Augmented Expression of Neuronal Nitric Oxide Synthase in the Atria Parasympathetically Decreases Heart Rate During Acute Myocardial Infarction in Rats

Yoshihito Takimoto, MD; Takeshi Aoyama, MD, PhD; Koichi Tanaka, PhD; Reiko Keyamura, MD; Yoshiki Yui, MD, PhD; Shigetake Sasayama, MD, PhD

Background—Nitric oxide (NO) synthesized within sinoatrial cells recently has been shown to participate in the autonomic control of heart rate. We hypothesized that NO in the neuronal cells in the heart was increased and parasympathetically regulated heart rate after myocardial infarction (MI).

Methods and Results—We examined heart rate dynamics and neuronal NO synthase (nNOS) expression and activities in the atria of rats with MI 1, 3, 7, and 14 days after MI (n = 7 to 22 for each group). Both the mRNA levels of nNOS in the atria determined by competitive reverse transcriptase–polymerase chain reaction and the protein levels determined by Western blotting were significantly increased compared with controls 1, 3, and 7 days after MI. nNOS activity in the atria 1 day after infarction was also increased in MI rats. nNOS immunoreactivity was observed in nerve fibers in the atria. After infusion of a specific inhibitor of nNOS and iNOS, 1-(2-trifluoromethylphenyl) imidazole (TRIM) (50 mg/kg IV), heart rate was significantly (P < 0.01) increased in MI rats compared with controls 1, 3, and 7 days after MI. The iNOS-specific inhibitor, 1400W (10 mg/kg SC), did not significantly affect the heart rate in rats with MI. The effect of TRIM was abolished by pretreatment with l-arginine (25 mg/kg IV) or by parasympathetic blockade with atropine but not by propranolol. There was a strong correlation (r = 0.837, P < 0.0001) between the nNOS protein expression and heart rate change after TRIM infusion.

Conclusions—These results indicate that increased nNOS parasympathetically decreased heart rate via the production of NO in rats with acute MI. (Circulation. 2002;105:490-496.)

Key Words: nitric oxide synthase • heart rate • myocardial infarction

Neuronal nitric oxide synthase (nNOS), first identified in the cerebella, has also been detected in the heart. nNOS immunoreactivity has been demonstrated in the ganglia and nerve fibers in the heart. Recently, several lines of evidence have demonstrated that nitric oxide (NO) synthesized from nNOS is one of the modulators of autonomic activities and decreases the heart rate in intact mammals. However, the role of nNOS in the autonomic control of heart rate is controversial. Moreover, its role in pathological conditions remains to be determined.

We recently reported that nNOS expression was increased in the ventricle during acute myocardial infarction (MI) in rats. We hypothesized that nNOS expression in the atria might also be increased and parasympathetically play a role in heart-rate regulation during acute MI. We examined nNOS expression and heart-rate dynamics using a rat MI model and found that nNOS expression was significantly increased and that augmented expression of nNOS parasympathetically attenuated the heart rate during acute MI.

Methods

Rat Myocardial Infarction Model

We performed animal experiments in accordance with the Declaration of Helsinki, and these were approved by our institutional ethics committee for animal experiments. MI was surgically induced in 8-week-old male Sprague-Dawley (IGS) rats (Charles River Japan Inc, Yokohama, Japan) weighing 280 to 320 g by ligation of the left anterior descending coronary artery, as described previously. Transthoracic echocardiography (Hewlett-Packard) was performed at each time point with a 7.5-MHz sector scan probe. At 1, 3, 7, and 14 days after the procedure, rats were sacrificed. Three thin transverse slices were cut from the apex to the base, and the infarct size was determined as described previously.

Quantitative Competitive Reverse Transcriptase–Polymerase Chain Reaction

Total cellular RNA was extracted from the atria using the acid guanidinium thiocyanate-phenol-chloroform method and was reverse-transcribed into cDNA according to the method described previously. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using primers complimentary to

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TABLE 1. Systolic Blood Pressure, Heart Rate, Body Weight, Heart Weight, Left Ventricular Dimension, and Fractional Shortening of Rats With Myocardial Infarction and of Sham-Operated Controls

