Modulation of the myocardial redox state by vagal nerve stimulation after experimental myocardial infarction

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the failing hearts. Secondly, redox regulation is mediated by both a decrease of cardiac NE spillover through attenuation of the cardiac sympathetic drives and a direct effect of acetylcholine (ACH) on the LV. Thirdly, NADPH oxidase is involved in the redox modulation by VNS. We tested these hypotheses in vivo using a murine model of CHF and in vitro using cultured neonatal rat cardiomyocytes.

Conventionally, it has been difficult to determine the free radical reactions or redox status in vivo, because free radicals and oxidants are unstable and highly reactive. The development of low frequency electron spin resonance (ESR) spectroscopy has allowed direct detection of free radicals and direct estimation of the redox status in living animals non-invasively. The advent of this technique has permitted the assessment of the contribution of free radicals in various pathological conditions. In this study, we used in vivo ESR spectroscopy to evaluate a cardiac redox alteration following VNS.

We herein demonstrated for the first time that short VNS modulates the cardiac redox status and adrenergic drive, and thereby suppresses free radical generation in the failing heart.

2. Methods

2.1 Animal model of heart failure

All procedures and animal care were approved by the Committee on Ethics of Animal Experiment, Kyushu University Graduate School of Medical and Pharmaceutical Sciences and performed in accordance with the Guideline for Animal Experiment of Kyushu University, and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

We used a murine model of CHF 28 days after induction of MI. The surgical procedure was described previously. Briefly, 8-10 week-old male CD-1 mice weighing 30-35 g were used. Under anaesthesia with pentobarbital sodium (30 mg/kg BW, i.p.), experimental MI was induced by ligation of the left coronary artery. Control mice received sham operation without coronary artery ligation. Mice were housed in a temperature- and humidity-controlled room and fed a commercial diet and provided water ad libitum.

2.2 Echocardiographic and haemodynamic measurements

CHF (n = 12) and sham mice (n = 12) underwent physiological evaluation by echocardiography and left heart catheterization as previously reported. Under light anaesthesia with tribromo-ethanol-amylene hydrate (2% Avertin, 8 μL/g BW, i.p.), two-dimensional targeted M-mode images were obtained from the short-axis view at the level of greatest LV dimension using a 7.5-MHz transducer connected to a dedicated ultrasonographic system (SSD-5500, ALOKA Co., Ltd., Tokyo, Japan). After echocardiography, a 1.4-F micromanometer-tipped catheter (Millar Instruments, Inc. Houston, TX, USA) was inserted into the right carotid artery and advanced into the LV for pressure measurement. Thereafter, mice were euthanized with overdose pentobarbital sodium. Heart and lungs were quickly excised and weighed.

2.3 Vagal nerve stimulation

CHF or control mice were randomly assigned to VNS (n = 6) and sham-stimulation (SS) group (n = 6). Under anaesthesia with pentobarbital sodium (30 μg/g BW, i.p.), the right vagal nerve was attached with a pair of stainless wire electrodes (Bioflex wire AS633; Cooner wire, Chatsworth, CA, USA) and covered with silicone gel for insulation and immobilization. The vagal nerve was stimulated with 10 Hz rectangular pulses of 1 ms duration for 15 min. Electrical voltage was optimized for each mouse so that the heart rate (HR) was reduced by 10% from baseline. In SS group, wire electrodes were implanted but VNS was not applied. Five minutes after VNS, mice subsequently underwent in vivo ESR analysis as described below. In a separate series of experiments, CHF mice were given intravenous injection of atropine sulphate (1 μg/g BW, Sigma-Aldrich, Inc., St Louis, MO, USA), followed by VNS, rectangular pulses of which were 10 Hz, and duration is 1 ms (Figure 1A). Electrical voltage was fixed at 500 mV, because muscarinic blockade abolishes the VNS induced HR reduction, and this electrical condition could reduce HR in almost all CHF and control mice.

