Discussion

The results of this theoretical analysis suggest that, in patients with hypoplastic RV, postoperative hemodynamics depends largely on the RV stiffness constant. PA/IVS, Ebstein's anomaly or their relatives are characterized by varying degrees of underdevelopment of RV. For a severely hypoplastic RV, the definitive treatment is single ventricular circulation. For a mildly hypoplastic RV, biventricular circulation is expected to have merit. Recently, 1.5VR has been proposed to reduce the surgical risk of 2VR. The use of 1.5VR has lowered the early or midterm mortality, and adequate growth of RV and the tricuspid valve has been documented in some patients [2]. However, the postoperative RV dysfunction or arrhythmic event has also been reported, in particular, when the patients are on the borderline of criteria between 1.5VR and Fontan operation [4, 5].

For the choice of surgical options among Fontan operation, 1.5VR, and 2VR, the previously used criteria were based on morphologic characteristics of the hypoplastic RV, such as RVEDV. However, simple anatomic indices may be inaccurate, since these values are dependent on the afterload and preload conditions. For that reason, the treatment strategy for hypoplastic RV based on the anatomic indices remains controversial. We focused on the intrinsic property of hypoplastic RV, i.e., RV stiffness constant. The fact that the RV stiffness constant, an index of chamber property, is relatively independent of the loading condition is important for the accurate prediction of postoperative hemodynamics. Based on the results of the present study, we propose that patients with hypoplastic RV can be classified into three groups according to the RV stiffness constant. The first group consists of patients with mild RV hypoplasia (RV stiffness constant <150% of normal), in whom enlargement of RV is expected after the operation. At the other extreme, the second group consists of patients with severe RV hypoplasia (RV stiffness constant >250%), in whom no RV reconstruction is expected to have merit. In addition, we have shown that there certainly exists a third group consisting of patients with intermediate RV hypoplasia (RV stiffness constant between 150 and 250%), who would benefit more from 1.5VR than from 2VR or Fontan operation.

Mild RV hypoplasia

When RV hypoplasia is mild (RV stiffness constant <150% of normal), systemic cardiac output is greater in 2VR than in 1.5VR or Fontan operation (APC or TAPC). Therefore, we recommend that 2VR should be chosen in the mild RV hypoplasia group. Although systemic cardiac output in 1.5VR is also greater than that in Fontan operation, SVC pressure (which is equal to PAP) is higher than that of APC. Accordingly, the upper part of the body is exposed to higher SVC pressure in 1.5VR, which may cause postoperative pleural effusion [2]. A large pressure gradient between SVC and IVC also results in abnormal venous collaterals from SVC to IVC [17–20], and they could effectively increase the venous return to RA in 1.5VR.

Intermediate RV hypoplasia

When RV hypoplasia is intermediate (RV stiffness constant between 150 and 250% of normal), systemic cardiac output in 1.5VR exceeds that in 2VR. Although SVC pressure is still higher in 1.5VR than in APC, RAP is lower in 1.5VR than in the other procedures. This condition is favorable to reduce supraventricular arrhythmias related to high RAP during the perioperative periods. This beneficial effect is not expected for 2VR since RAP in 2VR is higher than the atrial pressure of TCPC. Furthermore, 1.5VR is advantageous from the viewpoint of stressed blood volume because 1.5VR needs smaller stressed blood volume than does 2VR to maintain MAP (Fig. 3d).

In these patients, RVEDV in 1.5VR is relatively independent of the RV stiffness constant. However, abnormal systemic venous collateral channels might open after 1.5VR. These collateral channels would increase RV preload wastefully and decrease systemic cardiac output in the late postoperative phase. In such conditions, conversion to the Fontan circulation may be required in the late phase [4, 5]. Nevertheless, 1.5VR should be recommended for the intermediate RV hypoplasia group because high cardiac output and low RAP are anticipated.

Severe RV hypoplasia

When RV hypoplasia is severe (RV stiffness constant >250% of normal), neither 1.5VR and 2VR are expected to improve systemic cardiac output. In this condition, RVEDV is almost the same between 1.5VR and 2VR, and linearly decreases with an increase in the RV stiffness constant in spite of a rapid elevation in RAP. This indicates that RVEDV might be independent of the venous return to RA. Since RAP becomes higher than the atrial pressure of TCPC even in 1.5VR, supraventricular arrhythmias caused by high RAP are liable to occur [2, 5]. In this condition, 1.5VR is considered to have hemodynamics equivalent to APC and needs larger stressed blood volume than does TCPC to maintain systemic arterial pressure (Fig. 3d).

Therefore, TCPC should be chosen for patients with severe RV hypoplasia. In these patients, the arrhythmic events after TCPC are less than that after APC [21, 22]. Although a small pressure gradient between SVC and IVC

remains in 1.5VR, this may not be of clinical significance. Systemic venous collateral channels are expected to be rare, and an increase of RV volume after the operation is unlikely.

Clinical implication

The management strategy for patients with hypoplastic RV has been based on the morphological characteristics, which are dependent on the loading conditions. In contrast, we used a relatively load-independent index, RV stiffness constant, and simulated the postoperative hemodynamics. As a result, we identified the characteristics of hemodynamics after each of the surgical options, and clearly defined the indications of these operations.

Moreover, our results may be useful to theoretically speculate the reason for contrasting clinical findings. Chowdhury and colleagues [2] reported that the event rate of supraventricular arrhythmia was about 15% in the late postoperative phase of 1.5VR. On the other hand, Numata et al. [5] reported higher arrhythmic event rate. In the former report, the patients had a relatively high postoperative RV volume (45–75% of predicted normal RV; Fig. 3c) and a large pressure gradient between SVC and IVC (mean 7.6 mmHg; Fig. 3b) after 1.5VR. Indeed, there was significant pleural effusion in 22.7% of patients. Our results suggest that good systemic cardiac output, low IVC pressure, and high SVC pressure after 1.5VR can be expected under a condition of a relatively small RV stiffness constant. A great difference between SVC and IVC pressures may cause pleural effusion. Therefore, patients in the former report are likely to have low RV stiffness. In the latter report, the average RVEDV at 1 year after 1.5VR was about 50% of normal and there was no obvious collateral after the surgery in the patients examined. These data suggest a high RV stiffness (Fig. 3c), and a small difference between SVC and IVC pressures (Fig. 3b). Since higher arrhythmic event rate is likely to be associated with high RAP in patients with high RV stiffness, we can interpret the marked difference in arrhythmic event rate in these studies based on postoperative hemodynamics. Operations with 1.5 VR in potentially inappropriate patients (i.e., patients with stiffer RV) might impair long-term outcomes by continued high RAP-induced arrhythmia.

If we can assess the RV stiffness constant and other hemodynamic data in a catheter laboratory before operation, we will be able to select the most suitable operation for patients with hypoplastic RV. Recently, noninvasive methods for predicting LV chamber stiffness using echocardiography have been reported [23–25]. For example, LV chamber stiffness has been estimated from the deceleration time of LV early filling, effective mitral area and length. Such a method may be applied to estimate RV chamber stiffness using the deceleration time of RV early filling, effective tricuspid area and length. Moreover, it may be possible to choose an appropriate procedure for individual patients by performing simulation of postoperative hemodynamics from individual data using our model. Further clinical studies are needed to precisely assess the RV stiffness constant, including the above methods.

Limitations

A major limitation of this study is related to the parameters we used for the model. In our model, all parameters other than the RV stiffness constant are fixed. It is reported that RV end-systolic elastance as well as the RV stiffness constant depend upon RV histological changes such as RV hypertrophy [26]. The increase in RV end-systolic elastance moves the beneficial range of 1.5VR toward the stiffer range of the RV stiffness constant. The increase of heart rate also moves the range toward the stiffer range (Table 3). Moreover, ischemia caused by long-standing hypoxemia and hypertension of RV may influence other variables [6]. The existence of pulsatility of the pulmonary circulation may also affect the pulmonary vascular resistance [27]. Tricuspid regurgitation may also impair the postoperative hemodynamics. These limitations may be solved by using the preoperative data of individual patients. Santamore and Burkhoff have already reported the importance of ventricular interdependence using a computer model [13]. However, ventricular interdependence between small hypoplastic RV and relatively large left ventricle may be negligible.

Table 3 The influence of right ventricular end-systolic elastance and heart rate on the beneficial range of the one and a half ventricle repair

	Lower limit of RV stiffness constant (% of normal)	Upper limit of RV stiffness constant (% of normal)
$E_{es,RV} = 0.7$, HR = 75	150	250
$E_{es,RV} = 1.4$, HR = 75	200	300
$E_{es,RV} = 0.7$, HR = 100	175	275

RV Right ventricle, $E_{es,RV}$ right ventricular end-systolic elastance (mmHg/ml), *HR* heart rate (beats/min)

Conclusion

Using a model analysis, we have shown that the beneficial effect of 1.5VR depends on the RV stiffness constant. 1.5VR is the most beneficial for hypoplastic RV with 150–250% of normal RV stiffness constant. The beneficial range of 1.5VR may also be changed by individual parameters other than the RV stiffness constant, but the beneficial range certainly exists. Therefore, determination of management strategy should be based not only on the morphologic parameters but also on the physiologically determined properties.

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ORIGINAL CONTRIBUTION

A histamine H_2 receptor blocker ameliorates development of heart failure in dogs independently of β-adrenergic receptor blockade

Hiroyuki Takahama · Hiroshi Asanuma · Shoji Sanada · Masashi Fujita · Hideyuki Sasaki · Masakatsu Wakeno · Jiyoong Kim · Masanori Asakura · Seiji Takashima · Tetsuo Minamino · Kazuo Komamura · Masaru Sugimachi · Masafumi Kitakaze

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Abstract Histamine has a positive inotropic effect on ventricular myocardium and stimulation of histamine H_2 receptors increases the intracellular cAMP level via Gs protein, as dose stimulation of β-adrenergic receptors, and worsens heart failure. To test whether a histamine H_2 receptor blocker had a beneficial effect in addition to β-adrenergic receptor blockade, we investigated the cardioprotective effect of famotidine, a histamine H_2 receptor blocker, in dogs receiving a β-blocker. We induced heart failure in dogs by rapid ventricular pacing (230 beats/min). Animals received no drugs (control group), famotidine (1 mg/kg daily), carvedilol (0.1 mg/kg daily), or carvedilol plus famotidine. Both cardiac catheterization and echocardiography were performed before and 4 weeks after the initiation of pacing. Immunohistochemical studies showed the appearance of mast cells and histamine in the myocardium after 4 weeks of pacing. In the control group, the left ventricular ejection fraction (LVEF) was decreased after 4 weeks compared with before pacing (71 ± 2 vs. $27 \pm 2\%$, $p < 0.05$) and mean pulmonary capillary wedge pressure (PCWP) was increased (8 ± 1 vs. 19 ± 3 mmHg). Famotidine ameliorated the decrease of LVEF and increase of PCWP, while the combination of carvedilol plus famotidine further improved both parameters compared with the carvedilol groups. These beneficial effects of famotidine were associated with a decrease of the myocardial cAMP level. Histamine H_2 receptor blockade preserves cardiac systolic function in dogs with pacing-induced heart failure, even in the presence of β-adrenergic receptor blockade. This finding strengthens the rationale for using histamine H_2 blockers in the treatment of heart failure.

