The Role of $\alpha 7$ nAChR-Mediated Cholinergic Anti-Inflammatory Pathway in Vagal Nerve Regulated Atrial Fibrillation

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Summary

The aim was to investigate the role of the $\alpha 7$ nAChR-mediated cholinergic anti-inflammatory pathway in vagal nerve regulated atrial fibrillation (AF).

18 beagles (standard dogs for testing) were used in this study, and the effective refractory period (ERP) of atrium and pulmonary veins and AF inducibility were measured hourly during rapid atrial pacing at 800 beats/minute for 6 hours in all beagles. After cessation of 3 hours of RAP, the low-level vagal nerve stimulation (LL-VNS) group ($n = 6$) was given LL-VNS and injection of saline (0.5 mL/GP) into four GPs, the methyllycacetate (MLA, the antagonist of $\alpha 7$ nAChR) group ($n = 6$) was given LL-VNS and injection of MLA into four GPs, and the Control group ($n = 6$) was given saline into four GPs and the right cervical vagal nerve was exposed without stimulation. Then, the levels of the tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), acetylcholine (ACh), STAT3, and NF- κ B proteins were measured. During the first 3 hours of RAP, the ERPs gradually decreased while the dispersion of ERPs (dERPs) and AF inducibility gradually increased in all three groups. During the last 3 hours of 6 hours' RAP in this study, the ERPs in the LL-VNS group were higher, while the dERPs and AF inducibility were significantly lower when compared with the Control and MLA groups at the same time points. The levels of ACh in the serum and atrium in the LL-VNS and MLA groups were higher than in the Control group, and the levels of TNF- α and IL-6 were higher in the Control and MLA groups than in the LL-VNS group. The concentrations of STAT3 in RA and LA tissues were higher in the LL-VNS group while those of NF- κ B were lower.

In conclusion, the cholinergic anti-inflammatory pathway mediated by $\alpha 7$ nACh plays an important role in low-level vagal nerve-regulated AF.

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Key words: Neural regulation, Arrhythmia, Electrical remodeling, Inflammatory reaction, Inflammatory cytokines

Atrial fibrillation (AF) is one of the most common cardiac arrhythmias in clinical practice,¹⁾ and an epidemiological survey showed that AF significantly increases the incidence of stroke and mortality.²⁾ The current therapy in patients with drug-refractory AF is surgical operation or cardiac radiofrequency ablation,³⁾ but the long-term outcome of ablation for even the earliest stage of AF is disappointing.⁴⁾ Previous studies have confirmed that the autonomic nervous system (ANS) plays an important role in the generation of AF,⁵⁻⁷⁾ and low-level vagal nerve stimulation (LL-VNS) not only increases the atrial effective refractory period (ERP) and inhibits the in-

duction of AF caused by rapid atrial pacing (RAP) but also reduces the levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and other inflammatory factors.⁸⁾ In previous studies of ours, we also found that ANS could influence the levels of inflammatory factors, and low-level atrial ganglionic plexi (GP; which are contained in the epicardial fat pads) stimulation could decrease the levels of inflammatory factors in the serum.⁹⁻¹¹⁾

A previous study has confirmed that inflammatory factors such as TNF- α and IL-6 can lead to atrial electrical remodeling and thus, promote the progress of AF.^{12,13)} It has been demonstrated that acetylcholine (ACh) re-

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leased by vagal endings binds to the N-type ACh receptor in the $\alpha 7$ subunit ($\alpha 7$ nAChR) and activates $\alpha 7$ nAChR to inhibit the release of TNF- α and IL-6, which has a protective effect in some inflammatory diseases; this mechanism is known as the cholinergic anti-inflammatory pathway (CAP).¹¹⁾ Whether the effects of LL-VNS on the AF are related to $\alpha 7$ nAChR-mediated CAP is unknown. We suspected that in the present study, CAP mediated by $\alpha 7$ nAChR plays an important role in the effects of LL-VNS on atrial electrophysiology and AF induction after short-term RAP in beagles.

