Efferent vagal nerve stimulation induces tissue inhibitor of metalloproteinase-1 in myocardial ischemia-reperfusion injury in rabbit

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Departments of 1Cardiovascular Dynamics and 2Cardiac Physiology, National Cardiovascular Center Research Institute, Suita, Japan; and 3Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Science, Fukuoka, Japan

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Abstract

Efferent vagal nerve stimulation induces tissue inhibitor of metalloproteinase-1 in myocardial ischemia-reperfusion injury in rabbit. Am J Physiol Heart Circ Physiol 293:H2254–H2261, 2007. First published August 10, 2007; doi:10.1152/ajpheart.00490.2007.—Vagal nerve stimulation has been suggested to ameliorate left ventricular (LV) remodeling in heart failure. However, it is not known whether and to what degree vagal nerve stimulation affects matrix metalloproteinase (MMP) and tissue inhibitor of MMP (TIMP) in myocardium, which are known to play crucial roles in LV remodeling. We therefore investigated the effects of electrical stimulation of efferent vagal nerve on myocardial expression and activation of MMPs and TIMPs in a rabbit model of myocardial ischemia-reperfusion (I/R) injury. Anesthetized rabbits were subjected to 60 min of left coronary artery occlusion and 180 min of reperfusion with (I/R-VS, n = 8) or without vagal nerve stimulation (I/R, n = 7). Rabbits not subjected to coronary occlusion with (VS, n = 7) or without vagal stimulation (sham, n = 7) were used as controls. Total MMP-9 protein increased significantly after left coronary artery occlusion in I/R-VS and I/R to a similar degree compared with VS and sham values. Endogenous active MMP-9 protein level was significantly lower in I/R-VS compared with I/R. TIMP-1 mRNA expression was significantly increased in I/R-VS compared with the I/R, VS, and sham groups. TIMP-1 protein was significantly increased in I/R-VS and VS compared with the I/R and sham groups. Cardiac microdialysis technique demonstrated that topical perfusion of acetycholine increased dialysate TIMP-1 protein level, which was suppressed by coperfusion of atropine. Immunohistochemistry demonstrated a strong expression of TIMP-1 protein in cardiomyocytes around the dialysis probe used to perfuse acetycholine. In conclusion, in a rabbit model of myocardial I/R injury, vagal nerve stimulation induced TIMP-1 expression in cardiomyocytes and reduced active MMP-9 in myocardial I/R injury in rabbit.

METHODS

We used 49 Japanese white rabbits in this study (male, 2.5–3.0 kg). Care of the animals was in strict accordance with the guiding principles of the Physiological Society of Japan. All protocols were approved by the Animal Subjects Committee of the National Cardiovascular Center.

I/R Study

Experimental preparation. Anesthesia was induced by intravenous injection of pentobarbital sodium (35 mg/kg). Animals were tracheotomized, intubated, and mechanically ventilated. Arterial pH, PO2, and PCO2 were maintained within the physiological ranges by supplying oxygen and changing the respiratory rate. α-Chloralose (20 mg·kg−1·h−1) was continuously infused to maintain an appropriate level of anesthesia during the experiment. A catheter-tipped micromanometer (SPC-330A, Millar Instruments, Houston, TX) was inserted via the right femoral artery to measure arterial pressure (AP). After a median sternotomy, the heart was suspended in a pericardial...
cral. Another catheter-tipped micromanometer was introduced into the LV via the apex to measure LV pressure (LVP). Piezoelectric crystals (1 mm, Sonometrics, Ontario, Canada) were attached to the anterior and lateral walls of the LV using cyanoacrylate adhesive (3M, Vetbond, St. Paul, MN) to measure regional LV segmental length. A 4-0 prolene suture was passed around the main branch of the left anterior descending coronary artery (LAD), and a snare was formed by passing the ends of the thread through a small vinyl tube. A surface electrocardiogram (ECG) was recorded.

Bilateral cervical vagi were identified and transected at the neck. A pair of bipolar electrodes was attached at the cardiac end of the right vagal nerve. The duration of electrical pulse used to stimulate the vagal nerve was set at 4 ms. We adjusted the amplitude of the pulse in each animal to reduce heart rate (HR) by 30% from the baseline value at a stimulation frequency of 10 Hz. The resultant stimulation voltage was 2–4 V.