In another series of experiments, we performed fixed rate atrial pacing during VNS to determine the impact of bradycardia. In brief, after implantation of electrical wires, the left chest was opened under artificial ventilation. A pair of stainless wires was surgically attached with left atrium. After HR reached constant, VNS and fixed rate atrial pacing were simultaneously initiated. The vagal nerve was stimulated with rectangular pulses of 1-ms duration, 10 Hz, 500 mV for 15 min. Rectangular pulses for atrial pacing were also of 1-ms duration. After 15 min of VNS and atrial pacing, the chest was closed and mice subsequently underwent in vivo ESR.

To elucidate the role of nitric oxide (NO) pathway, we applied VNS in mice treated with Nn-nitro-L-arginine methyl ester (L-NAME) hydrochloride (Sigma-Aldrich). L-NAME (1 mg/kg/day) was administered to CHF mice through drinking water for 7 days prior to the experiment. The condition of VNS was same as in CHF + VNS mice.

2.4 In vivo electron spin resonance spectroscopy

We performed in vivo ESR spectroscopy to assess the myocardial redox state. This method is based on the theory that nitroxyl radicals are reduced to the corresponding hydroxyamine in the presence of free radicals in vivo, resulting in the disappearance of ESR signals. A semilogarithmic plot of time course of the ESR signals shows a linear decay curve, the rate of which (a reciprocal
number of time constant) is proportional to the amount of reductants including free radicals. Prior to in vivo ESR analysis, mice were given intravenous injection of 3-methoxy carbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (methoxy carbonyl-PROXYL) (0.3 μmol/L/g BW), a membrane permeable nitroxyl spin probe (oil : water ratio = 8.7) (Figure 1B), which was synthesized as described previously. Shortly thereafter, ESR spectra were recorded at regular intervals at chest region using L-band ESR spectrometer (JEOL Co., Ltd., Akishima, Japan) with a loop-gap resonator (33 mm i.d. and 30 mm in length). The power of the 1.1 GHz microwave was 1.67 mW.

First, as a validation study, we performed in vivo ESR in control and CHF mice, to which VNS were not applied, to determine whether the difference in signal decay could be observed between these mice. Secondly, we assessed the effect of two ROS scavengers, 1,2-dihydroxy-3,5-benzene disulfonic acid disodium salt monohydrate (tiron) (10 μmol/mouse, Dowel Molecular Laboratory Co. Ltd., Kumamoto, Japan) and dimethylthiourea (DMTU) (10 μmol/mouse, Sigma-Aldrich), on ESR signal decay. These chemicals were administrated intravenously prior to methoxy carbonyl-PROXYL injection. Thirdly, we performed in vivo ESR in CHF and control mice after VNS or SS.

2.5 Cardiac level of low molecular weight thiols

Cardiac level of low molecular weight thiols, which is mainly reduced glutathione (GSH), was measured by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method. After sham or vagal stimulation for 15 min (n = 6 each), CHF mice were euthanized and hearts were quickly excised. Non-infarcted LV was homogenized with 4% sulphosalicylic acid and 10 μL of supernatant was incubated with 125 μL of 1.5 mmol/L DTNB at 37°C for 15 min. Absorbance at 405 nm was measured. Thiold concentration was determined by calibration using 0–2 mmol/L GSH, and was expressed as mmol/L/mg tissue.

2.6 Cytochrome c reduction assay

NADPH oxidase activity was examined using superoxide dismutase inhibitable cytochrome c reduction assay. After sham or vagal stimulation for 15 min, mice were euthanized and the heart was quickly excised. Non-infarcted LV sample was immediately homogenized with phosphate buffer saline. Ten μL of the supernatants (final concentration 3 mg/mL) were diluted in 190 μL of assay buffer (300 mmol/L potassium phosphate, 0.1 mmol/L EDTA, 36 μmol/L cytochrome c, pH 7.8) in 96-well plates. NADPH (1 μmol/L) was added in the presence or absence of SOD (200 U/mL). Cytochrome c reduction was measured by reading the absorbance at 550 nm on a microplate reader. NADPH oxidase activity was calculated from the difference between the absorbance with or without SOD and the extinction coefficient (21.1 mmol/L/cm) for reduced cytochrome c. Cytochrome c reductase positive control (Sigma-Aldrich) was used to verify the specificity of the assay. Results were expressed as unit/mL, which was defined as reduction in 1.0 μmol of oxidized cytochrome c in the presence of 100 μmol/L NADPH per minute at pH 7.8 at 25°C.