Methods

Animal model preparation: This study was approved by the Animal Studies Subcommittee of Wuhan University School of Medicine and complied with the guidelines of the National Institutes of Health for the care and use of laboratory animals. Figure 1 shows the schematic diagram of the experimental model and protocol. All beagles were given an intramuscular injection of 25 mg/kg ketamine sulfate before being premeditated with pentobarbital sodium (30 mg/kg), intubated, and ventilated with room air supplemented with oxygen via a respirator (MAO01746, Harvard Apparatus, Holliston, MA, USA), normal saline at 50-100 mL/hour was infused to replace spontaneous fluid losses, and all efforts were made to minimize suffering.

Eighteen adult beagles (weight, 9-11 kg) were used in this study. The beagles were randomly assigned to three groups: the Control group ($n = 6$), the LL-VNS group ($n = 6$), and the methyllycaconitine (MLA) group ($n = 6$). Each beagle underwent RAP at 800 beats/minute for 6 hours. After cessation of 3 hours of RAP, the Control group was injected with saline into four GPs (0.5 mL/GP) and the right cervical vagal nerve was exposed without stimulation, the LL-VNS group was given LL-VNS combined with injection of saline into four GPs (0.5 mL/GP), and the MLA group was given MLA (an $\alpha 7$ nAChR inhibitor) into four GPs (0.5 mL/GP) combined with LL-VNS.

Electrophysiological measurements: After right- and left-sided thoracotomy of the fourth intercostal space of the beagles performed under anesthesia, four customized electrodes were sutured to the right atrial (RA) tissue, left atrial (LA) tissue, and right and left superior pulmonary veins (RSPV and LSPV, respectively). Beagles were subjected to RAP at 800 beats/minute for 6 hours, and the ERPs at all recording sites were measured hourly in a non-pacing and non-LL-VNS status. The ERPs were determined as previously described.¹⁴⁾ The dispersion of ERP was calculated as the maximum ERP minus the minimum ERP at all four recording sites. An S₁S₁ (120, 100, and 75 ms cycle length, lasting 5 seconds each, performed in triplicate for each frequency) programmed stimulus method was used to assess the inducibility and the mean duration of AF. AF was defined as irregular atrial rates faster than 500 beats/minute, with irregular atrioventricular conduction lasting longer than 5 seconds. AF times refers to the average number of times that AF was induced in every beagle within a group, respectively.

LL-VNS and MLA administration: In the MLA group, the MLA (2 mg; Abcam, UK) was dissolved in 2 mL of normal saline and injected equally into four GPs. In the LL-VNS and MLA groups, the vagal nerve on the right side of the neck was exposed and a pair of bipolar hook electrodes was attached to the vagal nerve. The electrodes were then connected to a constant current stimulator (S88; Grass Instruments, Quincy, MA, USA) with a stimulus isolation unit (model PSIU6; Grass Instruments) generating 2-Hz, 0.2-ms duration pulses. LL-VNS was performed during the last 3 hours of RAP. The lowest voltage level of LL-VNS that caused a heart rate decrease of 20% was considered to be the threshold and 80% of this voltage was then chosen as the voltage for LL-VNS.

ELISA: Four milliliters of venous blood was collected in ethylenediaminetetraacetic acid vacutainers from the Control, LL-VNS and MLA group beagles at baseline, 3 hours, and 6 hours. At the completion of the protocol, the hearts were quickly excised. Tissue specimens were obtained from the RA and LA tissues of every group. The tissue samples were homogenized in PBS buffer and centrifuged at $3000 \times g$ for 20 minutes at 4°C. The supernatants were then collected and the levels of TNF- α , IL-6, and ACh were examined by enzyme-linked immunosorbent assay (ELISA).

