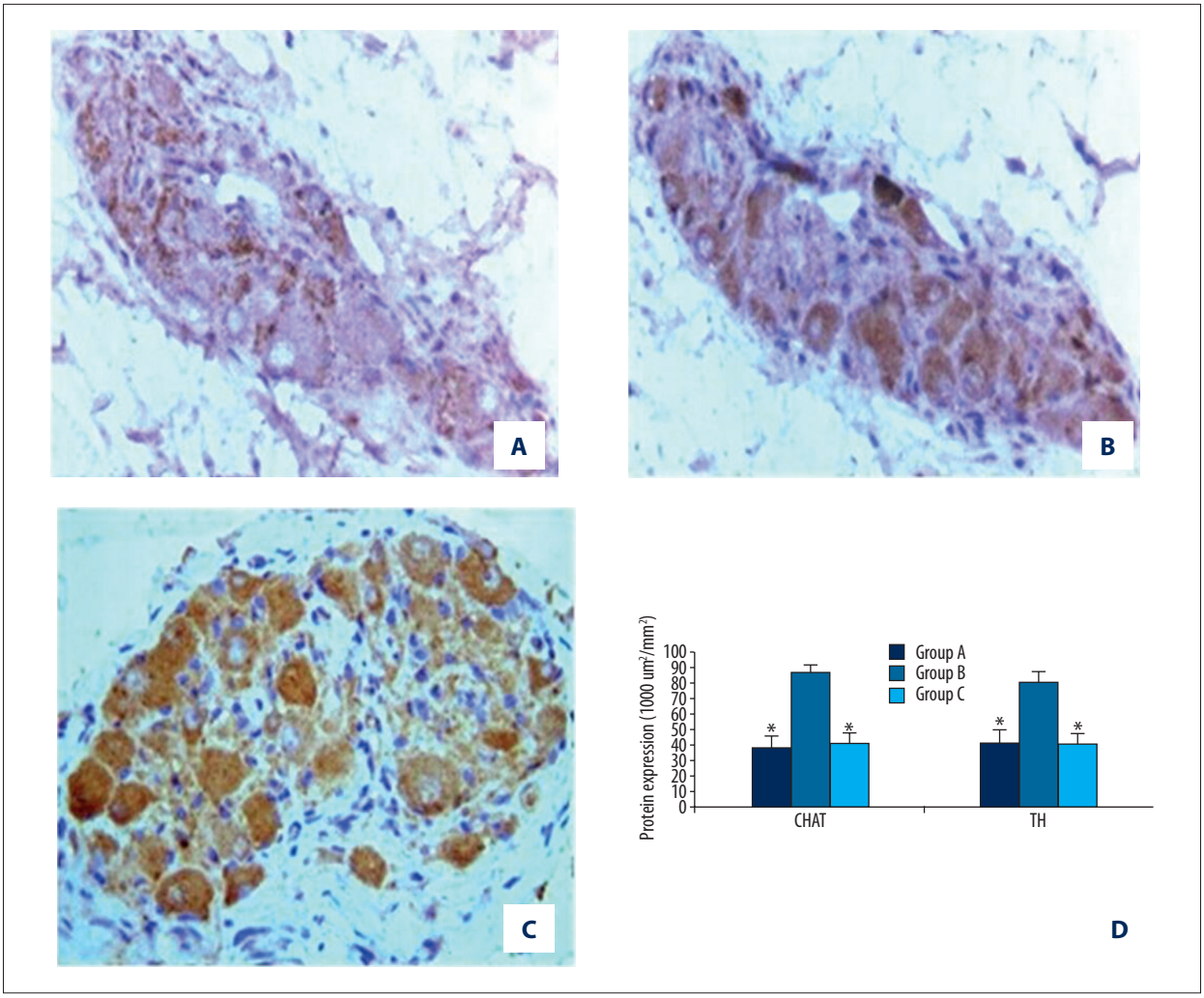

Figure 3. ARGP CHAT levels shown by immunohistochemistry staining. (**A–C**) ARGP CHAT staining in sham group (group A), empty vector gene control group (group B), and $G\alpha_{i2}$ctp gene experimental group (group C). The niger-brown-staining areas were positive ganglion cells and unpigmented areas were negative ganglion cells, while the blue-staining areas were cores. (**D**) TH and CHAT expression levels in ARGP. * *P*<0.05 compared with group B.

