Vagal nerve stimulation preserves right ventricular function in a rat model of right ventricular pressure overload

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Abstract

Vagal nerve stimulation (VNS) ameliorates pulmonary vascular remodeling and improves survival in a rat model of pulmonary hypertension (PH). However, the direct impact of VNS on right ventricular (RV) function, which is the key predictor of PH patients, remains unknown. We evaluated the effect of VNS among the three groups: pulmonary artery banding (PAB) with sham stimulation (SS), PAB with VNS, and control (no PAB). We stimulated the right cervical vagal nerve with an implantable pulse generator, initiated VNS 2 weeks after PAB, and stimulated for 2 weeks. Compared to SS, VNS increased cardiac index (VNS: 130 ± 10 vs. SS: 93 ± 7 ml/min/kg; p < 0.05) and end-systolic elastance assessed by RV pressure–volume analysis (VNS: 1.1 ± 0.1 vs. SS: 0.7 ± 0.1 mmHg/μl; p < 0.01), but decreased RV end-diastolic pressure (VNS: 4.5 ± 0.7 vs. SS: 7.7 ± 1.0 mmHg; p < 0.05). Furthermore, VNS significantly attenuated RV fibrosis and CD68-positive cell migration. In PAB rats, VNS improved RV function, and attenuated fibrosis, and migration of inflammatory cells. These results provide a rationale for VNS therapy as a novel approach for RV dysfunction in PH patients.

KEYWORDS

autonomic nerve activity, pulmonary artery banding, pulmonary hypertension, right ventricular dysfunction, vagal nerve stimulation

Right ventricular (RV) dysfunction is one of the leading causes of death in various cardiovascular diseases including pulmonary hypertension (PH).1 In the early stage of PH, RV pressure overload induces adaptive cardiac hypertrophy to maintain RV performance. As the disease progresses, RV dilatation associated with fibrotic...
remodeling reduces systolic and diastolic functions and eventually results in right heart failure and death.\(^2\) Despite recent developments in vasodilator treatment, there are no established pharmacological therapies that directly improve RV dysfunction in PH.\(^3\)

Based on the accumulated evidence on therapies for left ventricular dysfunction, \(\beta\)-blockers and renin-angiotensin system (RAS) inhibitors are widely approved for clinical use.\(^4\) Animal experiments have suggested the beneficial effects of those drugs on RV function.\(^5\)–\(^7\) However, in clinical studies, \(\beta\)-blockers worsened the hemodynamics and exercise capacity,\(^8\) and RAS inhibitors showed no benefits in PH patients with RV dysfunction.\(^9\)

Vagal nerve stimulation (VNS) is an interventional therapy that makes use of a device that directly and electrically activates the parasympathetic system and exerts multiple beneficial effects in several cardiovascular conditions.\(^10\) In animal experiments, VNS has shown significant therapeutic benefits after myocardial infarction (MI),\(^11\) lethal arrhythmias,\(^12\) and in chronic heart failure.\(^13\) Recently, we demonstrated in an experimental pulmonary arterial hypertension (PAH) rat model that VNS ameliorated pulmonary vascular remodeling and improved survival.\(^14\) In our study, VNS attenuated fibrotic RV remodeling and preserved RV function. However, whether the effects on RV came about through the reduction of afterload or were direct consequences of VNS on the RV has yet to be clarified.

The present study examined how VNS impacts RV function in pure RV failure using a rat model of RV pressure overload created by pulmonary artery banding (PAB). We examined whether VNS directly improves RV function in the model by assessing the RV pressure–volume (PV) relationship and histology.

**METHODS**

**Experimental procedure**

The Institutional Animal Care and Use Committee of Kyushu University, Japan approved all experimental procedures. We conducted animal experimentation and care in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

We used male Sprague Dawley rats weighing 180–200 g (SLC) \((n = 21)\). All rats were housed in a room maintained at constant temperature \((25 \pm 2°C)\) and a 12-h light/dark cycle and given food and water ad libitum during the entire experiment. We performed PAB to create a model of RV pressure overload. The procedure has been reported previously.\(^15\) Briefly, we anesthetized a rat with isoflurane \((3.0% \text{ in room air for induction}; 0.5% \text{ in room air for maintenance})\). We placed an 18-gauge needle (outer diameter, 1.3 mm) alongside the main pulmonary artery (PA) and tied the needle and the main PA together with a 3-0 nylon suture through a left thoracotomy. Removal of the needle left a constriction with a lumen size equal to the diameter of the needle. Sham-operated rats underwent the same procedure without tying the PA.

