Abstract—Atrial arrhythmia, which includes atrial fibrillation (AF) and atrial flutter (AFL), is common in patients with pulmonary arterial hypertension (PAH), who often have increased sympathetic nerve activity. Here, we tested the hypothesis that autonomic nerves play important roles in vulnerability to AF/AFL in PAH. The atrial effective refractory period and AF/AFL inducibility at baseline and after anterior right ganglionated plexi ablation were determined during left stellate ganglion stimulation or left renal sympathetic nerve stimulation in beagle dogs with or without PAH. Then, sympathetic nerve, β-adrenergic receptor densities and connexin 43 expression in atrial tissues were assessed. The sum of the window of vulnerability to AF/AFL was increased in the right atrium compared with the left atrium in the PAH dogs but not in the controls. The atrial effective refractory period dispersion was increased in the control dogs, but not in the PAH dogs, during left stellate ganglion stimulation. The voltage thresholds for inducing AF/AFL during anterior right ganglionated plexi stimulation were lower in the PAH dogs than in the controls. The AF/AFL inducibility was suppressed after ablation of the anterior right ganglionated plexi in the PAH dogs. The PAH dogs had higher sympathetic nerve and β1-adrenergic receptor densities, increased levels of nonphosphorylated connexin 43, and heterogeneous connexin 43 expression in the right atrium when compared with the control dogs. The anterior right ganglionated plexi play important roles in the induction of AF/AFL. AF/AFL induction was associated with right atrium substrate remodeling in dogs with PAH. (Hypertension. 2015;66:1042-1049. DOI: 10.1161/HYPERTENSIONAHA.115.05846.) • Online Data Supplement

Key Words: atrial fibrillation ■ atrial flutter ■ connexin 43 ■ hypertension ■ stellate ganglion

Supraventricular arrhythmias, such as atrial fibrillation (AF) and atrial flutter (AFL), are common in patients with pulmonary arterial hypertension (PAH) and are associated with poor outcomes.1,2 In 1 study, restoration and maintenance of sinus rhythm were invariably associated with clinical improvement and recovery.3 However, it is difficult to treat AF/AFL in patients with PAH because existing antiarrhythmic agents have substantial side effects in patients with PAH.

Previous studies have suggested that increased sympathetic nerve activation could contribute to disease severity in patients with PAH.4,5 A recent study by Chen et al6 demonstrated that pulmonary artery denervation decreased pulmonary arterial pressure and increased functional capacity. Our previous study showed that renal denervation attenuates pulmonary vascular remodeling and decreases pulmonary arterial pressure in experimental PAH.7 In a dog model with rapid atrial or ventricular pacing, a reduction in sympathetic tone or cardiac sympathetic denervation achieved by ablating extrinsic cardiac or renal sympathetic nerves was shown to be useful for controlling atrial arrhythmia.8–10 However, to the best of our knowledge, the effect of sympathetic nerve activity on vulnerability to atrial arrhythmia in PAH has not been evaluated. The purpose of this study was to test the hypothesis that the activity of intrinsic and extrinsic cardiac nerves has an important role in vulnerability to atrial arrhythmia in a model of PAH.

Methods

Additional methodological details for each of the sections below are described in the online-only Data Supplement.
Animal Model Preparation

This study was approved by the animal studies subcommittee of our institutional review board and was in compliance with the guidelines of the National Institutes of Health for the care and use of laboratory animals. Twenty-one beagles, weighing an average of 12.7±2.2 kg, were used in this study. Each beagle was given an intramuscular injection of 25 mg/kg ketamine sulfate before being premedicated with pentobarbital sodium.

In total, 14 of the 21 dogs were injected with pentobarbital sodium (30 mg/kg IV), intubated, and ventilated with room air supplemented with oxygen via a respirator (MAO01746, Harvard Apparatus, Holliston, MA). PAH was induced in these 14 dogs using monocrotaline as described previously. Hemodynamic parameters were measured as described in the online-only Data Supplement.

The atrial effective refractory period (AERP) was determined as previously described.11 Transthoracic two-dimensional and Doppler echocardiography were performed on the PAH dogs (IE33, S5-1, Philips, Holland) at baseline and again after 8 weeks. The protocols are described in the online-only Data Supplement.

Experimental Protocol

Seven normal dogs were assigned to the control group. Fourteen PAH dogs were assigned to PAH group 1 (n=7) or PAH group 2 (n=7). A flowchart of the experimental design is presented in Figure S1.

In each of the dogs from the control group and PAH group 1, the heart was exposed in a pericardial cradle by median sternotomy under anesthesia. Multielectrode catheters (Biosense-Webster, Diamond Bar, CA) were secured to allow recording from the left and right atrial appendages and the left and right atria (LA/RA). The atrial effective refractory period (AERP) was determined as previously described.11 Hemodynamic parameters were measured as described in the online-only Data Supplement.

