Nitric Oxide-Mediated Retinal Arteriolar and Arterial Dilatation Induced by Substance P

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Purpose. The present study was undertaken to compare vasodilatations caused by substance P in retinal arterioles in vivo and in the extraocular retinal central arteries in vitro, and to analyze the mechanisms of its action.

Methods. In the in vivo study, changes of the retinal arteriolar diameter were continuously measured using a retinal fundus camera. In the in vitro study, changes in the isometric tension were recorded in helical strips of extraocular retinal arteries with and without the endothelium, exposed to aerated bathing media.

Results. In anesthetized dogs, infusions of substance P into the carotid artery produced a dose-dependent dilatation of the intraocular retinal arteriole; the maximal response was obtained about 15 seconds later. The vasodilator response was significantly attenuated by treatment with N\textsuperscript{G}-nitro-L-arginine (L-NA), a nitric oxide (NO) synthase inhibitor, and the inhibition was reversed by L-arginine. On the other hand, vasodilatations caused by nitroglycerin were not influenced by L-NA and L-arginine. In the isolated retinal artery just before entering into the eyeball, the addition of substance P produced a concentration-dependent relaxation only when the endothelium of the strips was intact. Removal of the endothelium abolished the response. The peptide-induced relaxation was abolished by L-NA, whereas relaxations caused by NO and nitroglycerin were unaffected. The inhibitory effect of L-NA was reversed by L-arginine but not by D-arginine. Treatment with methylene blue or oxyhemoglobin abolished the relaxation induced by substance P, NO, and nitroglycerin.

Conclusions. Substance P-induced retinal arteriolar dilatation in vivo appears to be mediated by NO synthesized from L-arginine possibly in the endothelium. The endothelium-dependency would be supported by the findings obtained from isolated retinal arteries.


Release of vasodilator substance(s) from the endothelium, such as endothelium-derived relaxing factor (EDRF), has been demonstrated in a variety of blood vessels in response to acetylcholine, substance P, bradykinin, histamine, ATP, and so on.\textsuperscript{1-4} Angus and co-workers\textsuperscript{5,6} have demonstrated that EDRF-releasing agents increase the dog large femoral and coronary artery diameter in a totally endothelium-dependent manner. According to Young and Vatner,\textsuperscript{7} the iliac artery diameter in conscious dogs is increased by acetylcholine and epinephrine; the endothelium is required for the vasodilatation. The endothelium-dependent dilatation in perfused pial arterioles of the mouse and rat brain has also been reported by many investigators.\textsuperscript{8-11} In these studies, arteries and arterioles were placed under nonphysiological conditions such as exposure to artificial cerebrospinal fluid and subject to surgical invasions. In contrast, ocular fundus arteriolar dynamics can easily be accessed by fundus photography under physiological conditions.

The aims of the present study were to determine vasodilatation caused by substance P in the retinal arterioles in vivo and in the extraocular retinal central arteries\textsuperscript{12} in vitro and to analyze the mechanism of its action. N\textsuperscript{G}-nitro-L-arginine (L-NA), an NO synthase inhibitor,\textsuperscript{13,14} is used to clarify the involvement of NO produced from L-arginine. The arteriolar diameter was clearly seen in the fundus oculi under direct vi-
sion. This in vivo study is important because it shows time-to-time alterations in the diameter without any surgical invasion.

