Angiotensin-(1–7) attenuates atrial tachycardia-induced sympathetic nerve remodeling

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Abstract

Introduction: The effect of Angiotensin-(1–7) (Ang-(1–7)) on atrial autonomic remodeling is still unknown. We hypothesized that Ang-(1–7) could inhibit sympathetic nerve remodeling in a canine model of chronic atrial tachycardia.

Materials and methods: Eighteen dogs were randomly assigned to sham group, pacing group and Ang-(1–7) group. Rapid atrial pacing was maintained for 14 days in the pacing and Ang-(1–7) groups. Ang-(1–7) was administered intravenously in the Ang-(1–7) group. The atrial effective refractory period and atrial fibrillation inducibility level were measured at baseline and under sympathetic nerve stimulation after 14 days of measurement. The atrial sympathetic nerves labeled with tyrosine hydroxylase were detected using immunohistochemistry and Western blotting, and tyrosine hydroxylase and nerve growth factor mRNA levels were measured by reverse transcription polymerase chain reaction.

Results: Pacing shortened the atrial effective refractory period and increased the atrial fibrillation inducibility level at baseline and under sympathetic nerve stimulation. Ang-(1–7) treatment attenuated the shortening of the atrial effective refractory period and the increase in the atrial fibrillation inducibility level. Immunohistochemistry and Western blotting showed sympathetic nerve hyperinnervation in the pacing group, while Ang-(1–7) attenuated sympathetic nerve proliferation. Ang-(1–7) alleviated the pacing-induced increases in tyrosine hydroxylase and nerve growth factor mRNA expression levels.

Conclusion: Ang-(1–7) can attenuate pacing-induced atrial sympathetic hyperinnervation.

Keywords
Angiotensin-(1–7), chronic atrial pacing, sympathetic nerve remodeling, atrial fibrillation, tyrosine hydroxylase

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Introduction

Atrial fibrillation is the most common arrhythmia, and it is associated with a significant increase in morbidity and mortality.1 Recent studies have demonstrated that atrial sympathetic nerve hyperinnervation may increase atrial fibrillation susceptibility.2,3 Chronic atrial pacing could lead to sustained atrial fibrillation not only due to pacing-induced electrical and structural remodeling,4–6 but also due to autonomic nerve remodeling.7–10

It has been demonstrated that the renin–angiotensin system (RAS) is involved in the initiation and maintenance of atrial fibrillation.11 Angiotensin-(1–7) (Ang-(1–7)), as a novel member of the RAS, was confirmed to counterbalance the effects of Angiotensin II (Ang II).12,13 Our previous studies indicated that Ang-(1–7) could prevent atrial electrical and structural remodeling in dogs with chronic atrial tachycardia.14–16 The effects of Ang-(1–7) on atrial sympathetic nerve remodeling are still unknown. The purpose of this study was to investigate whether Ang-(1–7)
had a protective effect on sympathetic nerve remodeling in chronic atrial tachycardia in dogs. We labeled the sympathetic nerve with tyrosine hydroxylase (TH) as previously reported.\textsuperscript{10}

**Materials and methods**

*Preparation of the canine model*

All animal studies were approved by the experimental animal administration committee of Tianjin Medical University and Tianjin Municipal Commission for Experimental Animal Control. Eighteen mongrel dogs of either sex weighing between 12 and 14 kg were included in this study. All dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg) and ventilated with room air. Under sterile conditions, a modified unipolar J-shaped pacing lead (St. Jude Medical) was inserted through the right jugular vein and the distal end of the lead was positioned in the right atrium, as described in a previous study.\textsuperscript{14} Initial atrial capture was verified using an external stimulator (ALC-V8, Shanghai Alcott Biotech CO. LTD, and China). The proximal end of the pacing lead was then connected to a programmable pacemaker (made in Shanghai Fudan University, China), which was inserted into a subcutaneous pocket in the neck. The dogs in the pacing and Ang-(1–7) groups were paced at 500 beats/min for a period of 14 days. The dogs in the sham group received a pacemaker implant but did not undergo pacing. During the pacing, Ang-(1–7) (6 μg/kg per h) (Bachem AG, Bubendorf, Switzerland) was given intravenously through a jugular vein catheter by an ALZET® osmotic pump (DURECT Corporation, Cupertino, CA, USA) continuously during pacing for 14 days. The surface electrocardiogram was verified to ensure continuous 1:1 atrial capture every other day. Direct systolic blood pressure was measured during anesthesia before and after 14 days’ pacing by a multichannel physiological recorder (TOP 2001, Hongtong Inc., China).