Keywords Heart failure · Histamine · Histamine H_2 receptor blocker · β-Adrenergic receptor blocker

Introduction

Chronic heart failure (CHF) is one of the major causes of morbidity and mortality worldwide, and is characterized by neurohormonal imbalances that include activation of the sympathetic nervous system [9, 15]. β-Adrenergic receptor blockade is a established treatment of CHF because it protects the heart from the harmful effects of the sympathetic nervous system that are partly mediated via cyclic adenosine monophosphate (cAMP)-dependent pathways [2, 34]. Interestingly, histamine H_2 receptors are linked to Gs proteins that facilitate the production of cAMP (as are β-adrenergic receptors) and are expressed in the heart [18, 29, 33]. Histamine has a positive inotropic effect on human ventricular myocardium and chronotropic effects [3, 12], and also autonomic control of the heart [21]. Indeed, we previously reported that famotidine, a histamine H_2

H. Takahama · M. Wakeno · J. Kim · M. Asakura · M. Kitakaze (✉)
Department of Cardiovascular Medicine, National Cerebral and Cardiovascular Center, Suita, Osaka 565-8565, Japan
e-mail: kitakaze@zf6.so-net.ne.jp

H. Asanuma · H. Sasaki · K. Komamura · M. Sugimachi
Cardiovascular Dynamics Research Institute, National Cerebral and Cardiovascular Center, Suita, Japan

H. Asanuma
Department of Emergency Room Medicine, Kinki University School of Medicine, Osaka-Sayama, Japan

S. Sanada · M. Fujita · S. Takashima · T. Minamino
Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan

receptor blocker, protected the heart against ischemia-reperfusion injury in dogs [1] and also improved both symptoms of CHF and ventricular remodeling in the clinical setting [16]. Although the maximum inotropic effects of substances acting through cAMP were decreased in diseased myocardium [6], famotidine, a histamine H_2 receptor blocker, exerts negative effects on cardiac performance [13], the roles of the histamine would have remained unclear in the state of heart failure.

In addition, it is still unclear whether histamine H_2 receptor blockers have a protective effect against CHF by reducing the myocardial accumulation of cAMP and whether there is an additive effect of histamine H_2 receptor blockade in the presence of β-adrenergic receptor blockade.

Therefore, we investigated the effect of a histamine H_2 receptor blocker on cardiac performance and myocardial cAMP accumulation in dogs with pacing-induced heart failure, and also investigated whether there was an additive effect of combined histamine H_2 receptor blocker and β-blocker therapy on cardiac performance.

Methods

Materials

The histamine H_2 receptor blocker famotidine was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Carvedilol, a β-adrenergic receptor blocker, was obtained from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-histamine antibody was obtained from Progen (Queensland, Australia).

Animal preparation

Beagle dogs (Oriental Yeast Co. Ltd., Tokyo, Japan) weighing 8–10 kg were sedated with intravenous sodium pentobarbital at a dose of 25 mg/kg. After intubation with a cuffed endotracheal tube, anesthesia was maintained with 0.5–1% isoflurane and an equal mixture of air and oxygen. Ventilation was provided with a tidal volume of 22 mL/kg at a rate of 15 times per minute. A bipolar pacing lead (Model BT-45P, Star Medical Inc., Tokyo, Japan) was advanced under fluoroscopic guidance through the right jugular vein to the right ventricular (RV) apex and was connected to an external programmable pacemaker (VOO mode; Model SIP-501, Star Medical Inc., Tokyo, Japan) that was implanted in a subcutaneous pocket in the neck. The success of this procedure was confirmed by electrocardiography. Antibiotics were given after surgery, and the dogs were allowed to recover fully. Then heart failure was induced by rapid right ventricular pacing at a rate of 230 beats/min for 4 weeks as the model mimicking heart failure in human, as reported previously [22, 23, 27].

All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, 1996 revision) and were approved by the ethical committee for laboratory animal use of the National Cardiovascular Center in Japan.

Echocardiography

Transthoracic echocardiography was performed by using an echocardiographic system equipped with a 2–4 MHz phased-array transducer (SONOS 5500, Hewlett Packard, Massachusetts, USA) in conscious dogs before pacemaker implantation and 30 min after the cessation of RV pacing at 4 weeks. Good two-dimensional short-axis views of the left ventricle were obtained at the level of the papillary muscles for guided M-mode measurement of the left ventricular (LV) end-diastolic dimension (LVDd), LV end-systolic dimension (LVDs), LV fractional shortening (LVFS), and LV ejection fraction (LVEF). All measurements were made by two observers, who were blinded to the source of the tracings.

Hemodynamic studies

LV pressure and mean aortic pressure were measured by pressure amplifiers connected to a pig tail catheter (5F, Terumo Co. Ltd., Tokyo, Japan) that was inserted into the left ventricle from the left femoral artery. Pulmonary capillary wedge pressure (PCWP) was measured with a 7 Fr Swan-Ganz catheter (American Edwards Laboratories, California, USA). LV dP/dt was analyzed using software (Data viewer, Yokogawa Electric Corporation, Tokyo, Japan). These studies were performed both before and after 4 weeks of RV pacing or 4 weeks after pacemaker implantation in the sham group.

Measurement of the myocardial cAMP level

The myocardial cyclic AMP (cAMP) level was measured as described previously [8]. Briefly, a sample of frozen cardiac muscle was homogenized mechanically in 500 mL of frozen hydrochloric acid (0.1 N) with a mechanical homogenizer. The homogenate was thawed and centrifuged at $5{,}000 \times g$ at room temperature for 15 min and then a 100 mL aliquot of the supernatant was employed to measure cAMP with a sensitive radioimmunoassay (cyclic AMP kit; Yamasa Shoyu Co., Choshi, Japan).

Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously [24]. Briefly, myocardial tissue samples were fixed in 10% formalin and embedded in paraffin. Then

5-μm-thick sections were cut and preincubated with 3% hydrogen peroxide. Rabbit polyclonal anti-histamine antibody (1:1,000 dilution) was added, and incubation was done at room temperature overnight. Next, the sections were incubated with biotinylated anti-rabbit immunoglobulin for 30 min and subsequently with horseradish peroxidase-labeled streptavidin solution for 30 min. The slides were rinsed in tris-buffered saline after each incubation step. Peroxidase activity was visualized by incubation with diaminobenzidine hydrochloride solution.

Experimental protocols

Protocol 1: effects of famotidine on cardiac performance and myocardial cAMP accumulation in dogs with pacing-induced heart failure

After pacemaker implantation, the dogs were randomly assigned to a sham group ($n = 6$) without pacing, a control group ($n = 7$) with pacing only, and a famotidine group ($n = 5$) with pacing plus the daily oral administration of famotidine (1 mg/kg per day). The dose of famotidine was chosen on the basis of previous reports [30, 36]. Echocardiography and measurement of hemodynamic parameters were performed before and 4 weeks after pacemaker implantation. After the measurement of hemodynamic parameters, myocardial tissue samples were obtained and quickly placed into liquid nitrogen for storage at −80°C until measurement of cAMP levels.

Protocol 2: effects of famotidine on cardiac performance in dogs with pacing-induced heart failure under β-adrenergic receptor blockade

Next, we examined the additive effect of histamine H_2 receptor blockade on the development of CHF. After pacemaker implantation, the dogs were randomly assigned to a carvedilol group ($n = 6$) that received daily oral administration of carvedilol (0.1 mg/kg per day) or a carvedilol + famotidine group ($n = 6$) that received daily oral administration of both carvedilol (0.1 mg/kg per day) and famotidine (1 mg/kg per day).

Statistical analysis

Results are expressed as the mean ± SEM. Comparison of time-course changes between the groups was performed by two-way repeated measures analysis of variance (ANOVA). For comparison of mast cell counts and cAMP levels between the groups, the Mann–Whitney U test was performed. A p value < 0.05 was considered to represent statistical significance.

Results

Mast cells and histamine expression

Mast cells were detected in the myocardium by toluidine blue staining. Consistent with previous reports [8, 26], we observed an increase of mast cells in the failing hearts compared with the number of cells in the sham group (Fig. 1a). Immunohistochemical analysis showed an increase of histamine expression indicating increased degranulation of mast cells in failing hearts compared with the level in the sham group (Fig. 1b).

Effect of famotidine on cardiac performance and myocardial cAMP in dogs with pacing-induced heart failure

Both mean aortic pressure and heart rate before pacing were similar in the control group (104 ± 5 mmHg and

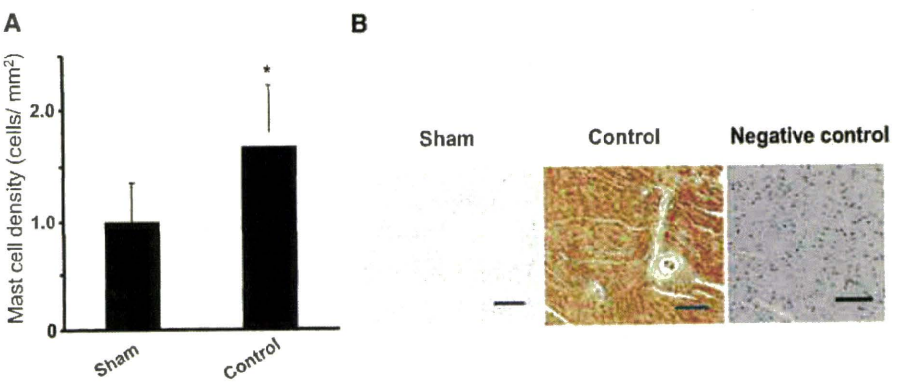

Fig. 1 Mast cell density and histamine expression in the failing heart. **a** Mast cell density in the heart. Values are the mean ± SEM. *$p < 0.05$ versus the sham group. **b** Immunostaining with an anti-histamine antibody. **a** Representative staining of a heart from the sham group. **b** Representative staining of a heart from the control group. **c** Negative control section incubated without the primary antibody. The *scale bars* indicate 50 μm