Western blotting: At the completion of the protocol, the hearts were quickly excised. Tissue specimens were obtained from the RA and LA tissues and temporarily stored at -80°C until needed. Expression of the Nuclear Transcription Factor- κ B (NF- κ B) and Signal Transducers and Transcriptional Activators 3 (STAT3) in the RA and LA were measured by western blot. The membranes were incubated with primary antibodies against NF- κ B (rabbit polyclonal anti-NF- κ B antibody used at 1:1000; Abcam) and STAT3 (rabbit polyclonal anti-STAT3 antibody used at 1:1000; Abcam). They were then washed in TBST three times, incubated with the secondary antibody for 1 hour at 37°C, and imaged using Immun-Star horseradish peroxidase substrate. The relative expression levels of the proteins were determined using an image analyzer software (AlphaEase FC, San Leandro, CA, USA).

Immunofluorescence assay: Immunofluorescence staining was used to assess the location of the $\alpha 7$ nAChR protein in the RA, LA, and GP tissues. After the experiment, the heart tissues in the Control group were collected and fixed with 4% formaldehyde diluted in warm PBS for 15 minutes at room temperature and washed three times in PBS. Then, the tissues were blocked in 5% normal serum/0.3% Triton X-100 in PBS for 1 hour at room temperature. Subsequently, the tissues were incubated with an antibody against $\alpha 7$ nAChR (1:100; ab10092; Abcam) overnight at 4°C. After being rinsed three times with PBS for 5 min each, the specimens were incubated in a 1:50 dilution of CY3-labeled goat anti-rabbit secondary antibody (1:50; AS-1109; Aspen) for 1 hour at room temperature in the dark and washed three times in PBS again. PI counterstaining was performed to stain the nuclei. Finally, the cover slips were mounted and images were viewed using an Olympus IX51 fluorescence microscope and Q-IMAGING Micropublisher.

Statistical analysis: The data are expressed as the mean $\pm$

Figure 1. A–D: Anatomical position of the four GPs. E: Experimental diagram of the experimental model and protocol, and experimental flow chart. ARGP indicates anterior right GP; IRGP, inferior right GP; SLGP, superior left GP; ILGP, inferior left GP; RAP, rapid atrial pacing; LL-VNS, low-level vagal nerve stimulation; ERP, effective refractory period; AF-D, atrial fibrillation duration; AF-ratio, atrial fibrillation inducibility ratio; MLA, methyllycaconitine; and GP, atrial ganglionic plexi.

Figure 2. Changes in the effective refractory period (ERP) and dispersion of ERP (dERP) during 6 hours of rapid atrial pacing (RAP) in the Control ($n = 6$), LL-VNS ($n = 6$), and MLA ($n = 6$) groups. **A–D:** The mean ERPs in the right atrium (RA), left atrium (LA), right upper pulmonary vein (RSPV), and left upper pulmonary vein (LSPV) were progressively shortened during the first 3 hours of RAP in all three groups, and compared with the baseline condition, the ERPs of all sites after cessation of 3 hours of RAP were significantly decreased in all three groups ($P < 0.05$ for all). **E:** The dERPs decreased during the last 3 hours of RAP in the Control and MLA groups. * $P < 0.05$ comparison between the baseline condition in the Control group; # $P < 0.05$ comparison between the third hour in the LL-VNS group; $\Delta P < 0.05$ comparison between the Control and LL-VNS groups at the same time point; $\&P < 0.05$ comparison between the MLA and LL-VNS groups at the same time point. There was no significant difference between Control and MLA groups.

standard deviation. A paired t -test was used for comparisons of continuous variables at the end of the study versus those at the baseline. One-way analysis of variance with Tukey tests were used to compare the means of continuous variables among multiple groups. All statistical tests were two-sided, and a P -value < 0.05 was required for statistical significance.

Results

Electrophysiology testing and AF induction: In the Control group, the ERPs at all recording sites gradually decreased during RAP (Figure 2A–D). In more detail, the

ERP at the RA in the Control group was shortened from 120.4 ± 3.85 ms at baseline to 104.8 ± 4.38 ms at the end of 3 hours of RAP, and then reached 102.4 ± 5.55 ms after cessation of 6 hours of RAP ($P < 0.05$ for all). In the LL-VNS group, the changes in ERPs at the first 3 hours at all recording sites gradually decreased during RAP, and then the ERPs increased gradually ($P < 0.05$ for all). In further detail, the ERP at the RA site in the LL-VNS group was shortened from 117.6 ± 7.79 ms at baseline to 101.6 ± 9.32 ms after cessation of 3 hours of RAP, and then increased to 124.8 ± 17.18 ms at the end of 6 hours of RAP ($P < 0.05$ for all). However, during the last 3 hours of RAP in the MLA group, the ERPs did not in-