2.7 Cardiac level of norepinephrine

Cardiac level of NE was measured by microdialysis and high performance liquid chromatography (HPLC). We used a transverse dialysis probe consisted of a dialysis fibre (2 mm in length, 220 μm in outer diameter, 200 μm in inner diameter, molecular weight cutoff at 50 kDa; OP-50-2, Eikom, Kyoto, Japan). Under light anaesthesia with pentobarbital sodium (20 mg/g BW, i.p.), mice were mechanically ventilated. After implantation of electrical wires, the left chest was opened. A dialysis probe was implanted into the non-infarcted LV. Ringer’s solution (Na+; 147 mmol/L, K+; 4 mmol/L, Ca2+; 1.26 mmol/L, Mg2+; 1 mmol/L, Cl−; 155.6 mmol/L) was pumped at a constant flow rate of 2 μL/min. Microdialysis session was started after a 30-min equilibration period. After collecting pre-VNS (baseline) samples for 30 min, VNS was started. From 15 min after VNS initiation, VNS samples were collected for 30 min. Finally, post-VNS samples were collected 15 min after the termination of VNS. NE concentration was assayed by HPLC, the condition of which was described in Supplementary Method.

2.8 Reactive oxygen species production in cultured neonatal rat ventricular cardiomyocytes

Primary cultures of cardiomyocytes were prepared from the ventricles of neonatal Wistar rats as described previously. Briefly, after digestion of the myocardial tissue with trypsin, cells were suspended in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich) containing 10% FBS and preplated twice in 100-mm culture dishes for 70 min each to reduce the number of non-myocytes. Non-adherent cells were plated in 12-well cultured plates at a density of 103 cells per mm2. Cardiomyocytes were maintained at 37°C in humidified air with 5% CO2. The culture medium was replaced by Hanks’ balanced salt solution with Ca2+ and Mg2+ but without phenol red (Gibco, Invitrogen, Carlsbad, CA, USA) 24 h before the experiments. On culture day 4, cells were exposed to NE (Sigma-Aldrich) in concentration from 0.01 to 100 μmol/L under the simultaneous incubation with prazosin hydrochloride (0.1 μmol/L) (Sigma-Aldrich) for 30 min to determine the ROS production from cardiomyocytes in response to β-adrenergic activation. H2O2 concentration in the culture medium was measured as described below. In a separate series of experiments, to elucidate the effect of ACh on the ROS production via β-adrenergic activation, cells were incubated with NE (10 μmol/L), NE + ACh (10 μmol/L) (Sigma-Aldrich), and NE + ACh + atropine hydrochloride (10 μmol/L) (Sigma-Aldrich). All NE-treated cells were incubated with 0.1 μmol/L prazosin. ACh and atropine were added 30 min prior to NE exposure. In the experiment to elucidate the ROS production within the myocytes, cells were incubated with 5 μmol/L of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich) at 37°C for 30 min. The fluorescence images were acquired with a microscope (BX50, Olympus Co. Ltd., Tokyo, Japan). Relative intensity for treated cells was determined by comparing with control cells. The experiment was repeated three times independently. For an experiment to determine the extracellular ROS production, after incubation at 37°C for 30 min, we collected the conditioned culture medium. The H2O2 concentration was measured by the method reported by Keston and Brandt. In brief, 10 μL of sample (n = 10 each) was reacted in vitro with 1 μmol/L DCFH-DA. Oxidation of DCFH-DA to the fluorescent 2’,7’-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) at 37°C for 30 min. The fluorescent images were acquired with a microscope (BX50, Olympus Co. Ltd., Tokyo, Japan). Relative intensity for treated cells was determined by comparing with control cells. The concentration of H2O2 was determined by calibration using 0–10 μmol/L H2O2.

