Atrial Fibrillation Induced by Applying Acetylcholine to Subadventitial Layer of Pulmonary Vein in Normal Canine Heart

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(Received October 8, 2009; Accepted November 17, 2009)

Objectives: We determined whether acetylcholine (Ach) application to the pulmonary vein (PV) wall could induce AF and clarified its mechanisms, and determined whether circumferential PV radiofrequency ablation (CPVA) could prevent Ach-induced AF in canine hearts.
Methods: Thirty seven beagle dogs were used for the study. Ach was injected into the subadventitial layer of the left superior PV (LSPV), at different distance from the LSPV-left atrium junction (LSPV-LA-J) to locate AF initiation. When AF was not induced by Ach alone, programmed electrical stimulation (S_1-S_2 method) was added to elicit AF. Atropine was injected at the same site of Ach injection to determine whether muscarine-receptor blockade suppressed AF, and CPVA at the LSPV-LA-J was performed using a newly devised basket electrode-catheter.
Results: AF was reproducibly induced by Ach injection in 19 of the 26 dogs (73%). S_1-S_2 method after Ach initiated AF in 5 of the remaining 7 dogs. Ach into the subadventitial layer of the LSPV, especially the distal portion, could elicit AF, which was preceded by pause (sinus arrest) ≧ 2.0 sec (37%) (pause-AF group), sinus bradycardia (32%) (brady-AF group) and sinus tachycardia (32%) (tachy-AF group).
The time from Ach injection to AF initiation and AF duration were not siginificantly different between pause-AF, brady-AF and tachy-AF groups. AF was not initiated by injecting Ach after atropine pretreatment. To eliminate AF, 1-6 (average 4.1 ± 1.2) CPVAs at the LSPV-LA-J were required.
Conclusions: Our observations suggest that local Ach application can initiate AF in PVs, preceded by a variety of modes such as pause, bradycardia or tachycardia, and an increase in vagal tone at the LSPV plays a critical role in eliciting AF in structurally normal heart.

Key words: atrial fibrillation, acetylcholine, pulmonary vein, canine heart

INTRODUCTION

Atrial fibrillation (AF) is the most common arrhythmia in humans and occasionally leads to critical problems such as ischemic stroke. AF is believed to be initiated mainly by the modification of neural mechanisms in patients with structurally normal hearts [1-2]. Experimentally, both direct vagal stimulation [3-5] and local application of acetylcholine (Ach) on the auricular surface [6] reportedly induce AF. Adrenergically mediated AF was also induced by adrenaline or isoproterenol infusion into sinus node artery [7]. However, Sharifov *et al*. [8] reported that AF could not be induced, even by intense stimulation by infusing adrenaline and isoproterenol into the sinus node artery, when the background cholinergic tone was suppressed by atropine, indicating that the cholinergic tone plays a critical role in these AF episodes. It has been shown that in a canine model, vagal denervation prevents AF induction [9]. In addition, Pappone *et al*. [10] reported that complete vagal denervation during circumferential pulmonary vein (PV) ablation (CPVA) reduced the recurrence of paroxysmal AF at 12 months.

It has long been known that PVs have independent pulsation, suggesting thier electrical automaticity [11]. It is also known that the left atrial myocardial sleeves extend into the outer layer of the PVs in humans and various other mammals [12-13]. Among the 4 PVs, the left superior PV (LSPV) has the most developed and extended sleeves [13]. Furthermore, previous studies suggested that the myocardial sleeves of the PV showed many spontaneous activities such as automaticity, or early after depolarization (EAD) or delayed after depolarization (DAD) [14-15]. There are differences in the electrophysiological and anatomical properties between cells at the distal end of the PV and those close to the LSPV-left atrial junction (LSPV-LA-J) [16-17]. That is, pace-making potentials were mainly observed in cells at the distal to the hearts [16] and anatomically clear cells resembling the sinus node cells, which were distributed mainly in the terminal portion of the PV sleeves, might potentially have pacemaking activity [17].

The purpose of the present in vivo study in canine model was to determine (1) whether Ach injection into the LSPV wall could induce AF, (2) if inducible, whether the location of the main site of AF initiation, distal or proximal part of the LSPV-LA-J could be specified, and (3) whether AF could be elicited, via direct vagal reflexes or changes in heart beats (study I). Another purpose was to determine whether pretreatment of muscarine (M2) -receptor blockade with

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atropine or CPVA at the LSPV-LA-J could prevent AF initiation by Ach injection in the LSPV wall (study II).

MATERIALS AND METHODS

Animals and surgical procedure

We anesthetized 37 adult beagle dogs (weighing 10–12 kg) with thiamylal (15 mg/kg; IV) and α-chloralose (50 mg/kg; IV). Drip infusion of α-chloralose was used to maintain anesthesia as needed. The dogs were intubated with a cuffed endotracheal tube and ventilated with a room-air respirator (Harvard model 670, USA). Arterial PaO_2, $PaCO_2$, pH and base excess were monitored throughout the experiment and adjusted by varying the tidal volume or O_2 flow or by administering sodium bicarbonate when needed. Positive expiratory pressure was applied to maintain PaO_2 in the normal range. The right common carotid artery and external jugular vein were cannulated to monitor arterial pressure and to administer physiological saline or drugs, respectively. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. The details of this procedure have been described in a previous paper [18]. The adipose and connective tissues surrounding the LA and LSPV were carefully separated and removed. Bipolar electrodes were sutured to the chest surface (Y-lead), the exposed surface of the LA appendage, LSPV (4 [LSPV-H] and 12 mm [LSPV-P] from the LSPV-LA-J) and left ventricle. Electrograms of the left ventricle was recorded to determine whether vasospastic constriction of the coronary artery was related to AF induction, which could not be initiated by direct Ach action on PVs. For this reason, we carefully observed changes in ST segment which was seen in the electrograms in the left ventricular leads. Electrograms of these sites were recorded throughout the experiment (RMP-6000; Nihon Kohden Co. Japan).

Experimental protocol

Study I (n = 26): In 14 dogs, Ach (200 to 400 μg/kg) was injected into the subadventitial layer of the LSPV at intervals of 4 mm in the location between 4 mm and 16 mm from the LSPV-LA-J to induce AF. The sequence of Ach injection in each dog was randomly selected by the envelope method. In 26 dogs, including the above 14, the cervical sympathetic and vagal trunks on both sides and stellate ganglia were transected to eliminate the Ach-induced reflex influence of the central nervous system on the heart. After AF initiation by Ach, we confirmed whether the induction of AF could be reproduced as follows. If AF terminated spontaneously within 10 min, the same Ach dose was injected using the same procedure 10 min after the termination. If AF persisted for more than 10 min, it was terminated by electrical cardioversion and Ach was injected 10 min after this termination.