Figure 3. Changes in AF inducibility and duration during 6 hours of rapid atrial pacing (RAP) in the Control ($n = 6$), LL-VNS ($n = 6$), and MLA ($n = 6$) groups. **A:** AF inducibility ratio during 6 hours of RAP in the three groups. **B:** Mean AF duration during 6 hours of RAP in the three groups. * $P < 0.05$ comparison between the baseline condition in the Control group; # $P < 0.05$ comparison between the baseline condition in the MLA group; § $P < 0.05$ comparison between the Control and LL-VNS groups at the same time point; ▲ $P < 0.05$ comparison between the third hours in the LL-VNS group; & $P < 0.05$ comparison between the MLA and LL-VNS groups at the same time points.

crease significantly when compared with the ERPs at the end of the 3 hours ($P = ns$ for all). When compared with the Control and MLA groups at the same time points during the last 3 hours of the experiment, the ERPs were higher in the LL-VNS group ($P < 0.05$ for all). In greater detail, after cessation of the 6 hours of RAP, the ERPs at RA in the Control and MLA groups (102.4 ± 5.55 and 109.5 ± 11.70 ms, respectively) were shorter than in the LL-VNS group (124.8 ± 17.18 ms; $P < 0.05$ for all) (Figure 2A). In the Control and MLA groups, a statistically significant increase was observed in the dispersion of ERP (dERP) during the 6 hours RAP, and after cessation of 4 hours, 5 hours, and 6 hours of RAP, significant differences were found in the dERPs in the LL-VNS group when compared with the Control and MLA groups at the same time points (Figure 2E).

As Figure 3A shows, there was no statistically significant difference among the three groups in terms of AF inducibility ratio and mean AF duration at the same time points during the first 3 hours. The AF inducibility ratios were higher in all three groups when compared with the baseline condition and after cessation of 3 hours of RAP (Control group: 6.7% versus 33.3%; LL-VNS group: 11.1% versus 51.1%; and MLA group: 8.9% versus 33.3%; $P < 0.05$ for all), and there was no significant difference in the AF inducibility ratios among the three groups at the same time points during the first 3 hours of the protocol ($P = ns$ for all) (Figure 3A). The results of the mean AF duration among the three groups were similar to those of the AF inducibility ratio (Figure 3B).

ELISA analyses: As Figure 4 shows, the plasma TNF- α and IL-6 concentrations increased significantly and the ACh levels decreased during RAP when compared with the baseline condition in the Control group. The serum TNF- α level increased from 18.1 ± 1.67 pg/mL at baseline to 21.8 ± 2.59 pg/mL after cessation of 3 hours of

RAP ($P < 0.05$), and then reached 22.3 ± 1.57 pg/mL after cessation of 6 hours of RAP ($P < 0.05$). The IL-6 level in the serum increased from 103.6 ± 6.14 pg/mL at baseline to 116.1 ± 8.20 pg/mL after cessation of 3 hours of RAP ($P < 0.05$), and then reached 117.5 ± 4.63 pg/mL after cessation of 6 hours of RAP ($P < 0.05$). The plasma ACh level decreased from 150.2 ± 4.24 μ g/mL at baseline to 135.2 ± 5.53 μ g/mL after cessation of 3 hours of RAP ($P < 0.05$), and then decreased to 126.4 ± 6.17 μ g/mL at the end of the 6th hour ($P < 0.05$). After cessation of 6 hours of RAP, the serum TNF- α and IL-6 levels were lower in the LL-VNS and MLA groups when compared with the Control group ($P < 0.05$ for all), but the ACh level was higher in the LL-VNS and MLA groups when compared with the Control group after cessation of 6 hours of RAP ($P < 0.05$ for all) (Figure 4A-C).