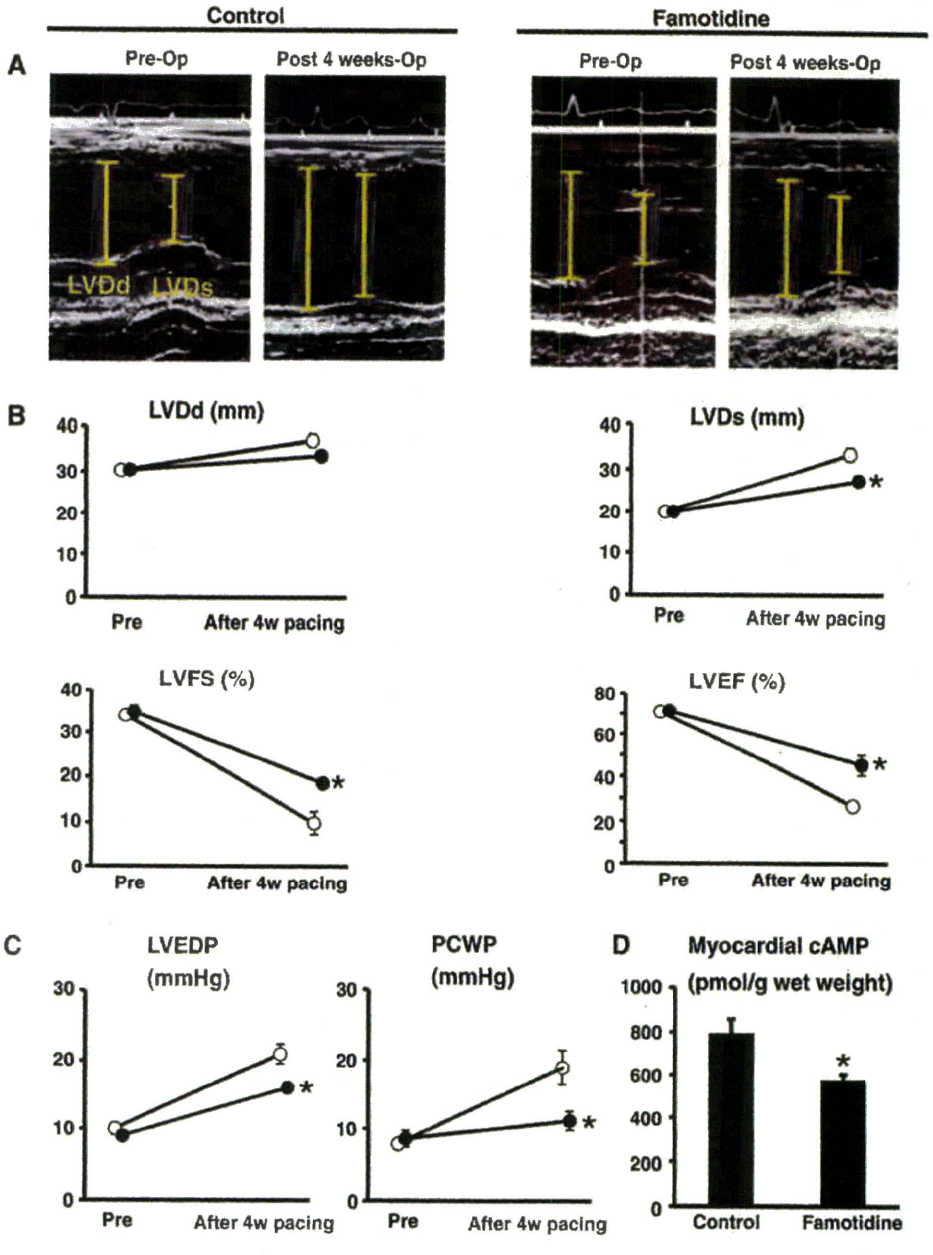

Fig. 2 Effects of famotidine on echocardiographic and hemodynamic parameters and myocardial cAMP levels. **a** Representative 2D echocardiograms. **b** Quantitative analysis of echocardiographic parameters in the control and famotidine groups. *Open* and *closed circles* indicate the control group and the famotidine group, respectively. **c** LVEDP and mean PCWP in the control and famotidine groups. **d** Myocardial cAMP levels in each group. All values are the mean ± SEM. *$p < 0.05$ versus the sham group

117 ± 7 bpm, respectively), and the famotidine group (101 ± 3 mmHg and 115 ± 8 bpm, respectively). These parameters did not significantly differ among the groups. LV d*P*/d*t* was 3,592 ± 512 mmHg/s in the control group and 3,981 ± 528 mmHg/s in the famotidine group. Four weeks after surgery, neither hemodynamic nor echocardiographic data showed any changes compared with the preoperative values in the sham group (data not shown). Four weeks after rapid RV pacing, an administration of famotidine significantly limited the increase of both LVDd and LVDs, as well as the decrease of both LVFS and LVEF (33.4 ± 0.8 mm, 27.4 ± 1.2 mm, 18.5 ± 2.6% and 45.4 ± 4.8%, respectively), compared with the findings in the control group (37.0 ± 1.4 mm, 33.4 ± 1.4 mm, 9.9 ± 1.0% and 26.7 ± 2.4%, respectively) (Fig. 2a, b). Four weeks after RV pacing, LV end-diastolic pressure (LVEDP) and PCWP of the famotidine group (16 ± 2 and 11 ± 1 mmHg, respectively), were both significantly lower compared with the values in the control group (21 ± 2 and 19 ± 2 mmHg and, respectively) (Fig. 2c). LV d*P*/d*t* after RV pacing was significantly preserved higher in the famotidine group (2,601 ± 216 mmHg/s) compared with that in control group (2,077 ± 124 mmHg/s) ($p < 0.05$).

The myocardial cAMP level was significantly higher in the control group (796 ± 111 pmol/g wet weight) compared with that in the sham group (597 ± 77 pmol/g wet

weight), while it was significantly lower in the famotidine group (577 ± 28 pmol/g wet weight) compared with the control group ($p < 0.05$) (Fig. 2d).

Additive effects of famotidine and a β-blocker on cardiac performance in dogs with pacing-induced heart failure

Before pacing, mean aortic pressure and heart rate were both similar in the carvedilol group (101 ± 7 mmHg and 111 ± 8 bpm, respectively), and the famotidine + carvedilol group (93 ± 2 mmHg, 106 ± 7 bpm, respectively), and these parameters did not significantly differ among the groups. LV d*P*/d*t* was 3,672 ± 417 mmHg/s in the carvedilol group and 3,941 ± 284 mmHg/s in the famotidine + carvedilol group. After rapid RV pacing for 4 weeks, both LVDd and LVDs were decreased and both LVFS and LVEF were increased (33 ± 0.4 mm, 25 ± 0.7 mm, 28 ± 2%, and 54 ± 3%, respectively), in the famotidine + carvedilol group compared with the respective values in the carvedilol group (34 ± 1 mm, 28 ± 1 mm, 23 ± 1%, and 38 ± 5%, respectively) (Fig. 3a).

Four weeks after RV pacing, LVEDP and PCWP of the carvedilol + famotidine group (12 ± 3 and 10 ± 4 mmHg, respectively), were both significantly reduced compared with the respective values in the carvedilol group (16 ± 2 and 15 ± 1 mmHg, respectively) (Fig. 3b). LV d*P*/d*t* after RV pacing was preserved higher in the famotidine + carvedilol group (3,382 ± 252 mmHg/s) compared with that in carvedilol group (2,740 ± 321 mmHg/s) ($p < 0.05$).

Furthermore, the myocardial cAMP level was significantly lower in the carvedilol + famotidine group (488 ± 45 pmol/g wet weight) compared with that in the carvedilol group (615 ± 28 pmol/g wet weight) (Fig. 3c).

Discussion

In the present study, we demonstrated that (1) myocardial histamine expression was increased by pacing-induced heart failure, (2) the histamine H_2 receptor blocker famotidine improved cardiac performance (gauged by echocardiographic and hemodynamic parameters) along with a reduction of myocardial cAMP accumulation, and (3) there was an additive effect of combined histamine H_2 receptor and β-adrenergic receptor blockade on cardiac performance in dogs with pacing-induced heart failure.

Impact of histamine blockade on cardiac failure

Histamine is one of the autacoids, and is stored and released by mast cells in the human heart, as well as in other organs or

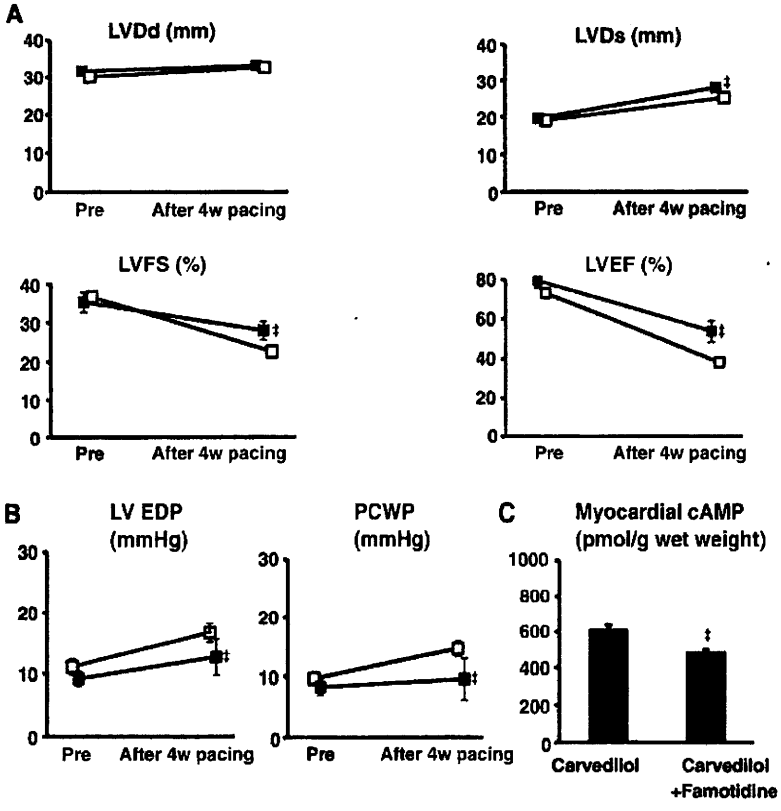

Fig. 3 Additive effect of famotidine and carvedilol on cardiac performance. **a** Quantitative analysis of echocardiographic parameters in the carvedilol and carvedilol + famotidine groups. *Open* and *closed squares* indicate the carvedilol group and the carvedilol + famotidine group, respectively. **b** LVEDP and mean PCWP in the carvedilol group and carvedilol + famotidine group. *Open* and *closed squares* indicate the carvedilol group and the carvedilol + famotidine group, respectively. **c** Myocardial cAMP levels in each group. All values are the mean ± SEM. *$p < 0.05$ versus the carvedilol group

tissues [8]. Recently, the number of mast cells and the myocardial histamine level were found to increase in the hearts of patients with idiopathic dilated cardiomyopathy or ischemic cardiomyopathy [26]. Histamine modulates various cellular functions via the activation of four different G-protein-coupled receptors (H_{1-4} receptors) [29]. As is well known, histamine H_2 receptors located on gastric cells promote the production of gastric acid [10] and histamine H_2 receptor blockers have been widely used for the treatment of peptic ulcer. Interestingly, histamine H_2 receptors are also expressed in canine and human ventricular myocardium [14, 18], although the expression levels were different among species [18]. Consisted with the previous studies, we confirmed the presence of histamine H_2 receptor in the canine heart using quantitative reverse-transcriptase PCR (data not shown).