In PAH group 2, the AERP and AF/AFL vulnerability at baseline or during LSGNS were measured as described for the control group and PAH group 1. Subsequently, all visible renal nerves entering the left kidney along the renal artery were isolated and ligated. The renal nerve bundle was placed on bipolar hook electrodes. During left renal artery stimulation, AF/AFL at each site was determined as described previously.12 During left stellate ganglion stimulation (LSGNS: 20 Hz, 2 ms) combined with atrial S2 stimulation, the AERP and AF/AFL vulnerability were measured at all sites in each dog. AF/AFL was defined as an irregular or regular atrial rate of >500/400 bpm, respectively, lasting for >5 s.

After AERP and AF/AFL vulnerability were measured at baseline, the left stellate ganglion was identified, and a pair of bipolar hook electrodes was attached to the left stellate ganglion as described previously.11 During left stellate ganglion stimulation (LSGNS: 20 Hz, 2 ms) combined with atrial S2 stimulation, the AERP and AF/AFL vulnerability were measured at all sites. The stellate ganglion stimulation threshold is defined as the current required to produce an increase of ≥20% in systolic blood pressure or heart rate.13 Next, high-frequency electric stimulation (Grass stimulator, 20 Hz, 0.1 ms duration) was applied to the anterior right ganglionated plexi (ARGP), the inferior right ganglionated plexi, the superior left ganglionated plexi, and the inferior left ganglionated plexi for 1 minute using incremental voltages up to the voltage that induced AF/AFL. The lowest sinus rate that was induced by GP stimulation at each voltage level was recorded as an indicator of cholinergic influence. After GP stimulation, RF energy was applied (50°C, 30–50 W, 30–80 s) to the ARGP, where high-frequency stimulation induced evoked vagal reflexes. Complete vagal denervation was defined arbitrarily as the abolition of all vagal reflexes evoked by high-frequency stimulation. LSGNS combined with atrial S2 stimulation was then performed again, and the AERP and WOV of AF/AFL at the 4 sites were measured. Next, the inferior right ganglionated plexi, superior left ganglionated plexi, and inferior left ganglionated plexi were ablated. The AERP and WOV of the AF/AFL at the 4 sites were measured again during LSGNS combined with atrial S2 stimulation.

In PAH group 2, the AERP and AF/AFL vulnerability at baseline or during LSGNS were measured as described for the control group and PAH group 1. Subsequently, all visible renal nerves entering the left kidney along the renal artery were isolated and ligated. The renal nerve bundle was placed on bipolar hook electrodes. During left renal artery stimulation, AF/AFL at each site was determined as described previously.12 During left stellate ganglion stimulation (LSGNS: 20 Hz, 2 ms) combined with atrial S2 stimulation, the AERP and AF/AFL vulnerability were measured at all sites in each dog. AF/AFL was defined as an irregular or regular atrial rate of >500/400 bpm, respectively, lasting for >5 s.

Table 1. Changes in the Atrial Effective Refractory Period at Different Stages in the 3 Groups (ms)

<table>
<thead>
<tr>
<th>Groups</th>
<th>RA</th>
<th>RAA</th>
<th>LA</th>
<th>LAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>126±3.4</td>
<td>125±3.1</td>
<td>126±3.2</td>
<td>127±2.8</td>
</tr>
<tr>
<td>LSGNS</td>
<td>123±3.8</td>
<td>122±4.1</td>
<td>110±5.5*</td>
<td>112±6.1*</td>
</tr>
<tr>
<td>After ARGP ablation LSGNS</td>
<td>123±4.1</td>
<td>122±4.7</td>
<td>112±6.1*</td>
<td>113±7.2*</td>
</tr>
<tr>
<td>After IRGP, SLGP, and ILGP ablation LSGNS</td>
<td>124±5.2</td>
<td>125±4.6</td>
<td>121±6.7</td>
<td>120±7.4</td>
</tr>
<tr>
<td>PAH group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>129±5.5</td>
<td>131±5.7</td>
<td>126±4.8</td>
<td>128±4.1</td>
</tr>
<tr>
<td>LSGNS</td>
<td>116±6.2*</td>
<td>117±6.4*</td>
<td>111±6.2*</td>
<td>114±6.5*</td>
</tr>
<tr>
<td>After ARGP ablation LSGNS</td>
<td>120±6.5*</td>
<td>121±5.8*</td>
<td>112±6.3*</td>
<td>115±6.5*</td>
</tr>
<tr>
<td>After IRGP, SLGP, and ILGP ablation LSGNS</td>
<td>124±6.2</td>
<td>126±6.5</td>
<td>122±5.5</td>
<td>125±6.2</td>
</tr>
<tr>
<td>PAH group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>129±6.2</td>
<td>132±5.3</td>
<td>126±4.1</td>
<td>125±4.4</td>
</tr>
<tr>
<td>LSGNS</td>
<td>115±4.5*</td>
<td>118±5.1*</td>
<td>112±5.7*</td>
<td>113±6.5*</td>
</tr>
<tr>
<td>LRSNS</td>
<td>127±6.7</td>
<td>131±7.1</td>
<td>125±4.7</td>
<td>126±4.9</td>
</tr>
<tr>
<td>After ARGP ablation LRSNS</td>
<td>126±5.8</td>
<td>133±7.4</td>
<td>126±5.5</td>
<td>124±5.8</td>
</tr>
<tr>
<td>After stellate ganglion cutting LRSNS</td>
<td>129±7.4</td>
<td>133±7.1</td>
<td>125±4.6</td>
<td>126±4.7</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SD. ARGP indicates anterior right ganglionated plexi; ILGP, inferior left ganglionated plexi; IRGP, inferior right ganglionated plexi; LA, left atria; LAA, left and right atrial appendage; LRSNS, left renal sympathetic nerve stimulation; LSGNS, left stellate ganglion stimulation; PAH, pulmonary arterial hypertension; RA, right atria; RAA, right atrial appendage; and SLGP, superior left ganglionated plexi.

