Mechanism of Neurally Induced Monkey Mesenteric Artery Relaxation and Contraction

Noboru Toda and Tomio Okamura

Physiological importance in vasodilator innervation alleviating noradrenergic neurogenic vasoconstriction has not been clarified. Isolated monkey mesenteric artery strips denuded of the endothelium responded to nerve stimulation by electrical pulses or nicotine with a contraction, which was potentiated by N\textsuperscript{G}-nitro-L-arginine, a nitric oxide synthesis inhibitor, but not by the \textit{d}-enantiomer. The potentiation was abolished by \textit{l}-arginine. \textit{N}\textsuperscript{G}-Nitro-L-arginine did not potentiate the response to exogenous norepinephrine nor did it increase the release of [\textsuperscript{3}H]norepinephrine from adrenergic nerves electrically stimulated. The contraction was reversed by treatment with phentolamine and guanethidine to a relaxation, which was abolished by \textit{N}\textsuperscript{G}-nitro-L-arginine. The inhibition was reversed by \textit{l}- but not \textit{d}-arginine. The relaxant response was not influenced by atropine, timolol, or indomethacin. These findings strongly suggest the importance of reciprocal nitric oxide-related (nitroxidergic) vasodilator and noradrenergic vasoconstrictor innervation in the regulation of monkey arterial tone.

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Noradrenergic innervation in the vascular wall plays a crucial role in the physiological control of vasomotor tone, thus regulating peripheral vascular resistance and blood distribution in organs and tissues. The vasoconstrictor innervation also participates in pathogenesis of vascular disorders such as vasospasm and hypertension. Despite a histochemical demonstration of cholinergic nervelike structures,\textsuperscript{1} the functional roles of these structures have not been clarified. Transmitter substances from noradrenergic and cholinergic nerves seem to act synergistically on smooth muscle since cholinergic nerve stimulation constricts bovine coronary arteries\textsuperscript{2} and canine veins.\textsuperscript{3} Nonadrenergic, noncholinergic vasodilator innervation has been demonstrated in human,\textsuperscript{4} monkey,\textsuperscript{4,5} dog,\textsuperscript{5,6} cat,\textsuperscript{7} and bovine\textsuperscript{8} cerebral arteries. Recent studies in our laboratory dealing with monkey and dog cerebral arteries have provided evidence that nitric oxide (NO) plays a crucial role in transmitting information from the nerve to arterial smooth muscle.\textsuperscript{9–12} Similar results were also obtained in rat anococcygeus muscle\textsuperscript{13} and dog ileocolonic junction.\textsuperscript{14} In the present study, we demonstrated the presence of nonadrenergic, noncholinergic vasodilator innervation in monkey mesenteric arteries, determined the vasodilator and vasoconstrictor nerve interaction, and clarified the involvement of NO in neurally induced vasodilatation by the use of \textit{N}\textsuperscript{G}-nitro-L-arginine (L-NA), an NO synthesis inhibitor.\textsuperscript{11,15}

\textbf{Methods}

Japanese monkeys (\textit{Macaca fuscata}, 4 years or older) of either sex, weighing 6–10 kg, were anesthetized with intramuscular injections of ketamine (40 mg/kg) and were killed by exsanguination from the carotid arteries. The age of wild monkeys was determined up to 7 years by the growth and the eruption of teeth. The superior mesenteric arteries of 0.4–0.6 mm o.d. were isolated. Helically cut strips of the arteries, denuded of the endothelium, were vertically fixed between hooks under a resting tension of 1 g in a muscle bath (20 ml capacity) containing a modified Ringer-Locke solution that was aerated with 95% O\textsubscript{2}-5% CO\textsubscript{2} and was maintained at 37°C. Endothelium denudation was determined by abolishment of acetylcholine-induced relaxations. Constituents of the solution were as follows (mM): NaCl 120, KCl 5.4, CaCl\textsubscript{2} 2.2, MgCl\textsubscript{2} 1.0, NaHCO\textsubscript{3} 25.0, and dextrose 5.6. The pH of the solution was 7.35–7.42. Before the start of experiments, all of the strips were allowed to equilibrate in the bathing media for 60–90 minutes, during which time the fluids were replaced every 10–15 minutes.