In case AF was not initiated after injecting Ach, Ach was again injected 10 min after the first injection. If AF was still not initiated, the LSPV-P was electrically stimulated using a programmed digital stimulator (Model SEN-3201; Nihon Kohden Co., Japan) with the S_1–S_2 method (cycle length of S1, 300–350 ms; pulse strength, 4 m A; and pulse duration, 2 msec) at twice the diastolic threshold, followed by continuously and gradually reducing the pacing cycle (at intervals of 10 ms) up to 200 ms to induce AF. Vagal reflexes were defined as asystole or atrio-ventricular (AV) block ≧ 2.0 s that occurred within a few seconds after Ach application, according to the report by Pappone *et al.* [10]. Blood pressure was monitored using the blood pressure transducer during the experiments.

Study II (n = 11): In 4 dogs in whom AF was induced by Ach injection, atropine was injected at the same site as Ach to determine whether M_2-receptor blockade effectively suppressed the initiation of AF. A basket electrode-catheter, the details of which are described below, was designed and manufactured by us (Fig. 1A). It was inserted into the PV lumen by squeezing the basket through the hilum of the LSPV and advancing the tip into the inner surface of the LSPV-LA-J, where 6 electrodes were attached to the intima of LSPV-LA-J by expanding the basket (Fig. 1B and 1C). Intravenous heparin sodium was continuously administered (2,000 U diluted in 500 ml of electrophysiological sodium) to prevent thrombus formation around the basket catheter. A preliminary experiment was separately conducted in 3 dogs to electrophysiologically determine the optimal electric current for CPVA through the new balloon catheter electrodes. A variety of ablation conditions such as watt (electric strength) and duration of time and impedance were adjusted as required. In these dogs, Ach was injected into the subadventitial layer of LSPV-P to initiate AF, and then catheter ablation was performed at the LSPV-LA-J.

The remaining 4 dogs were used in an experiment to confirm whether the application of radiofrequency energy to the LSPV-LA-J by using electrode-catheter was successful in eliminating the AF induced by Ach. First, AF initiation by Ach injection into the subadventitial layer of LSPV-P was confirmed. Ten min after its termination, AF was initiated again by injecting Ach at the same point. We applied the following optimal ablation conditions, as determined in the preliminary experiments; electrical strength, 10–15 watt; ablation period, 30 s; and electrical impedence at 2–3 Ω (67–68 Ω) which was lower than the pre-electrical discharge (about 70 Ω).

Manufactured basket catheter with expandable balloon-shaped electrodes

An ablation catheter was developed and manufactured for catheter ablation at the inner layer of the LSPV-LA-J, as described previously. The scheme of entire catheter, part of the electrodes including the connecting part of the ablation device (CAT-500; Central Technology Co., Japan) are shown in Fig. 1A and 1B. A steel wire was fixed through the central portion of the catheter and equipped with 6 mobile nonconducting polyethylene lines by which the circumference could be increased or decreased, and a silver electrode (width, 1 mm) was placed in the central part of each polyethylene line. The top of Fig. 1B shows the shrunken balloon electrodes and the bottom shows the expanded electrodes.

This study was reviewed and approved by the Animal Experimentation Committee, Isehara Campus, Tokai University. All experiments were performed in accordance with the Guidelines on Animal Use of

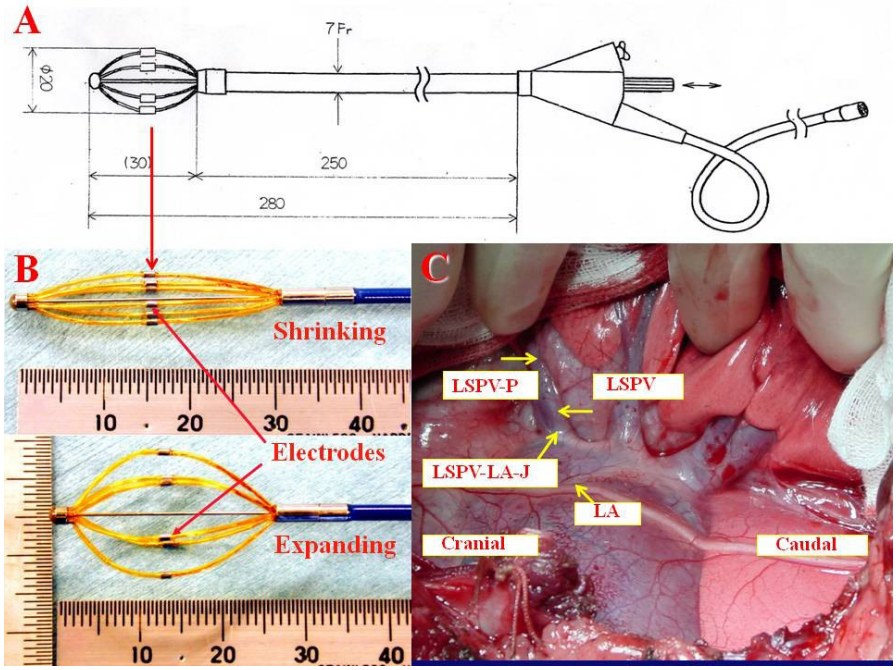


Fig. 1 Schema of entire catheter (A), part of basket electrodes (B) and part of left atrium (LA) and left superior pulmonary vein (LSPV) in the canine heart (C). The details are described in the text. LSPV-P, pulmonary portion of the LSPV; LSPV-LA-J, junction between the LSPV and the LA.

Tokai University School of Medicine, which conform to The Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Statistical Analysis

The variables are expressed as mean ± SD. Within-group comparisons were made using paired Student's *t* test. Comparisons in RR intervals and systolic blood pressure (SBP) before and after Ach injection, the time from Ach injection to AF initiation and the AF duration between pause-AF, brady-AF and tachy-AF groups, described below, were evaluated by analysis of variance with the Scheffé method. The results were significant at $p < 0.05$.