**Experimental protocol.** Thirty minutes were allowed for stabilization after the initial preparation and surgical procedures were completed. The animals were randomized into the following four groups: 1) sham group (n = 7), in which surgical preparation was conducted without coronary occlusion or vagal stimulation (VS); 2) VS group (n = 7), in which stimulation of the vagal nerve was started after baseline hemodynamics were obtained and continued during the experiment; 3) I/R group (n = 7), in which 60 min of LAD occlusion and 180 min of reperfusion were conducted; and 4) I/R-VS group (n = 8), in which stimulation of the vagal nerve was started 15 min before LAD occlusion and continued throughout 60 min of myocardial ischemia and 180 min of reperfusion.

Baseline hemodynamic data (baseline) were recorded in all groups. A second set of measurements of hemodynamic data (60 min) was obtained during the last 5 min of the 60-min observation period in the sham and VS groups or during the last 5 min of the 60-min ischemic period in the I/R and I/R-VS groups. A third set of measurements of hemodynamic data (240 min) was recorded during the last 5 min of the next 180-min observation period in the sham and VS groups or during the last 5 min of the 180-min reperfusion period in the I/R and I/R-VS groups.

At each time point, hemodynamic data were recorded under a steady-state condition. All data acquisitions were done at end expiration. Analog signals of AP, LVP, segmental length of the anterior-lateral wall of LV (risk area), and ECG were digitized at 200 Hz and stored in a computer for off-line analysis (Sonolab, Sonometrics).

Atrial appendages were dissected away, the remaining LV wall was snap frozen in liquid nitrogen and stored at −80°C.

**Myocardial protein extraction.** Approximately 200 mg of myocardial tissue sample obtained from the center of the risk area (anterior wall) of the LV free wall by an acid guanidium thiocyanate-phenol chloroform method (Isogen, Nippon Gene). First-strand cDNA was synthesized using reverse transcriptase with random hexamer primers from 1 μg of total RNA in a final volume of 20 μl, according to the manufacturer’s protocol (ReverTra Ace, Toyoobo).

**Real-time quantitative reverse transcription-PCR.** To analyze TIMP-1 gene expression in myocardial tissue, real-time polymerase chain reaction (PCR) amplification was performed with SYBR Premix Ex Taq (Perfect Real Time; TaKaRa, Japan) using the ABI PRISM 7500 sequence detection system (Applied Biosystems). For standardization and quantification, rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified simultaneously. The respective PCR primers were designed from GenBank databases (Table 1). The PCR consisted of initial treatments (50°C, 2 min; and 95°C, 10 min) followed by 40 three-step cycles (denaturation 94°C, 10 s; annealing 60°C, 10 s; and extension 72°C, 40 s). A cycle of PCR was performed, and dissociation curves were constructed to confirm the formation of the intended PCR products. Relative expression of TIMP-1 to the GAPDH levels was calculated as described previously (28, 45).

**Hemodynamic data analysis.** The following hemodynamic parameters were determined from hemodynamic data: HR, mean arterial pressure, maximum first derivative of LVP (LV dP/dt max), and fractional shortening of anterior-lateral wall (FS). End diastole and end systole were defined as the peak of R wave of ECG and the peak of minimum first derivative of LVP, respectively. FS was calculated as

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**Table 1. Probes used for real-time PCR**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sequence</th>
<th>Accession Number</th>
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<tbody>
<tr>
<td>TIMP-1</td>
<td>Forward</td>
<td>5’-CACCTCCTGGCACCTTTGTGCATCG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GGGCTAAGCTTTGTAACATCT-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5’-GGAGAAAGGCTGCTAAGTATGACG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-CACTGTTGGAATGCCAGGAAG-3’</td>
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**MMP-9 activity assay.** Bioactivity assay for MMP-9 was performed using the Biotrak activity assay system (GE Healthcare Bio-Sciences, Piscataway, NJ) following the manufacturer’s instructions (42). Briefly, supernatant samples were placed in microtiter wells coated with anti-MMP-9 (100 μl/well). The plates were incubated overnight at 4°C. The following day, p-aminophenylmercuric acetate was added to the wells for measuring “total” MMP-9 (pro- and active MMP-9). Buffer alone was added to the wells for measuring “active” (endogenous active MMP-9). MMP-9. Detection agent was then added to all wells (50 μl/well), and the plate was read at 405 nm (t = 0 min) and again after a 2-h incubation at 37°C. The value of MMP-9 was standardized by the protein concentration. All measurements were run in duplicate.