In the present study, the precise locations of histamine receptors in myocytes or vessels in the canine hearts remained unclear. However, since famotidine did not decrease blood pressure in this study, the accumulating lines of evidence would suggest that histamine H_2 blockers did not have the potent effect on the vessels compared with cardiomyocytes. On the other hand, since histamine H_1 receptors are abundantly expressed in the vessels in most animal species [29], histamine H_1 receptor blocker has the effects on vessels. In addition, stimulation of H_2 receptors transduces the intracellular signals via Gs protein, as does the stimulation of β-adrenergic receptors. Moreover, histamine has a positive inotropic effect on human ventricular myocardium and has been suggested to have a role in cardiovascular diseases [3, 11]. Although it was reported that the maximum inotropic effects of histamine receptor stimulation were less than those mediated by beta-adrenergic receptors [5, 35], the roles of histamine receptor blockade have remained unclear compared with those of beta-adrenergic receptor.

Based on these backgrounds, we previously proposed that histamine H_2 receptor blockers could provide a novel therapeutic strategy for heart failure, and we have reported that histamine H_2 blocker treatment may have a cardioprotective effect in patients with chronic heart failure [16]. In the present study, we found that myocardial histamine expression was increased in dogs with pacing-induced heart failure compared with that in sham dogs on immunohistochemical analysis. Also, the histamine H_2 receptor blocker, famotide, prevented the development of heart failure induced by rapid RV pacing and lessened the myocardial accumulation of cAMP. These findings suggest that histamine H_2 receptor blockade exerts a cardioprotective effect along with the amelioration of myocardial cAMP accumulation. Recently it was reported that increased cardiac adenylyl cyclase expression is associated with mortality after myocardial infarction in rats [31]. It has been controversial for the role of myocardial cAMP in the heart failure [17]. Although increased cAMP acutely induced the improvement of ventricular function, several trials with either beta-adrenergic agonists or PDE inhibitors have revealed an increase in mortality [17, 20]. In the present study, in the dogs with the drugs that decreases myocardial cAMP levels, the development of heart failure was substantially attenuated, however further investigation will be needed to solve the roles of cAMP in the onset and progression of heart failure.

Additive effects of histamine H_2 blocker and β-blocker therapy on cardiac performance

β-Blockers have long been established as useful agents for chronic heart failure [2, 7, 25, 32]. These drugs act by preventing intracellular Ca overload, because β-adrenergic stimulation promotes Ca overload via Gs protein [34]. Histamine H_2 receptor blockade also prevents Ca overload [28], so we hypothesized that the combination of a histamine H_2 receptor blocker and a β-blocker would exert a stronger cardioprotective effect than either agent alone. Almost all of the patients in our earlier study, which showed that histamine H_2 receptor blockers were effective for the treatment of CHF, were also on β-blocker therapy [16], suggesting that there was an additive effect of histamine H_2 receptor and β-adrenergic receptor blockade in patients with CHF. In the present study, we demonstrated that the combination of a histamine H_2 receptor blocker and a β-blocker prevented the development of heart failure compared with β-blocker monotherapy. Thus, histamine H_2 receptor blockers have a potential clinical role in the treatment of CHF.

Rationale of the present study

The present study provided strong experimental evidence that histamine H_2 receptor blockade improves the pathophysiology of CHF. We have already reported about the beneficial effects of famotidine in patients with heart failure [16]. However, clinical research may be confounded by unexpected errors due to (1) the influence of other drugs being used by patients with CHF, (2) variation in the severity of CHF between patients, and (3) variation in the duration of CHF. Therefore, it was important to prove that histamine H_2 receptor blockade improves CHF in a controlled experimental setting (canine cardiomyopathy model) to support the clinical use of famotidine for CHF. Furthermore, to determine the merit of famotidine in heart failure patients, the present study would be a basis to design a prospective randomized double-blinded study.

Limitations

There are several limitations in this study. First, carvedilol blocks alpha$_1$-, beta$_1$-, beta$_2$- receptors, decreased the cardiac effects of norepinephrine, and has additional antioxidant and antiproliferative effects [4, 19]. In the present study, we did not address that carvedilol has the pleiotropic effects and is not just a beta-blocker.

Second, we did not measure cardiac output as a cardiac contractive index in the present study. However, we have previously reported that our tachycardia-induced heart failure model in dogs using the same procedure of the present study, revealed the low output state that mimics heart failure in human [27]. Our untreated dogs in the heart failure group were strongly suggested in the low output state because of decreased the level of ejection fraction as much as our previous study. In the present study, we analyzed the values of dP/dt as the index of contractility by measuring LV pressure using a pig tail catheter.

In summary, despite these limitations, we demonstrated that the histamine H_2 receptor blockade preserves cardiac systolic function in dogs with pacing-induced heart failure, even in the presence of β-adrenergic receptor blockade. This finding strengthens the rationale for the beneficial effects of histamine H_2 blockers in the treatment of heart failure.

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Conflict of interest None.

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Early Short-Term Vagal Nerve Stimulation Attenuates Cardiac Remodeling After Reperfused Myocardial Infarction

KAZUNORI UEMURA, MD, PhD,[1] CAN ZHENG, PhD,[2] MEIHUA LI, PhD,[1]
TORU KAWADA, MD, PhD,[1] AND MASARU SUGIMACHI, MD, PhD[1]

Suita, Japan; Nankoku, Japan

ABSTRACT

Background: Vagal nerve stimulation (VS) has been suggested to be an effective adjunct to reperfusion therapy in myocardial infarction (MI). However, the effect of VS on left ventricular (LV) remodeling after reperfused MI has not been examined.

Methods and Results: We investigated the effects of early, brief VS on acute inflammatory reactions (study 1) and chronic LV remodeling (study 2) in a rabbit model of reperfused MI. In study 1, rabbits were subjected to 60-minute coronary artery occlusion followed by reperfusion alone (MI, n = 8) or treated with 24-hour VS (MI-VS, n = 8). At 24 hours after ischemia-reperfusion, MI-VS rabbits showed significantly decreased myocardial infiltration of neutrophils and reduced myocardial expressions of tumor necrosis factor-α and matrix metalloproteinase-8 and -9, compared with MI rabbits. Myocardial expression of interleukin-6 was not affected by VS. In study 2, rabbits were subjected to coronary occlusion and reperfusion alone (n = 16) or treated with VS for 3 days (n = 14). At 8 weeks after ischemia-reperfusion, MI-VS rabbits showed significantly improved LV dysfunction and dilatation, and significantly reduced infarct size, infarct wall thinning, and LV weight compared with MI rabbits.

Conclusion: Early, short-term VS attenuates LV remodeling after reperfused MI, which may be associated with suppression of acute inflammatory reactions. (*J Cardiac Fail 2010;16:689–699*)

Key Words: Hypertrophy, cardiac function, acute inflammatory response, matrix metalloproteinase.

Left ventricular (LV) myocardial remodeling that occurs after myocardial infarction (MI) leads to progressive LV dilation and eventually pump dysfunction, and is one of the major determinants of long-term survival after MI.[1] In patients with acute MI, reperfusion of the ischemic tissue is the primary therapeutic strategy, and reduction of infarct size afforded by reperfusion contributes to improved clinical outcomes.[2] However, a substantial population of patients with MI still develops LV remodeling and heart failure even after reperfusion therapy.[1,2] Therefore some novel therapeutic modality should be developed as an adjunct to reperfusion therapy to attenuate chronic LV remodeling and improve the long-term outcome of MI patients.

A previous communication from our laboratory reported that electrical stimulation of vagal nerve (VS) for 6 weeks ameliorated LV remodeling and improved survival in a rat model of non-reperfused MI.[3] A small pilot clinical study demonstrated the beneficial effect of long-term, 6-month VS on LV function in patients with heart failure.[4] Several acute phase experimental studies also indicated the cardioprotective effects of VS in myocardial ischemia-reperfusion injury.[5–7] All these results strongly suggest that VS may be an effective adjunct to reperfusion therapy in patients with MI. However, the antiremodeling effect of VS remains poorly characterized and the effect on reperfused MI has not been examined.

Inflammatory cytokines are important mediators of LV remodeling after MI. Tumor necrosis factor-α (TNF-α) is an essential cytokine that is produced in significant quantities within the infarcted myocardium very soon after MI, and contributes to LV remodeling by inducing intense local inflammatory response, matrix metalloproteinase (MMP) activities, and matrix degradation.[8,9] VS has been shown

From the [1]Department of Cardiovascular Dynamics, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, Suita, Japan and [2]Department of Cardiovascular Control, Kochi Medical School, Nankoku, Japan.

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Reprint requests: Kazunori Uemura, MD, PhD. Department of Cardiovascular Dynamics, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita 565-8565, Japan. Tel: +81-6-6833-5012 (ext. 2414); Fax: +81-6-6835-5403. E-mail: kuemura@ri.ncvc.go.jp

See page 698 for disclosure information.

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to attenuate hepatic and cardiac TNF-α synthesis in splanchnic artery reperfusion injury or lethal endotoxemia.[10,11] We[12] and LaCroix et al[13] have demonstrated that VS induces expression of tissue inhibitor of MMP (TIMP)-1 and reduces active MMP-9 in ischemic myocardium. Although these findings suggest that VS favorably modulates the inflammatory reactions in MI, the effects of VS on inflammatory responses including the expression of cytokines and MMPs as well as its association with the inflammatory cell infiltrations have not been investigated in the setting of MI.

Long-term VS requires permanent implantation of the entire stimulating system.[3,4] However, hemodynamically unstable patients with acute MI are poor surgical candidates. On the other hand, early brief VS via an intravascular[14] or a transcutaneous approach[11] without compromising the hemodynamic conditions would be feasible in acute clinical settings. Taking all these together, the objective of this study was to investigate the effects of early short-term VS on LV function and myocardial structural remodeling in a rabbit model of reperfused MI and their association with acute inflammatory reactions.

Methods

Animals

We used a total of 56 Japanese white rabbits in this study (male, 2.5 to 3.0 kg). The investigation conforms with 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). All protocols were approved by the Animal Subjects Committee of the National Cardiovascular Center (approval number: 8042). Rabbits were assigned to the MI group (left coronary artery occlusion and reperfusion only; n = 24), MI-VS group (left coronary artery occlusion and reperfusion plus early short-term VS; n = 22), and normal control (NC) group (no treatment; n = 10).

Implantation of Vagal Nerve Electrode

Rabbits in the MI-VS group were implanted with vagal nerve electrodes. Under general anesthesia (sodium pentobarbital, 35 mg/kg^{-1}) and mechanical ventilation, a pair of polyurethane-coated stainless steel wires for electrical stimulation was looped around the right vagal nerve in the neck region.[3] The electrode wires were tunneled beneath the skin, exited in the midscapular area, and connected to a radio-controlled pulse generator (SRG-3100, Nihon Kohden, Japan) placed in a nylon jacket. Rabbits in the MI group underwent sham surgery without implanting the electrode. The animals were allowed to recover for at least 1 week before induction of MI (Fig. 1).

