Blockade of neuronal nitric oxide synthase alters the baroreflex control of heart rate in the rabbit

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1Department of Physiology, Kagawa Medical University, Kagawa, J Japan; 2Department of Physiology, Gifu University School of Medicine, Gifu, J Japan; and 3Department of Physiology and Biophysics, University of Nebraska College of Medicine, Omaha, Nebraska 68198-4575

Murakami, Hiroshi, J un-Li Liu, Hirohito Yoneyama, Yasuhiro Nishida, Kenji Okada, Hiroaki Kosaka, Hiro-nobu Morita, and Irving H. Zucker. Blockade of neuronal nitric oxide synthase alters the baroreflex control of heart rate in the rabbit. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R181–R186, 1998.—In previous studies we used N\textsuperscript{G}-nitro-L-arginine (L-NNA) to investigate the role of nitric oxide (NO) in baroreflex control of heart rate (HR) and renal sympathetic nerve activity (RSNA). L-NNA increased resting mean arterial pressure (MAP), decreased HR, and did not change or slightly decreased RSNA. These changes complicated the assessment of the central effects of NO on the baroreflex control of HR and RSNA. Therefore, in the present study the effects of the relatively selective neuronal NO synthase inhibitor 7-nitroindazole (7-NI) on the baroreflex control of HR and RSNA were investigated in rabbits. Intra-peritoneal injection of 7-NI (50 mg/kg) had no effect on resting HR, MAP, or RSNA. 7-NI significantly reduced the lower plateau of the HR-MAP baroreflex curve from 140 ± 4 to 125 ± 4 and from 177 ± 10 to 120 ± 9 beats/min in conscious and anesthetized preparations, respectively (P < 0.05). In contrast, there was no significant difference in the RSNA-MAP curves before and after 7-NI administration in conscious or anesthetized preparations. These data suggest that blockade of neuronal NO synthase influences baroreflex control of HR but not of RSNA in rabbits.

sympathetic nerve activity; vagus; arterial pressure

NITRIC OXIDE (NO) is synthesized from L-arginine via the enzyme NO synthase (NOS) (24). At least three isofoms of NOS exist: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). eNOS mainly exists in endothelial cells and plays an important role in vasodilation (25). nNOS exists in large quantities in brain, spinal cord, sympathetic ganglia (28), and kidney. Therefore, it is conceivable that the majority of NO in the brain is synthesized by the action of nNOS. Because NO was identified as the endothelium-derived relaxing factor, there has been a vast amount of evidence suggesting that NO modulates the autonomic nervous system via central sites such as the nucleus tractus solitarius (10, 19, 29), the area postrema, the rostroventrolateral medulla (14, 32), and the caudal ventrolateral medulla (13, 30). In a previous study we demonstrated that inhibition of NO synthesis by N\textsuperscript{G}-nitro-L-arginine (L-NNA) enhances the baroreflex control of heart rate (HR) and renal sympathetic nerve activity (RSNA). Clear evidence was provided for a central focus of action in this study (18). Systemic administration of L-NNA resulted in a significant and profound decrease in resting HR. L-NNA is a nonspecific inhibitor of NOS; 7-nitroindazole (7-NI) is a relatively selective nNOS inhibitor (22, 23). It has been shown that 7-NI administered at 50 mg/kg decreased NOS activity within the forebrain to 45% after 30 min (33). 7-NI allowed us to provide further evidence for a role of nNOS in the control of baroreflex function and thereby the control of sympathetic nerve activity.

The aim of present study was to determine whether blockade of nNOS by 7-NI affects the baroreflex control of HR and RSNA. These experiments were carried out in conscious and anesthetized rabbits.

MATERIALS AND METHODS

Twenty-five New Zealand White rabbits (2.5–3.5 kg) were divided into groups: a normal unblocked group, an atropine-pretreated group, and a metoprolol-pretreated group. The experiments in these three groups were carried out under general anesthesia (see below). A fourth group was examined in the conscious state. All rabbits were fed and housed according to guidelines of the University of Nebraska Medical Center and at Kagawa Medical University. The studies were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conform to the "Guiding Principles for the Use and Care of Laboratory Animals" of the American Physiological Society and National Institutes of Health guidelines.