RESULTS

Study I: Induction of AF after Ach injection into LSPV wall

AF most frequently occurred in 12 mm from the LSPV-LA-J in the distal portion: 11 dogs (79%) had AF at 12 mm; 9 (64%) at 8 mm; 7 (50%) at 16 mm; and 6 (43%) at 4 mm. AF was reproducibly induced by Ach injection without programmed electrical stimulation in 19 of the 26 dogs tested (73%) (Table 1A). Programmed stimulation after Ach injection initiated AF in 5 of the remaining 7 dogs (Table 1B). The first AF initiated in each dog was investigated in the present study. As shown in Table 1A, among 19 AFs induced by Ach alone, pause (sinus arrest) ≧ 2.0 s was observed in 7 dogs (37%; pause-AF group; Fig. 2A-a and 2A-b). However, no atrioventricular (AV) block was seen during pauses. Sinus bradycardia followed by supraventricular premature contraction (SVPC) or followed by escape was seen in 6 dogs (32%; brady-AF group; Fig. 2B-a and 2B-b). Sinus tachycardia with subsequent SVPC was seen in 6 dogs (32%; tachy-AF group; Fig. 2C).

RR intervals (average of 5 beats) significantly increased and decreased just before AF initiation as compared to those just before Ach injection in brady-AF ($p < 0.001$) and tachy-AF ($p < 0.05$) groups, respectively. SBP was investigated just before and just after, 10 min after Ach injection, and just before AF initiation in the 3 groups. It significantly reduced just after and 10 s after Ach application and just before AF initiation as compared to SBP just before Ach application in pause- and tachy-AF groups ($p < 0.01$ in pause-AF and $p < 0.05$ in tachy-AF group) (Fig. 2A-a, 2C and 3). In the brady-AF group, there were no significant differences in BP between just before and just after or 10 s after Ach application (Fig. 2B-a and 3) although SBP just before AF initiation was significantly reduced as compared to other 3 periods ($p < 0.05$ in each) (Fig. 3). The mean time from Ach injection to AF initiation was 19.1 ± 19.6 s in pause-AF, 22.9 ± 36.7 s in brady-AF, and 50.7 ± 32.3 s in the tachy-AF group, and there were no significant differences among the 3 groups. The mean AF duration was 301.0 ± 89.4 s in pause-AF, 358.8 ± 185.7 s in brady-AF, and 330.3 ± 156.5 s in the tachy-AF group, and no significant differences were found. AF lasted more than 10 min in 1 dog in the brady-AF and 1 in the tachy-AF group.

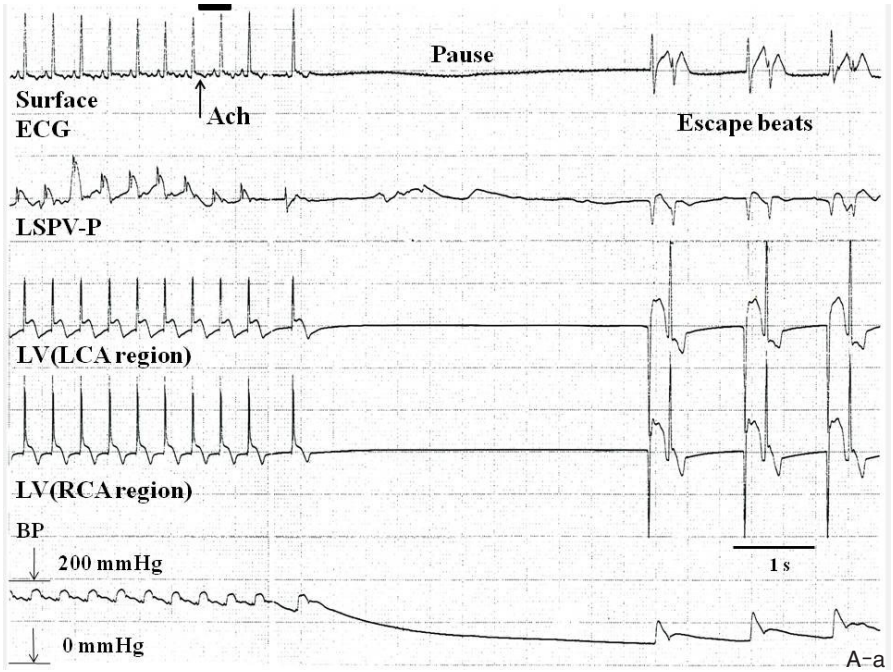
Of the 7 dogs, in whom AF was not initiated by Ach alone, AF was induced by programmed stimulation after Ach application in 5 (Fig. 4), and it was consistently generated with vulnerable periods after S2 stimulation in 4 of these 5 dogs (Table 1B).

Table 1A RR intervals and systolic blood pressure (SBP) before and after Ach injection, and the time from Ach injection to AF initiation and AF duration in 19 dogs, in whom AF was initiated by Ach application. Ach, acetylcholine; AF, atrial fibrillation; SVPC, supraventricular premature contraction; DC, direct current, which was applied to terminate AF over 600 s (10 min).

Dog No.	1, pause-AF 2, brady-AF 3, tachy-AF	RR intervals just before Ach (ms)	RR intervals just before AF (ms)	SBP just before Ach (mmHg)	SBP just after Ach (mmHg)	SBP 10 s after Ach (mmHg)	SBP just before AF (mmHg)	Time from Ach to AF (s)	AF duration (s)	Arrhythmia preceding AF
1	1	400	pause	128	80	72	115	7.5	345	Pause (2 sec) → escape → AF
2	1	480	pause	153	91	100	100	32	404	Pause (2.4 sec) → escape → AF
3	1	560	pause	120	78	65	100	5	222	Pause (2.5 sec) → escape → AF
4	1	520	pause	174	80	78	82	16.5	320	Pause (7 sec) → escape → AF
5	1	380	pause	165	128	130	115	58	303	Pause(4.5 sec) → escape → AF
6	1	400	pause	170	143	147	120	4.5	368	Pause (2 sec) → AF
7	1	480	pause	157	125	111	92	10	145	Pause (2 sec) → AF
8	2	420	520	154	154	145	90	23	over 600 → DC	Sinus bradycardia → escape → AF
9	2	400	480	150	148	147	125	15	365	Sinus bradycardia → SVPC → AF
10	2	420	520	135	135	138	100	96	212	Sinus bradycardia → escape → AF
11	2	480	580	148	146	139	130	2.5	276	Sinus bradycardia → SVPC → AF
12	2	390	480	149	141	142	120	2.3	143	Sinus bradycardia → SVPC → AF
13	2	340	420	137	128	130	135	3.5	557	Sinus bradycardia → SVPC → AF
14	3	300	280	126	111	120	122	5.1	158	Sinus tachycardia → SVPC → AF
15	3	360	320	155	145	134	114	85	385	Sinus tachycardia → SVPC → AF
16	3	540	480	175	150	145	125	60	301	Sinus tachycardia → SVPC → AF
17	3	520	380	163	138	140	134	70	over 600 → DC	Sinus tachycardia → SVPC → AF
18	3	400	320	186	142	139	120	68	203	Sinus tachycardia → SVPC → AF
19	3	320	280	140	133	128	125	16	335	Sinus tachycardia → SVPC → AF

