Monkey Central Retinal Artery Is Innervated by Nitroxidergic Vasodilator Nerves

Noboru Toda, Megumi Toda, Kazuhide Ayajiki, and Tomio Okamura

**Purpose.** To determine whether the monkey central retinal artery is innervated by vasodilator nerves and to analyze the mechanism underlying the neurogenic response.

**Methods.** Changes in isometric tension were recorded in helical strips of the arteries, which were stimulated by transmurally applied electrical pulses or nicotine. The presence of perivascular nerve fibers containing nitric oxide (NO) synthase immunoreactivity was determined histologically.

**Results.** Transmural electrical stimulation (5 Hz) and nicotine produced a relaxation of the arterial strips denuded of the endothelium, treated with prazosin, and contracted with prostaglandin F2α. The response was not influenced by timolol, atropine, and indomethacin, but it was abolished by methylene blue and oxyhemoglobin. N-[ω-nitro-L-arginine, a NO synthase inhibitor, abolished the neurogenic relaxation, and L-arginine restored the response. Antagonists of calcitonin gene-related peptide and vasoactive intestinal polypeptide in sufficient concentrations did not influence the response to nerve stimulation by nicotine. There were abundant nerve fibers and bundles containing NO synthase immunoreactivity in the adventitia.

**Conclusions.** Monkey central retinal arteries are innervated by NO synthase-containing nerves that liberate NO possibly as a neurotransmitter on excitation to produce muscular relaxation.
cal and chemical stimulation of perivascular nerves in isolated monkey central retinal arteries, to clarify the underlying mechanism, and to demonstrate histochemically the innervation. Nitric oxide, vasoactive intestinal polypeptide (VIP), and calcitonin gene-related peptide (CGRP) were thought to be candidates for the neurotransmitter. Nicotine was used as a chemical stimulant of nerves because it can release neurotransmitters in various blood vessels.\textsuperscript{12-14}

**METHODS**

All experimental procedures that used animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Our institutional review board approved the use of animal blood vessels in this study.

**Tension Recording**

Japanese monkeys (Macaca fuscata) of either sex, weighing 6 to 10 kg, were killed by bleeding from the common carotid arteries under anesthesia with intramuscular injections of ketamine (40 mg/kg) and then sodium thiopental (20 mg/kg). Eyeballs, with optic nerves and extraocular tissues attached, were removed rapidly from the orbital cavities. Central retinal arteries (0.3 to 0.4 mm outside diameter) located immediately before entering into the eyeball were isolated and cut into helical strips of approximately 20 mm in length. The endothelium was removed by gently rubbing the intimal surface with a cotton ball. Specimens were vertically fixed between hooks in a muscle bath (20 ml capacity) containing the modified Ringer–Locke solution, which was maintained at 37°C ± 0.3°C and was aerated with a mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The hook anchoring the upper end of the strips was connected to the lever of a displacement transducer (Nihon–kohden Kogyo, Tokyo, Japan). Some of the strips were placed between stimulating electrodes, and electrical pulses of 0.2 msec, at a frequency of 5 Hz for 40 seconds, were applied transmurally to stimulate perivascular nerves unless otherwise mentioned. The resting tension was adjusted to 1 g, which was optimal for inducing the maximal contraction. The composition of the solution was as follows (mmol/l): 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 7.37 to 7.44. Before the start of the experiments, all strips were allowed to equilibrate for 60 to 90 minutes in the bathing media, during which time the fluid was replaced every 10 to 15 minutes.