2.9 Statistical analysis

Data are presented as mean ± SEM. Significant differences were determined by one-way analysis of variance using the Tukey post hoc test. Myocardial NE concentrations before and during VNS were compared by a paired t-test, after confirming normal distribution. A P-value less than 0.05 was judged to represent a statistically significant difference.

3. Results

3.1 Animal characteristics

LV dimensions were significantly enlarged and systolic function was significantly reduced in CHF, compared with control mice (Table 1). Although there was no significant difference in HR, the values of mean aortic pressure, LV + dp/dt max
and $\Delta p/dt_{\text{max}}$ were lower, and LV end-diastolic pressure was higher in CHF than in control. Heart weight/body weight, LV weight/body weight, and lung weight have also increased in CHF. Four mice out of 12 had obvious pleural effusion, whereas no effusion was present in control mice.

### 3.2 Measurement of reactive oxygen species by in vivo electron spin resonance

The signal decay rate of methoxycarbonyl-PROXYL at the chest region in CHF mice was clearly enhanced compared with controls (Figure 2A). This increased signal decay rate was almost reversed by the treatment with tiron or DMTU (Figure 2B), suggesting that the total redox state was changed probably due to increased ROS production especially in the failing myocardium.

In the next VNS protocol, VNS elicited a similar HR reduction in both CHF and control mice. Although there were some minor differences in each animal, from 300 to 500 mV were needed to reduce HR by 10% from baseline. No HR reduction by electrical stimulation (500 mV) was observed in CHF mice treated with atropine.

In vivo ESR spectroscopy revealed that VNS normalized the enhanced signal decay in CHF mice. In control mice, VNS showed no effects on the signal decay rate. Furthermore, this vagal-mediated effect in CHF mice was abolished by the administration of atropine (Figure 2C). These results suggested that VNS normalized the altered redox state in the failing myocardium potentially through attenuating the over-production of ROS via muscarinic ACh receptor pathway.

As an additional series of experiments, we performed in vivo ESR in CHF mice under fixed rate atrial pacing to exclude the effects of bradycardia. Fixed rate pacing showed a trend to abolish the VNS effects (Figure 3A).

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of the chronic heart failure (CHF) mouse model</th>
<th>Control (n = 12)</th>
<th>CHF (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>40.0 ± 0.5</td>
<td>39.1 ± 0.8</td>
</tr>
<tr>
<td><strong>Echocardiographic data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>539 ± 13</td>
<td>535 ± 27</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.6 ± 0.1</td>
<td>5.9 ± 0.2*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.1 ± 0.3</td>
<td>5.5 ± 0.3*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>42.9 ± 1.0</td>
<td>6.8 ± 0.9*</td>
</tr>
<tr>
<td><strong>Haemodynamic data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean AoP (mmHg)</td>
<td>89 ± 3</td>
<td>76 ± 4*</td>
</tr>
<tr>
<td>LVdP/dt max (mmHg/s)</td>
<td>12900 ± 400</td>
<td>4900 ± 400*</td>
</tr>
<tr>
<td>$\Delta p/dt_{\text{max}}$ (mmHg/s)</td>
<td>−8600 ± 300</td>
<td>−3300 ± 200*</td>
</tr>
<tr>
<td><strong>Organ weight data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart wt/body wt (mg/g)</td>
<td>4.9 ± 0.1</td>
<td>10.8 ± 0.3*</td>
</tr>
<tr>
<td>LV wt/body wt (mg/g)</td>
<td>3.0 ± 0.1</td>
<td>4.0 ± 0.1*</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>183 ± 4</td>
<td>366 ± 20*</td>
</tr>
</tbody>
</table>

LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; AoP, aortic pressure; EDP, end-diastolic pressure; wt, weight. Values are mean ± SEM. 