**ELISA measurement of TIMP-1 and TIMP-2.** Commercially available ELISA kits (Daichi Fine Chemical, Toyama, Japan) were used to measure TIMP-1 and TIMP-2 levels in supernatants according to the manufacturer’s instructions (13, 17, 20). Briefly, standards and samples were incubated in microtiter wells coated with anti-TIMP-1 and anti-TIMP-2 antibody. Peroxidase-labeled antibodies directed to the respective TIMPs were added to the corresponding wells. Visualization of the presence of the peroxidase label was achieved using the o-phenylenediamine substrate (TIMP-1) or tetramethylbenzidine substrate (TIMP-2). The plates were read at 490 (TIMP-1) or 450 (TIMP-2) nm. Values of TIMPs were standardized by the protein concentration. Since the ELISA systems have some degree of interplate and interplate variability (<15%) (7), all measurements were run in duplicate to quadruplicate.

**Myocardial RNA extraction and reverse transcription.** Total RNA was extracted from the risk area (anterior wall) of the LV free wall by an acid guanidium thiocyanate-phenol chloroform method (Isogen, Nippon Gene). First-strand cDNA was synthesized using reverse transcriptase with random hexamer primers from 1 μg of total RNA in a final volume of 20 μl, according to the manufacturer’s protocol (ReverTra Ace, Toyobo).

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the ratio of systolic stroke change in segmental length and end-diastolic length of the anterior-lateral wall (36).

Cardiac Microdialysis Study

Experimental preparation. Experimental preparation was the same as described above in I/R Study, except that no coronary artery occlusion was performed. A microdialysis probe was implanted into the LV anterior wall. Heparin sodium (200 U/kg) was administered intravenously to prevent blood coagulation (19).

Dialysis technique. The materials and properties of the dialysis probe have been described (19). Briefly, we designed a hand-made long transverse dialysis probe. One end of a polyethylene tube (25 cm long, 0.5 mm OD, and 0.2 mm ID) was dilated with a 27-gauge needle (0.4 mm OD). Each end of the dialysis fiber (8 mm long, 0.215 mm OD, 0.175 mm ID, and 300 Å pore size; Evaflux type 5A, Kuraray Medical, Tokyo, Japan) was inserted into the polyethylene tube and glued.

Recovery of TIMP-1 passing through the dialysis fiber membrane was evaluated in vitro. The dialysis probe (n = 4) was immersed in Ringer solution (in mM; 147.0 NaCl, 4.0 KCl, and 2.25 CaCl2) containing Tween 20 (0.1%) and various concentrations of TIMP-1 (10–40 ng/ml, free form of human TIMP-1, Daiichi Fine Chemical). The dialysis probe was perfused with Ringer solution at a rate of 2.5 μl/min using a microinjection pump (model CMA/102, Carnegie Medicine). We measured the concentration of TIMP-1 in the dialysate sample using an ELISA kit. The relative recovery of TIMP-1 was calculated as the ratio of TIMP-1 concentration in dialysate to its concentration in the medium surrounding the probe (11, 22). The relative recovery of TIMP-1 was 11.1 ± 0.3%. Recovery was constant between probes and within the probe for the TIMP-1 concentration range studied.

A fine-guiding needle (25 mm long, 0.51 mm OD, and 0.25 mm ID) was used for implantation of the dialysis probes. The guiding needle was connected to the dialysis probe with a stainless steel rod (5 mm long and 0.25 mm OD). Experimental protocols were initiated 2 h after implanting the dialysis probe. The dialysate sampling period was set at 60 min and was performed taking into account the dead space volume between the dialysis membrane and the sample tube.