Isometric mechanical responses were displayed on an ink-writing oscillograph (Nihon–kohden Kogyo). The contractile response to 3 × 10\textsuperscript{-2} mol/l K\textsuperscript{+} was first obtained, and the arterial strips were washed repeatedly with fresh media and equilibrated. Strips were contracted partially with prostaglandin (PG) F\textsubscript{20} (5 to 25 × 10\textsuperscript{-6} mol/l); the contraction ranged between 25% and 44% of the contraction induced by 3 × 10\textsuperscript{-4} mol/l K\textsuperscript{+}. Removal of the endothelium was determined by abolishment of the relaxation induced by Ca\textsuperscript{2+} ionophore A23187 (10\textsuperscript{-7} mol/l). Transmural electrical stimulation was applied every 10 minutes to the strips treated with 10\textsuperscript{-5} mol/l prazosin until the response was stabilized; the application order was randomized. Nicotine, NO (acidified NaNO\textsubscript{2} solution), VIP, and CGRP in single concentrations were applied successively to the bathing media containing 10\textsuperscript{-5} mol/l prazosin, unless otherwise stated. At the end of each series of experiments, papaverine (10\textsuperscript{-4} mol/l) was added to attain the maximal relaxation, which was taken as 100% for the relaxation induced by nerve stimulation or agonists. Arterial strips had been treated for 15 to 20 minutes with blocking agents before the effect of electrical nerve stimulation or agonists was obtained.

**Histochemical Study**

The central retinal artery was fixed in ice-cold 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) containing 0.3% glutaraldehyde and 4% paraformaldehyde post-fixed overnight in 0.1 mol/l PBS with 4% paraformaldehyde, which was followed by cryoprotection in 15% sucrose. The fixed blocks were cut into sections (20 μm thick) in a cryostat (Cryotom, Nakagawa Seisakusho, Tokyo).

For immunohistochemical staining of NO synthase,\textsuperscript{6} the tissue sections were kept in 0.1 mol/l PBS containing 0.3% Triton X-100 at 4°C for 4 days. The specimens were exposed to affinity-purified rabbit antisera against rat cerebellum NO synthase (1:300) in PBS with 0.3% Triton X-100 at 4°C for 4 days. Subsequently, biotinylated goat anti-rabbit immunoglobulin G antibody and avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA) were conjugated to the primary antibody at room temperature for 1 hour each. Immunolabeled peroxidase was visualized by incubation at room temperature for 3 to 5 minutes with 5.6 × 10\textsuperscript{-4} mol/l 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan), 1.3 × 10\textsuperscript{-6} mol/l hydrogen peroxide, and 10\textsuperscript{-4} mol/l nickel ammonium sulfate. The specimens were mounted onto gelatin–chrome–alum-coated glass slides. After several washes with distilled water, the sections were air-dried and coverslipped with Entellan. An immunohistochemical control experiment, in which the antisera against NO synthase was excluded from the reaction mixture, gave no positive staining.
Statistics and Drugs Used

The results shown in the text, tables, and figures are expressed as mean values ± SE. Statistical analyses were made using the Student’s unpaired t-test and the Tukey’s method after one-way analysis of variance. Drugs used were N^o-nitro-L-arginine (L-NA), N^o-nitro-D-arginine (D-NA), VIP, CGRP, [8-37]CGRP (Peptide Institute, Minoh, Japan), L- and D-arginine, nicotine (base), methylene blue trihydrate, hexamethonium bromide (Nacalai Tesque, Kyoto, Japan), atropine sulfate (Tanabe, Osaka, Japan), timolol hydrochloride (Banyu, Tokyo, Japan), indomethacin, [4Cl-D-Phe^6, Leu^7]VIP (Sigma Chemical, St. Louis, MO), PGF_{2\alpha} (Upjohn, Tokyo, Japan), prazosin hydrochloride (Pfizer-Taito, Tokyo, Japan), tetrodotoxin (Sankyo, Tokyo, Japan), and papaverine hydrochloride (Dainippon, Osaka, Japan). Responses to NO were obtained by adding NaNO_2 solution adjusted at pH 2.15 and the concentrations of NaNO_2 in the bathing media were expressed as those of NO. Oxyhemoglobin was prepared according to the method of Martin et al.16

RESULTS

Arterial Response to Transmural Electrical Stimulation

In monkey central retinal arterial strips partially contracted with PGF_{2\alpha} and treated with 10^{-5} mol/l prazosin, transmural electrical stimulation at frequencies of 2, 5, and 20 Hz for 100, 40, and 10 seconds, respectively, produced frequency-related relaxations. Consistent and reproducible responses were induced at 5 Hz.