To administer VNS to freely moving rats, we attached a pair of electrodes to the right cervical vagal nerve and connected it to a neurostimulator (ANRE-210i; ANPEX) that was implanted subcutaneously at the back of the rat. We stimulated the vagal nerve at 20 Hz and 180-\(\mu\)s pulse and adjusted the current intensity to just below the symptom threshold \((82.9 \pm 10.2\ \mu\text{A})\) which we defined in our previous report.\(^14\) In that condition, VNS did not directly affect heart rate (HR) or arterial pressure (AP) under a conscious state.\(^14\)\(^,\)\(^16\) Our preliminary study indicated that stimulation stability worsened after 2 weeks. In addition, the battery limit of a small stimulator was around 2 weeks. For the first step to evaluate the chronic impact of VNS for a rat model of RV pressure overload, we performed VNS for 2 weeks in this experiment. There was no implantation-related death in this study, but one rat was excluded from the protocol because of device failure of the VNS system.

**Experimental protocols**

We performed PAB or sham operation at Week 0. Rats receiving sham operation were designated the control group \((\text{CTRL}; n = 5)\) (Figure 1). After 2 weeks, we
performed echocardiography on all the rats. We randomized PAB rats into two groups: sham stimulation (SS; \( n = 8 \)) and VNS (\( n = 8 \)), and implanted neurostimulators in all PAB rats. We initiated VNS in the VNS group for 2 weeks. In Week 4, we assessed autonomic function, hemodynamics, PV relationship, and RV histology and compared them among three groups.

**Echocardiography**

In Weeks 2 and 4, we performed echocardiography under general anesthesia (1.5% isoflurane inhalation). In the two-dimensional parasternal short-axis view, we measured RV diastolic (RVDd) and systolic dimensions (RVDs), and wall thickness (RVWT) at the level of the papillary muscles. We also measured tricuspid annular plane systole excursion (TAPSE) as an index of the systolic function of RV.17

**Heart rate variability**

We evaluated heart rate variability (HRV) under the conscious state at 2 weeks after initiation of VNS (Week 4) using an ECG telemetry system (TA11ETA-F10 Implant; Data Science International).14 We have repeatedly reported on the method for deriving stable HRV in freely moving rats.14,18 We used power spectral density (PSD; ms\(^2\)/Hz) to quantify the HRV. Beat-to-beat (RR) intervals were obtained from 10-min telemetric ECG recordings during the daytime. The RR-interval time series were used to derive PSD by Fourier transform. We plotted PSD for frequencies up to 3.0 Hz. The frequency range of 0.04–0.75 Hz was defined as the low frequency (LF) and 0.75–3.0 Hz as the high frequency (HF).

**Hemodynamic assessment and RV hypertrophy**

We evaluated hemodynamic data at the end of the protocol (Week 4). Data were recorded under general anesthesia with a mixture of urethane (250 mg/ml) + \( \alpha \)-chloralose (40 mg/ml) and mechanical ventilation. We placed a microtip PV catheter (FTH-1912B-8018; Transonic Inc.) in the RV via an apical puncture to measure the pressure of RV. We used a fluid-filled transducer system (DX-300; Nihon-Kohden) for AP measurement. A flow probe (2.5PS; Transonic Systems) was placed in the aortic root for measurements of cardiac output (CO) and cardiac index (CI; CO normalized by body weight). Each signal is obtained at 1000 Hz using a 16-bit analog-to-digital converter (Power Lab 16/35; AD Instruments) and stored in a dedicated laboratory computer system. RV systolic pressure (RVSP) and RV end-diastolic pressure (RVEDP) were estimated from the RV pressure by averaging over at least 10 sequential beats. Max \( +dP/dt \) and min \( -dP/dt \) were calculated from the first-time derivative of instantaneous RV pressure.