$P < 0.05$ and $*P < 0.01$ vs. control.
Furthermore, we also performed in vivo ESR analysis in CHF mice treated with L-NAME to clarify the involvement of NO. L-NAME treated mice showed a diminished HR response to VNS and a tendency to reduce the VNS induced improvements in myocardial redox state (Figure 3B).

### 3.3 Cardiac concentration of low molecular weight thiols

The myocardial concentration of low molecular weight thiols was significantly increased in CHF compared with control mice, and the increase was significantly attenuated by 15-min VNS (Figure 4A). This result also supported the observation that the redox status in the failing myocardium was altered by short application of VNS.

### 3.4 Nicotinamide adenin dinucleotide phosphate (NADPH) oxidase activity

Western blot analysis and cytochrome c reduction assay were performed on LV samples taken from control or CHF mice after the VNS or SS. Although the myocardial expression of p47phox, a cytosolic subunit of NADPH oxidase, was significantly increased in CHF mice, it was not altered after VNS (see Supplementary Methods and Supplementary material online, Figure S1A and B). Cytochrome c reduction assay revealed that myocardial NADPH oxidase activity was enhanced in CHF mice relative to control mice (0.51 ± 0.02 units/mL CHF + SS vs. 0.34 ± 0.03 units/mL control + SS, P < 0.05) and that there was a tendency for a reduction in NADPH oxidase activity by VNS (0.40 ± 0.03 units/mL CHF + VNS, P = 0.052) (Figure 4B).

### 3.5 Cardiac norepinephrine concentration

Cardiac NE concentration in CHF mice decreased significantly during VNS compared with before VNS (0.25 ± 0.07 ng/mL during VNS vs. 0.61 ± 0.12 ng/mL before VNS, P < 0.05) (Figure 5) and returned to baseline after the termination of VNS (0.43 ± 0.07 ng/mL after VNS vs. 0.25 ± 0.07 ng/mL during VNS, P < 0.05). Although it did not reach statistical significance, VNS induced reduction in NE level was also observed in control mice (0.47 ± 0.06 ng/mL before VNS, 0.36 ± 0.08 ng/mL during VNS, 0.42 ± 0.02 ng/mL after VNS, P > 0.05). These results suggested that VNS inhibited sympathetic nerve presynaptically especially in CHF mice.
3.6 β-Adrenergic receptor mediated reactive oxygen species production in cardiomyocytes

An increase in DCF fluorescence was observed 30 min after β-adrenergic receptor (β-AR) stimulation of cultured cardiomyocytes with 10 μmol/L NE. Furthermore, NE also increased the extracellular H₂O₂ release in a concentration-dependent manner (Figure 6A), confirming that β-AR stimulation increased the production of ROS in cardiomyocytes. The NE-induced DCFH oxidation was inhibited significantly by the addition of 10 μmol/L ACh, and this effect was abolished by atropine sulphate (Figure 6B), indicating that ACh directly inhibits the β-AR-stimulated ROS production in cardiomyocytes. The anti-oxidative effect of ACh was also demonstrated in NE-induced extracellular H₂O₂ release. ACh partially but significantly attenuated the NE-induced H₂O₂ release (46% reduction), which was also abolished by the addition of atropine sulphate (Figure 6C).

4. Discussion

The major findings demonstrated in the present study are that: (i) short VNS altered the myocardial redox status in CHF mice; (ii) this observation was mediated by both an inhibition of sympathetic drive and a direct action of ACh against free radical generation in the myocardium; and (iii) the subcellular mechanisms may involve NADPH oxidase activation, NO production, and myocardial oxygen consumption.