<table>
<thead>
<tr>
<th>Treatment (mol/l)</th>
<th>Number of Strips From Separate Monkeys</th>
<th>% Relaxation by TES or Nicotine*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>(10^{-7})</td>
<td>(10^{-7})</td>
<td>(10^{-7})</td>
</tr>
<tr>
<td>Timolol</td>
<td>4</td>
<td>24.5 ± 4.9</td>
</tr>
<tr>
<td>Atropine</td>
<td>4</td>
<td>25.5 ± 4.8</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3</td>
<td>23.2 ± 3.8</td>
</tr>
<tr>
<td>OxyHb 1.6 × 10^{-5}</td>
<td>3</td>
<td>28.5 ± 5.1</td>
</tr>
<tr>
<td>Tetrodotoxin 3 × 10^{-7}</td>
<td>12</td>
<td>23.9 ± 2.9</td>
</tr>
<tr>
<td>Nicotine</td>
<td>(10^{-7})</td>
<td>4</td>
</tr>
<tr>
<td>Atropine</td>
<td>(10^{-7})</td>
<td>4</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>(10^{-7})</td>
<td>4</td>
</tr>
<tr>
<td>[8-37]CGRP 10^{-7}</td>
<td>6</td>
<td>39.8 ± 5.1</td>
</tr>
<tr>
<td>VIP antagonist 10^{-6}</td>
<td>8</td>
<td>43.5 ± 5.0</td>
</tr>
<tr>
<td>OxyHb 1.6 × 10^{-5}</td>
<td>3</td>
<td>38.4 ± 6.9</td>
</tr>
<tr>
<td>Methylene blue 10^{-5}</td>
<td>3</td>
<td>41.3 ± 8.2</td>
</tr>
<tr>
<td>Hexamethonium 10^{-5}</td>
<td>8</td>
<td>46.3 ± 6.7</td>
</tr>
</tbody>
</table>

TES = transmural electrical stimulation.
VIP antagonist = [4Cl-D-Phe^6, Leu^7] VIP; OxyHb = oxyhemoglobin.
* Relaxations relative to those caused by 10^{-4} mol/l papaverine.
† Significantly different from control \((P < 0.05, \text{unpaired } t\text{-test})\).
‡ Significantly different from control \((P < 0.001, \text{unpaired } t\text{-test})\).
FIGURE 2. Modifications by D-NA (10^-6 mol/l), L-NA (10^-6 mol/l), L-NA + D-arginine (D-arg, 3 X 10^-4 mol/l), and L-NA + L-arginine (L-arg, 3 X 10^-4 mol/l) of the response to transmural electrical stimulation (TES, 5 Hz) of monkey central retinal arterial strips treated with 10^-7 mol/l prazosin. The strips were partially contracted with PGF2a. Relaxations induced by 10^-4 mol/l papaverine were taken as 100%. Significantly different from control (C), *P < 0.01; from the value with D-NA, **P < 0.01; and from the value with L-NA + L-arginine, ***P < 0.01 (Tukey’s method). The number of strips from separate monkeys was 12. Vertical bars represent SE.