Induction of MI

MI was induced in rabbits of the MI and MI-VS groups (Fig. 1). Under general anesthesia (sodium pentobarbital, 35 mg/kg^{-1}) and mechanical ventilation with room air, a left thoracotomy was performed. A 4-0 proline suture was passed around the circumflex coronary artery, and a snare was formed by passing the ends of the thread through a small vinyl tube.[12] Electrodes to record surface electrocardiogram were implanted subcutaneously. The circumflex tourniquet was tightened to completely stop blood flow as demonstrated by both electrocardiogram changes and visual blanching of the myocardium. In the MI-VS group, we started vagal nerve stimulation immediately after coronary occlusion using rectangular pulses of 1-ms duration at 20 Hz for 10 seconds every minute.[3] We adjusted the amplitude of the pulse in each animal to reduce heart rate (HR) by 10% from baseline value. Consequently, the amplitudes ranged from 2 to 8 V. In a preliminary study, we confirmed that VS at this intensity did not alter feeding behavior and did not evoke any sign of pain reaction. After 60 minutes of coronary occlusion, the tourniquet was released, allowing reperfusion in both groups. The chest wall was then closed, and the animal was allowed to recover.

After recovery from anesthesia of MI surgery, we checked HR response to VS in MI-VS rabbits by monitoring electrocardiogram at least twice per day under conscious condition. We readjusted the intensity of VS if necessary, because the response varied from day to day in an individual rabbit.

Experimental Protocols

We performed 2 studies (Fig. 1). In study 1, VS was continued for 24 hours in MI-VS rabbits, and the effects of VS on myocardial inflammatory reactions at 24 hours after coronary reperfusion were examined because myocardial expression of TNF and MMP-9 as well as infiltration of neutrophil have been shown to peak at around 24 hours after myocardial ischemia reperfusion.[15] In study 2, VS was continued for 3 days in MI-VS rabbits, and LV function and structure were examined at 8 weeks after coronary reperfusion.

Study 1: Acute Phase after MI

Study 1 consisted of 3 groups of rabbits: MI (n = 8), MI-VS (n = 8), and NC (n = 3). At 24 hours after coronary reperfusion, animals in the MI and MI-VS groups were euthanized and whole hearts were harvested.

Western Blot. Myocardial tissue sample obtained from the LV lateral wall (infarct region in MI and MI-VS rabbits) was homogenized in RIPA lysis buffer (Rockland, Gilbertsville, PA) containing proteinase inhibitor (Complete Mini, Roche, Basel, Switzerland). The homogenate was centrifuged at 4 °C at 2000*g* for 10 minutes and the resultant supernatant was further subjected to centrifugation at 12,000*g* for 20 minutes. Protein concentration of each supernatant sample was determined using a DC Protein assay kit (BioRad Hercules, CA).

Samples containing equal amounts of protein (25 μg) were separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Bio-Rad) and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). After blocking the membranes with BlockAce (Dainippon Pharmaceutical, Japan), TNF-α was detected with polyclonal antibody for TNF-α (sc-1348, Santa Cruz, CA) and donkey-anti-goat HRP (sc-2020, Santa Cruz). Interleukin-1β (IL-1β) was detected with polyclonal antibody for IL-1β (LS-C7719, LifeSpan Biosciences, Seattle, WA) and goat-anti-rabbit HRP (sc-2004, Santa Cruz). MMP-1, MMP-7, MMP-8, and β-actin were detected using monoclonal antibodies for MMP-1 (F-67, Daiichi Fine Chemical, Japan), MMP-7 (F82, Daiichi Fine Chemical), MMP-8 (F-83, Daiichi Fine Chemical), and β-actin (sc-47778, Santa Cruz), respectively, and goat-anti-mouse HRP (sc-2005, Santa Cruz). Protein bands were visualized with ECL Plus (GE Healthcare, UK), and analyzed using a densitometric analysis software (CS Analyzer 3.0, ATTO, Japan). Band densities were standardized to β-actin, and presented as percent

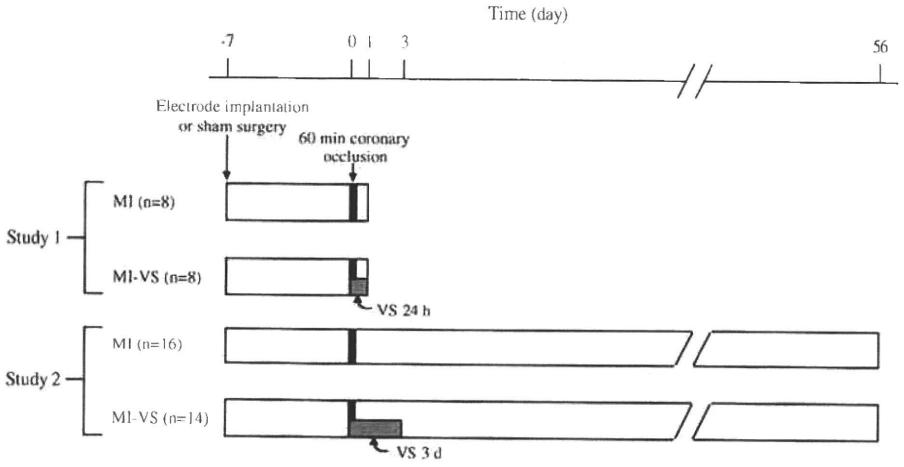

Fig. 1. Schematic representation of the protocols of Study 1 (acute phase myocardial infarction and reperfusion) and Study 2 (chronic phase after creation of reperfused myocardial infarction). MI, rabbits with reperfused myocardial infarction; MI-VS, MI rabbits treated with vagal nerve stimulation. Three rabbits in Study 1 and 7 rabbits in Study 2 were used as normal controls (not shown in this figure).

change compared with NC values, the means of which were arbitrarily set as 100%.

Enzyme-Linked Immunosorbent Assay. Myocardial tissue sample obtained from the LV lateral wall (infarct and peri-infarct regions in MI and MI-VS rabbits) was homogenized and processed as described previously.

Enzyme-linked immunosorbent assays for C-reactive protein (CRP) (KT-097, Kamiya Biomedical Company, Seattle, WA) and TIMP-1 (RPNJ409, Daiichi fine chemical) were performed using the supernatants of myocardial tissue homogenates from the infarct region according to the manufactures' instructions.[12] Enzyme-linked immunosorbent assays for IL-6 (900-033, Assay Designs, Ann Arbor, MI) was performed using the supernatants of myocardial tissue homogenates from the peri-infarct region. The reason for using the peri-infarct region instead of the infarct region for IL-6 measurement was based on a previous finding that IL-6 mRNA is intensely induced in myocytes of the viable border zone, and not the necrotic infarct zone in myocardial ischemia-reperfusion.[16]

Gelatin Zymography. Tissue sample from the LV lateral wall was homogenized in lysis buffer (50 mM Tris, pH 7.4). The homogenate was centrifuged at 2000*g* for 10 minutes at 4 °C and the supernatant was collected. Protein concentration of each supernatant sample was determined as described previously.

Gelatin zymography was performed to assess the relative contents of the gelatinases MMP-2 and MMP-9 as described previously.[12] The supernatants (30 μg protein) were loaded in Novex gels containing 0.1% gelatin (Invitrogen, Carlsbad, CA) and then electrophoresed. After renaturation, equilibration, and incubation for 20 hours at 37 °C in developing buffer, the gels were stained in 0.5% Coomassie Blue G-250. Gels were dried and scanned. MMP-2– and MMP-9–related bands were analyzed using the densitometric analysis software.

Determination of Neutrophil Infiltration. The middle ring slice of LV was embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin. We counted the numbers of neutrophils per field in the infarcted area. Neutrophil infiltration into ischemic myocardium was also quantified by evaluating myeloperoxidase activity. Tissue sample from the LV lateral wall was homogenized in potassium phosphate buffer (50 mM, pH 6.4) containing 0.5% hexadecyltrimethylammonium bromide (WAKO, Japan). The homogenate was centrifuged at 4 °C at 12,000*g* for 10 minutes and the resultant supernatant was reacted with 0.167 mg/mL of o-dianisidine dihydrochloride (Sigma-Aldrich, St. Louis, MO) and 0.0005% H_2O_2 in potassium phosphate buffer. The change in absorbance at 450 nm was measured spectrophotometrically over 2 minutes. One unit of myeloperoxidase activity was defined as that quantity of enzyme that hydrolyzed 1 μM of peroxide per minute at 25 °C.

Study 2: Chronic Phase after MI

Study 2 consisted of 3 groups of rabbits: MI (n = 16), MI-VS (n = 14), and NC (n = 7).

Echocardiography. Echocardiography was performed under conscious condition at baseline and at 3 days and 8 weeks after coronary reperfusion. Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 7-MHz transducer (Power Vision, TOSHIBA, Japan). LV dimensions were measured according to the American Society for Echocardiography leading-edge method for at least 3 consecutive cardiac cycles. Fractional shortening was calculated as (LVEDD-LVESD)/LVEDD×100, where LVEDD is LV end-diastolic diameter and LVESD is LV end-systolic diameter.

Hemodynamic and Plasma MMPs Measurements. Hemodynamic measurements were performed at 8 weeks after reperfusion. Under general anesthesia (sodium pentobarbital, 35 mg/kg^{-1}) and mechanical ventilation with room air, a 3F micromanometer-tipped catheter (Millar Instruments, Houston, TX) was inserted into the right carotid artery for measurement of mean arterial pressure. Next, the catheter was advanced into the LV for measurement of LV pressure. After completing these measurements, blood was sampled from the right carotid artery. The animal was euthanized. The whole heart was quickly excised and washed with cold phosphate-buffered saline.

Relative protein contents of MMP-2 and MMP-9 in plasma were measured with use of the gelatin zymography as described previously.

LV Passive Pressure-volume Relationship. The excised heart was perfused with a cold, hypocalcemic, hyperkalemic cardioplegic solution (NaCl: 130 mM, KCl: 20 mM, $CaCl_2$: 0.08

mM, lidocaine: 0.5 μg/mL; pH 7.3; 310 mOsm). The passive LV pressure-volume relationship of the arrested heart was measured as described previously.[17] In brief, a compliant water-filled latex balloon tied on a rigid Y-connector was placed in the left ventricle and secured at the mitral annulus with a purse-string suture. Pressure within each balloon was measured with a catheter-tipped micromanometer (Millar Instruments) as volume was progressively increased. Pressure was then plotted as a function of volume at each step, resulting in a passive pressure-volume relationship equivalent to the end-diastolic pressure-volume relationship of the beating heart.[17] The size of the left ventricle was indexed by the volume at which LV pressure reached 10 mm Hg (LVV_{10}).

Infarct Characterization. The coronary branch was reoccluded and 5 mL 0.25% Evans blue was injected from the aorta at 80 mm Hg. After the vasculature, right ventricular free wall, and atrial appendages were dissected, the left ventricle was weighed and fixed in 4% paraformaldehyde overnight. The left ventricle below the coronary artery ligation site was cut into ~5 transverse slices parallel to the atrioventricular ring. Each slice was photographed. The risk area unstained by the blue dye and the non-risk area stained by the blue dye were demarcated. For each slice, the risk area size was determined as the total circumference of the risk area divided by total LV circumference (in percent). The risk area sizes of all slices were averaged and expressed as the risk area size for each heart. The fixed slices from the apex, middle ring, and base were then embedded in paraffin and sectioned at a thickness of 5 μm.