Table 1B Five dogs in whom AF was induced by programmed electrical stimulation with S1–S2 method after acetylcholine (Ach) injection into the subadventtial layer of LSPV. In these 5 dogs, AF was not induced by Ach alone. AF, atrial fibrillation; LSPV-P, left superior pulmonary vein.

Dog No.	Progrramed Electrical Stimulation; S_1–S_2 method (S2/S1) (ms)	AF initiation after S1 or S2	AF duration (s)
20	250/300	3rd S1	36
21	240/350	S2	141
22	200/350	S2	591
23	180/350	S2	361
24	120/300	S2	over 600 → DC



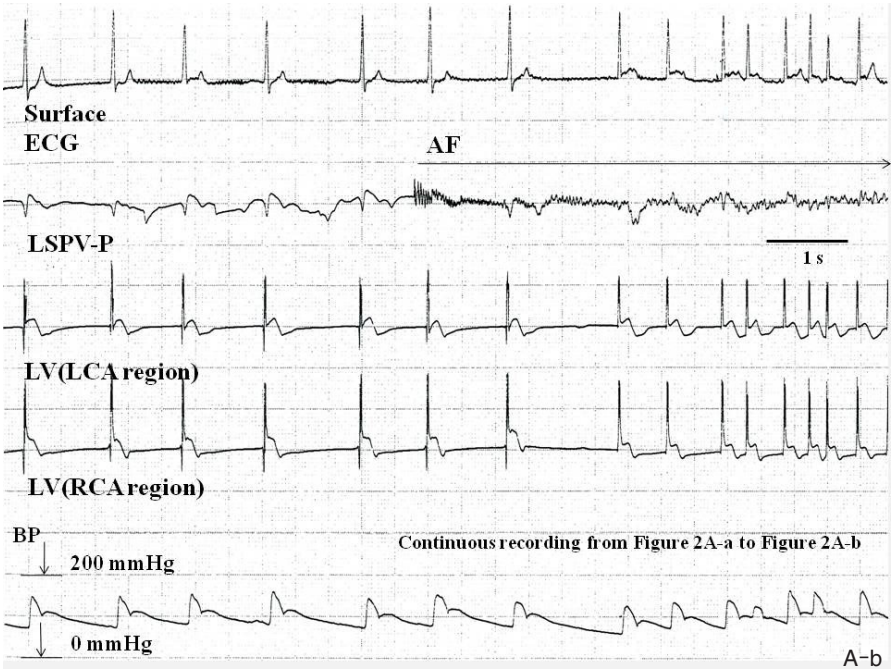


Fig. 2A–a and 2A–b Tracings of electrogram and blood pressure in a case of the pause-AF group. Ach injection into the subadventitial layer of the pulmonary portion of the left superior pulmonary vein (LSPV-P) caused pause (sinus arrest) followed by escape beats (Fig. 2A–a) with the subsequent atrial fibrillation (AF) (Fig. 2A–b). Also, blood pressure (BP) markedly reduced after Ach injection.