*P<0.01 compared with the baseline.
sympathetic nerve stimulation\textsuperscript{14} (LRSNS: 20 Hz, 2 ms) combined with atrial S\textsubscript{1}S\textsubscript{2} stimulation, the AERP and WOV of AF/AFL were measured again. The voltage that increased the systolic BP by 10\% was chosen for use in LRSNS. Next, ARGP ablation was performed. The WOV of AF/AFL was measured during LRSNS combined with atrial S\textsubscript{1}S\textsubscript{2} stimulation, and the bilateral stellate ganglions were cut. The WOV of AF/AFL was measured again during LRSNS combined with atrial S\textsubscript{1}S\textsubscript{2} stimulation.

Immunohistochemistry and Western Blotting

Tissue samples from 6 normal control dogs, 3 dogs in PAH group 1, and 3 dogs in PAH group 2 were used for immunohistochemistry and Western blotting. Sections (4 μm) were cut from paraffin blocks containing the RA and LA. Atrial sympathetic nerve densities, β-adrenergic receptor (AR) densities, and connexin 43 (Cx43) expression were evaluated as described in the online-only Data Supplement.

Statistical Analysis

The values are presented as the mean±SD. Details are provided in the online-only Data Supplement.

Results

Evidence of PAH

All the PAH dogs began to display rapid breathing and decreased appetite beginning at 10 days after injection of dehydromonocrotaline. The right atrial dimensions and right ventricular diastolic dimensions of the PAH dogs were significantly higher after 8 weeks than at baseline (P=0.02). In addition, the right ventricular lateral longitudinal strain was reduced after 8 weeks. Changes in the echocardiographic parameters and hemodynamic data are shown in Tables S1 and S2. Compared with their levels at baseline, pulmonary arterial pressure and right ventricular pressure were higher after 8 weeks in the PAH dogs.

Electrophysiological Testing and AF/AFL Induction

The AERP in the right atrial appendages and RA at baseline were increased in PAH groups 1 and 2 compared with the control group; however, this increase was not statistically significant (Table 1). The AF/AFL $\Sigma$WOV was 19.8±10.1 ms in PAH group 1 and 20.1±9.8 ms in PAH group 2 during AERP testing. The WOV was wider in the RA and right atrial appendages than in the LA and left right atrial appendages in PAH groups 1 and 2 (Table 2).

During ARGP stimulation, the dogs in the control group and PAH group 1 showed sinus rate slowing responses at similar voltages (heart rate slowing 1.9±0.4 V versus 1.7±0.5 V; P=0.55). However, the lowest voltage that induced AF/AFL was higher in the control group than in the PAH group 1 (2.5±0.5 V versus 1.8 V±0.5 V; P=0.03), inferior right ganglionated plexi, superior left ganglionated plexi, and inferior left ganglionated plexi stimulation had similar voltages in the sinus rate slowing response and AF/AFL vulnerability.