Experimental protocol. After baseline dialysate was sampled and baseline hemodynamic data were recorded, the animals were randomized into the following three groups: 1) VNS group (n = 5), in which electrical stimulation of vagal nerve was performed while the LV wall was perfused with Ringer solution via the dialysis probe; 2) ACh group (n = 8), in which the LV wall was perfused with Ringer solution containing ACh (1 mM); and 3) ACh-atropine (Atr) group (n = 7), in which the LV wall was perfused with Ringer solution containing ACh (1 mM) and Atr (0.2 mM). At 150 min after randomization, dialysate sampling and hemodynamic data recording were performed.

At the end of the experiment, the animal was euthanized. From selected hearts, transmural blocks of the LV free wall containing the dialysis probe were fixed in 4% paraformaldehyde for immunohistochemistry.

Immunohistochemistry and confocal microscopy. To investigate the distribution of TIMP-1, we performed confocal image analysis of LV tissue stained with anti-TIMP-1 antibody. Fixed blocks of LV tissues were washed in 0.1 mol/l phosphate buffer (pH 7.4), embedded in paraffin, and sectioned at a thickness of 5 μm. Sections were deparaffinized using xylene, rehydrated with serial grades of ethanol, and followed by hydration with distilled water. For antigen retrieval of TIMP-1 protein, specimens were immersed in a vessel filled with Target Retrieval Solution (pH 6.1; DAKO). The vessel containing the specimens was autoclaved at 121°C for 20 min. The slides were then allowed to cool at room temperature for 20 min to complete antigen unmasking. The sections were then incubated for 30 h with a mouse anti-TIMP-1 antibody (7-6C1, Daiichi Fine Chemical) diluted 1:5 and then incubated for 2 h in Alexa-488-conjugated goat anti-mouse Ig-G (Molecular Probes) diluted 1:200. Fluorescence of Alexa-488 was observed with a confocal laser-scanning microscope system (FV 300, Olympus). Reconstructed projection images were obtained from serial optical sections recorded at an interval of 0.5 μm.

Exclusion Criteria

Animals were excluded from the study when the following criteria were met: 1) in the I/R study, coronary artery occlusion did not produce substantial regional dysfunction (FS of the risk area after occlusion was not <20% of the baseline value); 2) intractable ventricular fibrillation or atrial tachycardia occurred; and 3) the animal died during the surgical procedure, and the protocol was not completed.

Statistical Analysis

All data are presented as means ± SE. Tukey-Welsch’s step-down multiple comparison test was used to determine the significance of differences among groups. P values <0.05 were considered statistically significant.

RESULTS

I/R Study

As shown in Fig. 1A,zymography of the myocardial extracts detected two bands at 92 and 72 kDa, corresponding to MMP-9 and MMP-2, respectively. Densitometric analysis demonstrated that relative MMP-9 level increased to a similar degree in the I/R and I/R-VS groups compared with the sham and VS groups (Fig. 1B). The relative MMP-2 level decreased in the I/R group compared with the sham and I/R-VS groups (Fig. 1C).

Bioactivity assays demonstrated that myocardial levels of total MMP-9 protein increased to a similar degree in the I/R and I/R-VS groups compared with the sham and VS groups (Fig. 2A). Levels of endogenous active MMP-9 protein also increased in the I/R and I/R-VS groups compared with the sham and VS groups (Fig. 2B). The level of active MMP-9 in the I/R-VS group was significantly lower than that in the I/R group (<50%, P < 0.01).

The myocardial level of TIMP-1 protein increased in the VS and I/R-VS groups compared with the sham and I/R groups (Fig. 3A). There was no significant difference in the myocardial level of TIMP-2 protein among the four groups (Fig. 3B). TIMP-1 mRNA as measured by real-time RT-PCR was increased in the I/R-VS group compared with the sham, VS, and I/R groups (Fig. 3C).

Table 2 summarizes the data of systemic hemodynamics and LV function during the I/R study. In the VS and I/R-VS groups, HR decreased significantly compared with sham and I/R values at 60 and 240 min. In the I/R and I/R-VS groups, FS was depressed during ischemia with only partial recovery after reperfusion. In the I/R and I/R-VS groups, sonomicrometry demonstrated early systolic bulging of the anterior LV wall during ischemia as reflected by negative FS at the 60-min time point. There was no significant difference in LV dP/dt max and FS between the I/R and I/R-VS groups at 60 and 240 min.