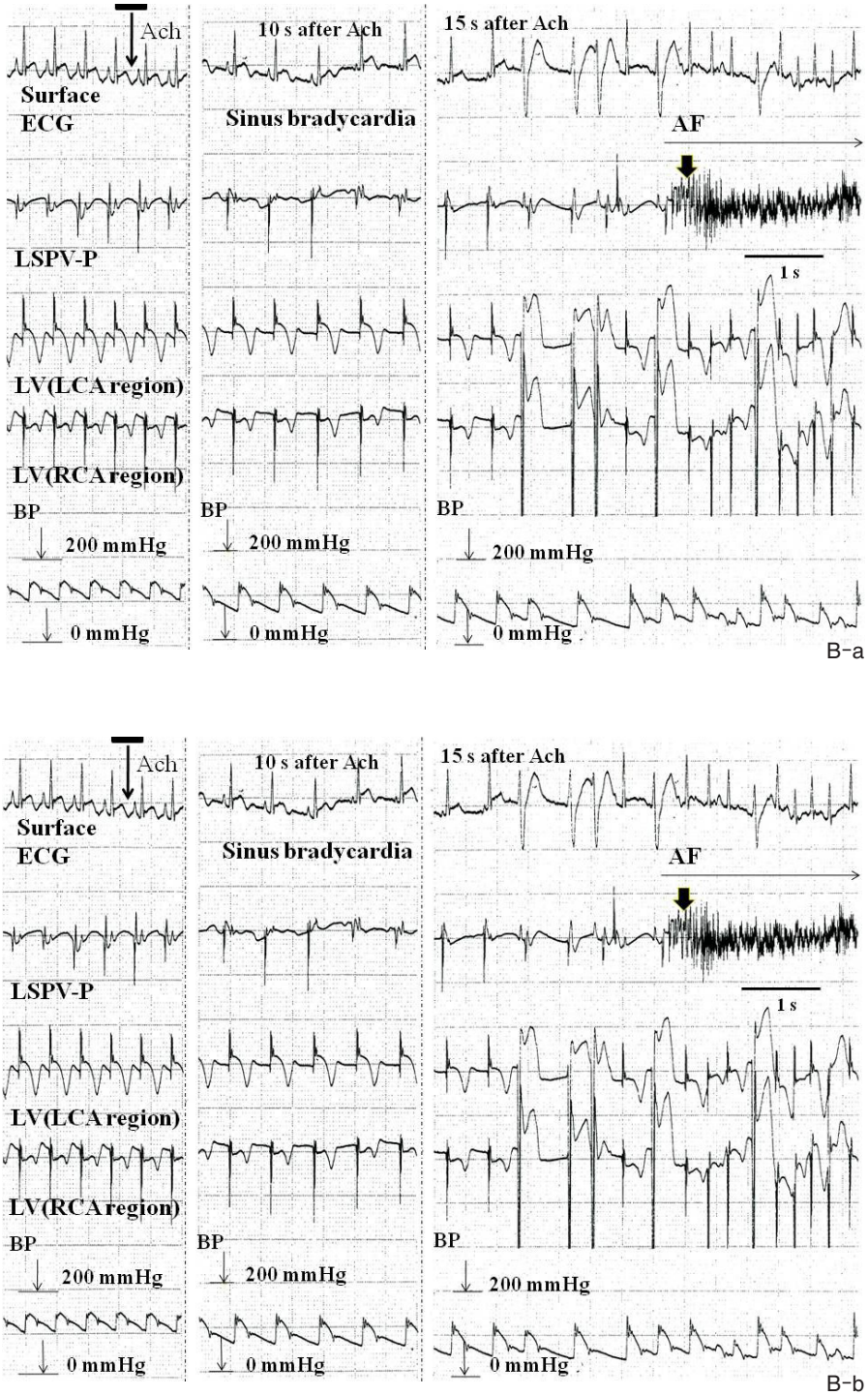


Fig. 2B-a and 2B-b A case of brady-AF group. Marked sinus bradycardia is seen 10 s after Ach injection and then followed by premature beats with the subsequent AF, and BP mildly reduced (Fig. 2B-a). AF occurred more than 15 s after Ach injection. Note that the signal of AF in this tracing of LSPV-P lead is very dense, as shown by an arrow. In this experiment, such dense signal in consistence with the beggining of AF was sometimes observed. This signal seems to be artefacts due to respiration because size of this signal looks to be modified by respiration periodicity and such signal disappeared with time as shown in the right panel of Figure 2B-b. However, cause of this phenomenon remains unclear in this study.

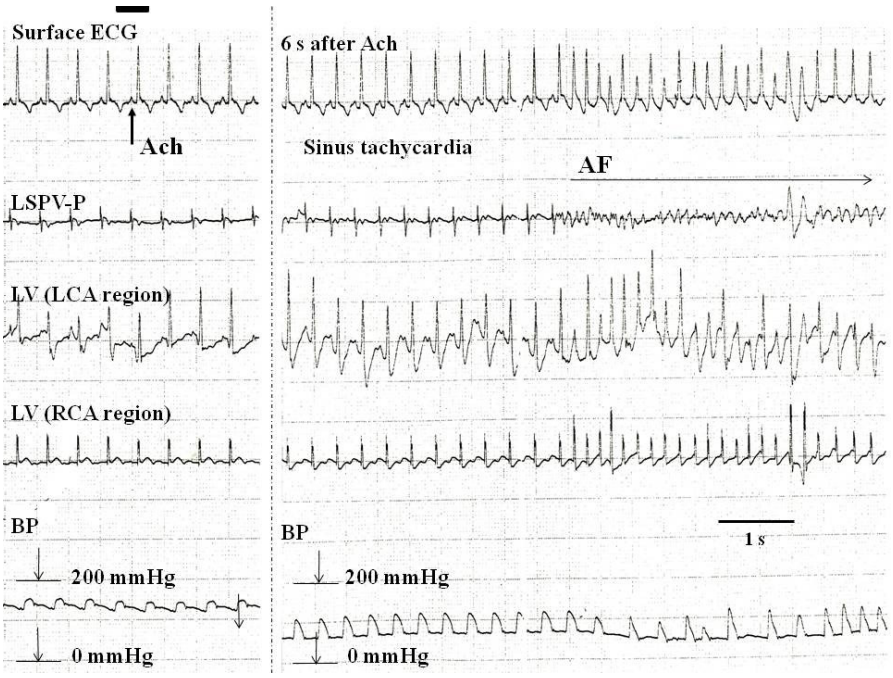


Fig. 2C A case of tachy-AF group. Sinus tachycardia occurred in 5 ~ 6 s after Ach injection followed by AF, and marked reduction in BP is seen.
LV, left ventricle; LCA region, myocardium distributed by blood flow from the left coronary artery (LCA). RCA region, myocardium distributed by blood flow from the right coronary artery (RCA).

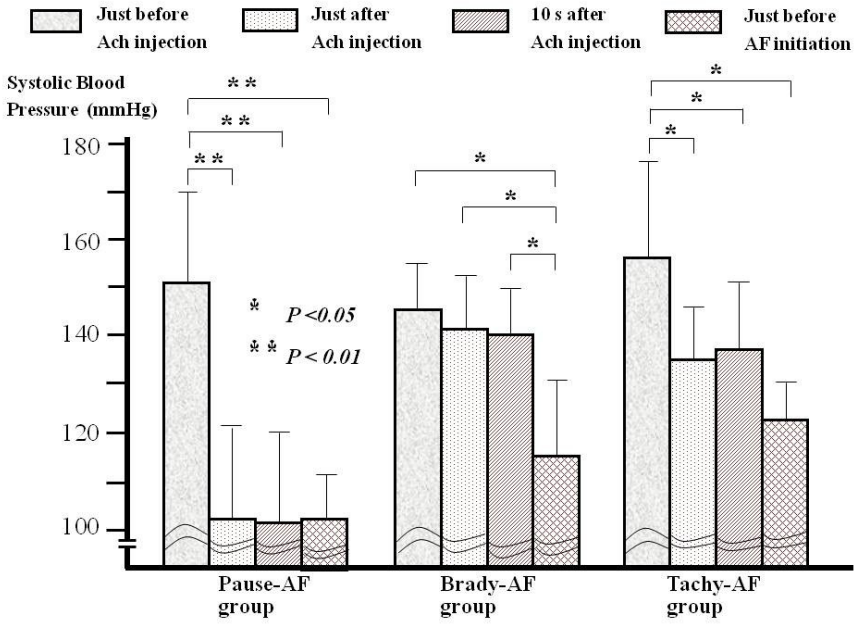


Fig. 3 Systolic blood pressure (SBP) changes before, just after and 10 s after Ach injection, and just before AF initiation. In the pause-AF group, pause often occurred within 1−2 s after Ach injection and SBP markedly reduced with the occurrence of pause. Low SBP persisted until AF initiation in a greater part of cases in this group. SBP in the brady-AF group did not significantly reduced just after and10 s after Ach injection. Note that a significant reduction is seen just after Ach injection in the tachy-AF group, which is different from SBP changes of the brady-AF group.