The concentrations of TNF- α and IL-6 in the RA and LA tissues were significantly higher in the Control and MLA groups than in the LL-VNS group ($P < 0.05$ for all; Figure 4D-F). No significant differences in the concentrations of TNF- α and IL-6 in the LA and RA tissues were observed between the Control and MLA groups ($P = ns$ for all). The levels of ACh in the RA and LA were significantly higher in the LL-VNS and MLA groups than in the Control group ($P < 0.05$ for all). No significant differences in the concentrations of ACh in the RA and LA tissues were observed between the LL-VNS and MLA groups (Figure 4D-F).

Western blot analyses: As shown in Figure 5A-C, the western blot results of atrial tissues from the three groups were compared. All immunoblot band intensity measurements were normalized to the intensity of the GAPDH band in the loaded sample. As shown in Figure 5C, the levels of STAT3 in the RA and LA samples were significantly higher in the LL-VNS group than in the Control and MLA groups ($P < 0.05$ for all). Compared with the Control and MLA groups, the levels of NF- κ B in the RA and LA tissues were lower in the LL-VNS group ($P < 0.05$ for all; Figure 5B).

Immunofluorescence assay analyses: As shown in Figure 6, an immunofluorescence assay was used to determine the expression of $\alpha 7$ nAChR in RA, LA, and GP tissues in all three groups. Cardiomyocytes appeared as dark

Figure 4. Changes in the plasma levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and acetylcholine (ACh) during 6 hours of rapid atrial pacing (RAP) in the Control ($n = 6$), LL-VNS ($n = 6$), and MLA ($n = 6$) groups. **A and B:** The levels of TNF- α and IL-6 increased when compared with the baseline condition and 3 hours of RAP in the three groups. The levels of TNF- α and IL-6 were lower in the LL-VNS and MLA groups at the end of the 6th hour when compared with the Control group. **C:** The levels of ACh decreased during pacing in the Control group, but they were higher in the LL-VNS and MLA groups at the end of the 6th hour when compared with the Control group. **D–F:** Changes in TNF- α , IL-6, and ACh in the atrial tissues of the Control ($n = 6$), LL-VNS ($n = 6$), and MLA ($n = 6$) groups. 1: * $P < 0.05$ comparison between the baseline condition in the Control group; * $P < 0.05$ comparison between the Control and LL-VNS groups at the same time points; # $P < 0.05$ comparison between the Control and MLA groups at the same time points. 2: $\Delta P < 0.05$ comparison between the Control and LL-VNS groups; $\Psi P < 0.05$ comparison between the MLA and LL-VNS groups.

red strip-cells, were neatly arranged, and their nuclei were blue and round (Figure 6A, B). Adipocytes in GP tissue appeared as vacuolate cells (Figure 6C). Finally, $\alpha 7nAChR$ appeared as bright red and evenly distributed among the cardiomyocytes and adipocytes. This results indicated that $\alpha 7nAChR$ was expressed in RA, LA and GPs tissues.

Discussion

This study explored the involvement of $\alpha 7nAChR$ in the effects of LL-VNS on atrial electrophysiology and AF induction after short-term RAP in experimental beagles. We provide evidence for the following: (1) LL-VNS prevented increases in the TNF- α and IL-6 levels in the atrium generated by RAP; (2) LL-VNS increased the ex-

pression of STAT3 and inhibited the expression of NF- κB ; and (3) the effect of LL-VNS on atrial electrophysiology and atrial inflammatory cytokines was reversed by the $\alpha 7nAChR$ inhibitor (MLA).

Recent studies have shown that the ANS plays an important role in the mechanism of AF, and inflammatory cytokines, such as TNF- α and IL-6, ACh, and other inflammatory factors may be linked to fibrosis and expression of current channel subunits that contribute to AF-associated electrical remodeling and thus, promote the progression of AF.⁹⁾ Mediators of the inflammatory response can alter atrial electrophysiology and structural substrates, thereby leading to increased vulnerability to AF. A previous study suggested that TNF- α can not only promote myocardial cell apoptosis and myocardial interstitial fibrosis but also inhibit L-type calcium channel cur-

Figure 5. Western blot results of the levels of NF-κB and STAT3 in the atrium tissues. **A:** Representative images of the results for NF-κB with a specific band at 65 kD, and for STAT3 with a specific band at 88 kD. GAPDH with a specific band at 37 kD was used as a reference. **B, C:** Relative expressions of NF-κB and STAT3 in the atrium tissues in the three groups. * $P < 0.05$ comparison between the Control group; [#] $P < 0.05$ comparison between the MLA group.