Although the level of $G\alpha_{i2}$ctp protein expression was slightly different between different parts of the atrium and pulmonary vein in group C (P>0.05) (Figure 5A), the atrial $G\alpha_{i2}$ctp protein expression was significantly higher in group C compared to that in group A and group B (P<0.05) (Figure 5B).

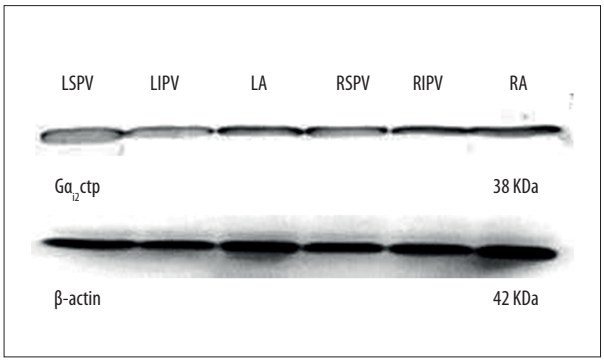

Figure 4. The electrophoretic band of $G\alpha_{i2}$ctp protein.

Discussion

Electrophysiological analysis confirmed that this technique prolonged the ERP of the atrium and pulmonary vein, and reduced the field potential of the tissue. Histology indicated that recombinant adenovirus-mediated overexpression of $G\alpha_{i2}$ctp reduced the expression of TH and CHAT in ARGP. At the molecular level, this gene transfer method can increase the expression of $G\alpha_{i2}$ctp in myocardial cells in the atrium. In summary,

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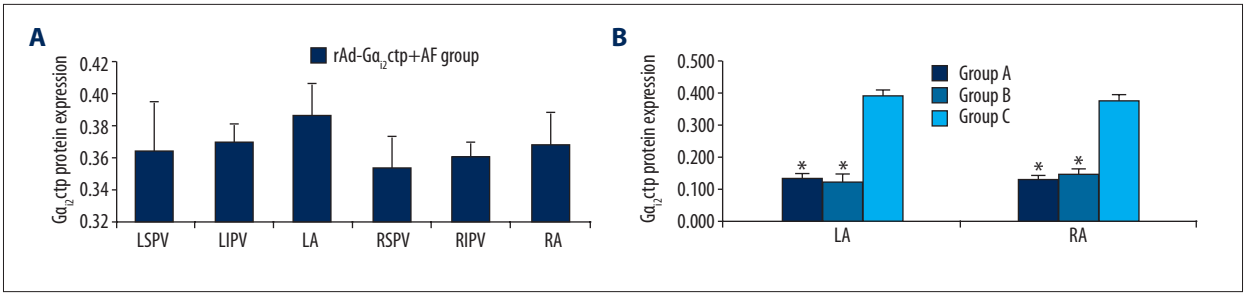

Figure 5. Different parts of $G\alpha_{i2}$ctp protein expression of the atrium and the pulmonary veins. (**A**) Level of $G\alpha_{i2}$ctp protein expression of different parts of the atrium and pulmonary vein in group C. (**B**) Atrial $G\alpha_{i2}$ctp protein expression in the 3 groups. * $P<0.05$ compared with group C.

this method can reverse the development and progression of autonomic nervous remodeling to reduce the incidence of AF.

Autonomic remodeling during AF

Studies in animal models and humans have confirmed that abnormal autonomic innervation is associated with AF. In a canine model of AF, Jayachandran et al. showed that the density of the sympathetic nerve was increased and it played a role in the induction and maintenance of AF following myocardial infarction [14–17]. Moreover, Ng et al. reported enhanced sympathetic and parasympathetic nerve growth in the left atrium in a canine model of heart failure and AF [18,19], whereas Choi et al. found that AF was triggered by endogenous cardiac discharges in dogs [20]. Similar findings were observed in human AF. In summary, these studies demonstrated that the changes in the autonomic nervous system during the development and maintenance of AF stemmed from autonomic remodeling.

Norepinephrine is the main neurotransmitter used by the sympathetic nervous system, and tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of norepinephrine. On the other hand, acetylcholine is the main vagal neurotransmitter, and choline acetyltransferase (CHAT) is the rate-limiting enzyme in the biosynthesis of acetylcholine. In the present study, the levels of TH and CHAT were significantly higher in group B than in group A, indicating enhanced sympathetic and vagal activities during RAP-induced AF, which is consistent with the findings reported in previous studies. However, $G\alpha_{i2}$ctp transduction in group C resulted in significantly reduced TH and CHAT levels compared with group B but not with group A, indicating that overexpression of $G\alpha_{i2}$ctp decreased vagal activity and reversed nerve remodeling in AF.