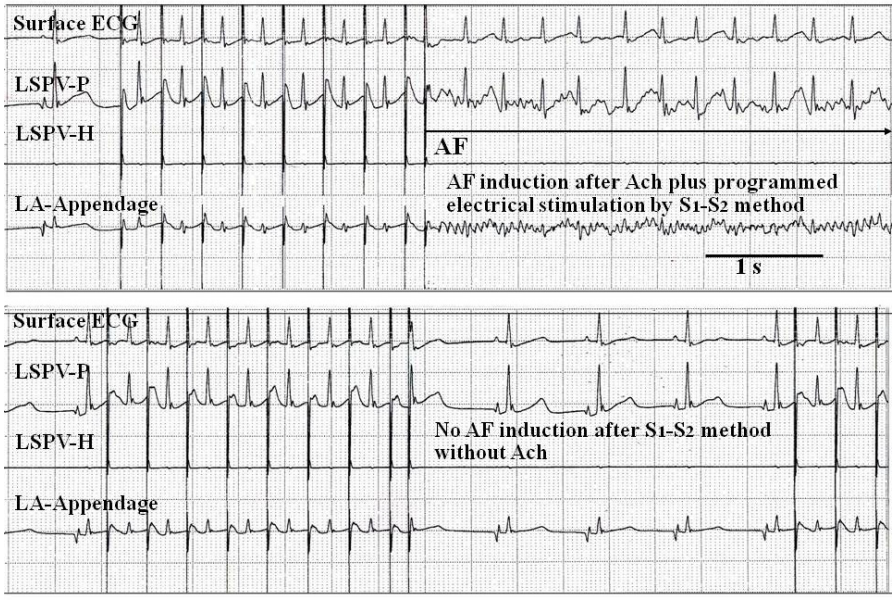


Fig. 4 AF induction after programmed electrical stimulation by S_1–S_2 method with prior acetylcholine (Ach) treatment. The top panel shows that premature S_2 stimulation after basic 8 consecutive S_1-stimulations elicited AF. Bottom panel shows that programmed electrical stimulation by S_1–S_2 method without prior Ach treatment did not elicit AF. LSPV-P, pulmonary region of the left superior pulmonary vein (LSPV); LSPV-H, cardiac region of the LSPV; LA-Appendage, Left atrial appendage; AF, atrial fibrillation.

Study II: Effects of muscarine receptor blockade and circumferential isolation of LSPV on AF initiation

In all the 4 dogs tested, AF was not initiated after atropine pretreatment (Fig. 5). The optimal ablation conditions at the LSPV-LA-J were described previously. At the LSPV-LA-J, 1–6 (average 4.1 ± 1.2) radiofrequency applications were required to eliminate AF, induced by Ach application to LSPV-P subadventitial layer. The top panel shows AF initiation after Ach injection before the CPVA at the LSPV-LA-J. The bottom panel shows that AF was not initiated after catheter ablation by using the newly manufactured catheter electrodes (Fig. 6).

DISCUSSION

Firstly, in this study, we developed a new experimental model for the induction of PV-mediated AF in structurally normal hearts by applying Ach into the subadventitial layer of the LSPV. The other major findings were (1) in some cases AF was initiated with marked slowing of the atrial rate in relation to the local reflex, whereas in the other cases AF initiation was preceded by a decrease or an increase in the heart rate followed by SVPCs or escape, (2) Ach-mediated AFs were more frequently initiated at the distal portion than at the proximal portion of the LSPV-LA-J, and (3) Ach-induced AFs could not be initiated at the LSPV by M2- receptor blockade and radiofrequency isolation, using a newly manufactured balloon electrode-catheter.

AF initiation by Ach injection into the subadventitial layer of the LSPV

It is well known that AF can be initiated by direct application of Ach on the atrial surface, infusion of Ach into the sinus node artery, or cervical vagal stimulation on both sides [3–8]. More than 90 % cases of clinical AF are reportedly preceded by premature contractions in the PV [19]. The myocardial fibers of the left atrium enter the PVs to form the myocardial sleeves, which are most developed in the LSPV of the 4 PVs [13]. We experimentally induced AF by locally injecting Ach into the subadventitial layer of the LSPV in this experiment, suggesting a strong relation between changes in the vagal tone and the initiation of AF in the LSPV. Furthermore, this AF initiation may be directly associated with the M_2-receptor in the LSPV, because prior administration of atropine suppressed Ach-induced AFs in the LSPV.

Initiation mechanism of Ach-induced AF in the LSPV

A previous study has shown that the incidence of the ablation-induced bradycardia-hypotension response was higher in PV areas than the ablation of other atrial tissues [20]. Hsieh *et al.* [21] reported severe bradycardia and hypotension in 6 of 37 patients (16%) undergoing focal PV ablation, and a majority of the ablation sites were inside the LSPV (4 patients). Lu *et al.* [22] described 2 patterns of clinical AF initiation: (1) episodes preceded by cycle length oscillation (53%) (cycle length was prolonged or shortened by 20% or more than the preceding beat) and (2) episodes initiated by a single premature beat preceding a relatively constant cycle length (47%). We could divide the AF initiation modes into 3 groups: pause-AF, brady-AF, and tachy-AF.

In the pause-AF group, pause was often followed by escape beats, resulting in the subsequent AF initiation. This group included sinus arrest as pauses and never showed atrioventricular (AV) block. The absence of