Two weeks after the pacemaker implant, all dogs were intubated and mechanically ventilated with room air. Under sterile conditions, a median sternotomy was performed. Six pairs of electrodes (diameter: 1.5 mm; distance between poles: 1.5 mm) were stitched onto the left and right atrial appendage (HLA, LLA, and LAA, respectively), and in the same sites of the right atrium (HRA, LRA, and RAA, respectively).

*Measurement of atrial effective refractory period and atrial fibrillation inducibility*

The atrial effective refractory period (AERP) at each site was measured by programmed pacing from the multi-channel electrophysiology recorder (TOP 2001, Hongtong Inc., China). There were eight sequential stimuli (S1–S1, cycle length 250 ms) followed by one premature stimulus (S2) with a doubling pacing threshold. The S1–S2 interval progressively decreased by 10 ms and, subsequently, 2 ms near the refractory value to ensure accurate recording. AERPs were the longest coupling interval that failed to capture the atrium.

Atrial fibrillation was defined as P-wave disappearance and rapid atrial activation with irregular ventricular response and a duration of more than 1 s. Atrial fibrillation was induced at the above six atrial sites with a 5-s continuous burst stimulation, at 10 Hz with four thresholds and 0.5-ms duration pulses. The burst stimulation was repeated three times at each site and was recorded whenever atrial fibrillation was successfully induced. If the atrial fibrillation duration time was longer than 60 s, rapid atrial pacing or electrical conversion was used to reverse atrial fibrillation to sinus rhythm, since a prolonged period of atrial fibrillation may induce transient atrial remodeling, provoke longer episodes of atrial fibrillation and affect subsequent measurements.

After basic electrophysiological examination, the AERPs and atrial fibrillation inducibility level were measured under sympathetic nerve stimulation (SNS). The bilateral stellate ganglia were carefully isolated from the surrounding tissue. Two insulated bipolar electrodes were attached to the dorsal and ventral ansae of both stellate ganglia, which were then stimulated continuously with pulses of 5 V for a duration of 0.5 ms and a frequency of 10 Hz. The stimulus intensity was adjusted by the heart rate (HR), such that a HR increase of at least 30% was maintained when compared with the basal sinus rhythm rate throughout the measurement of AERPs and atrial fibrillation inducibility level.

*Immunohistochemistry study*

After completing the electrophysiological measurement, the left atrial (LA) and right atrial (RA) tissues were immediately cut off and washed with cold 0.9% sodium chloride, while part of the tissue was fixed in formaldehyde and used in immunohistochemistry studies. The remaining tissue was rapidly frozen in liquid nitrogen and stored separately at −80°C for molecular biological studies. Immunohistochemistry was performed to examine the density and distribution of the sympathetic nerves (TH-positive) in the atria. Five-micrometer (μm) sections were cut from paraffin blocks of the LA and RA free walls, which were then stained with TH to label the sympathetic nerves. In brief, six sections from the LA and the RA were used to analyze the sympathetic nerve density per canine. We determined the fibrosis area using a computer-assisted image analysis system (Image-Pro Plus 7.0, Media Cybernetics, USA). The labels of all slides were covered,
so that the investigator who counted the nerves was blinded to the dog identification at the time of the nerve count. Each slide was examined under a microscope, and six fields with the highest density of sympathetic nerves were selected by the investigator. The computer automatically detected the stained nerves in these fields by their brown color, and automatically calculated the area occupied by the nerves in the field. The nerve density was the nerve-positive stained area divided by the total area examined (μm²/mm²). The mean nerve density in these six selected fields was used to represent the nerve density of that slide.