The 5-μm thick cross-sections of left ventricle were stained with Masson's trichrome and Sirius red. Using sections stained with Masson's trichrome, infarct size was determined as the total infarct circumference divided by total LV circumference (in percent). The thicknesses of septal (non-infarct area) and LV lateral walls (infarct area) were measured. The thinning ratio, an index of the extent of wall thinning in the infract, is calculated by dividing the infarct wall thickness by septal wall thickness. Cardiomyocyte cross-sectional areas were determined in the non-infarcted septal myocardium. Only cardiomyocytes cut in cross section were measured. Using sections stained with Sirius red, collagen densities in noninfarcted regions were determined as described previously.[18] Histological images were obtained with a microscope system (BZ 9000, Keyence, Japan), and analyzed using the National Institutes of Health Image software (Image J 1.37). The infarct sizes of the 3 sections (apex, middle ring, base) were averaged and expressed as the LV infarct size for each heart. The thickness of LV wall, cardiomyocyte cross-sectional area, and collagen density were determined in the section that most clearly transverses the infarct region.

Statistical Analyses

All data are presented as mean ± SEM values. Mortalities in the MI and MI-VS groups were compared using chi-square test. Between-group comparison of means obtained at a single time point was performed by Student's unpaired *t*-test or 1-way analysis of variance. Between-group comparisons of the changes of means over time were conducted using two-way repeated measure analysis of variance to examine any group-time effect. All analyses of variance showing significant differences were further analyzed by post hoc comparison using Student-Newman-Keuls test (Statistica, Statsoft, Inc., Tulsa, OK). *P* values less than .05 were considered statistically significant.

Results

Study 1: Acute Phase after MI

Body Weight and Mortality. Baseline body weight were comparable among the NC (2490 ± 38 g), MI (2656 ± 64 g), and MI-VS (2517 ± 31 g) groups. Two MI and 2 MI-VS rabbits died from arrhythmia at MI induction. There was no difference in mortality rate up to 24 hours after coronary reperfusion between the MI and MI-VS groups (25% vs. 25 %, P = NS).

HR. Changes of HR over time are summarized in Table 1. There were no significant differences in HR between the MI and MI-VS groups at baseline, after 30 minutes of coronary occlusion, and at 24 hours after coronary reperfusion. No significant time effects on HR were observed.

Myocardial Expression of Cytokines, MMPs, and CRP. Figure 2A shows representative Western blots for TNF-α in the infarcted myocardium. Densitometric analysis demonstrated that TNF-α protein level increased significantly in the MI and MI-VS groups compared with NC value, whereas TNF-α level in the MI-VS group was significantly lower than that in the MI group (<50%, $P < .05$, Fig. 2A). Myocardial protein expressions of IL-1β and IL-6 are summarized in Table 2. There were no significant differences in IL-1β level among the 3 groups. Myocardial levels of IL-6 increased significantly to similar degrees in the MI and MI-VS groups compared with NC values.

As shown in Fig. 2B, zymography of the myocardial extracts detected 2 bands at 92 kDa and 72 kDa corresponding to pro-MMP-9 and pro-MMP-2, respectively. In 2 of 11 MI hearts, but in none of 10 MI-VS hearts, a faint gelatinolytic band was also observed at 83 kDa, which presumably represents the active form of MMP-9.[19] To evaluate the relative content of MMP-9, we used the 92 kDa band (pro-MMP-9) because this band is representative of the global MMP-9 activity.[20] Relative MMP-9 level increased significantly in the MI and MI-VS groups compared with NC value. Level of MMP-9 in the MI-VS group was significantly lower than that in the MI group (<40%, $P < .05$, Fig. 2B). There were no significant differences in MMP-2 (pro-MMP-2) protein level among the 3 groups. Western

Table 1. Changes of Heart Rate with Time after Myocardial Ischemia-reperfusion (Studies 1 and 2)

	Baseline	30 Minutes	24 Hours	3 Days
Study 1				
MI	(n = 6)	277 ± 8	244 ± 24	257 ± 12
MI-VS	(n = 6)	256 ± 8	243 ± 6	250 ± 7
Study 2				
MI	(n = 11)	268 ± 10	245 ± 10	309 ± 11*,†
MI-VS	(n = 10)	264 ± 22	224 ± 11	313 ± 15†

MI, myocardial infarction; MI-VS, myocardial infarction treated with vagal nerve stimulation; 30 minutes, 30 minutes after coronary occlusion; 24 hours, 24 hours after coronary reperfusion; 3 days, 3 days after coronary reperfusion.

Heart rate data (beat/min) are means ± SEM.

*$P < .05$ versus baseline.

†$P < .01$ versus 30 minutes.

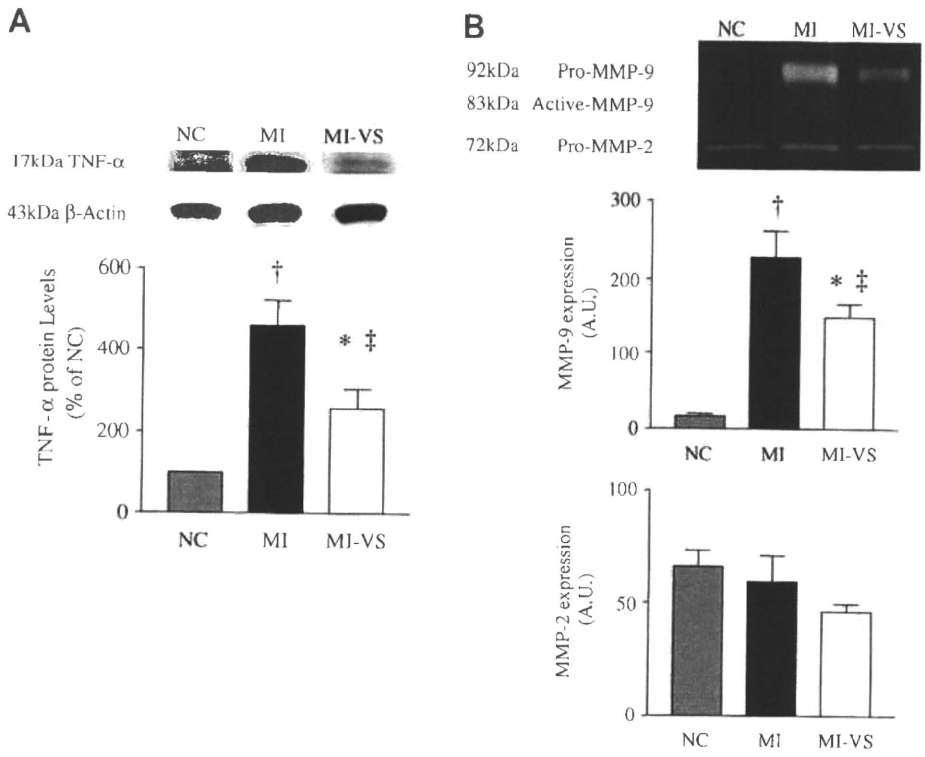

Fig. 2. Myocardial protein expressions of tumor necrosis factor-α (TNF-α) and matrix metalloproteinases (MMPs) in normal control rabbits (NC, n = 3), rabbits with reperfused myocardial infarction (MI) (n = 6), and MI rabbits treated with vagal nerve stimulation (MI-VS) rabbits (n = 6). (A) Representative Western blots of TNF-α (17 kDa) as well as corresponding β-actin bands (43 kDa) and band intensities normalized to NC values are shown. (B) Representative zymogram shows pro-MMP-9 band at 92 kDa and pro-MMP-2 band at 72 kDa. Note the faint 83 kDa band in MI hearts, which represents active MMP-9. Densitometric analysis of MMP-9 and MMP-2 contents expressed in integrated optical density (A.U.) relative to background. Data are means ± SEM. $^{*}P < .05$, $^{\dagger}P < .01$ versus NC. $^{\ddagger}P < .05$ versus MI.

blot analysis of other species of MMPs is summarized in Table 2. Myocardial protein levels of MMP-1 (50 kDa, pro-MMP-1) and MMP-7 (28 kDa, pro-MMP-7) decreased significantly to similar degrees in the MI and MI-VS groups compared to NC values. Myocardial protein level of MMP-8 (75 kDa, pro-MMP-8) increased significantly in the MI group compared with NC and MI-VS values (Table 2).

Table 2. Myocardial Protein Expression (Study 1)

	NC (n)	MI (n)	MI-VS (n)
IL-1β, % of NC	100 ± 6 (3)	79 ± 8 (6)	105 ± 25 (5)
IL-6, ng/g protein	52 ± 2 (3)	100 ± 7 (3) *	97 ± 13 (4)*
MMP-1, % of NC	100 ± 6 (3)	54 ± 8 (6) †	71 ± 12 (6)*
MMP-7, % of NC	100 ± 18 (3)	40 ± 10 (3) *	18 ± 2 (3)†
MMP-8, % of NC	100 ± 15 (3)	510 ± 82 (3) †	236 ± 65 (3)‡
TIMP-1, ng/g protein	39 ± 1 (3)	1394 ± 101 (6)†	1387 ± 164 (6)†
CRP, μg/g protein	13 ± 3 (3)	1926 ± 225 (6)†	1741 ± 114 (6)†

IL, interleukin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; CRP, C-reactive protein.

Data are means ± SEM. The number of hearts used for each experiment is given in parentheses.

$^{*}P < .05$.

$^{\dagger}P < .01$ versus NC.

$^{\ddagger}P < .05$ versus MI.

Myocardial level of TIMP-1 protein increased significantly to similar degrees in MI and MI-VS groups compared with NC values (Table 2).

Myocardial level of CRP increased significantly to similar degrees in the MI and MI-VS groups compared with NC values (Table 2).

Neutrophil Infiltration. No myocardial neutrophil infiltration was found in NC rabbits, whereas intense neutrophil infiltration into the infarcted myocardium was observed in MI rabbits (Fig. 3A). On the other hand, a significantly reduced neutrophil density in the infarcted myocardium was evident in MI-VS rabbits compared with MI animals (Fig. 3A, B). In accordance with neutrophil counts, myeloperoxidase activity in MI-VS rabbits was significantly reduced compared with MI animals (Fig. 3C).