Cardiac Microdialysis Study

Figure 4 presents dialysate TIMP-1 concentrations in response to electrical stimulation of the vagal nerve, to perfusion of ACh, and to perfusion of ACh with Atr. There were no
significant differences in baseline TIMP-1 concentrations among the three groups. At 150 min, dialysate TIMP-1 concentration was significantly higher in the VNS and ACh groups than in the ACh-Atr group ($P < 0.05$).

Figure 5 depicts representative microscopic findings of LV tissue around the microdialysis probes in the VNS, ACh, and ACh-Atr groups. Hematoxylin-eosin-stained sections demonstrated only a minimum hemorrhage around the dialysis probe (Fig. 5, A–C). TIMP-1-positive cardiomyocytes were detected sparsely but in diffuse distribution throughout the myocardium in the VNS group (Fig. 5D). TIMP-1-positive cardiomyocytes were detected over a relatively wide area around the dialysis probe in the ACh group (Fig. 5E). TIMP-1-positive cardiomyocytes were also detected but localized close to the dialysis probe in the ACh-Atr group (Fig. 5F). Immunoreactive signals of TIMP-1 were restricted to the cytoplasm of cardiomyocytes in all the groups (Fig. 5, G–I).

Table 3 summarizes the data of systemic hemodynamics and LV function during the cardiac microdialysis study. In the VNS group, HR decreased significantly compared with that in the ACh and ACh-Atr groups at 150 min. In the ACh and ACh-Atr groups, topical perfusion of ACh or ACh with Atr did not affect the systemic hemodynamics and the LV functions. Except for HR, there were no significant differences in other hemodynamic parameters among the three groups.

DISCUSSION

The major new findings of the present study were as follows. In ischemia-reperfused myocardium, stimulation of the efferent vagal nerve increased TIMP-1 mRNA and protein levels and reduced endogenous active MMP-9 protein. In normal myocardium, VNS or topical perfusion of ACh through a microdialysis probe increased dialysate TIMP-1 protein level. An increase in the dialysate TIMP-1 protein level induced by ACh perfusion was suppressed by coperfusion of Atr.

The robust increase in total MMP-9 levels after reperfusion in this study (Figs. 1B and 2A) might be mainly due to the...
infiltrated neutrophils. Although all cell types, including cardiomyocytes (25, 34) and endothelial cells (41), express MMP-9, neutrophil is an important source of MMP-9 after I/R (26). The level of endogenous active MMP-9 was lower in the I/R-VS group than in the I/R group (Fig. 2B). Increased expression of TIMP-1 by VNS (Fig. 3) likely inhibited the conversion of pro-MMP-9 to active MMP-9 and/or inhibited active MMP-9 itself more potently than in the case without VNS (14). Oxygen free radical induces expression and activation of MMP-9 (17, 41). Reduction of HR by VNS probably reduced myocardial oxygen consumption, ameliorated myocardial ischemia, and reduced oxygen free radicals (30). This may contribute to some extent to the reduction of active MMP-9 in the I/R-VS group.

In the I/R study, TIMP-1 mRNA was significantly higher in the I/R-VS group compared with the sham, VS, and I/R groups (Fig. 3C). TIMP-1 mRNA appeared higher in the VS and I/R groups compared with the sham group, although the differences were not significant. Stapel et al. (38) noted increased expression of TIMP-1 mRNA after myocardial I/R in mice. Proinflammatory cytokines such as interleukin-1β induced by