Surgical Procedures

Rabbits were anesthetized with a-chloralose (70 mg/kg iv) and urethane (700 mg/kg iv). An arterial catheter was inserted into the aorta via a femoral artery to record arterial pressure and measure blood gases, and a venous catheter was inserted into the inferior vena cava via a femoral vein to inject drugs during the experiment. A 5-Fr Tygon catheter was introduced into the abdomen to infuse 7-NI intraperitoneally. A left renal sympathetic nerve was isolated through a left flank incision, and RSNA was recorded. For conscious experiments, an anesthetic cocktail consisting of ketamine hydrochloride (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA; 58.8 mg/kg), acepromazine maleate (Fermenta Animal Health, Kansas City, MO; 1.2 mg/kg), and xylazine (Rompun, Miles, Shawnee Mission, KS; 5.9 mg/kg) in lactated Ringer solution was given by intramuscular injection (1 ml/kg), then the rabbits were chronically instrumented for recording of RSNA. For supplemental anesthesia, pentobarbital sodium (Abbott Laboratories, North Chicago, IL; 0.3–0.35 mg/kg) was injected intravenously via a marginal ear vein. The left renal sympathetic nerves were exposed through a flank incision using a retroperitoneal approach. The renal nerves were dissected from the surrounding tissue and renal artery. A pair of Teflon-coated stainless steel wire electrodes (A-M Systems, Everett, WA; 0.125 mm OD) were placed around the dissected renal nerves. To insulate the electrodes and the nerve from...
the surrounding tissue and to prevent the nerves from desiccation, the electrodes and the nerve assembly were covered with a two-component silicone gel (Wacker Sil-Gel, Munich, Germany). The electrodes were tunneled beneath the skin to the back and fixed between the shoulder blades. The flank incision was closed. Postoperatively, the rabbits were placed on an antibiotic regimen for 3 days (tylosin, Elano Animal Health, Indianapolis, IN; 5 mg/kg im). In addition, a chronic intraperitoneal catheter was implanted for administration of 7-NI. An arterial and a venous catheter were inserted at the time of the experiment, as described below.

Data Acquisition

The arterial catheter was connected to a pressure transducer (Hewlett-Packard) to measure mean arterial pressure (MAP). HR was determined using a Honeywell cardiotachometer that was triggered by the arterial pressure pulse. RSNA was recorded by preamplifying the signal using a Grass P16 preamplifier with the band-pass filters set between 100 Hz and 1 kHz. The amplified signal was displayed on a storage oscilloscope and passed through an audio amplifier and loudspeaker. The raw nerve activity was full-wave rectified and integrated using a voltage integrator (model 1801, Buxco Electronic, Sharon, CT). The signals were led to a Mac Lab data acquisition system (model 8s, AD Instruments, Milford, MA) and sampled at 100 Hz/channel. All sympathetic nerve recordings had a signal-to-noise ratio of at least 3.

Experimental Protocols

To investigate the effects of nNOS inhibition on baroreflex control of HR and RSNA, baroreflex curves were compared before and after intraperitoneal injection of 7-NI (50 mg/kg). Thirty minutes were allowed to elapse before the postblockade curve was constructed. 7-NI was dissolved in warm peanut oil (5 mg/ml). Baroreflex curves were generated by measuring the RSNA response to increases and decreases in arterial pressure by intravenous administration of phenylephrine or sodium nitroprusside. Phenylephrine (American Regent Laboratories, Shirley, NY; 30 µg/kg) or sodium nitroprusside (Hoffmann-La Roche, Nutley, NJ; 100 µg/kg) was administered to rabbits. The renal nerve electrodes were connected to the preamplifier to record RSNA. Baroreflex curves were compared before and after intraperitoneal infusion of 7-NI. As described above, 30 min were allowed to elapse before the postblockade curve was constructed. Baroreflex curves were generated as described above by measuring the HR and the RSNA response to increases and decreases in arterial pressure during intravenous administration of phenylephrine and sodium nitroprusside.