METHODS

Studies In Vivo

All experimental procedures that used animals conformed to the ARVO Resolution on the Use of Animals in Research. Mongrel dogs of either sex, weighing 8 to 13 kg, were anesthetized with sodium pentobarbital (25 mg/kg, intraperitoneally). Anesthesia was maintained throughout the experiment by supplemental doses of the barbiturate if necessary. An intratracheal catheter was intubated, and the animals were permitted to breathe spontaneously. Polyethylene catheter was inserted into the right femoral artery for monitoring arterial systolic and diastolic blood pressures and heart rate. Another catheter was inserted into the right femoral vein for injecting drugs systematically. The head of each animal was tightly fixed by a holder. The left eyeball used for experiments was immobilized by traction of the extraocular muscles. Phenylephrine was topically administered to the experimental eye for mydriasis. Agonists dissolved in 1 ml of saline were infused at a rate of 2 ml/min into the common carotid artery via a 22-gauge catheter. According to a method of fundus fluorescein angiography, we found that the dye reached the retinal arterioles 1 ~ 2 seconds after the infusion into the common carotid artery. L-NA (10 mg/kg) and L-arginine (500 mg/kg) dissolved in 10 ml of saline were infused intravenously using an infusion pump at a rate of 5 ml/min through the catheter placed in the femoral vein. Using a retinal fundus camera (TRU-WT3, Topcon, Tokyo, Japan) equipped with a color video camera (FCD-725, Ikegami, Osaka, Japan), 20° field was recorded on a videorecorder (GU-9, Sony, Tokyo) before and after the administration of drugs. Retinal images were acquired in real time directly. Observed changes of the arteriolar outside diameter under resting conditions was taken as 100%. Unless otherwise mentioned, vasodilatation induced by papaverine (2 mg/kg) injected at the end of experiments was taken as 100%. Intraphotographic reproducibility of the measurements was assessed by digitizing the same image on 10 separate occasions; the mean values of the variation in experiments with three different dogs were 3.2 ± 0.8%. Time-dependent changes were also assessed before and after intracarotid injections of saline; mean values of the variation from controls at 1, 3, 5, 10, and 20 seconds later were 2.8 ± 1.2%, 2.8 ± 0.3%, 3.3 ± 0.5%, -0.5 ± 0.4%, and 0.9 ± 1.1%, respectively.

Studies In Vitro

Mongrel dogs of either sex were anesthetized with intravenous injections of sodium pentobarbital (30 mg/kg) and killed by bleeding from the common carotid arteries. The eyeballs attached with optic nerves and extraocular tissues were rapidly removed from the orbital cavities. Extraocular muscles were removed, and the retinal central arteries (0.3 to 0.4 mm in outside diameter) just before entering into the eyeball were carefully isolated. The arteries were cut into helical strips approximately 10 mm long, with special care to preserve the endothelium. The specimens were vertically fixed between hooks in a muscle bath containing the modified Ringer-Locke solution maintained at 37 ± 0.3°C and aerated with a mixture of 95% O2 and 5% CO2. The hooks anchoring the upper end of the strips were connected to the lever of a force-displacement transducer (Nihonkohden-Kogyo Co., Tokyo). The resting tension was adjusted to 0.7 g, which is optimal for inducing the maximal contraction. The composition of the bathing medium was as follows (mM): NaCl 120, KCl 5.4, CaCl2 2.2, MgCl2 1.0, NaHCO3 25.0, and dextrose 5.6. The pH of the solution was 7.35 to 7.42. Before the start of experiments, all the strips were allowed to equilibrate for 60 to 90 minutes in the bathing media, during which time the medium was replaced three times every 10 to 15 minutes. Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Nihonkohden-Kogyo Co., Tokyo). The contractile response to 30 mmol/l K+ was first obtained, and the artery strips were repeatedly washed with fresh media and equilibrated. The strips were partially contracted with prostaglandin (PG) F2α (10⁻⁷ to 10⁻⁶ mol/l), the contraction being in a range between 20% and 40% of the contraction induced by 30 mmol/l K⁺ Substance P, nitric oxide (NO), and nitroglycerin in single concentrations were successively applied, unless otherwise mentioned. Concentration-response curves of substance P were obtained by applying single concentrations each time to avoid tachyphylaxis. After the strip was repeatedly washed and equilibrated, the peptide concentration was raised. At the end of each series of experiment, papaverine (10⁻⁴ mol/l) was applied to attain the maximal relaxation,¹⁶ which was taken as a standard for relaxation (100%). Preparations had been treated for 10 minutes with blocking agents before the effects of the agonist were obtained. The endothelium was removed by gently rubbing the intimal surface with a cotton ball. Removal of the endothelium was determined histologically by the silver stain-
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Statistics and Drugs Used

The results shown in the text and figures are expressed as mean values ± SEM. Statistical analyses were made using the Student’s paired and unpaired t -tests and the Tukey’s method after one-way analysis of variance. Drugs used were N G -nitro-L-arginine (L-NA), N C -nitro-D-arginine (D-NA), substance P (Peptide Institute Inc., Mino, Japan), Ca ++ ionophore A23187 (Boehringer Mannheim, GmbH, Germany), L-arginine, D-arginine, methylene blue trihydrate (Nacalai Tesque, Kyoto, Japan), PGF 2α (Ono Co., Osaka), indomethacin, dog hemoglobin (Sigma, St. Louis, MO), nitroglycerin (Nihon-Kayaku Co., Tokyo), dl-norepinephrine hydrochloride (Sankyo Co., Tokyo), papaverine hydrochloride (Dainippon Co., Osaka) and sodium pentobarbital (Abbott Laboratories, North Chicago, IL). Responses to NO were obtained by the addition of NaNO 2 solution adjusted at pH 2. 15 Oxyhemoglobin was prepared according to the method of Martin et al. 18