<table>
<thead>
<tr>
<th>Time After Infarction, days</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
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<tr>
<td><strong>Systolic BP, mm Hg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MI</td>
<td>129±1</td>
<td>111±2*</td>
<td>109±1*</td>
<td>115±1*</td>
<td>124±1*</td>
</tr>
<tr>
<td>Sham</td>
<td>127±2</td>
<td>124±2</td>
<td>119±2</td>
<td>135±2</td>
<td>135±1</td>
</tr>
<tr>
<td><strong>HR, beats/min</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td>371±2</td>
<td>479±3*</td>
<td>419±5*</td>
<td>397±4*</td>
<td>379±2</td>
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<td>Sham</td>
<td>366±5</td>
<td>402±8</td>
<td>375±2</td>
<td>377±6</td>
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<tr>
<td><strong>BW, g</strong></td>
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<tr>
<td>MI</td>
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<td>285±7</td>
<td>270±7</td>
<td>287±10</td>
<td>316±10</td>
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<tr>
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<td>309±18</td>
<td>270±3</td>
<td>278±7</td>
<td>284±13</td>
<td>317±8</td>
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<tr>
<td><strong>HW, mg</strong></td>
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<td></td>
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<tr>
<td>MI</td>
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<td>898±28</td>
<td>920±16†</td>
<td>854±26</td>
<td>936±29</td>
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<tr>
<td>Sham</td>
<td>...</td>
<td>818±25</td>
<td>831±39</td>
<td>846±48</td>
<td>957±27</td>
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<tr>
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<tr>
<td>MI</td>
<td>...</td>
<td>3.15±0.12</td>
<td>3.41±0.11†</td>
<td>2.98±0.05</td>
<td>2.96±0.04</td>
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<td>3.03±0.04</td>
<td>2.99±0.06</td>
<td>2.98±0.08</td>
<td>3.02±0.04</td>
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<td><strong>LVDd, mm</strong></td>
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<tr>
<td>MI</td>
<td>6.1±0.1</td>
<td>6.9±0.1*</td>
<td>7.3±0.1*</td>
<td>7.7±0.1*</td>
<td>8.3±0.1*</td>
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<td>6.4±0.1</td>
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<td><strong>LVDs, mm</strong></td>
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<tr>
<td>MI</td>
<td>3.3±0.1</td>
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<td>5.8±0.1*</td>
<td>6.2±0.1*</td>
<td>6.4±0.1*</td>
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<td>3.4±0.1</td>
<td>3.5±0.1</td>
<td>3.6±0.1</td>
<td>3.7±0.1</td>
</tr>
<tr>
<td><strong>FS, %</strong></td>
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<tr>
<td>MI</td>
<td>45.7±0.6</td>
<td>22.1±0.5*</td>
<td>21.3±0.6*</td>
<td>20.1±0.4*</td>
<td>19.9±0.7*</td>
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<tr>
<td>Sham</td>
<td>44.6±0.4</td>
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<td>41.1±0.8</td>
<td>42.2±0.5</td>
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<tr>
<td><strong>MI size, %</strong></td>
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<tr>
<td>MI</td>
<td>...</td>
<td>52.2±3.1</td>
<td>50.1±4.8</td>
<td>48.3±2.1</td>
<td>47.3±1.0</td>
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<td>11</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td><strong>No. of rats</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td>22</td>
<td>22</td>
<td>15</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Sham</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

BP indicates blood pressure; HR, heart rate; BW, body weight; HW, heart weight; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; and FS, fractional shortening.

*P<0.01; †P<0.05 for the infarct group compared with the respective noninfarcted control group at each time point.

Positions 2416 to 3033 of the rat nNOS cDNA sequence (sense, 5′-GTC TTC CAC CAG GAG ATG -3′; antisense, 5′-AAA GGC ACA GAA GTG GGG GTA -3′). The PCR amplification of the constitutively expressed GAPDH cDNA was used as a measure of the amount of input RNA. DNA fragments that shared the same primer template sequence with the target cDNA but contained a completely different smaller intervening sequence were prepared and used as DNA internal standards (mimics). Aliquots of sample cDNA mixed together with 6 serial dilutions of DNA mimics were coamplified as templates in the presence of primer pairs. PCR was performed as described previously. The PCR reaction products (20 μL) were analyzed via electrophoresis through 1.5% agarose gels containing 0.5 μg/mL ethidium bromide, and the results were quantified by scanning densitometry using the NIH Image computer software.

Western Blot Analysis

Lysates (60 μg) from rat atria were separated by 7.5% SDS-PAGE, and Western blotting was performed with anti-nNOS antibody (NOS1, R-20, Santa Cruz Biotechnology, Inc) according to the method described previously.11

Determination of NOS Activity

Total NOS activity was measured using a NOS Assay Kit (Cayman Chemical Co), [3H]-L-arginine (Amersham Pharmacia), and 10 mmol/L N-monomethyl-L-arginine (Wako Chemical) according to the manufacturer’s instructions. NOS activity in the presence of ethylene diamine tetra acetic acid (1 mmol/L) (Wako Chemical), an nNOS- and inducible NOS (iNOS)-specific inhibitor, 1-(2-trifluoromethylphenyl) imidazole (TRIM) (100 μmol/L) (Tocris Cookson Inc), or iNOS-specific inhibitor 1400W (2 μmol/L) (Cal-

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biochem) was also determined. Each NOS isoform activity was calculated from these measures.