Table 2. WOV of the AF/AFL Changes at Different Stages in the 3 Groups (ms)

<table>
<thead>
<tr>
<th>Groups</th>
<th>RA</th>
<th>RAA</th>
<th>LA</th>
<th>LAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LSGNS</td>
<td>9.2±4.2*</td>
<td>10.5±6.4*</td>
<td>13.4±6.4*</td>
<td>13.2±7.1*</td>
</tr>
<tr>
<td>After ARGP ablation LSGNS</td>
<td>12.5±7.2*</td>
<td>14.3±7.7*</td>
<td>21.1±8.1*</td>
<td>19.1±8.6*</td>
</tr>
<tr>
<td>After IRGP, SLGP and ILGP ablation LSGNS</td>
<td>5.2±3.1†</td>
<td>6.2±4.6†</td>
<td>6.3±4.1†</td>
<td>6.7±3.4†</td>
</tr>
<tr>
<td>PAH group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>24.2±6.1‡</td>
<td>23.2±5.9§</td>
<td>13.2±3.8</td>
<td>14.2±4.1</td>
</tr>
<tr>
<td>LSGNS</td>
<td>71.8±12.2‡</td>
<td>71.2±11.4§</td>
<td>51.4±9.4*</td>
<td>54.2±9.1*</td>
</tr>
<tr>
<td>After ARGP ablation LSGNS</td>
<td>41.3±11.3‡</td>
<td>39.3±10.5§</td>
<td>37.2±12.3§</td>
<td>32.5±9.5†</td>
</tr>
<tr>
<td>After IRGP, SLGP and ILGP ablation LSGNS</td>
<td>42.7±10.2‡</td>
<td>37.1±8.5§</td>
<td>36.6±8.5†</td>
<td>35.2±9.2‖</td>
</tr>
<tr>
<td>PAH group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>25.5±5.3‡</td>
<td>22.6±5.1§</td>
<td>14.1±4.5</td>
<td>13.7±4.6</td>
</tr>
<tr>
<td>LSGNS</td>
<td>74.2±11.3‡</td>
<td>68.5±9.5§</td>
<td>54.7±8.9*</td>
<td>51.1±9.5*</td>
</tr>
<tr>
<td>LRSNS</td>
<td>47.7±8.3‡</td>
<td>45.4±7.5§</td>
<td>33.7±7.2‖</td>
<td>31.5±7.5‖</td>
</tr>
<tr>
<td>After ARGP ablation LRSNS</td>
<td>32.1±5.4†</td>
<td></td>
<td></td>
<td>31.3±4.1§¶</td>
</tr>
<tr>
<td>LRSNS</td>
<td>24.1±6.1¶</td>
<td>21.8±5.5§</td>
<td>14.7±3.2#</td>
<td>14.5±5.5#</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SD. AF indicates atrial fibrillation; AFL, atrial flutter; ARGP, anterior right ganglionated plexi; ILGP, inferior left ganglionated plexi; IRGP, inferior right ganglionated plexi; LA, left atria; LAA, left and right atrial appendage; LRSNS, left renal sympathetic nerve stimulation; LSGNS, left stellate ganglion stimulation; PAH, pulmonary arterial hypertension; RA, right atria; RAA, right atrial appendage; SLGP, superior left ganglionated plexi; and WOV, window of vulnerability.

*P<0.01 compared with the baseline.
†P<0.05 compared with LSGNS after ARGP ablation.
‡P<0.01 compared with LA.
§P<0.01 compared with LAA.
||P<0.01 compared with LSGNS.
¶P<0.05 compared with LRSNS.
#P<0.05 compared with LRSNS after ARGP ablation.
between the control group and PAH group 1. During LSGNS, the AERP decreased at the LA and left right atrial appendages in the control group and at all 4 sites in PAH group 1, whereas the dAERP increased in the control group but not in the PAH group 1. The $\Sigma WOV$ was 12.2±6.3 ms in the control group and 63.4±22.1 ms in the PAH group 1 ($P<0.01$). After ARGP ablation, the $\Sigma WOV$ decreased further than it did before the stellate ganglion was bilaterally cut (14.6±9.2 ms versus 26.3±11.2 ms; $P<0.05$) during LRSNS combined with atrial S1S2 stimulation. There were no significant differences in the dAERP at baseline, during LSGNS or during LRSNS.

**Histological Findings**

Representative RA sections stained for TH and GAP43 are shown in Figure 1. The atrial nerve density for each group was expressed as the mean of the nerve densities in the RA. The densities of TH-positive nerves and GAP43-positive nerves within the RA were significantly higher in the PAH dogs than in the control dogs ($P<0.01$). There was no significant difference in nerve densities in the LA between the control and PAH dogs.

Figure 2 shows the results of immunostaining for Cx43 expression in the RA. The heterogeneity of Cx43 expression, expressed as the means±SD for different portions of the heart, was 3347±812 and 5131±1583 $\mu m^2/mm^2$ for the subepicardial RA in control dogs and dogs with PAH, respectively. Cx43 distribution in the RA was significantly more heterogeneous in PAH dogs than in control dogs ($P<0.05$). There was no significant difference in the heterogeneity of Cx43 expression in the LA between the control and PAH dogs.