RESULTS

Effects of Substance P and Nitroglycerin In Vivo

Intraarterial infusions of substance P (0.15 to 150 ng/kg) produced a dose-dependent vasodilatation of the intraocular retinal arterioles, whereas those of norepinephrine (1 to 10 μg/kg) produced a dose-related vasoconstriction (Fig. 1). Drugs were applied about every 10 minutes, and the dose-response curves were obtained by raising the doses. Typical responses to substance P are shown in Figure 2. The maximal response to the peptide was attained about 15 seconds after the infusion, and the vasodilatation abolished within 1 minute (Fig. 3). On the other hand, mean blood pressure was not influenced for 20 seconds by the infusion, and then it was significantly lowered (Fig. 3). The administration of the vehicle showed no vaso-motor effects.

Vasodilator responses to substance P (1.5 ng/kg) and nitroglycerin (4 μg/kg) were compared in seven dogs. The maximal response was attained about 15 seconds after the infusion of the peptide and 20 seconds after the nitroglycerin infusion (Fig. 4). The response to substance P was significantly attenuated by treatment with L-NA (10 mg/kg, intravenously), and the inhibition was reversed by L-arginine (500 mg/kg, intravenously) (Fig. 4, upper panel). On the other hand, the vasodilatation caused by nitroglycerin was not influenced by L-NA and L-arginine (Fig. 4, lower panel).

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The intravenous infusion of L-NA (10 mg/kg) produced a vasoconstriction (6.6 ± 0.7% decrease in diameter, n = 4, P < 0.001), which was reversed by the administration of L-arginine (500 mg/kg) (6.5 ± 0.5% increase in diameter, n = 4, P < 0.001) from the level attained by L-NA.

Effects of Substance P, NO, and Nitroglycerin In Vitro

The addition of substance P in concentrations ranging from 10^{-10} to 10^{-7} mol/l produced a concentration-dependent relaxation in retinal arterial strips with the endothelium, partially contracted with PGF_{2\alpha}. Removal of the endothelium almost abolished the relaxation (Fig. 5).

Substance P (10^{-8} mol/l)-induced relaxations were not influenced by 10^{-6} mol/l indomethacin in the strips with intact endothelium; mean values of the maximal relaxation before and after the treatment were 84.1 ± 6.7% and 72.6 ± 7.6% (n = 7) relative to those induced by 10^{-4} mol/l papaverine, respectively. In the endothelium-intact arterial strips treated with indomethacin, relaxant responses to substance P (10^{-8} mol/l), NO (10^{-2} mol/l), and nitroglycerin (10^{-6} mol/l) were compared before and after treatment with L-NA (10^{-4} and 10^{-5} mol/l) and L-arginine (10^{-3} mol/l). Typical responses are illustrated in Figure 6. The relaxation elicited by substance P was attenuated by 10^{-6} mol/l L-NA and abolished at 10^{-5} mol/l, whereas relaxations by NO and nitroglycerin were unaffected. In contrast to L-NA, D-NA (10^{-5} mol/l) was without effect. The peptide-induced response suppressed at 10^{-5} mol/l L-NA was partially restored by additional treatment with 10^{-3} mol/l L-arginine, but not D-arginine. Quantitative data are summarized in Figure 7. Treatment with 10^{-5} mol/l methylene blue or 1.6 x 10^{-5} mol/l oxyhemoglobin abolished the relaxation induced by substance P (n = 4), NO (n = 4) and nitroglycerin (n = 4).

The addition of L-NA in concentrations up to 10^{-5} mol/l did not produce contractions of retinal arterial strips with the endothelium under resting conditions and when partially contracted with PGF_{2\alpha} (n = 3).
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**FIGURE 5.** Concentration (Conc.)-relaxant response curves for substance P in isolated dog retinal central arteries with (●) and without the endothelium (○). The strips were partially contracted with PGF2α (3 × 10⁻⁷ M). Relaxations induced by 10⁻⁴ M papaverine were taken as 100%; mean absolute values in the strips with and without the endothelium were 260 ± 9 mg (n = 5) and 270 ± 10 mg (n = 5), respectively. Vertical bars represent SEM.