After catheterization, we dissected the RV from the LV and interventricular septum (S). We calculated the Fulton index, the weight ratio of RV to (LV + S), for assessment of RV hypertrophy (RVH).15

**PV analysis of the right ventricle**

To investigate the RV function independent of the load conditions, we performed the PV analysis of RV. After evaluating hemodynamic data, we acquired the data of both pressure and volume of RV simultaneously using a microtip PV catheter and plotted the PV relationship. To obtain multiple PV loops, we occluded the inferior vena cava to reduce the preload and determined the slope of the end-systolic PV relationship (end-systolic elastance; \( E_{es} \)).19 Although PAB decouples the ventricular afterload and pulmonary vascular characteristics in this model, we calculated the effective arterial elastance (\( E_a \)) as an index of RV afterload as follows:

\[
E_a = \frac{P_{es}}{EDV - ESV},
\]

where \( P_{es} \) stands for end-systolic pressure.20

We also plotted the end-diastolic PV relationship, which is approximated by an exponential curve of the following equation:

\[
P = \alpha e^{\beta V},
\]

where \( \alpha \) and \( \beta \) represent coefficients of diastolic properties. We then calculated end-diastolic elastance (\( E_{ed} \)) as follows:

\[
E_{ed} = \alpha \beta e^{\beta (EDV - V_0)},
\]

where \( E_{ed} \) is the slope of the end-diastolic PV relationship at the point of original end-diastolic volume.20 We also evaluated RV stiffness as a parameter of diastolic function, which was estimated by dividing end-diastolic pressure by end-diastolic volume.21

The effective RV ejection fraction (RVEF) in the PV loop of RV was calculated as follows:
\[ \text{RVEF} = \frac{\text{EDV} - \text{ESV}}{\text{EDV} - V_0}. \]

**Plasma norepinephrine and brain natriuretic peptide**

We collected blood samples from the carotid artery after hemodynamic studies. We measured plasma norepinephrine (NE) level as an index of sympathetic activation by high-performance liquid chromatography (SRL).\(^\text{14}\) We assayed plasma brain natriuretic peptide (BNP) level using a BNP 45 Rat ELISA Kit (ab108816; Abcam).\(^\text{14}\)

**Histopathological analysis**

After catheterization, rats were euthanized by exsanguination under isoflurane (5.0% in room air). The heart was harvested and immediately fixed in 10% buffered paraformaldehyde, embedded in paraffin, and cut into 5-\(\mu\)m thick sections. We stained serial sections with hematoxylin and eosin or Masson trichrome.

To evaluate cardiomyocyte hypertrophy in the RV, we measured the cardiomyocyte cross-sectional area by tracing the outline of 80–120 cells stained by hematoxylin and eosin in each section.\(^\text{15}\) To evaluate RV fibrosis, Masson trichrome staining was performed on serial sections of the RV. The percentage of the fibrotic area was quantified on digitized images: the blue-stained tissue area was expressed as a percentage of the total surface area of RV.\(^\text{15}\)

**Immunohistochemical analysis**

We used 5-\(\mu\)m thick sections for immunohistochemical staining. All sections were blocked with 5% skim milk in phosphate-buffered saline, and incubated with primary antibody at 4°C overnight. The antibodies used were anti-mouse CD68 (1:400 dilution; ab31360; Abcam) and anti-CD34 antibody (1:100 dilution; LS-C150289; LS Bio). We incubated each section with a biotinylated secondary antibody before horseradish peroxidase-labeled streptavidin. We acquired images with an upright microscope (BX63; Olympus).

To obtain a CD68-positive macrophage count, the number of CD68-positive cells in the RV tissue section was counted and normalized by area (cell/mm\(^2\)).\(^\text{15}\) Capillary density in RV was expressed as the number of capillaries per section area at \(\times400\) magnification, measured in at least three randomly chosen areas per ventricle where cardiomyocytes were transversally sectioned.\(^\text{15}\)

**Statistical analysis**

Differences among the three groups were tested by one-way analysis of variance, followed by a post hoc Tukey–Kramer test. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software). Data are expressed as mean \(\pm\) SEM. Differences were considered significant when \(p<0.05\).

**RESULTS**

**Effects of VNS on autonomic function**

In this study, we assessed HRV and plasma NE levels as markers of autonomic function. Representative traces of the PSD of HRV in individual rats are shown in Figure 2. The HF component, indicating parasympathetic activity, decreased in SS compared to
CTRL. However, VNS somewhat restored HF compared to SS. Table 1 compares HRV and plasma NE levels among the three groups. There was no difference in HR among the three groups. Meanwhile, higher HF was observed in the VNS group than in the SS group, indicating parasympathetic activation in VNS in PAB rats. The LF/HF ratio and plasma NE level trended lower in VNS than in the SS group, but the differences were not significant.