Study 2: Chronic Phase after MI

Body Weight and Survival. Baseline body weights were comparable among the NC (2693 ± 184 g), MI (2530 ± 38 g), and MI-VS (2462 ± 24 g) groups. At 3 days after coronary reperfusion, body weights decreased to the same extent in both the MI (2325 ± 38 g) and MI-VS (2361 ± 53 g) groups. At 8 weeks after reperfusion, body weights increased to similar degrees in the MI (2843 ± 69 g) and

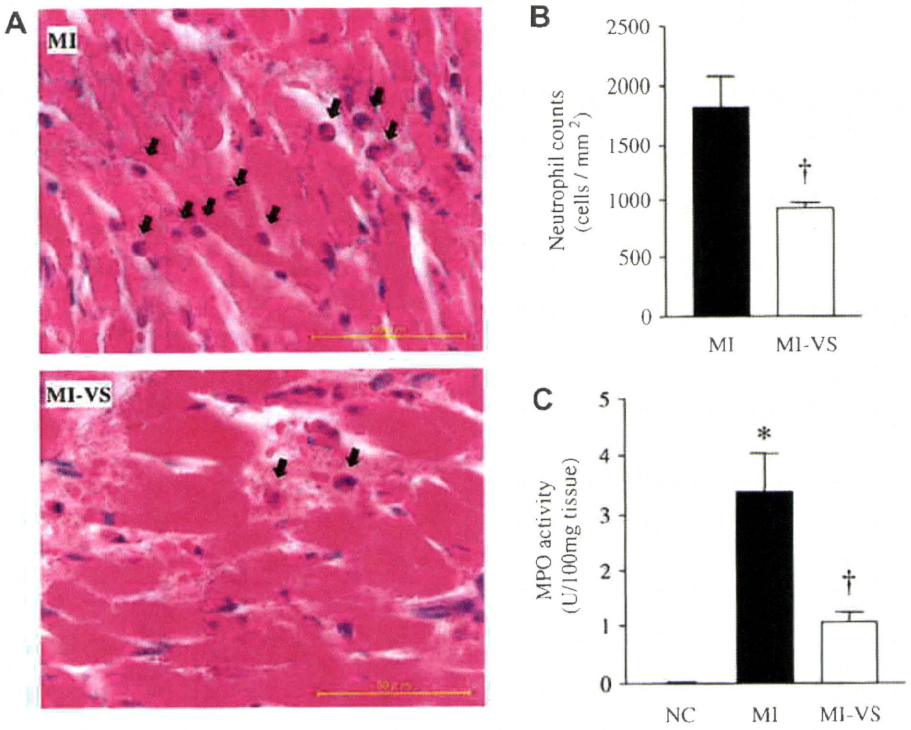

Fig. 3. (A) Photomicrographs of hematoxylin-eosin stained left ventricle cross-sections in infarcted regions obtained from rabbits with reperfused myocardial infarction (MI) and MI rabbits treated with vagal nerve stimulation (MI-VS) rabbits at 24 hours after coronary reperfusion. Arrows indicate infiltrating neutrophils. Bars = 50 μm. (B) Neutrophil counts of sections in infarct regions from MI (n = 6) and MI-VS (n = 6) rabbits. (C) Myocardial myeloperoxidase (MPO) activity in NC rabbits (n = 3), and in the infarct regions from MI (n = 4) and MI-VS (n = 4) rabbits. $^{*}P < .01$ versus NC, $^{\dagger}P < 0.01$ versus MI.

MI-VS (2899 ± 53 g) groups compared with the respective baseline values.

Five MI and four MI-VS rabbits died and the mortality rate up to 8 weeks after coronary reperfusion was comparable between the MI and MI-VS groups (31% versus 29%, P = NS). Of these deaths, 2 MI (13%) and 3 MI-VS (21%) rabbits died from arrhythmia at MI induction (P = NS).

Hemodynamics and LV Function. HR at 3 days after coronary reperfusion significantly increased in both the MI and MI-VS groups from their respective baseline values (Table 1). There were no significant differences in HR between the MI and MI-VS groups at baseline, after 30 minutes of coronary occlusion, and at 3 days after coronary reperfusion.

Baseline LV diameters and fractional shortening were similar in the MI and MI-VS groups (Table 3). At 3 days after coronary reperfusion, LV fractional shortening was reduced and LVESD was increased from baseline in both the MI and MI-VS groups to similar degrees. However, further deterioration of LV fractional shortening at 8 weeks after reperfusion observed in MI rabbits was prevented in MI-VS rabbits (Table 3). At 8 weeks after coronary reperfusion, MI-VS rabbits showed significantly smaller LVESD and LVEDD compared with MI rabbits. Data of invasive hemodynamic study are summarized in Table 4. Because 2 rabbits in the MI group with severely depressed LV function (LVEDD >20 mm, LV fractional shortening <14%) developed cardiac arrest during the induction of anesthesia, they were excluded from the invasive hemodynamic study. LV end-diastolic pressure was significantly increased in MI rabbits, which was significantly attenuated in MI-VS rabbits.

LV Passive Pressure-volume Relationship and LV Weight. Plots of ex vivo LV passive pressure-volume relationship are shown in Fig. 4A. A marked difference

Table 3. Changes in Echocardiographic Parameters with Time (Study 2)

	Baseline	3 Days	8 Weeks
LVESD, mm			
MI	7.8 ± 0.2	10.9 ± 0.2†	15.5 ± 0.6†,§
MI-VS	7.9 ± 0.4	9.8 ± 0.5*	11.4 ± 1.1$^{\dagger,\#}$
LVEDD, mm			
MI	13.0 ± 0.3	14.6 ± 0.3	19.2 ± 0.6†,§
MI-VS	12.8 ± 0.3	13.3 ± 0.5	15.5 ± 1.0$^{\dagger,\ddagger,\#}$
FS, %			
MI	39.9 ± 1.3	24.9 ± 1.5†	19.6 ± 1.6†,‡
MI-VS	38.6 ± 1.9	27.1 ± 1.2†	27.2 ± 2.6$^{\dagger,\|\|}$

3 d, 3 days after coronary reperfusion; 8 w, 8 weeks after coronary reperfusion; LVESD, left ventricular (LV) end-systolic diameter; LVEDD, LV end-diastolic diameter; FS, LV fractional shortening.

Data are means ± SEM.

n = 11 in MI group.

n = 10 in MI-VS group.

$^{*}P < .05$.

$^{\dagger}P < .01$ versus baseline.

$^{\ddagger}P < .05$.

$^{\S}P < .01$ versus 3 days.

$^{\|}P < .05$.

$^{\#}P < .01$ versus MI.

Table 4. Invasive Hemodynamic Parameters 8 Weeks after Coronary Reperfusion (Study 2)

	NC	MI	MI-VS
HR, beat/min	335 ± 14	320 ± 5	322 ± 5
MAP, mm Hg	110 ± 3	112 ± 3	112 ± 4
LV dP/dt_{max}, mm Hg/s	4622 ± 234	4546 ± 229	4770 ± 348
LV EDP, mm Hg	4 ± 1	16 ± 3*	7 ± 2†

NC, normal control; HR, heart rate; MAP, mean arterial pressure; LV dP/dt_{max}, the maximum first derivative of left ventricular pressure; LV EDP, left ventricular end-diastolic pressure.

Data are means ± SEM.

n = 7 in NC group, n = 9 in MI group, n = 10 in MI-VS group.

*$P < .01$ versus NC.

†$P < .05$ versus MI.

between NC and MI hearts is evident, whereas the average curve derived from MI-VS hearts is close to that of NC hearts. As shown in Fig. 4B, LV size, indexed by LVV_{10}, of MI-VS hearts was significantly smaller than that of MI hearts ($P < .01$) and reached values close to those of NC hearts. LV weight normalized by body weight increased significantly in both MI and MI-VS hearts compared with NC value (Fig. 4C), but was significantly lower in MI-VS hearts than in MI hearts ($P < .01$).

Histomorphologic Analysis of LV. The risk area sizes were comparable in MI and MI-VS hearts (47 ± 3% vs. 51 ± 5%, P = NS). A transverse LV section demonstrated a smaller LV cavity in the heart receiving VS (Fig. 5A). The LV infarct size was significantly reduced in MI-VS hearts ($P < .05$) compared with MI hearts as shown in Fig. 5B. Because the risk area sizes were comparable in MI and MI-VS hearts, the reduction of infarct size seen in MI-VS rabbits was due to the VS treatment, not insufficient ischemic insults. Wall thickness in LV septum (non-infarct region) was comparable in MI and MI-VS hearts, whereas LV infarct wall thickness was significantly greater in MI-VS hearts than in MI hearts. This resulted in higher thinning ratios in MI-VS hearts than in MI hearts (Fig. 5B). Myocyte hypertrophy in the septum was attenuated in MI-VS hearts as demonstrated by significantly reduced myocyte cross-sectional area compared with MI hearts. Collagen densities in viable myocardial tissue were similar in MI and MI-VS hearts (11 ± 1% versus 9 ± 0%, P = NS).

Plasma MMP. Relative MMP-9 level in plasma was comparable among NC (156 ± 19 A.U., n = 6), MI (147 ± 28 A.U., n = 7), and MI-VS (146 ± 22 A.U., n = 7) groups. Relative MMP-2 level in the MI-VS group (164 ± 20 A.U.) was significantly lower compared with those in the NC (226 ± 17 A.U.) and MI (230 ± 16 A.U.) groups ($P < .05$).

Subgroup Analysis of Effects of VS on LV Remodeling in Large MI. Because the progression of LV remodeling is problematic, especially in patients with large infarct

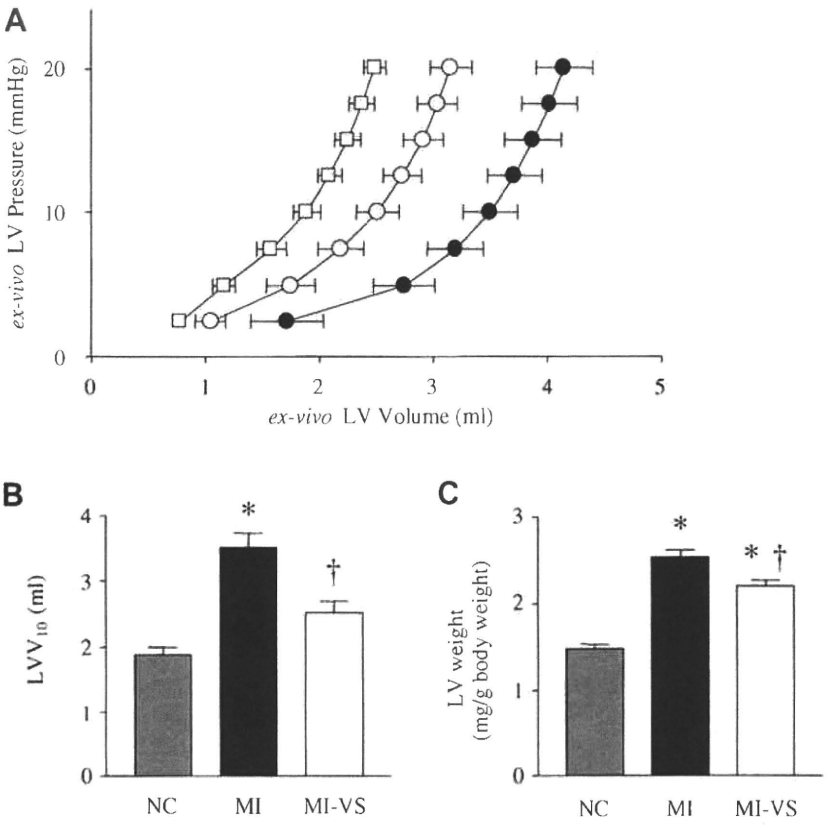

Fig. 4. Ex vivo left ventricular (LV) examinations of normal control (NC) (n = 7), reperfused myocardial infarction (MI) (n = 11), and MI rabbits treated with vagal nerve stimulation (MI-VS) (n = 10) hearts. (A) LV passive pressure-volume relationship in NC (□), MI (●), and MI-VS (○) hearts. (B) Ex vivo LV volume at LV pressure of 10 mm Hg (LVV_{10}). (C) LV weight normalized by body weight. *$P < .01$ versus NC. †$P < .01$ versus MI.