### Table 2. Hemodynamic parameters during I/R study

<table>
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<tr>
<th></th>
<th>Baseline</th>
<th>60 min</th>
<th>240 min</th>
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<tbody>
<tr>
<td><strong>HR, beats/min</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sham</td>
<td>317±9</td>
<td>334±7</td>
<td>326±9</td>
</tr>
<tr>
<td>VS</td>
<td>281±14</td>
<td>215±17†</td>
<td>238±19‡</td>
</tr>
<tr>
<td>I/R</td>
<td>306±9</td>
<td>316±9</td>
<td>314±8</td>
</tr>
<tr>
<td>I/R-VS</td>
<td>301±7</td>
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<td>228±8‡</td>
</tr>
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<td><strong>MAP, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>92±3</td>
<td>93±4</td>
<td>92±3</td>
</tr>
<tr>
<td>VS</td>
<td>98±4</td>
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<td>89±5</td>
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<td>I/R</td>
<td>102±3</td>
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<td>I/R-VS</td>
<td>99±4</td>
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<td>83±2</td>
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<td><strong>LV dP/dt max, mmHg/s</strong></td>
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<tr>
<td>Sham</td>
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<td>5,308±388</td>
<td>4,819±339</td>
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<td>4,514±467</td>
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<td>4,549±250</td>
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<td><strong>FS, %</strong></td>
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<tr>
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<td>10.8±0.9</td>
<td>10.1±1.0</td>
<td>9.3±1.0</td>
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<tr>
<td>VS</td>
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<td>11.1±1.2</td>
<td>10.4±1.6</td>
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<tr>
<td>I/R</td>
<td>8.7±0.8</td>
<td>-0.6±0.6†</td>
<td>0.1±0.8†</td>
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<tr>
<td>I/R-VS</td>
<td>8.5±1.3</td>
<td>-0.6±0.4†</td>
<td>1.5±0.7†</td>
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</table>

Values are means ± SE. Sham group, no myocardial ischemia and no vagal stimulation (VS); VS group, no myocardial ischemia with VS; I/R group, myocardial ischemia-reperfusion (I/R); I/R-VS, myocardial I/R with VS; HR, heart rate; MAP, mean arterial pressure; LV dP/dt max, maximum first derivative of left ventricular (LV) pressure; FS, fractional shortening of anterior wall (risk area). *P < 0.01 vs. sham; †P < 0.01 vs. VS; ‡P < 0.01 vs. I/R.
protein levels in the VS and I/R-VS groups were significantly increased by 10.2 ± 0.3 compared with baseline (150 min) in the VNS group, whereas those in the ACh-Atr group did not change significantly compared with baseline (150 min). The differences in TIMP-1 levels among the four groups were significant (ACh > ACh-Atr > VNS > baseline). In addition, TIMP-1 levels were higher in the I/R and I/R-VS groups than in the sham and VNS groups. These findings suggest that VNS may induce TIMP-1 expression in myocardial ischemia, and that the induction of TIMP-1 mRNA in the I/R-VS group. TIMP-1 and myocardial ischemia likely exerted an additive effect on myocardial ischemia are known to induce TIMP-1 (4). VNS was implanted with microdialysis probe.

Fig. 5. Representative microscopic finding of left ventricular (LV) tissue implanted with microdialysis probe. A–C: hematoxylin and eosin-stained section of LV tissue perfused with Ringer solution under vagal nerve stimulation (VNS; A), perfused with ACh (B), and perfused with ACh and Atr (ACh-Atr; C). D–F: anti-TIMP-1 antibody (green)-immunostained sections of LV tissue perfused with Ringer solution under VNS (D), perfused with ACh (E), and perfused with ACh-Atr (F). G–I: higher magnifications of D–F, respectively. Arrows indicate dialysis probes. Bar = 100 μm.

myocardial ischemia are known to induce TIMP-1 (4). VNS and myocardial ischemia likely exerted an additive effect on the induction of TIMP-1 mRNA in the I/R-VS group. TIMP-1 protein levels in the VS and I/R-VS groups were significantly elevated compared with the sham and I/R groups (Fig. 3A). Figure 3, A and C, indicates dissociation between TIMP-1 mRNA and protein synthesis among the four groups. If the TIMP-1 protein level had correlated with the mRNA level, TIMP-1 protein level in the I/R and I/R-VS groups should have been higher than those presented in Fig. 3. In myocardial ischemia, protein synthesis decreases owing to the inhibition of peptide chain elongation (8, 18). This may have partially inhibited TIMP-1 protein synthesis in the I/R and I/R-VS groups.