Cardiac parasympathetic nerve may play a defensive role in the pathogenesis of various heart diseases. According to the previous studies, VNS not only reduces the occurrence of lethal ventricular tachyarrhythmia but also attenuates the development of cardiac remodelling. In addition to these effects, the present study demonstrated that VNS suppresses myocardial ROS over-production. ROS cause cardiac apoptosis and activate several maladaptive cascades, which in turn lead to further dysfunction of cardiomyocytes. Therefore, the vagal-mediated anti-oxidative effects in the failing heart may provide an important mechanism contributing to the anti-remodelling action of chronic VNS.

4.1 Alteration of myocardial redox state by vagal nerve stimulation

We used in vivo ESR spectroscopy with a spin probe to measure the excess amount of ROS generation or estimate the redox status in living animals. CHF mice with marked LV systolic dysfunction showed enhanced ESR signal decay compared with controls. The increased signal decay rate in CHF mice indicates alteration of the redox status, probably due to excess ROS generation, because administration of antioxidants normalized the accelerated signal decay. Strikingly, the enhanced signal decay was almost normalized by...
15-min VNS, which was not observed in control mice. Therefore, these results indicated that a short VNS suppressed the enhanced ROS generation especially in the failing heart. Furthermore, the VNS-induced effects are mediated by muscarinic ACh receptors, because administration of atropine sulphate blocked the VNS-induced effects. Moreover, the cardiac level of low molecular weight thiols, which was mainly reduced form of GSH, was altered in parallel with the ESR signal decay as a result of VNS, suggesting modulation in myocardial redox state. However, the increased GSH in the failing myocardium is in contrast to the previous reports.25,26 These controversial findings may be due to contribution of various factors, including experimental conditions and animal species employed, namely the difference in HF models, details of which are unknown at the moment.

4.2 Antioxidant effects by inhibition of norepinephrine and release of acetylcholine

Regarding autonomic innervation in the heart, previous studies both in animals and in humans have demonstrated that NE released by cardiac sympathetic nerve can be suppressed by parasympathetic activation via muscarinic receptor located at adrenergic nerve terminals.14 This effect is more prominent especially under a condition of enhanced adrenergic drive.15,16 Furthermore, the high concentration of NE is cardiotoxic27 and induces apoptosis,8 playing a central role at the formation of a vicious cycle in the pathogenesis of HF. In this study, VNS decreased the cardiac NE level in vivo, suggesting sympathetic inhibition. In addition, in cardiomyocytes, NE generated ROS in a concentration-dependent manner. Taken together, sympatho-inhibition may be one mechanism of vagally mediated antioxidative effects in the failing heart.

It is well known that ACh released from vagal terminals also counteracts AR signalling within cardiomyocytes. Adenylcyclase which is activated by Gs protein coupled with β-AR is inhibited by pertussis toxin-sensitive Gi/o protein coupled with M2 muscarinic receptor.28 VNS increases the interstitial ACh level in the heart.29 In this study, β-adrenoceptor-mediated ROS production in cardiomyocytes was suppressed by the co-incubation with ACh. Therefore, the increased interstitial ACh evoked by VNS may also protect heart from oxidative stress in vivo. However, since NE-induced ROS production was partially inhibited by ACh, the direct effect of ACh does not fully explain our in vivo ESR results, indicating that other mechanisms may intervene between the anti-oxidative effects and VNS.

4.3 Involvement of nitric oxide and NADPH oxidase

One potential mechanism is the involvement of NO. It is well established that NO and NO-based signal transduction pathways modulate myocardial physiological function.30 Altered production of reactive oxygen and nitrogen species, which is defined as redox disequilibrium, is one of the major characteristics of failing myocardium.30 Indeed, in vivo ESR spectroscopy in our study revealed that chronic treatment with L-NAME attenuated the VNS-induced effects. Therefore, NO may mediate the normalization of myocardial redox disequilibrium by VNS. Dedkova et al.31 demonstrated that ACh increases NO production in cardiomyocytes. NO itself may act as a ROS scavenger at low physiologic levels.32 Furthermore, possible involvement of neuronal NO synthase should be taken into consideration. Extensive studies by Mohan and colleagues13 have shown that neuronal NO facilitates vagal neurotransmission and bradycardia via cGMP-dependent pathway. We also observed diminished HR response to VNS in CHF mice treated with L-NAME. Although we could not speculate to what extent myocardial or neuronal NO production contributes, NO and NO-based signal transduction pathways play important roles in the VNS induced myocardial redox modulation.