Measurement of NOS Activity

In two other groups the medulla oblongata was taken 40 min after administration of 7-NI (n = 5) or vehicle (n = 5). The tissues were rapidly frozen and stored at −80°C until analysis. Tissue was homogenized in 3.5 ml/g of 50 mM trishydroxymethyl)aminomethane-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15 µM pepstatin A, and 20 µM leupeptin. The homogenate was centrifuged at 20,000 g for 45 min, and the supernatants were passed over a column of Dowex 50W-X8 (Na+ form) to remove the endogenous arginine. The eluates were used for the measurements of NOS activity and protein concentration. All supplemental data described above were performed at 4°C. The conversion of L-[14C]arginine to L-[14C]citrulline by NOS was measured in the supernatants of the tissues as described by Salter et al. (27). Briefly, 10 µl of tissue supernatant were incubated with 90 µl of assay buffer solution containing 0.5 µCi/ml L-[14C]arginine, 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4, 50 mM/l valine, 2 mM/l NADPH, 1 mM/l CaCl2, 1 mM/l L-citrulline, 3 mM/l flavin adenine dinucleotide, and 3 µmol/l flavin mononucleotide. HR or RSNA (16). The peak slope (or maximum gain) was determined by taking the first derivative of the baroreflex curve described by Eq. 1. The first derivative is described by Eq. 2.

\[ \text{HR or RSNA} = \frac{A}{1 + \exp[B(MAP - C)]]} + D \] (1)

where A is HR or RSNA range, B is the slope coefficient, C is the pressure at the midpoint of the range, and D is minimum HR or RSNA (16). The peak slope (or maximum gain) was determined by taking the first derivative of the baroreflex curve described by Eq. 1. The first derivative is described by Eq. 2.

\[ \text{slope} = \frac{A \times B \times \exp[B(MAP - C)]}{(1 + \exp[B(MAP - C)])^2} \] (2)

Values are means ± SE. RSNA is expressed as percentage of maximal activity. Data were analyzed using a one-way analysis of variance for repeated measures comparing more than two sets of mean data. When the F ratio exceeded the critical value, Fisher’s protected least-significant difference test was applied to test the significance of the differences among the values of each group. To evaluate the baseline
and 7-NI, respectively. MAP, mean arterial pressure; bpm, beats/min.

After 7-NI, HR range was significantly increased to 160 ± 8 beats/min compared to control state after pretreatment with atropine and after 7-NI. These results indicate that blockade of nNOS increased the range mainly because of a reduction of the minimum HR. There was a slight increase in the maximum gain, but this parameter did not reach statistical significance. All changes were completely reversed after L-arginine administration (Table 1). In fact, L-arginine reduced the maximum gain below the control level.

**Effects of 7-NI on the Baroreflex Control of HR in Anesthetized Rabbits After Autonomic Blockade**

 Pretreatment with atropine. Composite baroreflex curves generated during control, after pretreatment with atropine, and after 7-NI are shown in Fig. 2. Pretreatment with atropine increased the minimum HR from 178 ± 8 to 248 ± 3 beats/min (Table 1; P < 0.05). Intraperitoneal administration of 7-NI evoked a decrease in the minimum HR to 229 ± 3 beats/min (P < 0.05; Table 1). The maximum gain after atropine was significantly reduced and was restored toward control by 7-NI (Table 1).

 Pretreatment with metoprolol. Composite baroreflex curves generated during control, after pretreatment with metoprolol, and after 7-NI are shown in Fig. 3.

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### Table 1. Resting MAP, HR, percentage of maximum RSNA, and parameters of logistic function curves in anesthetized rabbits