Figure 6. ($\times 200$) Expression of $\alpha 7$ nAChR in the atrium tissue and GP samples in the Control group. **A:** Expression of $\alpha 7$ nAChR in RA; **B:** Expression of $\alpha 7$ nAChR in LA; **C:** Expression of $\alpha 7$ nAChR in GP. Arrows indicate the expression of $\alpha 7$ nAChR.

rent and shorten the atrial ERP by reducing the expression of an L-type calcium channel, and IL-6 can also induce myocardial cell necrosis and apoptosis, which in turn can promote atrial electrical remodeling and increase the inducibility of AF.¹⁶⁾

Our recent studies have shown that renal sympathetic denervation and low-level atrial GP stimulation can reduce the levels of inflammatory factors as well as suppress the inducibility of AF.^{11,17,18)} In the present study, our aim was to investigate the how $\alpha 7$ nAChR-mediated CAP is involved in vagal nerve-regulated AF; therefore, we had to confirm that the $\alpha 7$ nAChR was expressed in the atrial tissues and GP samples of normal beagles. Hence, we chose four GPs to be injected with saline and MLA based on the results of immunofluorescence assays showing that $\alpha 7$ nAChR was expressed in GP samples and atrial tissues in the Control group. Recently, studies have shown that LL-VNS can increase atrial ERP, decrease atrial dERP and the inducibility of AF, and in turn suppress the atrial electrical remodeling caused by RAP.^{19,20)} Furthermore, Po, *et al.* found that low-level transcutaneous vagal nerve stimulation not only suppressed the atrial electrical remodeling and inhibited the occurrence of AF but also reduced the serum levels of inflammatory factors such as TNF- α and IL-6 in patients with AF.²¹⁾ We have also reported that acute RAP can increase the levels of inflammatory factors and atrial electrical remodeling, and that median nerve stimulation can prevent these changes.²²⁾ What's more, Stavrakis, *et al.* showed that LL-VNS not only increases ERP and inhibits the induction of AF caused by acute RAP but also reduces the levels of TNF- α , IL-6, and other inflammatory factors.⁸⁾ A previous study demonstrated that ACh released by vagal endings binds to $\alpha 7$ nAChR and activates this receptor to inhibit the release of TNF- α and IL-6, which has a protective effect on some inflammatory diseases.¹⁵⁾ Previous study has shown that ACh significantly attenuates the release of pro-inflammatory cytokines, and that $\alpha 7$ nAChR activation modulates multiple intracellular signal transduction cascades and suppresses the transcription of pro-inflammatory cytokines.²³⁾

The results of this study show that during the first 3 hours RAP the ERPs at all recording sites decreased gradually and the dERP and inducibility of AF increased in all three groups tested, and that there were no significant differences at the same time points among the three groups. However, during the last 3 hours of RAP, the ERPs at the recording sites in the LL-VNS group increased significantly when compared with the end of 3 hours of RAP, and were also statistically higher than in the Control and MLA groups at the same time points. In addition, the vulnerability to AF in the LL-VNS group was lower than in the Control and MLA groups at the same time points during the last 3 hours. Therefore, RAP can increase the vulnerability to AF, and this effect can be prevented by LL-VNS as well as by the $\alpha 7$ nAChR inhibitor MLA, which can suppress the effects of LL-VNS on atrial electrical remodeling that may be associated to CAP. Furthermore, when compared with the baseline condition, the levels of TNF- α and IL-6 in the plasma increased gradually in the Control group while that of ACh de-