**Western Blot Studies**

Figure 3 compares Western blots of atrial tissue samples from control and PAH dogs. All immunoblot band intensity measurements were normalized to the intensity of the $\beta$-actin band in the loaded sample. As shown in Figure 3A and 3B, the level of Cx43 protein was reduced in the RA ($P=0.02$), but not in the LA ($P=0.34$), of the PAH dogs compared with the control dogs. Conversely, the ratio of nonphosphorylated Cx43 protein to total Cx43 protein was increased in the RA of the PAH dogs (38±12% versus 59±18%; $P=0.03$), but not in the LA ($P=0.41$; Figure 3A and 3C). The $\beta_1$-AR density was increased in the RA in the PAH dogs (Figure 4) but not in the LA. There was no change in $\beta_2$-AR density in any region of the PAH dogs.

Figure 1. A, Histological sections of tyrosine hydroxylase (TH)-positive and growth-associated protein (GAP)43-positive atrial nerves in control and pulmonary arterial hypertension (PAH) dogs. B, Representative images of TH and GAP43 staining in control and PAH dogs (original magnification: ×400). #P<0.01 vs control dogs.

Figure 2. Heterogeneity of connexin 43 (Cx43) expression in the right atria in control and pulmonary arterial hypertension (PAH) dogs (original magnification: ×400).
Discussion

This study explored the influence of intrinsic and extrinsic cardiac nerves on AF/AFL vulnerability in experimental PAH. We provide evidence for the following: (1) the ARGP, as an intrinsic cardiac ganglion nerve, plays an important role in determining AF/AFL vulnerability in experimental PAH; (2) the effects of LRSNS on AF/AFL vulnerability are closely associated with the left stellate ganglion nerve and ARGP; and (3) high densities of sympathetic nerves and β₁-ARs, as well as greater heterogeneity in Cx43 expression in the RA, are essential for AF/AFL vulnerability in PAH dogs.

PAH is characterized by elevations in pulmonary arterial pressure and pulmonary vascular resistance and is associated with a relatively high incidence of AF/AFL. Increases in pulmonary vascular resistance lead to increased right ventricular afterload, resulting in right atrial dilation. Studies have demonstrated that sympathetic nerve activity is an important causal factor in PAH development. In an animal model of PAH, sympathetic nervous system activity is markedly increased, and neurohumoral dysfunction contributes to the development of excessive muscularization and pulmonary artery fibrosis. To the best of our knowledge, the relationship between the incidence of AF/AFL and the activity of the autonomic nerves in PAH has not been evaluated. To evaluate the impact of autonomic nerve activity on AF/AFL vulnerability in PAH, we used an experimental canine model of PAH to investigate the association of AF/AFL vulnerability with the intervening intrinsic and extrinsic cardiac nerves.

We determined that the WOV of AF/AFL was wider in PAH dogs than in control dogs. The RA had greater AF/AFL vulnerability than the LA at baseline and during LSGNS or LRSNS in PAH dogs. Furthermore, the RA of the PAH dogs contained a higher level of unphosphorylated Cx43 and more heterogeneous Cx43 expression than the RA of the control dogs. Previous studies have demonstrated that heterogeneous Cx43 expression may enhance susceptibility to arrhythmia, as it is associated with dispersed impulse conduction. Taken together, our findings suggest that regional changes and greater heterogeneity of Cx43 expression provide the substrate for AF/AFL vulnerability in PAH dogs. However, AF/AFL vulnerability was suppressed during LRSNS after the left stellate ganglion was cut. In a recent study, Huang et al demonstrated that renal nerve stimulation facilitates ischemia-induced ventricular arrhythmia by increasing left stellate ganglion activity. Left stellate ganglion ablation attenuates these arrhythmias. In another report, Tsai et al found that renal denervation was associated with reduced left stellate ganglion nerve activity and atrial tachycardia episodes in ambulatory dogs. These results suggest that the left stellate ganglion plays an important role in the relationship between renal sympathetic nerve activity and atrial arrhythmia.

The other major findings of this study were that ARGP function facilitated AF vulnerability and that ARGP ablation
attenuated AF/AFL vulnerability in an experimental model of PAH. Furthermore, PAH dogs had high densities of sympathetic nerves and β₁-ARs in the RA. Previous clinical studies have indicated that GP ablation with or without PV isolation can successfully treat the paroxysmal form of AF. Experimental studies have demonstrated that partial GP ablation is less effective than more complete GP ablation; additionally, partial GP ablation may increase the incidence of AF by exacerbating the heterogeneity of refractoriness. In this study, we determined that AF/AFL vulnerability was facilitated and that dAERP increased during LSGNS after ARGP ablation in normal dogs. The increased AF vulnerability during LSGNS may be related to exacerbated refractory heterogeneity after partial GP ablation. However, in this study, we determined that AF/AFL vulnerability was suppressed during LSGNS after GP ablation in PAH dogs. Previous studies have shown that the ARGP is located between the right superior pulmonary vein and the right atrial junction, extending to the RA and LA. The GPs are particularly well innervated with both adrenergic and vagal nerve endings and contain efferent cholinergic and adrenergic neurons that affect the atrial myocardium. ARGP ablation decreased right atrial sympathetic nerve activity in the PAH dogs in our study, which may have attenuated AF/AFL vulnerability during LSGNS.