**Effects of VNS on heart weight and echocardiographic findings**

There was no death of animals in the three groups during this study. As shown in Table 2, body weight did not differ among the groups. Compared to CTRL, the weights of RV, liver, and spleen, and RVH were significantly increased in SS. VNS did not significantly change those parameters compared to SS.

Figure 3 shows the transition of echocardiographic parameters from Weeks 2 to 4. PAB significantly increased RVDd, RVDs, and RVWT, and decreased TAPSE at Week 2, indicating the induction of RV dysfunction by PAB. Compared to SS, VNS significantly attenuated the PAB-induced increases in RVDd and RVDs, and a decrease in TAPSE in Week 4, suggesting the effect of VNS in preserving RV function and remodeling.

**Effects of VNS on hemodynamics and plasma BNP level**

Shown in Figure 4 are hemodynamic parameters obtained from catheterization analysis and plasma BNP level in three groups after the 2-week VNS period. Compared to SS, VNS did not change HR (VNS: 352 ± 26 vs. SS: 327 ± 33 bpm; \( p = 0.38 \)), mean AP (VNS: 103 ± 15 vs. SS: 85 ± 16 mmHg; \( p = 0.09 \)), and RVSP (VNS: 125 ± 7 vs. SS: 110 ± 7 mmHg; \( p = 0.20 \)). VNS decreased RVEDP (VNS: 4.5 ± 0.7 vs. SS: 7.7 ± 1.0 mmHg; \( p < 0.05 \)) and increased RV Max +dP/dt (VNS: 6133 ± 519 vs. SS: 4465 ± 500 mmHg/s; \( p < 0.05 \)), while there was no difference in RV min −dP/dt (VNS: −4653 ± 709 vs. SS: −3717 ± 946 mmHg/s; \( p = 0.08 \)) compared to SS. Moreover, VNS significantly increased CI (VNS: 130 ± 10 vs. SS: 93 ± 7 ml/min/kg; \( p < 0.05 \)) and decreased plasma BNP level (VNS: 340 ± 24 vs. SS: 529 ± 58 pg/ml; \( p < 0.05 \)).

**Effects of VNS on RV function**

Figure 5 shows the representative plots of RV PV loop analysis of CTRL, SS, and VNS rats. PAB markedly increased RV pressure and dilated RV. Thus, the PV loop in PAB (SS and VNS) groups showed a rightward shift and vertical increment. Table 3 shows the pooled data of RV PV loop analysis. Both EDV and ESV were apparently smaller in VNS than in SS, although the differences were not statistically significant.

### Table 1

| Heart rate variability (HRV) and plasma norepinephrine concentration. |
|---------------------------|-----------------|-----------------|-----------------|
|                           | CTRL            | SS              | VNS             | \( p \) Value  |
| \( n \)                   | 4               | 5               | 5               | 0.865          |
| HR (bpm)                  | 347 ± 17        | 356 ± 12        | 357 ± 16        |               |
| HF (ms\(^2\))             | 4.9 ± 1.0       | 0.3 ± 0.1**     | 3.1 ± 0.7\(^7\) | 0.002         |
| LF (ms\(^2\))             | 10.1 ± 1.8      | 1.4 ± 0.4**     | 3.2 ± 1.0       | <0.001        |
| LF/HF ratio               | 2.6 ± 0.9       | 6.4 ± 3.7       | 1.2 ± 0.4       | 0.295         |
| SDNN (ms)                 | 8.2 ± 1.1       | 3.4 ± 0.7       | 7.0 ± 1.8       | 0.061         |
| \( n \)                   | 5               | 8               | 7               |               |
| Plasma NE (pg/ml)         | 376 ± 111       | 955 ± 352       | 558 ± 107       | 0.299         |

Note: Values are expressed as mean ± SEM. Differences were tested by one-way analysis of variance, followed by a post hoc Tukey-Kramer test. **\( p < 0.01 \) vs. CTRL. \( p < 0.05 \) vs. SS.

Abbreviations: CTRL, control rats without pulmonary artery banding (PAB); HF, high-frequency component of HRV (0.04–0.73 Hz); HR, heart rate; LF, the low-frequency component of HRV (0.73–2.0 Hz); plasma NE, plasma norepinephrine concentration; SDNN, the standard deviation of the normal to normal interval; SS, rats with PAB and sham stimulation; VNS, rats with PAB and vagal nerve stimulation.