Western blotting
LA and RA tissues from each group were prepared for Western blotting analysis. Protein was extracted with total protein extraction buffer. An equal amount of protein was loaded onto a 6% sodium dodecyl sulfate (SDS) denaturing polyacrylamide gel, separated by electrophoresis, transferred onto a polyvinylidene fluoride membrane (Merck Millipore, Billerica, MA, USA) and incubated with a specific primary antibody at 4°C overnight. The membranes were then washed and subsequently incubated with the secondary antibody conjugated to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence. Protein levels of TH were expressed as a ratio against the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Antibody to TH (ab93291) was purchased from Abcam, Inc. (Cambridge, UK) and anti-rabbit IgG HRP conjugate (W4011) was purchased from Promega, Inc. (Madison, USA).

Reverse transcription polymerase chain reaction
One aliquot of each tissue sample was used to investigate the mRNA expression of TH and nerve growth factor (NGF). In brief, 100 mg of tissue was homogenized in 1 ml of TRIzol reagent (Invitrogen, USA). RNA was then extracted with chloroform, precipitated in isopropyl alcohol and subsequently dissolved in diethyl pyrocarbonate-treated water. The integrity of each sample was confirmed by analysis on a denaturing agarose gel. The concentration of total RNA was determined spectrophotometrically at a wavelength of 260 nm, and the RNA was stored at −80°C for later analysis. Specific oligonucleotide primer pairs used for the amplification of GAPDH, TH and NGF were designed according to the sequences obtained from GeneBank. The primers specific (forward/reverse) for each protein were: 5’-GGCGTGAACCATGAAGATAT-3’/5’-GTCGAGTGGATGGATGTT-3’ (GAPDH), 5’-TCTCCAGTGCAAGGCAGTACAT-3’/5’-CGAACTCCTACTGTGAACCA-3’ (TH), and 5’-ACAGGACTCA CAG GACGAA-3’/5’-TCCAGTGGATGATCGAT-3’ (NGF). A 200 ng quantity of total RNA was converted to cDNA and subjected to reverse transcription polymerase chain reaction (RT-PCR) using a commercially available kit (TaKaRa, China). PCR was performed using the SYBR-Green PCR kit as described by the manufacturer (TransGen, Beijing, China). The PCR consisted of 44 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s. The relative mRNA expression levels were normalized to the GAPDH gene according to the 2−ΔΔCt method. All the PCR experiments were carried out in triplicate within each experiment, and the experiments were replicated at least three times.

Statistical analysis
All data were expressed as mean ± SD. Statistical comparisons among groups were performed with one-way analyses of variance (ANOVA). Subsequently, if significant effects were indicated by ANOVA, all significant data were analyzed with a t-test to evaluate the differences between individual mean values. The inducible rate of atrial fibrillation was analyzed with the exact probability test. A p<0.05 was considered statistically significant.

Results
HR and blood pressure
There was no significant difference in HR and systolic blood pressure between the three groups at baseline (p>0.05), and the HR and systolic blood pressure were not changed by atrial pacing in any of the groups (p>0.05; Table 1).

AERPs
Two weeks of chronic atrial pacing markedly shortened the AERPs in the pacing group compared with the sham group (p<0.05). The Ang-(1–7) treatment attenuated the pacing-induced AERPs. Shortening at the sites of the HRA, HLA and LLA reached statistical significance (p<0.05). SNS did not change the AERPs in the sham group (p>0.05), but significantly decreased the AERPs in the pacing group. Ang-(1–7) attenuated the pacing-induced AERPs. The AERP shortening under SNS, at the sites of the HRA, LRA, HLA and LLA, reached statistical significance, as compared with the pacing group (p<0.05; Table 2).

Inducible rate of atrial fibrillation
Compared with the sham group, the pacing group showed a significantly elevated atrial fibrillation inducibility level (p<0.05). The Ang-(1–7) treatment reduced the atrial fibrillation inducibility level compared with that in the pacing group (p<0.05), though it was still higher than that in the sham group (p<0.05). SNS increased the atrial fibrillation inducibility level.
fibrillation inducibility level only in the pacing group (p<0.05) and not in the Ang-(1–7) group (p>0.05; Table 3).

**Sympathetic nerve density**

Immunohistochemical staining showed that the density of TH-positive nerves in the RA and LA were all significantly increased in the pacing group compared with that in the sham group (LA: 3032±463 vs. 1613±725; RA: 3621±752 vs. 1180±441, p<0.05). Ang-(1–7) attenuated the atrial pacing-induced atrial sympathetic hyperinnervation (LA: 3032 ± 463 vs. 2156 ± 873; RA: 3621±752 vs. 2621±752, p<0.05; Table 4 and Figure 1).