Arterial Response to Nicotine, NO, CGRP, and VIP

The addition of nicotine (10^-4 mol/l) and NO (10^-6 mol/l) elicited transient relaxations of central retinal arterial strips contracted with PGF2a and treated with 10^-5 mol/l prazosin (Fig. 3). This concentration of nicotine was used because submaximal and reproducible responses were obtained when the single concentration was applied in each series of experiments.5 The nicotine-induced relaxation was not inhibited by atropine, timolol, indomethacin, [4Cl-D-Phe6, Leu17]-VIP (10^-6 mol/l), a VIP receptor antagonist,17 and [8-37]CGRP (10^-7 mol/l), a CGRP receptor antagonist,18 but it was abolished by oxyhemoglobin (1.6 X 10^-5 mol/l), methylene blue (10^-5 mol/l), and hexamethonium (10^-5 mol/l) (Table 1). Figure 4 illustrates typical recordings for the inability of VIP and CGRP antagonists to attenuate the response to nicotine. The relaxation also was abolished by L-NA but not by D-NA (Fig. 5). L-arginine restored the response, but the D-enantiomer was ineffective. The quantitative data are presented in Figure 5.

FIGURE 3. Typical tracings of the response to nicotine (N; 10^-4 mol/l) and nitric oxide (10^-6 mol/l) of a monkey central retinal arterial strip before (control) and after treatment with D-NA (10^-6 mol/l), L-NA (10^-6 mol/l), and L-NA + L-arginine (L-arg, 3 X 10^-4 mol/l). The strip was treated with 10^-5 mol/l prazosin and was contracted partially with PGF2a (2 X 10^-7 mol/l). PA represents papaverine (10^-4 mol/l) that produced the maximal relaxation.
Nitric Oxide Nerve in Monkey Retinal Artery

Control

[8-37]CGRP

After wash

VIP

[4CI-D-Phe6, Leu17] VIP

10 min

FIGURE 4. Typical tracings of the response to nicotine (N, $10^{-5}$ mol/l), calcitonin gene-related peptide (CGRP, $10^{-10}$ mol/l) and vasoactive intestinal peptide (VIP, $10^{-10}$ mol/l) in the absence (control) and presence of [8-37]CGRP ($10^{-7}$ mol/l) or [4CI-D-Phe6, Leu17]VIP ($10^{-6}$ mol/l) in monkey central retinal arterial strips obtained from the same monkey; one was used for studies on CGRP (upper three tracings), and the other was used for studies on VIP (lower two tracings). After the addition of papaverine, the strip was repeatedly washed with fresh media. "After wash" means "after replacement of the [8-37]CGRP containing media with the drug-free media. The strips were treated with $10^{-5}$ mol/l prazosin and was contracted partially with PGF2α ($10^{-7}$ mol/l). PA represents papaverine ($10^{-4}$ mol/l) that produced the maximal relaxation.

FIGURE 5. Modifications by D-NA ($10^{-6}$ mol/l), L-NA ($10^{-6}$ mol/l), L-NA + D-arginine (D-arg, $3 \times 10^{-4}$ mol/l), and L-NA + L-arginine (L-arg, $3 \times 10^{-4}$ mol/l) of the response to nicotine ($10^{-4}$ mol/l) of monkey central retinal arterial strips treated with $10^{-5}$ mol/l prazosin. The strips were contracted partially with PGF2α. Relaxations induced by $10^{-4}$ mol/l papaverine were taken as 100%. Significantly different from control (C), $P < 0.01$; from the value with D-NA, $P < 0.01$; and from the value with L-NA + L-arginine, $P < 0.01$ (Tukey's method). The number of strips from separate monkeys was 10. Vertical bars represent SE.

The NO ($10^{-6}$ mol/l)-induced relaxation was not influenced by L-NA and L-arginine but was abolished by oxyhemoglobin and methylele blue (Table 2).

The addition of CGRP ($10^{-11}$ to $10^{-9}$ mol/l) and VIP ($10^{-11}$ to $10^{-7}$ mol/l) caused concentration-related relaxations in the arterial strips contracted with PGF2α. To avoid tachyphylaxis, a single concentration ($10^{-10}$ mol/l), sufficient to cause submaximal relaxation, was used for the analysis of susceptibility to pharmacologic antagonists. Treatment with L-NA or oxyhemoglobin did not significantly alter the response to these peptides (Table 2). However, [8-37]CGRP ($10^{-7}$ mol/l) almost abolished the CGRP-induced relaxation, and [4CI-D-Phe6, Leu17]VIP ($10^{-6}$ mol/l) significantly attenuated the response to VIP (Fig. 4; Table 2).