### Table 2

| Body and organ weights. |
|--------------------------|-----------------|-----------------|-----------------|
|                           | CTRL            | SS              | VNS             | \( p \) Value  |
| \( n \)                   | 5               | 8               | 7               | 0.600          |
| BW (g)                   | 346 ± 12        | 359 ± 16        | 345 ± 8         |               |
| RV weight (mg)            | 178 ± 10        | 482 ± 32**      | 469 ± 12        | <0.001        |
| LV + S weight (mg)        | 640 ± 12        | 682 ± 30        | 666 ± 16        | 0.505         |
| RVH                      | 0.28 ± 0.01     | 0.71 ± 0.04**   | 0.71 ± 0.02     | <0.001        |
| Liver (g)                | 10.4 ± 0.2      | 12.7 ± 0.3*     | 11.1 ± 0.2      | 0.047         |
| Spleen (mg)              | 570 ± 7         | 731 ± 22*       | 641 ± 20        | 0.046         |

Note: Values are expressed as mean ± SEM. Differences were tested by one-way analysis of variance, followed by post hoc Tukey-Kramer test. \( *p < 0.05 \) and **\( p < 0.01 \) vs. CTRL.

Abbreviations: BW, body weight; CTRL, control rats without pulmonary artery banding (PAB); LV + S, left ventricle plus septum; RV, right ventricle; RVH, ratio of RV/LV + S; SS, rats with PAB and sham stimulation; VNS, rats with PAB and vagal nerve stimulation.
End-systolic elastance ($E_{es}$), which is the slope of the end-systolic pressure–volume relationship (ESPVR), is an index reflecting the load-insensitive cardiac systolic function. PAB significantly increased $E_{es}$ compared to CTRL, and VNS further increased $E_{es}$ compared to SS (Figure 5; Table 3). The volume intercept of ESPVR ($V_0$) did not differ significantly among the three groups. The effective arterial elastance ($E_a$) increased significantly in SS compared to CTRL and was further augmented in VNS. Since $E_{es}$ and $E_a$ in each intervention showed the same trend, the effective RVEF obtained from the PV loop did not change significantly among the three groups.

As representative PV loops indicated, the stiffness was significantly lower in VNS than that in SS. Meanwhile, $E_{ed}$, which is the slope of EDPVR at the operating point, did not differ significantly between SS and VNS.

**Effects of chronic VNS on RV histology**

Compared to CTRL rats, PAB significantly increased the cross-sectional area of cardiomyocytes and decreased the capillary density of RV in SS rats. Similar trends were observed in VNS rats, and there were no significant differences compared to SS (Figure 6a,b). Meanwhile, VNS significantly decreased fibrotic area compared to SS (VNS: 4.9 ± 0.4% vs. SS: 7.8 ± 0.7%; $p < 0.05$; Figure 6c). We also assessed the migration of CD68-positive cells, which is known to play a key role in VNS effects. VNS significantly attenuated the PAB-induced migration of CD68-positive cells (VNS: 6.0 ± 0.8 vs. SS: 10.7 ± 1.6 cells/mm²; $p < 0.05$; Figure 6d).

**DISCUSSION**

This study aimed to investigate the impact of VNS on RV function assessed by PV loop analysis in RV pressure-overloaded rats. We found that VNS improved RV systolic function and attenuated fibrotic remodeling and invasion of CD68-positive inflammatory cells without changing RVH.

**Effect of VNS on autonomic balance**

Autonomic imbalance in patients with RV dysfunction associated with PAH is known to predict clinical outcomes.20,22 Since VNS directly stimulates the parasympathetic vagal nerve, we expected that VNS would restore the autonomic imbalance in PAB rats. We stimulated the vagal nerve at just below the symptom.
threshold. Therefore, VNS in this setting had no direct effect on HR under the conscious state.

As shown in Table 1, we found significant decreases in HF and LF components of HRV in SS rats compared to CTRL rats. In a rat model of transaortic constriction-induced left ventricular hypertrophy (LVH), the heart–brain interactions via vagal afferent and efferent pathways are altered. In the LVH rats, excitation of cardiac vagal neurons (CVNs) is decreased due to increased frequency of inhibitory GABAergic neurotransmission to CVNs in the dorsal motor nucleus of the vagus (DMNX) and diminished frequency of excitatory neurotransmission to CVNs in both the nucleus ambiguous and DMNX. Thus, left-sided ventricular hypertrophy induces parasympathetic deactivation. We previously reported decreases in LF and HF components of HRV in both monocrotaline-induced PH and Sugen/hypoxia PH models. Our present results are consistent with previous reports and indicate that RVH caused by PAB also induces autonomic imbalance.