All of the arterial strips, except for those used for experiments with nicotine and NO, were placed between stimulating electrodes. The gap between the strip and the electrodes was wide enough to allow...
undisturbed contractions and relaxations and yet sufficiently narrow to stimulate intramural nerve terminals effectively. A train of 0.2 msec square pulses of supramaximal intensity (10 V) were applied transmurally at frequencies of 2, 5, and 20 Hz for 100, 40, and 10 seconds, respectively. Isometric contractions and relaxations were recorded on an ink-writing oscillograph (Nihonkohden Co., Tokyo). The contractile response to 30 mM K⁺ was first obtained; the strips were then washed three times with fresh media and equilibrated for 30–40 minutes. To examine the relaxant response, the strips were partially contracted with prostaglandin F₂α (PGF₂α) (5×10⁻⁸ to 10⁻⁷ M); the contractions were in a range between 25% and 40% of the contraction induced by 30 mM K⁺. Transmural electrical stimulation was applied repeatedly at intervals of 10 minutes until steady responses were obtained, and then blocking agents were applied. At the end of each series, tetrodotoxin (3×10⁻⁷ M) was applied to confirm the neurally induced response. Nicotine in a concentration of 10⁻⁴ M was applied directly to the bathing media, and the strips were repeatedly washed. After reproducibility of the response to nicotine was determined, preparations were treated for about 30 minutes with blocking agents. Nicotine and NO in single concentrations were applied, and at the end, papaverine (10⁻⁴ M) was added to attain the maximal relaxation. Relaxations induced by agonists relative to those caused by papaverine were presented. Contractions induced by 30 mM K⁺ were taken as standards (100%) for the contractile responses to electrical and chemical stimuli. Isotope experiments were carried out on helical strips of monkey mesenteric arteries as previously described. The tissue was preincubated for 60 minutes at 37°C with 0.5 μM [³H]norepinephrine (specific activity of 1,616.9 gigabecquerel/mmol). It was then superfused with the modified Ringer-Locke solution containing cocaine (3×10⁻³ M) and corticosterone (4×10⁻³ M) at a rate of 1 ml/min. The strips were electrically stimulated five times for 3 minutes each at a frequency of 5 Hz. Stimulations were applied after 126 (S₁), 144 (S₂), 162 (S₃), 180 (S₄), and 198 (S₅) minutes of superfusion. The stimulation-evoked overflow of total tritium was calculated as percent of the tissue tritium content at the time of stimulation. L-NA (5×10⁻⁶ M) was added 12 minutes before S₄. The effect of L-NA on the stimulation-evoked [³H] overflow was expressed as the ratio between the overflow evoked by S₄ and that evoked by S₁. The ratios were compared with those obtained in the absence of treatment with the drug.

The content of cyclic GMP in monkey mesenteric artery strips denuded of the endothelium was measured. After 20 minutes equilibration in the bathing media, the arterial strips were exposed for 1 minute to nicotine (10⁻⁴ M) with or without hexamethonium and then were plunged into liquid nitrogen. The tissues were homogenized in 1.5 ml of 6% trichloroacetic acid at 0°C with a glass homogenizer. After centrifugation at 800g for 10 minutes, an ether extraction procedure was carried out on the supernatant. An aliquot of the extract was then used for determination of cyclic GMP by the use of a commercial radioimmunoassay kit (Yamasa Co., Chiba, Japan).