**Immunohistochemistry**

The rats were reanesthetized and perfused through the left ventricle with 100 mL of 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Two blocks of the atrium known to contain ganglionic cell bodies were removed. One was the region around the sinoatrial node and the other near the pulmonary veins and the atrioventricular node. After rinsing with phosphate buffered saline (PBS), they were stored in 20% sucrose in PBS for 2 hours. Free-floating sections of 40-μm thickness were preincubated with PBS containing 10% normal donkey serum, 0.2% BSA, and 0.3% Triton X-100 for 60 minutes at room temperature. The nNOS antibody was applied at a dilution of 1:500 in PBS containing 0.2% BSA and 0.1% Triton X-100 for 48 hours at 4°C. After rinsing with PBS, the sections were incubated with Cy3-conjugated anti-rabbit IgG produced in donkey (Jackson) diluted 1:500 in PBS for 90 minutes at room temperature. The tissue was viewed with an Olympus AX-80 fluorescence microscope using a filter with an excitation range of 560 to 596 nm and an emission range of 610 to 655 nm.

**Heart-Rate Dynamics and Autonomic Blockade**

The basal heart rate and systolic blood pressure of each animal were measured by a tail-cuff method in the fully conscious drug-free state. Rats with MI or sham operation received atropine 0.5 mg/kg IP or 1 mg/kg propranolol IV, and heart rate and blood pressure were measured every 2 minutes for 30 minutes 1 and 14 days after infarction. We used TRIM or 1400W to determine the effect of nNOS or iNOS on the heart rate regulation. After the measurement of baseline heart rate and blood pressure, rats received TRIM 50 mg/kg IV or 1400W 10 mg/kg SC, and heart rate and blood pressure were measured every 2 hour for 1 hour, 1, 3, 7, and 14 days after the operation. After the administration of L-arginine (25 mg/kg IV) or the autonomic blockade with atropine or propranolol, rats also received TRIM to determine the effect of nNOS on the autonomic heart-rate regulation.

**Statistics**

Values are presented as mean±SEM. The measurements from rats with MI were compared with those of sham-operated rats by the Student’s unpaired t test. The significance of difference among the means of various groups was analyzed by one-way ANOVA with post-hoc comparisons by the Tukey-Kramer test. Relationships between 2 variables were tested by linear regression analysis. Significance was taken as P<0.05.

**Results**

**Heart Weight, Body Weight, and Echocardiographic Study**

The heart weight and the heart-weight-to-body-weight ratio of the myocardial infarction group 3 days after infarction, by which time inflammation and edema had developed within the infarct, were significantly increased (P<0.05) compared with those of the sham-operated control group. The heart weights of MI rats 7 days after infarction were decreased because of the shrinkage of infarcted tissue. All animals developed large MIs, with an infarct size ranging from 38.9% to 62.5% of the left ventricle’s circumference. Left ventricular diastolic and systolic dimensions were rapidly increased during the acute phase of MI, and additional changes were observed by 2 weeks (P<0.01 at each time point compared with sham-operated rats). Fractional shortening was rapidly decreased during the acute phase of MI because of akinesis of the anterior wall and then gradually decreased during the chronic phase (P<0.01 at each time point compared with sham-operated rats) (Table 1).

**Quantitative Reverse Transcriptase-PCR of nNOS mRNA**

The mRNA levels of nNOS were 13.4×10⁵, 13.3×10⁵, and 5.8×10⁵ copies 1, 3, and 7 days after infarction in rats with MI 1, 3, and 7 days after infarction.
MI, respectively, and <1×10^7 in rats 14 days after MI and sham-operated rats (Figure 1). There was no difference in the expression levels of GAPDH in each group (data not shown). Thus, the nNOS mRNA expression level was significantly increased during acute MI.