In the cardiac microdialysis study, the ACh-induced release of TIMP-1 was mediated by muscarinic ACh receptors because Atr blocked the increase in TIMP-1 in response to ACh stimulation (Fig. 4). TIMP-1 was produced by cardiomyocytes (Fig. 5, G–I). These findings suggest that VNS may induce TIMP-1 mRNA expression through muscarinic ACh receptors in cardiomyocytes and increase TIMP-1 protein content in myocardium. The distribution of TIMP-1-positive cardiomyocytes was different among the three groups (Fig. 5, D–F). This may reflect differences in the distribution of ACh among the three groups. ACh probably had a diffuse distribution in the myocardium in the VNS group but was concentrated around the dialysis probe in ACh group, whereas the effect of ACh concentrated around the dialysis probe was antagonized by Atr in the ACh-Atr group.

In addition to cardiomyocytes (25, 34), a variety of cell types, such as fibroblasts (14) and endothelial cells (6), produces and secretes TIMP-1. TIMP-1 expression in these cell types is low in the basal condition but is transcriptionally induced by various agents, including the cytokines, serum, growth factors, and phorbol esters (14). The signal transduction pathway from muscarinic ACh receptor stimulation to the induction of the TIMP-1 gene is not clear. Further elucidation of this is not in the scope of this study. ACh increases the production of nitric oxide from cardiomyocytes (9). Nitric oxide induces TIMP-1 gene expression by activating the transforming growth factor-β/Smad signaling pathway in glomerular mesangial cells in the kidney (2). These mechanisms may be involved in the increases in TIMP-1 mRNA and protein induced by VNS in myocardial I/R observed in the present study. Further studies are clearly required to elucidate these issues.

Myocardial expression of TIMP-2 was not modified by VNS (Fig. 3B). Contrary to the highly responsive nature of TIMP-1 expression to stimuli, TIMP-2 expression is, for the most part, constitutive (14). Previous studies demonstrated that ischemic injury or change in loading condition had little effect on myocardial expression of TIMP-2 (24, 25, 29). Myocardial content of MMP-2 decreased after I/R, and the decrease was inhibited by VNS (Fig. 1C). Cheung et al. (5) demonstrated that MMP-2 was released from the myocardium into the coronary effluent following myocardial I/R, resulting in the depletion of myocardial content of MMP-2.

In the present study, VNS did not prevent contractile dysfunction after I/R (Table 2). Actions of MMP and TIMP did not seem to be responsible for acute mechanical changes. Lu et al. (29) demonstrated that treatment with the MMP inhibitor failed to prevent acute myocardial dysfunction and regional expansion after I/R injury. The duration of reperfusion in our study (180 min) and that in Lu et al. (90 min) (29) may be too short to detect a significant influence of MMP and TIMP on regional LV function, which may become evident after a longer period of reperfusion.