In PAB model rats, VNS significantly increased HF components of HRV. In addition, VNS apparently decreased LF/HF ratio and plasma NE level compared to SS, although the difference was not significant. These data suggest that VNS may improve autonomic balance by activating the parasympathetic nervous system in PAB rats.

**Therapeutic effects of VNS on RV dysfunction**

Several reports have demonstrated the beneficial effects of chronic VNS in experimental animal models of
cardiovascular disease. Li et al. and Nishizaki et al. showed that chronic VNS significantly attenuated cardiac remodeling and heart failure after large MI in rat models. Chapleau et al. demonstrated that VNS reduced blood pressure by improving endothelial dysfunction via anti-inflammatory response and by enhancing eNOS expression in stroke-prone spontaneously hypertensive rats. However, they showed that VNS did not affect LVH and function in their model. We previously demonstrated in severe PAH model rats that VNS ameliorated pulmonary vascular remodeling and improved survival, associated with improved RV function. Since we were not able to differentiate whether the direct or indirect effect of VNS improved RV function, the mechanism by which VNS impacted RV function remained to be elucidated.

This report is the first to demonstrate the therapeutic effects of VNS in RV pressure-overloaded rats. Compared to SS, VNS significantly increased TAPSE (Figure 3) and Max +dP/dt (Figure 4) with the reduction of RV dimensions. We also performed PV loop analysis (Figure 5) to obtain $E_{es}$, a load-insensitive index of systolic function, and found that VNS significantly increased $E_{es}$ compared to SS. These data suggest that VNS improves RV systolic function in PAB rats. In PH patients, increased RV systolic function and wall thickness are adaptive reactions to high afterload. Continuous high afterload due to PH dilates the RV and deteriorates RV systolic function resulting in decompensated RV failure. These trends are also detected in our PAB model rats (Table 3). Andersen et al. demonstrated that RVH precedes dilatation and increases $E_{es}$ as a compensatory mechanism to overcome pressure overload by PAB. In the chronic phase of PAB, as in PAH patients, PAB rats show further RV dilatation.

**FIGURE 5** Effects of vagal nerve stimulation (VNS) on pressure–volume loop analysis. Representative plots of pressure–volume loops in each group; CTRL, control rat without pulmonary artery banding (green); SS, a rat with pulmonary artery banding and sham stimulation (blue); VNS, a rat with pulmonary artery banding and VNS (red). Multiple loops are acquired by occlusion of the inferior vena cava. The slope of the loops was determined as the end-systolic pressure–volume relationship (end-systolic elastance; $E_{es}$).

| Parameters of pressure–volume loop analysis of the right ventricle. |
|-------------------|--------|--------|----------|--------|
|                   | CTRL   | SS     | VNS      | p Value |
| $n$               | 5      | 7      | 5        |         |
| EDV (μl)          | 222 ± 18 | 386 ± 29** | 312 ± 25 | 0.002  |
| ESV (μl)          | 102 ± 13 | 187 ± 22** | 159 ± 23 | 0.036  |
| $V_o$ (μl)        | 32.9 ± 8.5 | 88.1 ± 26.0 | 99.1 ± 25.5 | 0.157  |
| $E_{es}$ (mmHg/μl)| 0.30 ± 0.03 | 0.65 ± 0.06** | 1.06 ± 0.12† | <0.001 |
| $E_a$ (mmHg/μl)   | 0.26 ± 0.04 | 0.72 ± 0.05* | 1.22 ± 0.20† | <0.001 |
| RVEF (%)          | 54.8 ± 3.7 | 52.2 ± 3.1 | 50.1 ± 4.3 | 0.699  |
| $E_{cd}$ (mmHg/μl)| 0.035 ± 0.011 | 0.070 ± 0.019 | 0.064 ± 0.011 | 0.305  |
| Stiffness (mmHg/ml)| 5.53 ± 1.6 | 18.6 ± 1.2** | 12.2 ± 2.3† | <0.001 |
| $\alpha$ (mmHg)  | 0.042 ± 0.025 | 0.859 ± 0.226* | 0.210 ± 0.157 | 0.013  |
| $\beta$           | 0.026 ± 0.004 | 0.011 ± 0.003* | 0.023 ± 0.006 | 0.038  |

Note: Values are expressed as mean ± SEM. Differences were tested by one-way analysis of variance, followed by post hoc Tukey–Kramer test. *p < 0.05 and **p < 0.01 vs. CTRL. †p < 0.05 and ‡p < 0.01 vs. SS.