In this study, PAH dogs had higher dAERP at baseline compared with control dogs. Viswanathan et al previously indicated that the degree of gap junction coupling is an important determinant of heterogeneity in action potential duration and dispersion of repolarization. The higher dAERP found in PAH dogs may be associated with the increased quantity of unphosphorylated Cx43 and the greater heterogeneity of Cx43 expression in the RA. During LSGNS, dAERP increased in the controls, but not in the PAH dogs. These results suggested that LSGNS could not further increase dAERP in the atrium after electric and substrate remodeling in this experimental canine model of PAH.

Clinical Implications

Atrial tachyarrhythmias, such as AF and AFL, are common in patients with PAH and are often associated with the worsening of heart failure and a decline in patient clinical status. Previous studies have implicated increased sympathetic nervous system activity and the renin–angiotensin–aldosterone system (RAAS) in the pathogenesis of PAH. More recently, in a pilot study in humans, pulmonary artery denervation was found to decrease pulmonary artery pressure in patients with idiopathic PAH who were not responding optimally to medical therapy. In a recent study, we determined that renal denervation attenuates pulmonary vascular remodeling and decreases pulmonary arterial pressure in experimental PAH. Other studies have demonstrated that renal denervation or GP ablation is potentially efficacious for the treatment of AF patients. Taken together, our findings suggest that renal denervation and ARGP ablation are effective alternative treatments for atrial tachyarrhythmia in PAH.

Study Limitations

This study has several limitations. First, we did not monitor stellate ganglion nerve activity, renal sympathetic discharge activity, or atrial GP activity. Thus, we could not determine whether stellate ganglion nerve activity or GP activity were increased in the PAH dogs or during LRSNS. Second, we did not perform hemodynamic studies during electrophysiologic examination or after GP ablation. We measured femoral artery blood pressure before and after GP ablation and found no obvious changes. The question of whether atrial GP ablation has a long-term impact on hemodynamics and AF/AFL vulnerability in the evaluated beagle monocrotaline model of PAH should be further investigated. Third, we did not investigate histological changes in the atria of humans who have died from PAH. To our knowledge, little is known about the histological changes that occur in the atria of humans with PAH. The question of whether atrial tissues in these individuals undergo substrate remodeling, such as autonomic nerve remodeling, and changes in Cx43 expression should be further investigated. In previous studies, monocrotaline has been commonly used to create PAH models. It is not clear whether the changes observed in this study occur in other experimental types of PAH. The stimulation of afferent renal nerves has different effects on sympathetic nerve activities in different animal species; however, the effects of afferent renal nerve stimulation have not been determined in primates. Recent studies have shown that renal denervation substantially reduces single-unit muscle sympathetic nerve activity in patients with resistant hypertension and decreased renal noradrenaline spillover. In a recent study, we found that renal denervation attenuates changes in plasma neurohormone levels in the activated RAAS and the sympathetic nervous system but has no obvious effects on the normal physiology of RAAS or the sympathetic nervous system in dogs. We speculate that renal denervation does not significantly affect RAAS activity in healthy individuals with normal sympathetic nerve activity but that it may decrease RAAS activity during heart failure, resistant hypertension, or PAH, which causes sympathetic nerve hyperactivity. In this study, we found that the right atrium, but not the left atrium, had high densities of sympathetic nerves in PAH dogs. We did not further investigate the mechanism of PAH-related increases in the right atrial nerves. This is the limitation of this study.

Conclusions

AF/AFL was easily induced in the RA at baseline and during LSGNS or LRSNS in experimental PAH. Higher AF/AFL vulnerability was observed in the PAH canines, which may have been associated with substrate remodeling in the RA. The effects of LRSNS on AF/AFL inducibility were closely associated with the left stellate ganglion nerve and ARGP. ARGP ablation decreased AF/AFL inducibility in the PAH model.