Table 3. Hemodynamic parameters during cardiac microdialysis study

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
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<tbody>
<tr>
<td>HR, beat/min</td>
<td></td>
<td></td>
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<tr>
<td>VNS</td>
<td>286 ± 7</td>
<td>227 ± 7†</td>
</tr>
<tr>
<td>ACh</td>
<td>303 ± 16</td>
<td>308 ± 9</td>
</tr>
<tr>
<td>ACh-Atr</td>
<td>304 ± 14</td>
<td>298 ± 16</td>
</tr>
<tr>
<td>MAP, mmHg</td>
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<td></td>
</tr>
<tr>
<td>VNS</td>
<td>101 ± 8</td>
<td>103 ± 8</td>
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<tr>
<td>ACh</td>
<td>93 ± 3</td>
<td>100 ± 4</td>
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<td>ACh-Atr</td>
<td>87 ± 3</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>LV dp/dtmax, mmHg/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VNS</td>
<td>5,050 ± 588</td>
<td>4,768 ± 475</td>
</tr>
<tr>
<td>ACh</td>
<td>5,203 ± 345</td>
<td>5,488 ± 400</td>
</tr>
<tr>
<td>ACh-Atr</td>
<td>4,519 ± 269</td>
<td>4,718 ± 450</td>
</tr>
<tr>
<td>FS, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VNS</td>
<td>7.4 ± 1.8</td>
<td>7.2 ± 1.9</td>
</tr>
<tr>
<td>ACh</td>
<td>5.0 ± 1.2</td>
<td>4.9 ± 1.2</td>
</tr>
<tr>
<td>ACh-Atr</td>
<td>5.4 ± 0.5</td>
<td>5.0 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. VNS group. LV tissue was perfused with Ringer solution via a dialysis probe under vagal nerve stimulation; ACh group, LV tissue was perfused with Ringer solution containing ACh (1 mM) via a dialysis probe; ACh-Atr group, LV tissue was perfused with ACh (1 mM) and atropine (0.2 mM) via a dialysis probe. *P < 0.01 vs. ACh; †P < 0.01 vs. ACh-Atr.
Several previous studies (10, 35, 39) demonstrated that targeted deletion of MMP-9 and/or the upregulation of TIMP-1 reduced infarct size, prevented LV rupture, and ameliorated LV remodeling after MI. Conversely, the expression of other MMPs, such as MMP-2, has been shown to be important in the myocardial healing that occurs in the later phases after ischemic injury (10). These observations suggest that the beneficial effect of VNS on LV remodeling after MI observed in our previous study (23) may be in part mediated through the modified expression of MMPs and TIMPs as noted in the present study.

Except for the post-MI LV remodeling, MMPs and TIMPs contribute to the progression of various cardiovascular disorders, including expansion and rupture of aortic aneurysm (44), progression of acute viral myocarditis (15), and restenosis after coronary intervention (12). Local overexpression of TIMP-1 prevented the expansion and rupture of aortic aneurysm in rats (3) or prevented cardiac injury and dysfunction during experimental viral myocarditis in mice (15). VNS may be an effective biological inducer of TIMP-1 for the treatment of these disorders.

**Limitation**

The present study examined a limited number of MMP and TIMP species over a very short duration after myocardial I/R. A number of MMP and TIMP species are expressed in the myocardium, and several have been identified to be upregulated in cardiac disorders (24). Myocardial MMP-1 (collagenase) is induced by I/R (29). The actions of MMP-1 are inhibited in part by TIMP-1 (31). These suggest that VNS may inhibit the activity of MMP-1 in myocardial I/R injury. Further studies to define the effect of VNS on the profile of MMPs and TIMPs expressed in the myocardium are warranted.

In the present study, VNS was started 15 min before coronary occlusion. We did not examine whether VNS started after the coronary artery occlusion or whether reperfusion is capable of increasing myocardial TIMP-1. The pretreatment strategy as adopted in this study is unrealistic in clinical practice. Therefore, further studies are required to examine the time factor of VNS.

Concentration of ACh perfused through the dialysis probe in this study (1 mM) was substantially higher than the dialysate concentration of endogenous ACh released from the myocardium (<20 nM) (1). The ACh concentration within the myocardial interstitium might have been elevated over the supra-physiological range in the present microdialysis study. However, even if the interstitial concentration of ACh was unphysiologically high, Atr blocked the increase in TIMP-1 expression in response to ACh stimulation. Therefore, it is fair to say that TIMP-1 expression in response to ACh stimulation is mediated through the muscarinic ACh receptor.

TIMP-1 binds with MMPs to form a rather high molecular weight complex. Our preliminary in vitro experiment demonstrated that the relative recovery of TIMP-1/lipocalin/MMP-9 complex (Calbiochem, La Jolla, CA) was 3.8 ± 1.3% (range 0–5.5%) and was lower than that for free TIMP-1 (11.1 ± 0.3%) (see Methods). Although the presence of MMPs, especially MMP-9, could affect the measurement of TIMP-1 within the myocardium by our microdialysis method, this probably does not affect the conclusion drawn from the cardiac microdialysis study, because the study was conducted in a heart free of I/R, which might contain low levels of myocardial MMP-9 as inferred from the results of the I/R study.

**CONCLUSION**

In a rabbit model of myocardial I/R injury, VNS induced TIMP-1 expression in cardiomyocytes and reduced active MMP-9.

**GRANTS**

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**REFERENCES**