The results shown in the text, figures, and tables are expressed as mean±SEM. Statistical analyses were made using Student’s paired and unpaired t test and Tukey’s method after one-way analysis of variance. Drugs used were N⁶-nitro-L-arginine (L-NA), N⁶-nitro-D-arginine (D-NA) (Peptide Institute, Minoh, Japan), L-arginine and D-arginine, nicotine (Nacalai Tesque, Kyoto, Japan), tetrodotoxin, dl-norepinephrine hydrochloride (Sankyo, Tokyo), PGF₂α (Ono, Osaka), indomethacin, guanethidine sulfate (Sigma Chemical Co., St. Louis, Mo.), atropine sulfate (Tanabe, Tokyo), timolol maleate (Banyu, Tokyo), phentolamine mesylate (CIAB-GEIGY, Japan, Takarazuka, Japan), hexamethonium bromide (Yamanouchi, Tokyo), and papaverine hydrochloride (Dainippon, Osaka). Responses to NO were obtained by adding the NaN₃ solution adjusted at pH 2. Concentrations of acidified NaN₃ in the muscle bath were presented as those of NO. Oxyhemoglobin (oxyHb) was prepared by addition of 10-fold molar excess of the reducing agent, sodium dithionite, to a 1 mM solution of commercial Hb (Sigma) in distilled water. Sodium dithionite was then removed by extensive dialysis.

Results

Effects of Transmural Electrical Stimulation on Mesenteric Arteries

Transmural electrical stimulation (2, 5, and 20 Hz) produced a frequency-related contraction in the monkey mesenteric artery strips denuded of the
endothelium, under resting conditions and when partially contracted with PGF$_2\alpha$ (49±21, 135±20, and 238±39 mg, n=5, respectively). The contraction was markedly suppressed or abolished by treatment with 10$^{-7}$ M phentolamine (n=18), 10$^{-6}$ M guanethidine (n=5), or 3×10$^{-7}$ M tetrodotoxin (all the strips used). The stimulation-induced contraction was potentiated by treatment with 10$^{-6}$ M L-NA, as shown in Figure 1. L-NA did not contract the arteries under resting conditions. The potentiating effect was not influenced by 3×10$^{-4}$ M d-arginine (n=4) but was antagonized by treatment with 3×10$^{-4}$ M L-arginine (Figure 1). The typical result is illustrated in Figure 2, upper tracing. D-NA (10$^{-6}$ M) did not alter the response (Figure 1). Contractions of the artery strips in response to exogenously applied norepinephrine (2×10$^{-8}$, 10$^{-7}$, and 5×10$^{-7}$ M) were not significantly increased by 10$^{-6}$ and 10$^{-5}$ M L-NA (Figure 3). In the strips from the same monkeys, contractions induced by 5 Hz stimulation averaged 93±26 mg (n=7) under resting conditions.

The [3H] overflow evoked by transmural electrical stimulation (5 Hz for 3 minutes) from superfused monkey mesenteric artery strips previously incubated in the bathing media containing [3H]norepinephrine was not increased by treatment with 5×10$^{-6}$ M L-NA. The overflow ratio S$_2$/S$_3$ was 1.00±0.06 in five control series of experiments and 0.87±0.02 in five L-NA-treated series.

In the arteries treated with 10$^{-7}$ M phentolamine (Figure 2) or 10$^{-6}$ M guanethidine (Figure 4) and partially contracted with PGF$_2\alpha$ transmural electrical stimulation produced a relaxation or a slight contraction followed by a relaxation; relaxations by the stimulation at 2, 5, and 20 Hz averaged 61.2±2.2% (n=4), 14.4±1.8% (n=24), and 20.3±4.7% (n=4), respectively. Treatment with 10$^{-6}$ M L-NA markedly suppressed the relaxation at 5 Hz stimulation (Figure 4) or reversed it to a contraction (Figure 2, lower tracing). The quantitative data are summarized in Figure 5. The inhibitory effect was antagonized by L-arginine (3×10$^{-4}$ M) (Figure 5) but not by D-arginine (from 3.0±1.2% contraction relative to that caused by 30 mM K$^+$ to 3.1±1.2%, n=4). The stimulation (5 Hz)-induced relaxations were abolished by 3×10$^{-7}$ M tetrodotoxin (all of the preparations used) and 1.6×10$^{-5}$ M oxyHb (n=5) but were unaffected by treatment with 10$^{-7}$ M timolol, 10$^{-7}$ M atropine, and 10$^{-6}$ M indomethacin (Table 1). L-Arginine (3×10$^{-4}$ M) did not relax the control and L-NA-treated strips. NO (10$^{-7}$ M)-induced relaxations were not significantly influenced by 10$^{-6}$ M L-NA (59.0±4.5 versus 51.9±5.2%, n=10).