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>RSNA, %maximum</th>
<th>HR Range, beats/min</th>
<th>BP50, mmHg</th>
<th>Min HR, beats/min</th>
<th>Max Gain, beats·min⁻¹·mmHg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93 ± 5</td>
<td>268 ± 9</td>
<td>43 ± 6</td>
<td>121 ± 8</td>
<td>103 ± 3</td>
<td>177 ± 10</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>7-NI</td>
<td>87 ± 4</td>
<td>262 ± 6</td>
<td>46 ± 6</td>
<td>160 ± 13</td>
<td>112 ± 2</td>
<td>120 ± 9*</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>L-Arg</td>
<td>77 ± 6</td>
<td>240 ± 11</td>
<td>30 ± 4</td>
<td>82 ± 10†</td>
<td>105 ± 4</td>
<td>165 ± 4†</td>
<td>2.0 ± 0.3†</td>
</tr>
<tr>
<td>Control</td>
<td>101 ± 2</td>
<td>273 ± 10</td>
<td>26 ± 3</td>
<td>137 ± 10</td>
<td>113 ± 5</td>
<td>178 ± 8</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Atropine</td>
<td>91 ± 7†</td>
<td>290 ± 6</td>
<td>31 ± 2</td>
<td>61 ± 6*</td>
<td>103 ± 3</td>
<td>248 ± 3*</td>
<td>101 ± 0.2*</td>
</tr>
<tr>
<td>7-NI</td>
<td>98 ± 1†</td>
<td>291 ± 7</td>
<td>28 ± 4</td>
<td>75 ± 4†</td>
<td>122 ± 2†</td>
<td>229 ± 3†</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>99 ± 3</td>
<td>279 ± 6</td>
<td>33 ± 4</td>
<td>130 ± 16</td>
<td>110 ± 5</td>
<td>202 ± 7†</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Metoprol</td>
<td>86 ± 5</td>
<td>208 ± 14*</td>
<td>35 ± 4</td>
<td>40 ± 6*</td>
<td>100 ± 8</td>
<td>181 ± 8†</td>
<td>0.6 ± 0.1†</td>
</tr>
<tr>
<td>7-NI</td>
<td>90 ± 5</td>
<td>215 ± 14*</td>
<td>44 ± 5</td>
<td>76 ± 12*</td>
<td>110 ± 4</td>
<td>158 ± 5†</td>
<td>1.6 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. 7-NI, 7-nitroindazole; MAP, mean arterial pressure; HR, heart rate; RSNA, renal sympathetic nerve activity; BP50, pressure at midpoint of range; Min HR, minimum heart rate; Max gain, maximum gain. *Significantly different from control for each group; †significantly different from treatment.
Pretreatment with metoprolol reduced minimum HR from 202 ± 6 to 181 ± 6 beats/min (P, 0.05). Intraperitoneal injection of 7-NI caused a further and significant reduction in minimum HR to 158 ± 5 beats/min (P, 0.05; Table 1). 7-NI increased the maximum gain toward the control level (Table 1).

Effects of 7-NI on the Baroreflex Control of RSNA in Anesthetized Rabbits

Composite baroreflex curves of RSNA generated before and after intraperitoneal administration of 7-NI in anesthetized rabbits are shown in Fig. 4. Although there was some shift of the curve to the left and a slight reduction in maximum gain, 7-NI had little effect on the baroreflex control of RSNA in this group of rabbits.

Effects of 7-NI on the Baroreflex Control of HR and RSNA in Conscious Rabbits

Composite baroreflex curves relating HR to MAP generated before and after intraperitoneal administration of 7-NI in conscious rabbits are shown in Fig. 5. The only difference observed in this group of rabbits after 7-NI was a significant decrease in minimum HR from 140 ± 4 to 125 ± 4 mmHg (P, 0.05). Composite baroreflex curves relating RSNA to MAP before and after intraperitoneal administration of 7-NI in conscious rabbits are shown in Fig. 6. 7-NI had no effects on the baroreflex control of RSNA in this group of rabbits.

Effects of 7-NI on the NOS Activity of Medulla Oblongata

NOS activity in the medulla was significantly lower in the 7-NI-treated than in the vehicle-treated group.

Fig. 3. Composite baroreflex curves for HR control generated in control state after pretreatment with metoprolol and after 7-NI + metoprolol (Meto) in anesthetized rabbits (n = 7). Metoprolol decreased minimum HR. Addition of 7-NI further decreased minimum HR. ○, Control; □, metoprolol; ◊, metoprolol + 7-NI; ●, ◊, and □, mean resting values for control, metoprolol, and metoprolol + 7-NI.

Fig. 4. Composite baroreflex curves for control of renal sympathetic nerve activity (RSNA) generated before and after intraperitoneal infusion of 7-NI in anesthetized rabbits (n = 6). 7-NI had no effect on baroreflex control of RSNA. ○, Control; □, 7-NI; ● and ◊, mean resting values for control and 7-NI, respectively.

Fig. 5. Composite baroreflex curves for HR control generated before and after intraperitoneal injection of 7-NI in conscious rabbits (n = 6). 7-NI reduced minimum HR in a manner similar to that observed in anesthetized rabbits. ○, Control; □, 7-NI; ● and ◊, mean resting values for control and 7-NI, respectively.

Fig. 6. Composite baroreflex curves for control of RSNA generated before and after intraperitoneal injection of 7-NI in conscious rabbits (n = 6). 7-NI had no effect on baroreflex control of RSNA. ○, Control; □, 7-NI; ● and ◊, mean resting values for control and 7-NI, respectively.
202 ± 21 vs. 334 ± 18 pmol·min⁻¹·mg protein⁻¹ (P < 0.05).