Perspectives

AF/AFL ablation might be an intriguing option for patients but it is unknown whether the substrate for AF/AFL in patients...
with PAH rests in the LA or the RA. This study confirms that the RA had higher AF/AFL vulnerability than the LA in the PAH dogs. High densities of sympathetic nerves, $\beta_1$-AR and more heterogeneity of Cx43 expression in the RA provide the substrate for AF/AFL vulnerability. Furthermore, we also found that the ARGP ablation, but not the other intrinsic cardiac ganglion nerves ablation, attenuates AF/AFL vulnerability in experimental PAH dogs. ARGP ablation could offer a novel therapeutic pathway, which would potentially target AF/AFL in PAH.

Acknowledgments
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Disclosures
None.

References


### Novelty and Significance

#### What Is New?
- Anterior right ganglionated plexi ablation is an effective alternative treatment for atrial tachyarrhythmia in an animal model of pulmonary arterial hypertension. The effects of left renal sympathetic nerve stimulation on atrial fibrillation/atrial flutter inducibility were closely associated with the left stellate ganglion nerve and anterior right ganglionated plexi.

#### What Is Relevant?
- High densities of sympathetic nerves and β₁-adrenergic receptor, as well as a greater quantity of unphosphorylated connexin 43 and more heterogeneous connexin 43 expression in the right atria provide substrate for atrial fibrillation/atrial flutter vulnerability in pulmonary arterial hypertension.

#### Summary
- Anterior right ganglionated plexi ablation plays an important role in atrial fibrillation/atrial flutter inducibility, and atrial fibrillation/atrial flutter vulnerability is associated with substrate remodeling in the right atria in pulmonary arterial hypertension dogs.
Effects of Intrinsic and Extrinsic Cardiac Nerves on Atrial Arrhythmia in Experimental Pulmonary Artery Hypertension

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Effects of Intrinsic and Extrinsic Cardiac Nerves on Atrial Arrhythmia in Experimental Pulmonary Artery Hypertension

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Short title: Autonomic nerve and atrial arrhythmia in PAH

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Methods

Measurements of hemodynamic parameters

Continuous electrocardiographic (ECG) monitoring was performed using leads I, II and III. Briefly, beagles were injected with 2 mg/kg dehydromonocrotaline via a Swan-Ganz pulmonary artery catheter inserted into the right atrium. Then, all of the beagles were allowed to recover for 8 weeks. The pulmonary capillary wedge pressure (PCWP), pulmonary arterial systolic pressure (PASP), pulmonary artery mean pressure (PAMP), right ventricular systolic pressure (RVSP) and right ventricular mean pressure (RVMP) were measured in all of the beagles at baseline and after 8 weeks.

Transthoracic 2D and Doppler echocardiography measurement

Standard 2-D short- and long-parasternal views and 4-, 2-, and 3-chamber apical views were obtained. Left atrial dimension (LAD), right atrial dimension (RAD), left ventricular diastolic dimension (LVDD), and right ventricular diastolic dimension (RVDD) were measured using Simpson’s biplane formula. All of the volumes were measured in triplicate, and the averages were reported. Right ventricular end-systolic longitudinal strain was analyzed by 2-dimensional speckle tracking. The left ventricular stroke volume (LVSV) was calculated from the difference between the left ventricular end-diastolic and end-systolic volumes. The left ventricular ejection fraction (LVEF) was calculated from the stroke volume divided by the left ventricular end-diastolic volume. An independent echocardiography expert reviewed the images and parameters.

Immunohistochemistry and Western blotting

Tissue sections were stained with monoclonal rabbit anti-tyrosine hydroxylase (TH) antibodies (Abcam, Inc., UK; used at a dilution of 1:500) to label sympathetic nerves, and monoclonal rabbit anti-growth-associated protein 43 (GAP43) (Millipore, Inc., USA; used at a dilution of 1:200) was used to label growing nerve cones. The sections were also stained with monoclonal goat anti-connexin 43 (Cx43) (Santa Inc.; used at a dilution of 1:500). The heterogeneity of Cx43 expression was quantified by transforming photomicrographs of subepicardial Cx43. The mean density of these slides was determined using computer-assisted IPP 6.0 software and was used as a measure of Cx43 heterogeneity. Each slide was examined under a microscope with a 40× objective, and the three fields with the highest densities were selected. Density was determined by dividing the positive area by the total area examined. The mean density in these three selected fields was used to represent the slide density.

Immunoblotting was performed to visualize beta-adrenergic receptors (anti-β1 and anti-β2 adrenergic receptor antibodies; Abcam, Inc., UK); Cx 43 (anti-Cx43 antibodies; Abcam, Inc., UK; rabbit anti-actin antibody, Santa, Inc., USA) and nonphosphorylated Cx43 (antisera against the nonphosphorylated form of Cx43; Invitrogen, Carlsbad, CA, USA), as previously described. Relative protein expression was determined with image analyzer software (AlphaEase FC, USA).