creased; however, following LL-VNS or at the end of the protocol in the LL-VNS and MLA groups, the levels of TNF- α and IL-6 decreased while those of ACh increased in the LL-VNS and MLA groups. We also found that the levels of TNF- α and IL-6 in atrium tissues in the Control group were significantly higher than in the LL-VNS group. However, MLA reversed the effects of LL-VNS on the atrial inflammatory cytokines. We also found that the levels of ACh in atrium tissues in the Control group were lower than in the LL-VNS group, but that MLA could not reverse the effects of LL-VNS on atrial ACh levels. Why are the levels of TNF- α and IL-6 in the atrial samples not similar to those in the serum? We thought that LL-VNS could inhibit the release of TNF- α and IL-6 throughout the body, so the concentrations of TNF- α and IL-6 in the plasma decreased because of the effects of LL-VNS on the whole body. However, MLA was injected into four GPs, which was expected to work locally in the heart rather than in the whole body. Therefore, MLA inhibited the effects of LL-VNS on the levels of inflammatory factors in the atrial tissues, and the levels of inflammatory factors in atrial tissues were different than those in the plasma.

A previous study suggested that $\alpha 7$ nAChR-mediated CAP is mainly mediated by NF- κ B and JAK2/STAT3.²⁴⁾ NF- κ B is a transcription factor of pro-inflammatory cytokines, and after activation, NF- κ B translocates into the nucleus, where it mediates the production of inflammatory cytokines. After activation, $\alpha 7$ nAChR can inhibit NF- κ B inhibitory protein phosphorylation, which has an anti-inflammatory effect by inhibiting NF- κ B. The intracellular domain changes after activating $\alpha 7$ nAChR, so that JAK2 phosphorylates, which can activate the STAT3 of the pathway downstream, and then can play an anti-inflammatory effect.²⁵⁾ In our study, the western blot results showed that the levels of STAT3 in the atrial tissues were higher in the LL-VNS group than in the Control and MLA group; however, the expression of NF- κ B in the atrial tissues was lower in the LL-VNS group than in the Control and MLA groups. In our study, we did not compare the expression level of $\alpha 7$ nAChR among the three groups; whether and how LL-VNS or MLA could increase or decrease its levels in atrial tissues needs further investigation. Our results showed that LL-VNS could suppress the atrial electrical remodeling and AF inducibility by activating $\alpha 7$ nAChR-mediated CAP, and that $\alpha 7$ nAChR plays an important role in the effects of LL-VNS on atrial electrophysiology and AF induction after short-term RAP in beagles.

Limitations of the study: This study has several limitations. First, we did not measure the changes in atrial ion currents, such as Na⁺ and Ca²⁺ currents, as well as the changes in atrial hemodynamic parameters. Previous studies have shown that atrial electrical remodeling induced by RAP is mediated by rate-induced intracellular calcium overload. Therefore, it is unclear whether $\alpha 7$ nAChR-mediated CAP changed atrial ion currents in this study. Second, the optimal concentration of MLA was not determined. We did not investigate the potential effects of MLA on neurons found within the intrinsic cardiac nervous system. Importantly, the concentration of MLA effectively inhibits $\alpha 7$ nAChR and provides evidence that LL-

VNS suppresses AF by activating the $\alpha 7$ nAChR-mediated CAP. Finally, perhaps the long-lasting and deep anesthesia influenced the ERP and AF inducibility. Whether the anesthesia or the operation influenced the vagal nerve activity is not known. However, in this study, the dose of pentobarbital sodium administered and the anesthesia time were similar among the three groups. Therefore, our data is comparable.

Conclusions

In this study, we demonstrated, for the first time, that LL-VNS substantially prevented atrial electrical remodeling and AF vulnerability induced by short-term RAP by mediating the $\alpha 7$ nAChR-mediated CAP. Blocking $\alpha 7$ nAChR can suppress the effects of LL-VNS on atrial electrical remodeling and AF vulnerability. Our results support the notion that the effects of LL-VNS on atrial electrical remodeling and AF vulnerability have an essential relationship with $\alpha 7$ nAChR-mediated CAP.

Disclosure

Conflicts of interest: None declared.

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