**The expression of TH protein**

Western blotting showed that the level of TH protein was significantly increased in the pacing group compared with that in the sham group (p<0.05). In the sham group, the LA exhibited a higher level of TH protein, although this increase was not significant (p>0.05). In the pacing group, both the LA and RA TH protein levels were significantly increased (p<0.05), with the increase in the RA TH protein level being greater than the increase in the LA TH protein level, and the absolute increase in values reached statistical significance (RA vs. LA, p<0.05). Ang-(1–7) treatment reduced both the LA and RA TH protein levels compared with those in the pacing group (p<0.05), though they were still higher than those in the sham group (p>0.05; Figure 2).

**mRNA levels of TH and NGF**

RT-PCR showed that the TH and NGF mRNA levels were significantly increased in the pacing group (p<0.05), and that Ang-(1–7) treatment reduced the mRNA levels of TH.

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**Table 1.** Hemodynamic parameters before and after pacing in each group (X±s).

| Groups       | Heart rate (beats/min) | Systolic blood pressure (mmHg) |
|--------------|------------------------|---------------------------------|
|              | Before pacing          | After pacing                   | Before pacing          | After pacing                   |
| Sham         | 165 ± 9                | 167 ± 12                       | 134 ± 7                | 138 ± 6                       |
| Pacing       | 171 ± 11               | 174 ± 16                       | 140 ± 10               | 138 ± 8                       |
| Ang-(1–7)    | 168 ± 10               | 166 ± 11                       | 135 ± 6                | 136 ± 9                       |
| F            | 0.593                  | 1.016                          | 1.160                  | 0.074                          |
| p            | 0.565                  | 0.386                          | 0.340                  | 0.929                          |

**Table 2.** AERPs among the three groups at baseline and under SNS.

| AERP (X±s, ms) | Group | HRA | LRA | RAA | HLA | LLA | LAA |
|----------------|-------|-----|-----|-----|-----|-----|-----|
| **Baseline**   |       |     |     |     |     |     |     |
| S              | 110.4±8.8 | 105.6±7.2 | 106.8±8.3 | 101±7.9 | 101.0±5.5 | 102.0±8.9 |
| P              | 90.4±16.9* | 87.1±6.1*  | 88.7±10.7*  | 85.1±6.9* | 81.3±12.9* | 85.6±3.9* |
| A              | 106.4±8.8*** | 95.7±6.8  | 94.7±10.6  | 96.7±8.7** | 93.3±7.3**  | 94.3±9.6   |
| **SNS**        |       |     |     |     |     |     |     |
| S              | 103.6±8.6   | 108.2±8.2  | 101.8±7.5  | 92.9±9.7  | 102.0±6.0  | 104.7±7.9  |
| P              | 79.1±14.5*** | 75.6±8.6*** | 76.7±12.6*** | 74.0±10.4*** | 69.8±10.2*** | 76.9±9.8*** |
| A              | 95.2±8.4**** | 88.7±8.4**** | 86.7±8.6  | 92.0±12.6**** | 87.7±8.2**** | 81.7±9.6   |

**Table 3.** The atrial fibrillation inducibility level among the three groups (X±s).

| AF inducible rate (%) | Sham | Pacing | Ang-(1–7) |
|-----------------------|------|--------|-----------|
| Baseline              | 9.6±3.6 | 32.7±7.6* | 17.6±4.5*  |
| SNS                   | 11.0±4.5 | 40.8±4.7* | 20.5±4.9*  |

The AF inducibility level was significantly elevated in the pacing group compared with that in the sham group (p<0.05). The AF inducibility level in the Ang-(1–7) group was significantly decreased compared with that in the pacing group (p<0.05). SNS significantly increased the AF inducibility level in the pacing group (p<0.05). However, in the Ang-(1–7) group, SNS did not significantly increase the AF inducibility level as compared with that at baseline (p>0.05).