Abbreviations: CTRL, control rats without pulmonary artery banding (PAB); $E_{es}$, effective arterial elastance; EDV, end-diastolic volume; $E_{cd}$, end-diastolic elastance; $E_{es}$, end-systolic elastance; ESV, end-systolic volume; RVEF, right ventricular ejection fraction; SS, rats with PAB and sham stimulation; VNS, rats with PAB and vagal nerve stimulation; $V_o$, volume intercept of end-systolic pressure–volume relationship.
and worsening of hemodynamics. In our study, PAB significantly increased $E_{es}$ indicating the adaptation of RV systolic function to pressure overload. The finding that VNS further augmented $E_{es}$ compared to SS suggests that VNS augments the adaptive $E_{es}$ increase after PAB.

We also assessed the impact of VNS on diastolic function in PAB rats. VNS significantly decreased stiffness but did not alter $E_{ed}$ or parameters of EDPVR (Table 3). These data support that VNS did not change diastolic function significantly in PAB rats. Various alterations of diastolic function in PAB rats have been reported.27 In the early adaptive phase of PAB, a drastic increase of RV systolic pressure induces adaptive hypertrophy and concentric remodeling and steepens EDPVR. Meanwhile, as shown in the alteration of $E_{es}$, continuous RV pressure elevation shifts EDPVR rightward and induces eccentric remodeling. Thus, PAB rats show both concentric and eccentric remodeling within a short period. In our model, PV loop analysis at 4 weeks after PAB revealed a definite rightward shift of EDPVR, suggesting a mixed phase of concentric and eccentric remodeling. Since RV systolic pressure remained higher in VNS, the intervention did not change EDPVR significantly.

**FIGURE 6** Effects of chronic vagal nerve stimulation (VNS) on histology of RV. Representative photomicrographs of (a) hematoxylin and eosin (HE) staining, (b) CD34 immunostaining, (c) Masson trichrome staining (MT), and (d) CD68-positive cells are shown ($n = 5$ in each group). CTRL, control rats without pulmonary artery banding; SS, rats with pulmonary artery banding and sham stimulation; VNS, rats with pulmonary artery banding and VNS. Data are expressed as mean ± SEM. Differences were tested by one-way analysis of variance, followed by post hoc Tukey–Kramer test. **$p < 0.01$ vs. CTRL. †$p < 0.05$ and ‡$p < 0.01$ vs. SS.
In the present study, VNS increased CI but decreased RVEDP (Figure 4d,g), suggesting the improvement of the RV pumping performance. In addition, plasma BNP level, which reflects disease severity in PH, decreased significantly in VNS rats compared to SS rats (Figure 4h), demonstrating a net improvement of hemodynamics. Taken together with the effects of VNS on RV and hemodynamics in PAB rats, we speculate that VNS has the potential to prevent the worsening of RV failure in RV pressure overload.

The impact of VNS on RV histopathology

In this study, significant hypertrophy of cardiomyocytes was demonstrated in PAB rats (Figure 6a). Other groups and we have reported similar hypertrophic changes in cardiomyocytes after PAB. However, VNS did not attenuate the hypertrophic changes in PAB rats. The capillary density was inversely related to the RVH and was not improved in VNS compared to SS rats (Figure 6b). Since hypertrophic remodeling is an adaptation against increased RV afterload, VNS did not suppress adaptive hypertrophy in our experiment, possibly because RV pressure was maintained at a high level. In addition, the absence of change in cell hypertrophy in the VNS group is consistent with the lack of changes in RV wall thickness measured by echocardiography (Figure 3c) and EDPVR measured by PV loop analysis (Table 3). The results may depend on the degree of stenosis in the PAB, the timing of VNS initiation, the duration of VNS treatment, and the timing of evaluation. Rol et al. reported the effect of a 3-week treatment with nintedanib, a tyrosine kinase inhibitor, on SUS416/hypoxia rats in the advanced stage. Although nintedanib did not affect pulmonary vascular remodeling, it attenuated RVH and fibrosis, consequently improving RV function. Further investigations are needed to understand whether VNS improves hypertrophic remodeling in RV pressure overload.