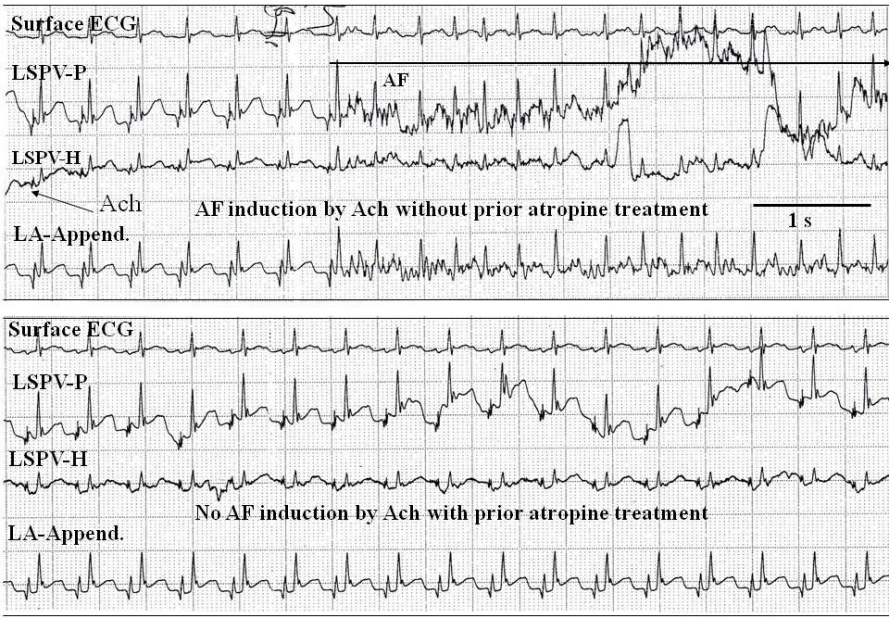


Fig. 5 No AF induction by Ach with prior atropine treatment. Top panel shows AF induction by Ach without prior atropine treatment. However, AF is not induced any more by Ach with prior treatment of atropine, as shown in the bottom panel. LSPV-P, pulmonary region of the left superior pulmonary vein (LSPV); LSPV-H, cardiac region of the LSPV; LA-Appendage, Left atrial appendage; AF, atrial fibrillation.

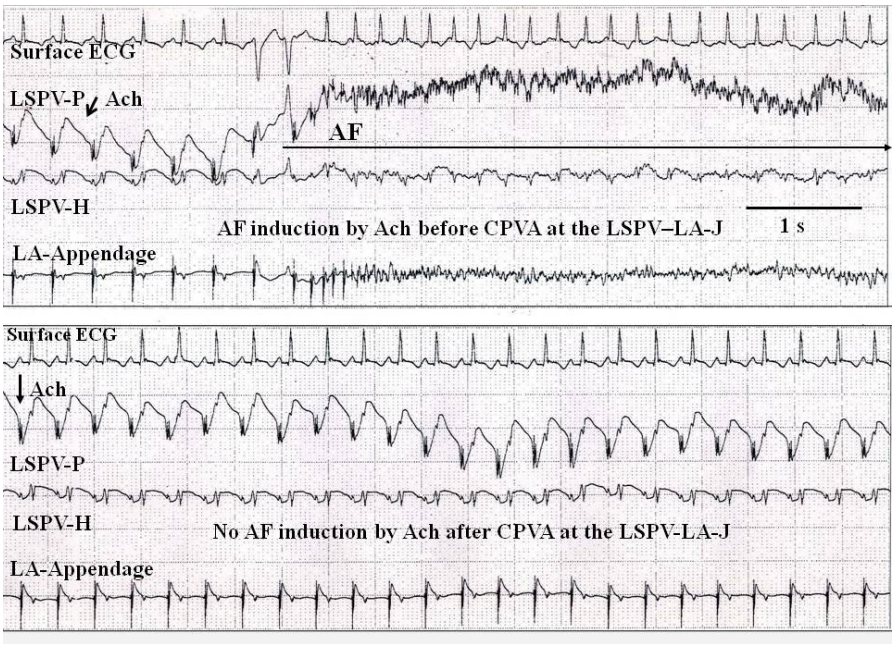


Fig. 6 No AF induction by Ach after CPVA at the LSPV-LA-J. AF was induced by Ach injection before the CPVA at the LSPV-LA-J, as shown in the top panel, but it was not generated by the same procedure after the CPVA, as shown in the bottom panel. AF, atrial fibrillation; Ach, acetylcholine; CPVA, circumferential pulmonary vein ablation; LSPV-LA-J, junction between the left superior pulmonary vein and the left atrium. LSPV-P, pulmonary region of the left superior pulmonary vein (LSPV); LSPV-H, cardiac region of the LSPV; LA-Appendage, Left atrial appendage.

Explanation of Abbreviations;

Ach, acetylcholine
AF, atrial fibrillation
AV block, atrioventricular block
CPVA, circumferential pulmonary vein ablation
DAD, delayed after depolarization
EAD, early after depolarization
PV, pulmonary vein
LSPV, left superior pulmonary vein
LSPV-H, heart portion of the LSPV
LSPV-P, pulmonary portion of LSPV
LSPV-LA-J, junction between LSPV and left atrium
M_2-receptor, muscarine receptor
SBP, systolic blood pressure
SVPC, supraventricular premature contraction

an AV block in this group may be explained by the fact that we injected Ach into the left-side of the LSPV wall, and there is a report that the stimulation of the left-side of PV areas predominantly affects the sinus node, whereas stimulation of right PV areas preferentially affects the AV node [10].

The pause in this group occurred within 1–2 s after Ach application. It may be due to the local reflex, but not due to direct Ach action on sinus node, because Ach does not seem to reach the sinus node through circulatory pathway within a second. In addition, the reflex may not be related to the central nervous pathway since we performed vagotomy and sympathectomy on the both sides in the cervical region. In the brady-AF group, we observed gradual prolongation of the RR intervals and gradual occurrence of hypotension several decade seconds after Ach injection, and the subsequent SVPC or escape beats, resulting in AF initiation. Namely, we did not observe hypotension immediately after Ach injection in this group, which was different from the pause-AF group. Therefore, Ach, which ran into sinus node through the circulatory pathway, might have directly suppressed the sinus node and induced bradycardia. Clinically, we often observe the strong relation between sinus node dysfunction and AF initiation. From our observations, in pause-AF and brady-AF groups, Ach may preferentially affect myocardial sleeves of PV after suppression of sinus node and induce SVPC or escape beats, followed by AF initiation. Animal model studies have demonstrated that vagal activity increases vulnerability for AF by shortening the PV effective refractory periods and increasing spatial PV heterogeneity [4, 23–24]. Therefore, reentry is suggested to be at least one mechanism for AF initiation in these 2 groups.