DISCUSSION

In the present study we investigated the effects of nNOS inhibition on the baroreflex control of HR and RSNA in anesthetized and conscious rabbits with the use of the relatively selective nNOS inhibitor 7-NI. The new findings of the present study are as follows. 1) Blockade of nNOS decreased the minimum HR of the baroreflex in conscious and anesthetized rabbits without changing the resting MAP and HR. 2) The mechanisms of the decrease in minimum HR involve sympathetic and parasympathetic pathways. The effects of 7-NI on minimum HR are not likely to be due to a direct SA nodal effect, since we previously showed that inhibition of NO synthesis with L-NNA had no effect on resting or baroreflex-mediated HR changes after combined cholinergic and β-blockade (18). 3) Blockade of nNOS had no effect on baroreflex control of RSNA in conscious or anesthetized rabbits.

The central pathways involved in the modulation of the baroreflex are complex. In addition to the nucleus tractus solitarius, the rostroventrolateral medulla, the nucleus ambiguous, and the dorsal motor nucleus of the vagus and several other important areas of the brain stem and hypothalamus can modulate baroreflex function. According to recent studies, NO can influence baroreflex function at several of these sites in vivo studies. Blockade of nNOS decreased the minimum HR of the baroreflex in conscious and anesthetized rabbits without changing the resting MAP and HR. The mechanisms of the decrease in minimum HR involve sympathetic and parasympathetic pathways. The effects of 7-NI on minimum HR are not likely to be due to a direct SA nodal effect, since we previously showed that inhibition of NO synthesis with L-NNA had no effect on resting or baroreflex-mediated HR changes after combined cholinergic and β-blockade (18). 3) Blockade of nNOS had no effect on baroreflex control of RSNA in conscious or anesthetized rabbits.

Several studies have assessed the effects of anesthesia on baroreflex function (4, 20). To determine the effects of anesthesia, we repeated these experiments in conscious rabbits. There was no significant effect on resting MAP or HR after intraperitoneal administration of 7-NI. Although the magnitude of the change in minimum HR was smaller in the conscious state than in the anesthetized state, the results were qualitatively similar in conscious and anesthetized conditions. The finding that 7-NI affects the baroreflex control of HR but not of RSNA is surprising and suggests that the central modulation of baroreflex function by NO is targeted primarily to those structures that regulate autonomic outflow to the heart rather than to the kidney. NO may have heterogenous effects on sympathetic outflow to different beds, such as may be exhibited here. Recently, Hirai et al. (11, 12) clearly showed that NO inhibition increased RSNA but had the opposite effect on lumbar sympathetic nerve activity in anesthetized rats. The lack of effect of nNOS inhibition on RSNA in this study is in good agreement with the results of our previous study carried out in conscious rabbits (18). In that study we also found a greater effect of NO inhibition on baroreflex control of HR than of RSNA. The lack of an effect on resting RSNA in this study as in the previous one may reflect specificity of the sites in the brain that are modulated by NO or may reflect the lack of an endogenous excitatory pathway that is permissive for sympathoexcitation after NO inhibition. We recently provided evidence for such an effect (17). When arterial pressure was kept constant in conscious rabbits, N-nitro-L-arginine methyl ester failed to increase RSNA unless plasma levels of angiotensin II (ANG II) were elevated. ANG II can act as a central sympathoexcitatory agent (2, 26). ANG II levels may not be sufficiently high in normal animals to facilitate the sympathoexcitation when NO synthesis is blocked. It is possible that the inhibition of NOS activity was not sufficient to affect the baroreflex control of RSNA in the present study. The inhibition of NOS activity after 7-NI was ~40% in the present study, significantly less than in the vehicle-treated rabbits.

In summary, the present study indicates that endogenous brain NO decreases the baroreflex control of HR but not of RSNA. Cardiac sympathetic and parasympathetic components are involved in the HR effects.

PERSPECTIVES

The present study was designed to elucidate the central effects of NO on the baroreflex control of HR and RSNA. The conclusion of our study indicated that NO had small but significant effects on the baroreflex control of HR. The importance of these findings is that NO has central effects on the regulation of the circulation. In some pathological situations, NO is decreased or increased. It appears that clarification of the central effects of NO in such a pathophysiological state is more important and should be examined in the future.

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