Effects of Nicotine on Mesenteric Arteries

The addition of nicotine (10$^{-4}$ M) produced a phasic contraction in the monkey mesenteric arteries denuded of the endothelium, which was potentiated by treatment with 10$^{-6}$ M L-NA (Figure 1). The
potentiation was reversed by 3×10^{-4} \text{M} \text{L-arginine. The}
\text{arteries treated with 10^{-7} \text{M} \text{phenolamine or 10^{-6} \text{M} \text{guanethidine and contracted with PGF}_{2\alpha}}
\text{responded to nicotine with a relaxation, which was}
\text{abolished or converted to a slight contraction by 10^{-6} \text{M L-NA (Figure 6). The inhibition was reversed by}
3×10^{-4} \text{M L-arginine but not by its D-enantiomer (n=4). Typical responses are shown in Figure 7. The}
\text{nicotine-induced relaxation was not influenced by 10^{-7} \text{M timolol and 10^{-7} \text{M atropine (Table 1) but}
\text{were abolished by 10^{-5} \text{M hexamethonium (n=6).}

\text{The level of cyclic GMP in monkey mesenteric}
\text{artery strips without the endothelium was measured.}
\text{Nicotine (10^{-4} \text{M}) significantly increased cyclic GMP}
\text{in the strips, and treatment with 10^{-5} \text{M hexametho}
\text{num abolished the stimulating effect of nicotine. The}
\text{results are summarized in Table 2.}

**Discussion**

\text{Vasoconstrictor responses to noradrenergic nerve}
\text{stimulation by electrical pulses or nicotine were}
\text{potentiated by L-NA in monkey mesenteric arteries}
\text{(present study) and by N^{\text{G}}\text{-monomethyl-L-arginine, the other NO synthesis inhibitor,}}^{19}
\text{in dog mesenteric arteries}^{9} \text{but not by the D-enantiomers. The potentiation was abolished by the addition of L-arginine but}
\text{not D-arginine. The [H] overflow evoked by adrenergic nerve stimulation from artery strips previously incubated in [H]norepinephrine was not increased by L-NA nor was the contractile response to exogenously applied norepinephrine. Therefore, the induced potentiation of the neurally induced contraction would be due to a suppression of vasodilatation}
\text{possibly mediated by NO rather than an increase in the}
\text{stimulation-induced release of norepinephrine and the sensitivity of arteries to the amine. Treatment with bretylium}^{9} \text{or guanethidine, adrenergic neuron blockers, and 6-hydroxydopamine (N. Toda}
\text{and T. Okamura, unpublished data) abolished the contractile response to adrenergic nerve stimulation but did not inhibit the relaxation elicited by the dilator nerve stimulation, suggesting that norepinephrine and the vasodilator transmitter do not coexist in the same nerve.}

\text{Relaxant responses to transmural electrical stimulation}
\text{and nicotine in monkey mesenteric artery strips treated with phenolamine or guanethidine were not influenced by timolol and atropine but were abolished by tetrodotoxin (for electrical stimulation) or hexamethonium (for nicotine). The responses were abolished or converted to contractions by treatment with L-NA, the inhibitory effect being antagonized by L-arginine but not D-arginine. Similar results were also obtained in monkey and dog cerebral arteries that responded to the nerve stimulation only with relaxations}\^{9,12} \text{even in the absence of blockade of adrenergic nerves and a-adrenergic receptors. The relaxations were abolished by hemoglobin and methylene blue.}^{19} \text{In the present study, nicotine increased the level of cyclic GMP in the monkey mesenteric arteries without the endothelium; the effect was abolished by hexamethonium. It may therefore be concluded that the neurally induced relaxation is associated with NO released from nonadrenergic, noncholinergic nerves that activate guanylate cyclase and increase the synthesis of cyclic GMP. Recent