Histologic Study

The extraocular central retinal artery, close to the portion used for the mechanical study, was subjected to the histochemical analysis of neurons containing NO synthase immunoreactivity. Figure 6 illustrates nerve bundles and fibers (A and B) in the adventitia and some fine fibers in the outer layer of media (A) in the monkey arteries. Similar results were obtained from two additional monkeys.

DISCUSSION

After treatment with an α1-adrenoceptor antagonist, monkey central retinal arterial strips denuded of the endothelium responded to transmural electrical stimulation and nicotine with relaxation; the responses were abolished by tetrodotoxin and hexamethonium, respectively, suggesting that perivascular nerves can be stimulated electrically and chemically. Treatment with pharmacologic inhibitors, including timolol, at-
ropine, and indomethacin, in sufficient concentrations did not inhibit the neurogenic response; therefore, the involvement of β-adrenoceptors, muscarinic receptors, and cyclooxygenase products was excluded. Oxyhemoglobin, a scavenger of NO, and methylene blue, an inhibitor of soluble guanylate cyclase, abolished the response to electrical stimulation and nicotine, as well as the relaxation induced by exogenously applied NO. These findings indicate that the induced response seems to be mediated by NO and cyclic guanosine monophosphate.

Relaxant responses to transmural electrical stimulation and nicotine were abolished by treatment with L-NA in a dose sufficient to suppress the production and release of NO from the endothelium. D-NA was without effect. The inhibitory effect of L-NA was reversed by L-arginine but not by D-arginine. Similar stereospecific actions of these compounds were observed in the neurogenic vasodilatation seen in the canine and monkey cerebral arteries. The response to NO was not affected by L-NA. These findings, together with those mentioned above as actions of hemoglobin and methylene blue, strongly support the hypothesis that NO produced from L-arginine in nerve terminals is liberated as a neurotransmitter and activates soluble guanylate cyclase in smooth muscle to increase the production of cyclic guanosine monophosphate, resulting in muscle relaxation. Histochemical study demonstrated the presence of nerve fibers and bundles containing NO synthase immunoreactivity in the adventitia and outer layer of the media of the monkey central retinal artery, as seen in canine and monkey cerebral arteries and canine and rat retinal arteries, which supports our hypothesis that NO is a neurotransmitter of vasodilator nerves. The nerve is nitrooxidergic.

Histologic studies have demonstrated perivascular nerve networks containing immunoreactivity of VIP, CGRP, and substance P in blood vessels of the brain and eye. These substances were thought to act as vasodilator neurotransmitters. In fact, VIP, CGRP (in the current study) and substance P proved to be potent vasodilators in the central retinal artery. However, the involvement of substance P is excluded from the neurotransmitter candidates because the relaxation caused by this peptide is endothelium dependent, whereas the response to nerve stimulation is not influenced by endothelium denudation (current study). Therefore, studies on VIP and CGRP antagonists were carried out to determine whether VIP and CGRP participate in neurogenic relaxation. The antagonists in concentrations sufficient to attenuate significantly the response to VIP and CGRP did not alter the response to nicotine. In addition to the fact that the neurally induced relaxation was abolished completely by the NO synthase inhibitor, these findings led us to conclude that the peptides used did not contribute to the transmittal of information from the nerve to smooth muscle when the nerve was stimulated under the experimental conditions. In our earlier studies, the same conclusion was drawn in isolated canine arteries made unresponsive to the peptides by