AF: atrial fibrillation; Angiotensin-(1–7); SNS: sympathetic nerve stimulation.
and NGF compared with those in the pacing group \((p<0.05)\); however, the levels were still higher than those in the sham group \((p<0.05); Figure 3).
sympathetic nerve sprouting and proliferation were found in the pacing group, and SNS decreased the AERPs and increased the atrial fibrillation inducibility level. Our findings are consistent with those of previous studies that demonstrated that cardiac sympathetic innervation plays a critical role in the dynamics of atrial fibrillation initiation and maintenance. The catecholamines released by the proliferation sympathetic nerve endings were found to increase the heterogeneity of the AERPs and elevate the atrial fibrillation inducibility level. Sympathetic hyperinnervation in the pulmonary vein provides a substrate for the trigger of rapid focal sources, thereby inducing some atrial fibrillation episodes. Heterogeneous sympathetic innervation also facilitates sustained atrial fibrillation. These findings indicate that sympathetic hyperinnervation may play an important role in the generation and maintenance of atrial fibrillation.

**RAS-induced atrial sympathetic hyperinnervation**

The RAS is widely distributed in the sympathetic nerve system and throughout the central nervous system, the peripheral nerve ganglia and even sympathetic nerve endings. Activation of the RAS can directly affect the brain and sympathetic nerve ganglia, thereby increasing sympathetic outflow and causing excessive sympathoexcitation. NGF is the main factor that stimulates the growth of sympathetic nerves, and the RAS stimulates the synthesis of NGF, thereby promoting the growth of sympathetic nerves and resulting in sympathetic nerve hyperinnervation. In the present study, significantly increased NGF mRNA expression levels were observed. During atrial fibrillation, the RAS has been shown to increase the mechanical stretch of atrial myocardial cells, resulting in the increased growth of sympathetic neurocytes. Thus, the RAS plays an important role in the pathogenesis of sympathetic nerve sprouting and hyperinnervation.

**Ang-(1–7) attenuates sympathetic nerve remodeling**

Ang-(1–7) inhibits the RAS and attenuates sympathetic nerve remodeling. Gironacci et al. demonstrated that Ang-(1–7) significantly decreased potassium-induced norepinephrine release in the hypothalamus by stimulating the Ang II receptor. Moreover, Ang-(1–7) was reported to inhibit the evoked release of norepinephrine from the hypothalamus. Byku also showed that Ang-(1–7) modulates sympathetic neurotransmitter overflow in a manner that might oppose the effects of Ang II on sympathetic neurotransmission. Ang II receptor blockers have been shown to inhibit the RAS and abolish the stretch-induced increase in NGF expression, thereby preventing stretch-induced neural growth. Furthermore, Ang-(1–7) might abolish stretch-induced NGF expression and inhibit sympathetic nerve growth by counterbalancing the effects of Ang II. In the present study, we found that Ang-(1–7) inhibits the activity of the sympathetic nerves and attenuates both SNS-induced shortening of the AERPs and the increased atrial fibrillation inducibility level.

Our study showed, for the first time, that Ang-(1–7) could significantly attenuate atrial sympathetic hyperinnervation in a chronic atrial paced canine atrial fibrillation model. Electrical, structural and autonomic remodeling are the three main atrial remodeling mechanisms that act as the foundation of atrial fibrillation initiation and maintenance. Ang-(1–7) effectively suppresses all three of those mechanisms and has been speculated to act as an...
effective agent in the treatment of atrial fibrillation; however, further clinical studies are necessary to verify this hypothesis.

**Study limitations**

Pentobarbital is known to prolong the AERP compared with the state of non-anesthesia, which may affect the atrial fibrillation inducibility level. We did not measure the levels of Ang-(1–7), which might have influenced the outcomes of this study. Unfortunately, we were unable to obtain specific canine NGF antibodies that could be used to measure the changes in NGF protein expression levels.

**Conclusion**

Ang-(1–7) significantly attenuated atrial sympathetic hyperinnervation in a chronic atrial pacing-induced canine atrial fibrillation model. These results might help to elucidate the relationship between the RAS and atrial fibrillation.

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Wenfeng Shangguan and Wen Shi contributed equally to this work and should be considered co-first authors.

**Declaration of conflicting interests**

The authors declare that there is no conflict of interest.

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