**TABLE 1. Effects of Inhibitors on Relaxant Response to Transmural Electrical Stimulation and Nicotine of Monkey Mesenteric Artery Strips Denuded of Endothelium and Treated With Phenolamine**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relaxation (%)* induced by TES (5 Hz)</th>
<th>Nicotine (10^{-4} \text{M})</th>
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<tr>
<td></td>
<td>n Control Treated n Control Treated</td>
<td></td>
</tr>
<tr>
<td>D-NA (10^{-6} \text{M})</td>
<td>6 16.5±3.2 16.8±3.5</td>
<td>4 17.3±2.0 14.0±1.6</td>
</tr>
<tr>
<td>Atropine (10^{-7} \text{M})</td>
<td>5 17.8±4.9 19.6±5.4</td>
<td>4 15.6±2.3 16.5±3.3</td>
</tr>
<tr>
<td>Timolol (10^{-7} \text{M})</td>
<td>5 18.2±4.7 18.6±5.6</td>
<td>5 17.6±2.2 17.6±3.8</td>
</tr>
<tr>
<td>IM (10^{-4} \text{M})</td>
<td>4 11.5±3.9 13.3±4.1</td>
<td>... ...</td>
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TES, transmural electrical stimulation; n, number of strips from separate monkeys; D-NA, N^{\text{G}}-nitro-D-arginine; IM, indomethacin.

*Relaxations relative to those induced by 10^{-4} \text{M} \text{papaverine.
immunohistochemical study by Bredt et al.\textsuperscript{20} indicates that NO synthase–containing nerves are located in the adventitia of rat large cerebral arteries but not in the media. They provided supportive evidence for our hypothesis that NO liberated from the nerves (putatively nitroxidergic) transmits vasodilator information to smooth muscle cells.

The role of cholinergic innervation in vasodilatation has not been clarified. However, our data obtained so far strongly suggest that vasodilator nitroxidergic and vasoconstrictor noradrenergic nerves participate importantly in the regulation of vascular tone by balancing dilatation and constriction. In most vasculatures, neuronal vasoconstriction predominates over the vasodilatation. In the present and previous (Reference 11 and N. Toda and T. Okamura, unpublished data) studies, L-NA markedly suppressed the vasodilator nerve function in monkey mesenteric, superficial temporal, and cerebral arter-

### Table 2. Cyclic GMP Levels of Monkey Mesenteric Artery Strips Denuded of Endothelium and Exposed to Control Media and Those Containing Nicotine or Nicotine Plus Hexamethonium

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Cyclic GMP (fmol/mg wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>2.62±0.48</td>
</tr>
<tr>
<td>Nicotine</td>
<td>5</td>
<td>5.32±0.53††</td>
</tr>
<tr>
<td>Nicotine+Ca</td>
<td>5</td>
<td>2.72±0.34</td>
</tr>
</tbody>
</table>

\(n\), Number of artery strips from separate monkeys; Ca, hexamethonium.

*Frozen tissue weight.

†p<0.01 significantly different from control.

‡p<0.01 significantly different (Tukey's method) from value with nicotine plus hexamethonium.