Statistical analysis

The echocardiography results of the PAH dogs were compared for the post-study
period using the paired T-test, and two-sample independent Student’s $t$ tests were used to compare the means of the two groups. ANOVAs in the form of Newman-Keuls tests were used to compare the means of continuous variables among multiple groups, and any significant difference was further analyzed using the Tukey–Kramer test. All of the statistical tests were two-sided, and a probability value $<0.05$ was required for statistical significance.

Reference:

**Table S1:** Changes in echocardiographic parameters at baseline and after 8 weeks in PAH group 1 and PAH group 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Different stages</th>
<th>RAD (mm)</th>
<th>LAD (mm)</th>
<th>RVDD (mm)</th>
<th>LVDD (mm)</th>
<th>RV longitudinal strain (%)</th>
<th>LSVV (ml)</th>
<th>LVEF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH group 1</td>
<td>Baseline</td>
<td>16.2±1.4</td>
<td>16.1±1.4</td>
<td>11.4±0.5</td>
<td>29.2±2.6</td>
<td>21.0±0.9</td>
<td>15.4±1.1</td>
<td>61±2.8</td>
</tr>
<tr>
<td></td>
<td>After 8 weeks</td>
<td>19.1±1.3*</td>
<td>16.8±1.5</td>
<td>13.7±1.0*</td>
<td>28.1±2.5</td>
<td>16.7±1.2*</td>
<td>13.4±1.5†</td>
<td>60±2.1</td>
</tr>
<tr>
<td>PAH group 2</td>
<td>Baseline</td>
<td>16.5±1.3</td>
<td>16.9±1.3</td>
<td>11.2±0.7</td>
<td>28.7±2.5</td>
<td>20.7±1.0</td>
<td>15.1±0.9</td>
<td>62±2.3</td>
</tr>
<tr>
<td></td>
<td>After 8 weeks</td>
<td>19.2±1.5*</td>
<td>16.8±1.4</td>
<td>13.9±1.1*</td>
<td>27.1±2.1</td>
<td>17.1±0.8*</td>
<td>13.3±1.4†</td>
<td>61±2.4</td>
</tr>
</tbody>
</table>

PAH, pulmonary arterial hypertension; RAD, right atrial dimension; LAD, left atrial dimension; RVDD, right ventricular diastolic dimension; LVEE, left ventricular diastolic dimension; RV, right ventricular; LSVV, left ventricular stroke volume; LVEF, left ventricular ejection fraction. Data are presented as the mean ± SD. *P<0.01 compared with the baseline; †P<0.05 compared with the baseline.
**Table S2:** Changes in hemodynamic parameters at baseline and after 8 weeks in PAH group 1 and PAH group 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Different stages</th>
<th>RVSP (mmHg)</th>
<th>RVMP (mmHg)</th>
<th>PASP (mmHg)</th>
<th>PAMP (mmHg)</th>
<th>PCWP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH group 1</td>
<td>Baseline</td>
<td>22± 6.3</td>
<td>13± 4.6</td>
<td>25± 7.6</td>
<td>16± 3.6</td>
<td>8.3±1.7</td>
</tr>
<tr>
<td></td>
<td>After 8 weeks</td>
<td>45± 8.9*</td>
<td>26± 6.5*</td>
<td>47± 11.6*</td>
<td>34± 7.9*</td>
<td>8.5±1.8</td>
</tr>
<tr>
<td>PAH group 2</td>
<td>Baseline</td>
<td>22± 6.1</td>
<td>12± 4.2</td>
<td>24± 7.5</td>
<td>15± 3.4</td>
<td>8.1±1.6</td>
</tr>
<tr>
<td></td>
<td>After 8 weeks</td>
<td>44± 8.8*</td>
<td>25± 6.5*</td>
<td>48± 11.2*</td>
<td>34± 8.1*</td>
<td>8.7±1.7</td>
</tr>
</tbody>
</table>

PAH, pulmonary arterial hypertension; RVSP, right ventricular systolic pressure; RVMP, right ventricular mean pressure; PASP, pulmonary arterial systolic pressure; PAMP, pulmonary artery mean pressure; PCWP, pulmonary capillary wedge pressure.

Data are presented as the mean ± SD. * P<0.01 for PAH compared with the baseline.
Figure S1  The experimental protocol.

AERP: atrial effective refractory period; AF: atrial fibrillation; AFL: atrial flutter; LSGNS: left stellate ganglion stimulation; LRSNS: left renal sympathetic nerve stimulation; ARGP: anterior right ganglionated plexi; IRGP: inferior right ganglionated plexi; SLGP: superior left ganglionated plexi; ILGP: inferior left ganglionated plexi.
**Figure S2** Changes in the dispersion of the atrial effective refractory period in the control group and PAH group 1.

# P<0.01 vs. baseline in the control group; † P<0.01 vs. LSGNS after GPs ablation