Intervention of autonomic remodeling can reverse electrical remodeling in AF

Several studies have elucidated the effect of intervention of autonomic remodeling on electrical remodeling in AF. Sheng et al. demonstrated that low-level vagus nerve stimulation inhibited cardiac autonomic activity and prevented electrical remodeling in acute AF [21]. Similarly, Dietrich et al. showed that subcutaneous low-level stimulation of the vagus nerve reversed electrical remodeling in a canine model of RAP-induced AF [22,23]. Both studies demonstrated that inhibition of vagal activity reversed atrial electrical remodeling.

In the present study, atrial ERP shortened and dispersion clearly increased in group B compared to group A, indicating that the model of electrical remodeling was successfully built in AF [24,25]. In group C compared to group B but not group A, ERP was significantly prolonged and dispersion was apparently decreased, indicating that overexpression of $G\alpha_{i2}$ctp decreased vagal activity and reversed electrical remodeling in AF.

Inhibitory mechanism of $G\alpha_i$ctp gene overexpression against electrical remodeling in AF

In previous studies, it was shown that vagal activity is increased in AF [26,27]. Vagal activity is dependent on the spatial heterogeneity of acetylcholine, which is synthesized from acetyl-CoA and choline by the action of CHAT. Acetylcholine mainly accumulates in the synaptic knob of cholinergic nerves, and, upon release, it binds to choline receptors to continue signal propagation [28]. The M_2 muscarinic receptor is the predominant choline receptor expressed on myocardial cells [29], which, upon binding with acetylcholine, activates the breakdown of $G\alpha_{i/o}\beta_\gamma$ into $G\alpha_{i/o}$ and $G\beta_\gamma$. Since $G\alpha_{i/o}$ can lead to attenuation of L-type calcium channels and inwardly rectifying potassium channels (If), while $G\beta_\gamma$ can activate acetylcholine-sensitive potassium channels ($I\alpha_{Ch}$), which eventually results in delayed signal conduction in the sinus node and atrioventricular (AV) node, and shortened atrial ERP with increased dispersion, the latter of which acts as the substrate for recurrent and increased induction of AF [30–32].

High $G\alpha_{i2}$ expression in the AV node inhibited basal AV conduction, prolonged the duration of AV node conduction, and reduced the ventricular rate during AF in the absence of a complete heart block [33,34] because given that $G\alpha_{i2}$ is structurally

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similar to $G\alpha_i$, it can competitively inhibit the binding between $G\alpha_i$ and M_2R, interfere with the degradation of the $G\alpha_{i/o}\beta_\gamma$ trimer, and block signal transduction, thereby leading to the absence of free $G\alpha_{i/o}$ and $G\beta_\gamma$, prolonged atrial ERP with reduced dispersion, and lower AF induction [35–37]. Therefore, it is believed that this is the mechanism by which $G\alpha_{i2}$ctp ($G\alpha_{i2}$ peptide) overexpression reduced vagal activity and prevented the development and progression of AF.

In this study, atrial $G\alpha_{i2}$ctp protein expression was significantly higher in group C compared to that in group A and group B. Although the level of $G\alpha_{i2}$ctp protein expression was slightly different between different parts of the atrium and pulmonary vein, these differences were not statistically significant, indicating that the $G\alpha_{i2}$ctp gene was successfully transduced into the canine atrium. Furthermore, comparison with the control groups demonstrated that the results found in group C were solely caused by overexpression of the $G\alpha_{i2}$ctp gene in the atrium, as the effects of surgery and gene transduction were excluded in group A and group B, respectively.

Our study provides an effective genetic target for cardiac ablation at the molecular level for the effective treatment of AF. However, our study has some limitations. The model of AF induced by left atrial appendage fast pacing is different from human AF. The effects of $G\alpha_{i2}$ctp gene overexpression in dogs with untreated AF might be different from that in patients receiving antiarrhythmic drugs. Therefore, further investigations are needed.

Conclusions

$G\alpha_{i2}$ctp gene overexpression may be a new "selective drug" method to regulate autonomic nerve activity to prevent vagally-mediated AF.

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