FIGURE 5. Bar graph shows responses to transmural electrical stimulation (5 Hz) of monkey mesenteric artery strips denuded of endothelium and partially contracted with prostaglandin F\(_{2\alpha}\) before (C\(_0\)) and after treatment with 10\(^{-7}\) M phentolamine. In phentolamine-treated strips, the stimulation produced a transient contraction (hatched column) followed by a relaxation (open column). Responses in control media (C) were compared with those after treatment with N\(^6\)-nitro-L-arginine (L-NA) (10\(^{-6}\) M) alone and L-NA+L-arginine (L-arg.) (3\times10\(^{-4}\) M). Contractions induced by 30 mM K\(^+\) were taken as 100% contraction; the mean absolute value was 1,360±404 mg (n=9). Relaxations induced by 10\(^{-4}\) M papaverine were taken as 100% relaxation; mean absolute values in control media and those containing L-NA and L-NA+L-arg. were 327±39, 374±37, and 342±40 mg (n=9), respectively. Significantly different from the value before phentolamine, °p<0.01, °p<0.05. Significantly different from the value with L-NA, °p<0.01, °p<0.05 (Tukey's method). Number of strips from separate monkeys, 9. Vertical bars represent SEM.

FIGURE 6. Bar graph shows responses to 10\(^{-4}\) M nicotine of monkey mesenteric artery strips denuded of endothelium and partially contracted with prostaglandin F\(_{2\alpha}\) before (C\(_0\)) and after treatment with 10\(^{-7}\) M phentolamine. In phentolamine-treated strips, nicotine produced a transient contraction (hatched column) followed by a relaxation (open column). Responses in control media (C) were compared with those after treatment with N\(^6\)-nitro-L-arginine (L-NA) (10\(^{-6}\) M) alone and L-NA+L-arginine (L-arg.) (3\times10\(^{-4}\) M). Contractions induced by 30 mM K\(^+\) were taken as 100% contraction; the mean absolute value was 1,136±246 mg (n=14). Relaxations induced by 10\(^{-4}\) M papaverine were taken as 100% relaxation; mean absolute values in control media and those containing L-NA and L-NA+L-arg. were 266±43, 278±47, and 281±67 mg (n=14), respectively. Significantly different from the value before phentolamine, °p<0.01, °p<0.05. Significantly different from the value with L-NA, °p<0.01, °p<0.05 (Tukey's method). Number of strips from separate monkeys, 14. Vertical bars represent SEM.

FIGURE 7. Representative tracings show modifications by N\(^6\)-nitro-L-arginine (L-NA) and L-NA+L-arginine (L-arg.) (3\times10\(^{-4}\) M) of the responses to nicotine (N) (10\(^{-4}\) M) and nitric oxide (NO) (10\(^{-7}\) and 10\(^{-6}\) M) of a monkey mesenteric artery strip without endothelium. Strip was partially contracted with 10\(^{-4}\) M prostaglandin F\(_{2\alpha}\) and treated with 10\(^{-7}\) M phentolamine. PA, 10\(^{-4}\) M papaverine.
ies, rendering noradrenergic nerve function more predominant in the former two arteries. We used mesenteric and temporal arteries as representatives of visceral and skeletal muscle/cutaneous arteries, respectively. Studies are underway to determine whether the other visceral, skeletal muscle, and cutaneous arteries are also innervated by nonadrenergic, noncholinergic vasodilator nerves. Systemic blood pressure in anesthetized monkeys was significantly raised by intravenous injections of L-Na, and the effect was reversed by L-arginine (N. Toda and T. Okamura, unpublished data). In anesthetized rabbits and guinea pigs and conscious rats, infused into the brachial artery decreases forearm blood flow in healthy volunteers. These authors postulated that increased vascular resistance induced by the synthesis inhibitors is due to a suppression of basal release of endothelium-derived NO that dilates arterioles. Although their idea is one of possible explanations for the generation of hypertension associated with NO synthetase inhibition, the depression of L-NA of the nitrooxidergic vasodilator nerve function may also play an important role in the generation of hypertension. Our hypothesis would be validated by additional evidence demonstrating that such a vasodilator innervation participates also in the regulation of other vasculatures and resistance vessels.

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References


Key Words • nitric oxide • monkey • adrenergic nerve • N°-nitro-L-arginine • mesenteric artery • nitrooxidergic nerve
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