Among the several myocardial sources of oxidative stress, NADPH oxidase has been clarified as one of the major sources in the failing myocardium. NADPH oxidase isoform Nox2, which is abundantly expressed in cardiomyocytes, is regulated by cytosolic components, p47phox, p67phox, p40phox, and rac.6 It is reported that both NADPH oxidase activity and p47phox expression are enhanced in the diseased myocardium not only in animal models34 but also in humans.35 Expression of gp47phox and NADPH oxidase activity were also enhanced in the LV of CHF mice. VNS did not alter the protein level of gp47phox, whereas the enhanced NADPH oxidase activity was attenuated by VNS. This observation would confirm our result that VNS resets the myocardial redox imbalance. It is natural that enzymatic reactions or activations are involved in the acute myocardial redox modulation. Although the mechanism how VNS attenuated NADPH oxidase activity is uncertain, NO is suggested to inhibit NADPH oxidases.36 Therefore, VNS induced NADPH oxidase regulation may also be mediated by NO.

Regarding the cardiac mechanics and energetics, decrease in HR reduces myocardial oxygen consumption.37 This may contribute to the VNS-induced redox alteration to some extent, because fixed rate atrial pacing partially inhibited the VNS effects. There are several mechanisms to explain this partial inhibition. As previously reported, inotropic effects of VNS were influenced by the presence of sympathetic drive.13 Without sympathetic drive, it is mainly mediated by indirect effect of bradycardia via force frequency mechanism.37 In contrast, with sympathetic drive, inotropic effects of VNS are also mediated by direct effects which are independent of HR. Therefore, partial inhibition by atrial pacing may be attributed to the inhibition of indirect bradycardic effects. Although it is difficult to define the extent of contribution of bradycardia, reduction in myocardial oxygen consumption, at least in part, plays a role in myocardial redox modulation by VNS.

4.4 Limitations

There are several limitations and unsolved questions. First, care should be paid to interpret that the enhanced signal decay is due to the increase in ROS, because reductants such as ascorbic acid, GSH, and NO are known to reduce nitroxy radicals in vivo. However, the accelerated signal decay in CHF mice was normalized by the administration of antioxidants. Therefore, it appears to be reasonable postulate that VNS suppresses ROS-overproduction and modulates the myocardial redox status in CHF mice. Secondly, we stimulated vagal nerve for 15 min and the electrical voltage was optimized to reduce HR by 10%. We did not examine details of the influence of duration or strength, which remains for future study. Thirdly, involvements of
central nervous system (CNS) and anaesthetic agents remain unknown. Inputs to CNS via vagal afferents have been reported to modulate several neuronal reflexes and the activities of autonomic nervous system. Since we also observed both the pressor and depressor responses to vagal afferent stimulation in rats (data not shown), alteration of CNS function may affect the present results. Furthermore, anaesthetic agents suppress autonomic nervous activity. The myocardial NE levels, therefore, might be different from that in conscious animals. Fourthly, the concentration of NE used in cultured cardiomyocyte experiments was much higher than physiological plasma NE concentration in vivo. Therefore, these issues, the reason of which are not clarified in the current study in vitro, have to be taken into consideration to explain the pathophysiological mechanism responsible for the regulation in vivo.

4.5 Conclusions
In conclusion, in a mouse model of chronic HF, VNS modulates the cardiac redox status and adrenergic drive thereby suppressing ROS generation in the failing heart.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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