TABLE 2. Modification by Inhibitors of the Relaxant Response to NO (10^-6 mol/l), CGRP (10^-10 mol/l), and VIP (10^-10 mol/l) in Monkey Central Retinal Arterial Strips Contracted With PGF_2α

<table>
<thead>
<tr>
<th>Treatment (mol/l)</th>
<th>Number of Strips From Separate Monkeys</th>
<th>% Relaxation by Vasodilators*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>NO</td>
<td>9</td>
<td>47.0 ± 5.6</td>
</tr>
<tr>
<td>L-NA 10^-6</td>
<td>9</td>
<td>47.0 ± 5.6</td>
</tr>
<tr>
<td>L-NA + L-arg</td>
<td>9</td>
<td>54.6 ± 6.8</td>
</tr>
<tr>
<td>OxyHb 1.6 × 10^-5</td>
<td>5</td>
<td>43.6 ± 6.1</td>
</tr>
<tr>
<td>Methylene blue 10^-5</td>
<td>3</td>
<td>62.0 ± 3.2</td>
</tr>
<tr>
<td>CGRP</td>
<td>3</td>
<td>58.5 ± 6.5</td>
</tr>
<tr>
<td>L-NA 10^-6</td>
<td>6</td>
<td>60.4 ± 7.5</td>
</tr>
<tr>
<td>OxyHb 1.6 × 10^-5</td>
<td>5</td>
<td>46.8 ± 7.8</td>
</tr>
<tr>
<td>[8-37]CGRP 10^-7</td>
<td>5</td>
<td>50.0 ± 7.3</td>
</tr>
<tr>
<td>VIP</td>
<td>7</td>
<td>38.9 ± 4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Relaxations relative to those caused by 10^-4 mol/l papaverine. L-arg = 3 × 10^-4 mol/l L-arginine; OxyHb = oxyhemoglobin; VIP antagonist = [4C1-D-Phe'' Leu'7]VIP; NO = nitric oxide; CGRP = calcitonin gene-related peptide; VIP = vasoactive intestinal peptide.
† Significantly different from control (P < 0.001, unpaired t-test).
‡ Significantly different from control (P < 0.01, unpaired t-test).
Nitric Oxide Nerve in Monkey Retinal Artery

FIGURE 6. Histochemical demonstration of nerve bundles (arrows) and fibers (arrowheads) containing nitric oxide synthase immunoreactivity in the adventitia and outer layer of the media of the central retinal arteries from two monkeys (A,B). L = lumen. Bar = 50 μm.

the application of high concentrations of these compounds. In addition, the possibility that VIP and CGRP produced retinal arterial relaxations by a mediation of NO could be ruled out because the responses to these peptides were not influenced by L-NA and oxyhemoglobin in concentrations sufficient to abolish the neurogenic relaxation and the response to exogenous NO, respectively, in endothelium-denuded strips.

The current study proves that the monkey central retinal artery is innervated by vasodilator nerves that possibly liberate NO as a neurotransmitter. Because monkey and human vasculature show a similar responsiveness to chemical and physical stimuli, the hypothesis proposed by the current study can be extrapolated to humans. The possible involvement of neurogenic NO in the human and bovine posterior ciliary artery dilatation has been reported. In anesthetized cats, the involvement of NO released from perivascular nerves in choroidal blood flow increase is suggested. In canine and monkey cerebral arteries in vivo, the suppression of NO synthesis in the perivascular nerve and endothelium by NO synthase inhibitors is expected to be one of the mechanisms underlying vasospasm after subarachnoid hemorrhage. Determining the physiological role and pathophysiological implication of nitrooxidergic vasodilator innervation in the human retinal artery would be intriguing for the development of therapeutic maneuvers for retinal circulatory disorders.

Key Words

central retinal artery, monkey, nicotine, nitric oxide (NO), vasodilator nerve

Acknowledgments

The authors thank Kazuhide Yoshida for assistance in the histochemical study.

References

13. Toda N. Relaxant responses to transmural stimulation