Western Blotting of nNOS
Western blotting showed 160-kDa bands in the atria of rats with MI and in the sham-operated rats. No other minor bands were observed. As a positive control for nNOS, 20 μg of lysates of rat cerebellar tissues were used in each experiment (Figure 2A). nNOS protein levels were expressed as the fold increase compared with positive controls. The protein levels of nNOS were 1.2-, 1.3-, 0.7-, and 0.4-fold compared with positive controls in the rats with MI and 0.3-, 0.4-, 0.4-, and 0.4-fold in the sham-operated rats 1, 3, 7, and 14 days after infarction, respectively. Protein expression of nNOS was significantly (*P<0.01) increased in the rats with MI 1, 3, and 7 days after infarction compared with the sham-operated rats (Figure 2B).

NOS Activity
Total and nNOS activities were significantly increased in the atria of rats with MI 1 day after infarction compared with those in sham-operated rats. NOS activity in the presence of TRIM but not 1400W was significantly decreased in the atria of rats with MI (data not shown). Endothelial NOS (eNOS) and iNOS activity did not change in the atria after MI (Table 2).

Immunohistochemistry of nNOS
There were nNOS immunoreactive nerve fibers in the region around the sinoatrial node and the region near the pulmonary veins and the atroventricular node. The number of nNOS immunoreactive nerve fibers was increased compared with sham-operated rats (data not shown). Cardiomyocytes were not stained with nNOS antibody (Figure 3).

Effects of TRIM and 1400W on Heart Rate
Rats with MI had a significantly (*P<0.01) higher basal heart rate compared with sham-operated rats 1, 3, and 7 days after the procedure (Table 1). Heart rate changes after the infusion of TRIM were 66±12, 59±4, 32±3, and 6±2 bpm in rats with MI and 8±4, 6±3, 6±2, and 4±3 bpm in sham-operated rats 1, 3, 7, and 14 days after the procedure, respectively (Figure 4A). Compared with the effect of TRIM on heart rate in sham-operated rats, the infusion of TRIM led to significantly (*P<0.01) larger changes in rats with MI 1, 3, and 7 days after infarction. After the administration of L-arginine, the heart rate was changed from 466±10 to 477±21 in rats with MI and from 401±15 to 404±9 in controls 1 day after infarction. The heart rate was not significantly increased in infarcted rats (from 477±21 to 490±27) or in sham-operated rats (from 404±9 to 401±9) after the additional injection of TRIM. Thus, the effect of TRIM was abolished by the previous administration of L-arginine (Figure 4B). There was no significant heart rate change after the administration of 1400W in rats with MI or sham-operated rats 1 day after the procedure (Figure 4B).

Table 2. NOS Activity in the Atria

<table>
<thead>
<tr>
<th></th>
<th>MI (n=6)</th>
<th>Sham (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total, nmol/g per min</td>
<td>1.43±0.04*</td>
<td>0.56±0.10</td>
</tr>
<tr>
<td>nNOS, nmol/g per min</td>
<td>0.86±0.13†</td>
<td>0.14±0.09</td>
</tr>
<tr>
<td>eNOS, nmol/g per min</td>
<td>0.45±0.19</td>
<td>0.38±0.07</td>
</tr>
<tr>
<td>iNOS, nmol/g per min</td>
<td>0.12±0.03</td>
<td>0.04±0.02</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
*P<0.01; †P<0.05 for the infarct group compared with the noninfarcted control group.

Figure 3. Immunohistochemistry of nNOS. There were nNOS immunoreactive nerve fibers in the region around the sinoatrial node (A and B) and the bundle of nerve fibers in the region near the pulmonary veins and the atroventricular node (C and D). Cardiomyocytes were not stained with nNOS antibody. Bars=50 μm.
Autonomic Blockade

Administration of atropine led to a similar increase in heart rate; 118±13 and 106±9 bpm in rats with MI (M) compared with that of sham-operated rats (S) 1, 3, and 7 days after the procedure. Before administration of L-arginine (L-Arg) abolished the effect of TRIM on heart rate 1 day after infarction. 1400W did not affect the heart rate in rats with MI and controls.

After the parasympathetic blockade with atropine, TRIM was infused into rats, and the heart rate was not significantly increased either in rats with MI (7±5 bpm) or controls (0±4 bpm) 1 day after infarction (Figures 5C and 5E). Administration of TRIM with pretreatment of propranolol led to a significant (P<0.01) heart rate increase in rats with MI (55±3 bpm) compared with sham-operated rats (14±3 bpm) 1 day after infarction (Figures 5D and 5E).

Correlation of the Amount of nNOS Protein and the Effect of TRIM on Heart Rate

We plotted the relationships between the nNOS protein level and the heart rate change after TRIM infusion for each animal. As shown in Figure 6, there was a strong correlation (Y=69.263×−14.338, r=0.837, P<0.0001) between these two measures.