In the tachy-AF group, SBP reduced immediately after Ach injection with the subsequent increase in heart rate. However, AFs in this group mostly occurred a decade second or more following sinus tachycardia after Ach injection. Extremely rapid reaction of RR interval within 1 s after blood pressure changes is known to be based on the vagal tone moduration [25]. An increase in heart rate in this group could be interpreted as an immedeate withdrawal of vagal activity after reduction in SBP just after Ach application, and then an increase in sympathetic tone may add and maintain rapid HR [25]. In this process, automaticity may be enhanced in PVs with sympathetic stimulation [26]. On the other hand, Kumagai *et al.* [27] used the multipolar catheter to demonstrate the complex patterns of activation and block produced within PV sleeves during rapid pacing, and hypothesized that in this situation, the reentry mechanism may be the initiating factor to induce ectopic beats followed by AF. Therefore, the mechanism of AF induction in the tachy-AF group may be enhanced automaticity or reentry in PVs. Otherwise, although previous reports indicated the possibility of automaticity via EAD or DAD, which was induced by isoproterenol with isolated PV cells of rabbits [14], or with combined Ach and norepinephrine stimulation to isolated superfused canine PVs [15], this study could not determine whether this EAD or DAD was related to AF initiation in this experiments.

In this study, AF was easily initiated by programmed stimuration in dogs in whom Ach administration alone could not induce AF. It has been demonstrated that the structural and electrophysiological properties of the PV are favorable for reentry and reentry within the PV can be readily induced by programmed stimulation [27–28]. Reentry is the suggested mechanism for initiating AF in these dogs of our experiment.

Distal or proximal location of AF initiation in the LSPV

As mentioned earlier, the bradycardia-hypotension response by vagal stimulation is reportedly higher after ablation of the PV areas than after ablation of other atrial tissues [20]. The observation suggests that vagal influences are much stronger in the PVs than atrial ones. There are sinus-node like cells in the PVs which may be capable of independent pace making activity and may easily be influenced by changes in autonomic tone [16]. These sinus-node like cells are known to be denser in the distal end of the PVs than near the LSPV-LA-J [17]. However, the preferred location of AF induction in the PVs by vagal influences has not been studied. The present study indicated that Ach-induced AF was more pronounced in the portion distal to rather than proximal to the LSPV-LA-J, and might be associated with the results of the previous investigation demonstrating dence sinus-node like cells capable of independent pacemaker in the distal portion of the PVs [16–17]. However, this experiment could not clarify whether these sinus node-like cells was directly related to the AF initiation after applying Ach at the distal portion of PV.

Effects of CPVA to the LSPV-LA-J on AF initiation

Pappone *et al.* [10] reported that vagal reflexes were particularly elicited in LSPV-LA-J, suggesting vagal innervation in this area. Chevalier *et al.* [29] reported several gradients of autonomic innervation in the PV, with nerves more abundant in the proximal PV than the distal PV and more abundant in the epicardium than the endocardium. Recently, Tan *et al.* [30] indicated that the PV antrum within 5 mm of the PV-LA junction, rather than further away in the atria or distal to the PV, was the most densely innervated and therefore the optimal location for autonomic nerve modulation procedures such as radiofrequency ablation. In the present study, AF was more frequently initiated by Ach injection in the distal portion of the LSPV than in the proximal portion, and was terminated by CPVA of the LSPV-LA J in all the 3 groups: pause-AF, brady-AF and tachy-AF.

According to the report by Armour *et al.* [31], autonomic nerves in the PV-LA area are concentrated in the superior left atrial ganglionated plexus and the intrinsic cardiac neurons extend toward the periphery of the PV cells. There is a report that atrial autonomic ganglia are distributed mainly around the PV antrum comprising sympathetic and parasympathetic fibers that share the same ganglionated plexus [32]. Therefore, in the present experiment, Ach injection may be similar to stimulate the peripheral portion of the vagal nerve in the LSPV and CPVA is considered to eliminate AF by denervation of the ganglionated plexus around LSPV-LA-J. On the other hand,

previous studies demonstrated that the ectopic beats originating from the PV propagated to the LA with a characteristically long conduction time, often with a conduction delay or block within the PV or at the PV-LA junction [19, 33]. Therefore, there seems to be at least 2 mechanisms for the prevention of AF initiatuon. One is that the conduction of ectopic beats from the LSPV into the LA could be interrupted at the LAPV-LA-J, where CPVA is performed. The other is that vagal activities around the LAPV-LA-J are suppressed by the damage to the superior left atrial ganglionated plexus during CPVA because LSPV-LA J ablation itself promotes left atrial autonomic denervation [32].

Clinical implications

The PVs is clinically common site in which focal paroxysmal AF occurs [19]. The role of autonomic nervous system in relation to the arrhythmia in PVs is poorly understood although there are strong evidence for increased vagal or increased sympathetic tone as initiators of AF [1]. The present experiment, although limited to structurally normal heart in dogs, suggests that the increased level of Ach in PV wall initiates AF. Clinically, an increase in vagal tone has a potential role initiating AF and vagal denervation reduces the recurrence of paroxysmal AF from the PVs [9−10]. The fact that Ach-mediated AF occurred after a slowing (pause-AF and brady-AF) and an accelerating (tachy-AF) heart rate may partly explain nighttime initiating AF and daytime initiating AF, respectively [34]. In addition, our observations suggests that CPVA at the LSPV-LA-J could eliminate not only AF initiation but also autonomic reflexes, because heart rate changes such as pause, bradycardia and tachycardia, wnich were initiated after Ach injection, did not occur after the CPVA.

Study limitations

Firstly, although we could classify AF initiation modes induced by Ach application into 3 groups, which were pause-AF, brady-AF and tachy-AF, mechanism differences in AF initiation for each mode remain unclear. With this regards, use of multipolar electrodes over PVs may be a useful method to determine reentry mechanism by clarifying conduction route and the presence or absence of conduction delay or block. Second, this study demonstrated the elimination of AF by CPVA at the LSPV-LA J, but could not determine which procedure, vagal denervation only, conduction block only on PV or both, was more preferable to eliminate AF initiation. Further studies are needed to clarify these limitations.

Conclusion

Ach application into the subadventitial layer of the LSPV, especially to the distal portion, could easily elicit AF, which was preceded by pause, sinus bradycardia, or sinus tachycardia in structurally normal canine hearts. Radiofrequency catheter ablation around the endocardial site of the LSPV-LA-J, in which the dense ganglionated plexus of sympathetic and vagal nerves exists [31], could prevent Ach-induced AF in the LSPV. These observations suggest that local Ach application can initiate AF not only in the atria but also in the PVs, and an increase in vagal tone at the LSPV-LA-J plays a critical role in eliciting AF in normal hearts.

ACKNOWLEDGMENTS

We deeply appreciate Ms Y. Shinozaki, Y. Takahari, K. Naito, and K. Iwao in Teaching and Research Support Center for their expert technical assistances.

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