Continued pressure overload of the heart causes progressive fibrosis and results in ventricular systolic dysfunction and irreversible eccentric remodeling. Oka et al. demonstrated that chronic pressure overload-induced LV sterile inflammation, causing fibrotic remodeling and systolic dysfunction in mice with transverse aortic constriction. We also reported that TLR9–NF-κB-mediated sterile inflammation played a crucial role in PAB-induced RV dysfunction. In that report, PAB gradually activated the TLR9–NF-κB pathway and induced the migration of CD68-positive macrophages as well as fibrosis. In this study, VNS significantly attenuated the fibrotic area and the number of CD68-positive macrophages in the RV. Efferent VNS is known to increase the release of acetylcholine and exert an anti-inflammatory effect, redox modulation, reduction of oxygen consumption, calcium regulation, and nitric oxygen production. In contrast, afferent VNS exerts inhibition of sympathoexcitation. Furthermore, Borovikova et al. reported the importance of the anti-inflammatory effect of VNS-mediated organ protection. We conjecture that the suppression of fibrosis mediated by the VNS-activated anti-inflammatory effect may be involved in preserving RV systolic function.

Clinical relevance

In this study, we demonstrated the direct beneficial effects of VNS on RV function in a rat model of RV pressure overload. Drugs that reduce pulmonary vascular resistance are the first-line treatment for PH. However, in patients with severe PH, drugs do not provide effective PVR reduction, and patients die from progressive right heart failure. RV ejection fraction is a major prognostic factor in severe PH, indicating that prevention of worsening RV systolic function improves clinical outcome in severe PH. Based on our present data and previous reports, we propose VNS as a potential therapeutic strategy for RV dysfunction in PH patients. Since the feasibility of VNS has already been confirmed, our findings may contribute greatly to the development of device therapy and the expansion of the clinical applicability of VNS.

VNS at a higher intensity may confer greater therapeutic benefit through stronger activation of the parasympathetic nervous system. On the other hand, high-intensity VNS could result in adverse effects such as neural fiber damage, hemodynamic disruption, and respiratory abnormality. To achieve maximal benefits and avoid adverse effects, titration for each individual needs to be performed.

Limitation

There are some limitations in this study. First, we performed 2-week VNS in this study. Due to the limitation of the battery, we were not able to deliver long-term stable stimulation. Hence, we cannot prove that longer VNS results in further improvement of hemodynamics. Second, patients with RV dysfunction due to PH have already been given monotherapy or combination therapy with PH-specific drugs. The effect of VNS in the setting of ongoing treatment with PH-specific drugs has not been studied. Third, in terms of
pathophysiology, there are many differences between a rat model of PAB and the pressure overload caused by clinical PH. Thus, we could not directly extrapolate our results to human PH and clinical translation of VNS. However, PAB has been reported as a relevant model to study RV dysfunction due to PAH. In addition, several PH interventions have been evaluated by using a PAB model. Fourth, the mechanism by which VNS confers benefit for the pressure-overloaded ventricle remains unknown. Considering previous reports indicating the powerful anti-inflammatory effects of VNS, the attenuation of RV pressure overload-induced inflammation may play an important role in the beneficial effects of VNS in PAB rats. Further detailed investigations are required to optimize this treatment.

In conclusion, in a rat model of PAB-induced RV failure due to pressure overload, we demonstrated that VNS improved RV function, which was accompanied by attenuation of fibrotic remodeling. Since there is no good therapeutic option to manage RV failure due to persistent PH, VNS may be a potential therapeutic strategy for severe PH patients.

**AUTHOR CONTRIBUTIONS**

Keimei Yoshida, Keita Saku, Kohtaro Abe, and Kenji Sunagawa conceived and designed the study. Kohtaro Abe and Hiroyuki Tsutsui optimized methodology. Keimei Yoshida and Keita Saku collected data. Keimei Yoshida and Keita Saku analyzed and visualized data. Keimei Yoshida and Keita Saku wrote the original manuscript. Harm Jan Bogaard and Kenji Sunagawa revised critically for important intellectual content. Kohtaro Abe and Kenji Sunagawa supervised the project. Keimei Yoshida, Keita Saku, Harm Jan Bogaard, Kohtaro Abe, Kenji Sunagawa, and Hiroyuki Tsutsui approved the manuscript submitted.

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**ETHICS STATEMENT**

The Institutional Animal Care and Use Committee of Kyushu University, Japan, approved all experimental procedures. We conducted the animal experimentation and care in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

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