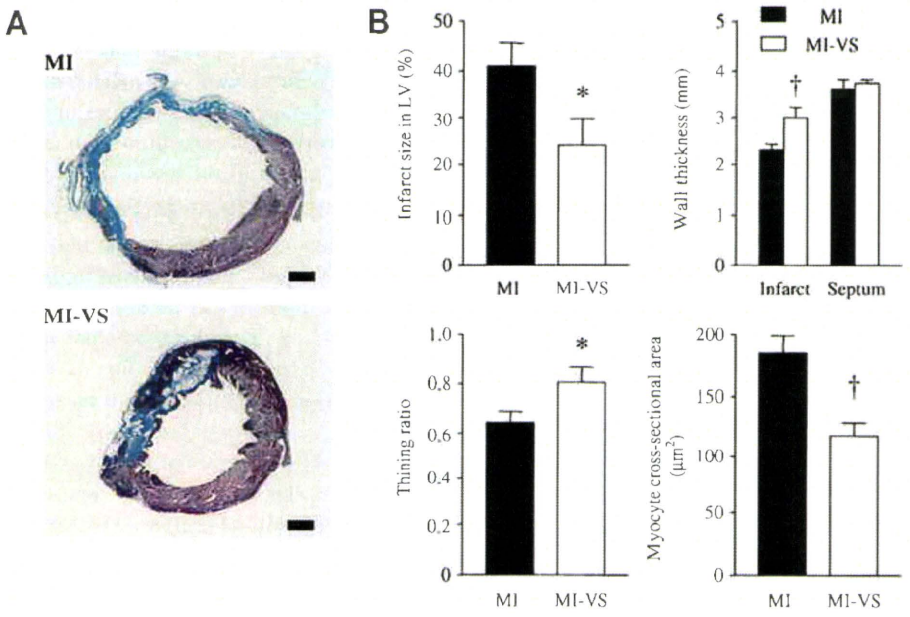

Fig. 5. (A) Transverse LV sections obtained from reperfused myocardial infarction (MI) and MI rabbits treated with vagal nerve stimulation (MI-VS) hearts 8 weeks post-MI. The sections were stained with Masson's trichrome. Blue stained area indicates scared infarct area. Bars = 3 mm. (B) Histomorphometric analyses of LVs in MI (n = 11) and MI-VS (n = 10) hearts. $^{*}P < .05$, $^{\dagger}P < .01$ versus MI.

size,[1] we evaluated the effects of VS on the parameters of LV remodeling (LV weight and LVEDD at 8 weeks after coronary reperfusion) in animals with large infarct size (>30% of LV, 8 MI. and 4 MI-VS rabbits). LV infarct size was comparable in MI and MI-VS hearts (49 ± 3% versus 44 ± 6%, P = NS). However, LV weight was significantly lower in MI-VS hearts than in MI hearts (2.2 ± 0.1 versus 2.6 ± 0.1 mg/g body weight, $P < .05$). There was a strong trend of reduction in LVEDD in MI-VS hearts compared with MI hearts, although the difference did not reach statistical significance (15.1 ± 2.0 versus 18.9 ± 0.8 mm, $P = .052$).

Discussion

The major new findings of the present study were as follows. In the acute inflammatory phase of reperfused MI, VS decreased TNF-α protein level and suppressed neutrophil infiltration in the infarcted myocardium. In the chronic phase of reperfused MI, VS markedly attenuated LV dysfunction and remodeling, even though VS was limited to a short period early after MI. Although several acute experimental studies examined the cardioprotective effects of VS in reperfused MI, they lacked detailed assessment of LV function and structure as done in this study.[5–7]

Cardioprotective Effects of VS and Cytokine Expressions

The beneficial effects of VS on reperfused MI are primarily attributable to the reduction of infarct size.[1,21] Several cardioprotective mechanisms of VS in ischemic myocardium have been reported previously. In a neutrophil-free isolated heart preparation, VS attenuated ischemia-reperfusion injury by protecting the myocytic mitochondria.[5] In a non-reperfused MI model, VS protected cardiomyocytes by upregulating hypoxia-inducible factor-1α pathway, and reduced infarct size.[22] Acetylcholine, the principal vagal neurotransmitter, mediated these direct protective effects on cardiomyocyte through the muscarinic acetylcholine receptor pathway.[5,22] These mechanisms may have contributed to the reduction of infarct size by VS in the present study. In addition, suppression of myocardial neutrophil infiltration in acute MI phase might also contribute to the reduction of infarct size by VS. Although reperfusion of the ischemic myocardium is necessary to salvage viable myocytes from eventual death, reperfusion also causes tissue damage.[9] Reperfusion injury in the acute phase of MI shares many characteristics with inflammatory reactions. Neutrophils feature prominently in this inflammatory reaction.[9] It is well known that in the reperfused ischemic myocardium, upregulated TNF-α accelerates the infiltration of neutrophils.[8,23] In this study, VS suppressed neutrophil infiltration into the infarcted myocardium possibly through inhibition of TNF-α expression, which might suppress the neutrophil-induced myocyte injury, thereby reducing the infarct size. Acetylcholine attenuates the release of TNF-α from macrophages through the nicotinic acetylcholine receptor pathway.[10,11] Cardiac mast cell is an important source of TNF-α in ischemic myocardium,[23] and expresses the nicotinic receptor.[24] In our model, VS might have directly reduced TNF-α expression on cardiac mast cells via the nicotinic acetylcholine receptor pathway.

Myocardial IL-1β protein content was not changed in our MI model. Previous experimental study also reported

similar results and suggested an insignificant role of IL-1β as an upstream inducer of post-reperfusion inflammatory reactions.[23] Myocardial IL-6 protein expression was not affected by VS in this study. Previous clinical study indicated that VS decreased plasma IL-6 concentration in patients with advanced heart failure, but the reduction was transient and noted only at 3 months after commencing VS.[4] Although the increase in plasma IL-6 has been associated with progression of LV contractile dysfunction in patients with heart failure, recent experimental research suggests that IL-6 expression in viable myocardium may play a pivotal role in cytoprotection.[25] Several biochemical and hemodynamic factors have been shown to regulate IL-6 expression after MI. IL-6 mRNA expression in mononuclear cells infiltrating the infarcted myocardium is upregulated by TNF-α.[23] Gwechenberger et al[16] have shown the reperfusion-dependent expression of IL-6 mRNA in cardiomyocytes in the viable border zone. Perfusion dependency of IL-6 expression was also observed in patients with reperfused MI, where plasma IL-6 concentration positively correlated with ischemic myocardial collateral flow.[26] In the present study, VS-induced TNF-α reduction might have decreased myocardial IL-6 expression. On the other hand, the beneficial effect of VS on myocardial perfusion[27] or the direct cardiomyocyte-protecting effect of VS[5] might have increased de novo synthesis of IL-6 in the jeopardized but viable myocardium. In VS, all these factors may operate simultaneously.

Myocardial CRP expression increased drastically after MI, which was not affected by VS in this study. After MI, CRP is produced from the liver partly as a response to stimulation by IL-6 released from the damaged heart.[28] Because we found that cardiac expression of IL-6 was similar in the MI and MI-VS groups, it is reasonable that myocardial CRP content was not different between the 2 groups in the present experiment. CRP-mediated complement activation in the myocardium was associated with increase in infarct size and LV remodeling after MI in several experimental and clinical studies,[28,29] although this association remains controversial.[30,31]

Our previous studies suggest that bradycardia plays a significant role in cardioprotection by VS.[3] However, the present results indicate that the cardioprotective effect of VS does not necessarily require strong bradycardia. Huston et al demonstrated that in a mouse model of sepsis, mild intensity of VS drastically reduces serum TNF-α without inducing bradycardia.[11] Furthermore, the cardioprotective effect of VS through protecting myocytic mitochondria is also independent of the degree of bradycardia.[5] Increasing VS intensity to attain strong bradycardia can cause unwanted side effects such as local pain in animals and also in patients.[3,4] Although the degree of bradycardia has been the primary parameter used in adjusting the intensity of VS,[4] additional parameters may be required for deciding the proper therapeutic strategy of VS in clinical application.

MMP Inhibition and LV Remodeling. Subgroup analysis of study 2 demonstrated that VS attenuated LV hypertrophy when comparing MI and MI-VS hearts with similarly large infarct size. This finding indicates that VS indeed confers an antiremodeling effect beyond that through reduction in infarct size. Reduction of MMP-9 activity in the infarcted myocardium early after MI may contribute to this antiremodeling effect of VS.[18,32–34] In addition to the loss of contractile cardiomyocytes, pathological degradation and reconstitution of extracellular matrix contribute to the progression of LV remodeling after MI, where MMP and TIMP play crucial roles. After MI, pharmacological MMP inhibition attenuated LV remodeling without affecting infarct size even if given for a short period.[18,33] Suppression of the infiltration of neutrophil, an important source of MMP-9 after myocardial ischemia reperfusion,[32] may be one reason of the reduction of MMP-9 contents by VS in infarct observed in this study. VS did not change the collagen content of the viable myocardium at 8 weeks after MI in spite of reduced MMP-9 expression early after MI. Several studies also reported that MMP inhibition improved LV remodeling without changing the collagen contents within the infarcts or in viable tissues after MI.[18,33] Lindsey et al demonstrated in a rabbit model of MI that inhibition of MMP activities attenuated LV dilatation and preserved the infarct wall thickness and the thinning ratio without changing the tissue collagen content.[33] Their findings are almost compatible to the present results. These observations suggest that a noncollagenolytic mechanism may also play a critical role in regulating LV remodeling. Plasma MMP-9 concentration measured 8 weeks after MI was not reduced in MI-VS rabbits compared with MI rabbits and controls. This indicates that the MMP-9 suppressive effect of VS delivered early after MI did not last long. This might be beneficial in terms of LV remodeling. A previous experimental study[34] demonstrated that MMP inhibition early after MI conferred beneficial effects on LV remodeling, but chronic prolonged MMP inhibition was associated with adverse effects on LV remodeling. MMP-8, a neutrophil collagenase, has been shown to increase in cardiac tissue after MI and relate to LV rupture in MI patients.[35] In this study, suppression of neutrophil infiltration is probably the primary reason for the reduction of myocardial MMP-8 contents by VS.

MMP-1 is mainly produced by cardiac fibroblasts. MMP-2 is mainly expressed in cardiomyocytes after ischemic injury.[35] MMP-7 is expressed in macrophages and cardiomyocytes after MI.[36] After reperfused MI, myocardial protein contents of MMP-1, -2, and -7 were not affected by VS. Taken together, VS appears to specifically affect neutrophil-associated MMPs.

Myocardial TIMP-1 protein level was not affected by VS in this study, which seems inconsistent with our previous finding.[12] TIMP-1 level increased drastically after 24 hours of coronary reperfusion in the present study (>1200 ng/g protein, compared with that observed after 3 hours of reperfusion in rabbits in our previous study (<500 ng/g protein).[12] On the other hand, the intensity of VS in this study was rather mild compared with that in the previous