Discussion

We found that nNOS expression in both mRNA and protein levels and its activity were increased in the atria of the rat during the acute phase of MI. This is the first in vivo model in which nNOS expression was increased in the atria. We analyzed the effect of nNOS on heart rate using this novel model of rat MI. The administration of TRIM, but not 1400W, increased the heart rate, and this effect of TRIM was
abolished by the pretreatment with L-arginine. The heart rate was not increased additionally by the treatment of TRIM after parasympathetic blockade with atropine. This evidence suggests that nNOS parasympathetically decreased the heart rate via the production of NO in rats with acute MI.

It has been reported that the muscarinic control of heart rate is attenuated by inhibitors of the NO/cGMP pathway. Han et al. reported that muscarinic regulation of L-type calcium current in cardiac myocytes is absent in mice deficient in eNOS. In contrast, the chronotropic responses to both β-adrenergic and muscarinic agonist were not altered in isolated cardiac tissue preparations in a study using the same mouse. Thus, the role of eNOS in the muscarinic regulation of L-type calcium current is controversial. Tanaka et al. reported that the parasympathetic preganglionic nerve fibers originating from the vagus nerve contained nNOS in the guinea pig heart. Several lines of evidence suggest that endogenous NO synthesized from nNOS in the heart decreases heart rate. As to the mechanism of heart rate reduction by nNOS, there are two possibilities. First, Sears et al. reported that specific inhibition of nNOS with TRIM significantly enhanced the magnitude of the change in heart rate with sympathetic nerve stimulation. Choate et al. reported that NO inhibited the positive chronotropic response evoked by cardiac sympathetic nerve stimulation via a cGMP-dependent pathway in the isolated guinea pig double atrial/right stellate ganglion preparation. These studies suggest that endogenous NO plays an inhibitory role in cardiac sympathetic neurotransmission. Second, Conlon et al. reported that vagally evoked frequency-dependent bradycardia was significantly attenuated by infusion of TRIM. Sears et al. also demonstrated that the NO precursor significantly enhanced the decrease in heart rate seen with right vagal nerve stimulation. These results suggest that NO in the heart parasympathetically decreases the heart rate. On the other hand, Elvan et al. reported that NO played a stimulating role in mediating vagal neurotransmission and an inhibitory role in mediating sympathetic neurotransmission using open-chest dogs. The quantitative differences among these studies may result from the effects of endogenous nitric oxide on the vagal control of heart rate being critically dependent on the availability of nNOS. However, the expression level of nNOS was not determined in these studies. In the present study, we measured the expression level and activity of nNOS in the atria and showed that they were increased in the atria during acute MI, whereas they were low in control rats. Administration of TRIM, but not 1400W, increased the heart rate in rats with acute MI. After parasympathetic blockade with atropine, the heart rate was increased and no additional heart rate increase was observed by TRIM. In contrast, after β-adrenergic blockade with propranolol, TRIM increased the heart rate to the same degree as TRIM infusion without autonomic blockade. Our findings strongly suggest the latter possibility that NO synthesized from nNOS facilitates vagal chronotropic actions on the heart in vivo and are compatible with the findings of the study of Jumrussirikul et al. that NO within sinoatrial cells participates in the cholinergic control of heart rate using mice deficient in nNOS.

Immunohistochemical analyses showed that nNOS immunoreactivity was observed in the nerve fibers in the region around the sinoatrial node and the region near the pulmonary veins and the atrioventricular node. Because ganglia in these regions were not stained with nNOS antibody and the administration of atropine blocked the effects of TRIM, nNOS immunoreactive nerve fibers seem to be preganglionic fibers of the vagal nerve. It has been reported that nNOS existed in the nerve fibers and cardiomyocytes in the heart. In the present study, we observed nNOS immunoreactivity only in the nerve fibers. This evidence suggested that nNOS in the parasympathetic nerve plays an important role in heart rate regulation after MI.

NO in the central nervous system is also responsible for the autonomic regulation of the cardiovascular system. Despite possibly different modes of action of NO in different brain regions, the overall sympathoinhibitory role of NO observed by several studies in vivo seems to be predominant at least under physiological conditions. Although we did not determine the nNOS levels in the central nervous system, inhibition of nNOS in the brain by TRIM could activate sympathetic tone, resulting in an increased heart rate. However, in the present study we showed that nNOS protein expression in the atria has a strong correlation with the heart rate changes after the administration of TRIM. Furthermore, atropine administration abolished the effects of TRIM on heart rate. Thus, TRIM seems to inhibit the activity of nNOS in the atria, but not in the brain, and increases the heart rate.

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References


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