**DISCUSSION**

The present study demonstrated vasodilator and vasoconstrictor responses of dog intraocular retinal arterioles in vivo by means of retinal fundus camera, video recorder, and computerized digitizing system. Vasodilatation induced by substance P and vasoconstricton caused by norepinephrine were dose-dependent. The vasodilatation by a submaximal dose of substance P was significantly attenuated by L-NA, and the inhibition was reversed by the administration of L-arginine. On the other hand, the vasodilator response to nitroglycerin was not influenced by L-NA. These findings strongly suggest that endogenous NO or an NO analog, like S-nitrosothiol, plays an important role in the substance P-induced retinal arteriolar dilatation. Studies on the EDRF-mediated response of arterioles have been reported: the endothelium-dependent dilatation by acetylcholine or bradykinin in artificial cerebrospinal fluid-perfused pial arterioles of the mouse brain with a cranial window technique, and the dilatation by acetylcholine in rat mesenteric microvessels with a myographical technique. Acetylcholine and other EDRF-releasing agents increase the dog’s femoral and coronary artery diameters only when the endothelium

**FIGURE 6.** Typical recordings of the response to substance P (SP, ○, 10⁻⁸ M), nitric oxide (NO, ●, 10⁻⁷ M), and nitroglycerin (NTG, △, 10⁻⁸ M) of a dog retinal artery strip with intact endothelium. Responses of the strip were obtained before (control) and after treatment with N⁵-nitro-L-arginine (L-NA, 10⁻⁶ and 10⁻⁵ M) and 10⁻⁵ M L-NA plus L-arginine (L-arg, 10⁻³ M). The strip was treated with 10⁻⁶ M indomethacin and partially contracted with PGF2α (3 × 5 × 10⁻⁷ M). PA represents 10⁻⁴ M papaverine that produced maximal relaxation.
In the in vitro study, relaxations induced by substance P in dog retinal arteries were abolished by removal of the endothelium, whereas those associated with NO and nitroglycerin were not influenced. The substance P-induced relaxation was not affected by treatment with indomethacin, suggesting that cyclooxygenase products are not involved. However, the peptide seems partially to elicit a relaxation by mediation of the vasodilator PGs, such as PG12, produced in smooth muscle cells in other arteries isolated from smooth muscle cells in other arteries isolated from dogs, including middle cerebral arterial and superficial temporal arteries (Enokibori et al., unpublished data, 1993). The relaxation caused by substance P was suppressed or abolished by L-NA and oxyhemoglobin, an NO scavenger, and the inhibitory effect of L-NA was reversed by additional treatment with L-arginine. On the other hand, relaxations by NO and nitroglycerin were unaffected by L-NA but were abolished by oxyhemoglobin and methylene blue, an inhibitor of soluble guanylate cyclase. These findings strongly suggest that relaxations caused by substance P in the retinal artery are exclusively mediated by endothelium-derived NO, which activates soluble guanylate cyclase and increases the synthesis of cyclic GMP in smooth muscle cells. These results would allow us to consider that substance P-induced vasodilatation in vivo is mediated by NO derived also from the endothelium in retinal arterioles that are located just distal of the retinal central artery used in the present study in vitro.

The intravenous infusion of L-NA produced a retinal vasoconstriction, which was reversed by L-arginine. This finding may suggest that the vasoconstriction is associated with a suppression of basal release of NO. However, it cannot be concluded that endogenous NO responsible for dilating the basal vascular tone in retinal arterioles is derived from the endothelium or nitroxidergic nerves or both. Recent in vivo studies have demonstrated that intravenously applied L-NA and L-NMMA increase peripheral vascular resistance in the dog, rabbit, guinea pig, and rat. These authors have postulated that the hypertension and increased vascular resistance are associated with a suppression of the basal release of NO from the endothelium. On the other hand, hypertension associated with L-NA in anesthetized dogs has been depressed by treatment with hexamethonium; thus, we have postulated that the induced response is caused by elimination of nitroxidergic vasodilator nerve function. Whether the L-NA-induced retinal arteriolar constriction in vivo is the result of a depression of NO released from the endothelium or vasodilator nerve was not determined in the present study.

In summary, it appears that substance P-induced relaxations in the isolated dog retinal artery are associated exclusively with NO released from the endothelium, and vasodilatation caused by the peptide in vivo is mediated by NO derived possibly from the endothelium. The method introduced here for in vivo experiments shows time-to-time alterations in the diameter of retinal arterioles without any surgical invasion. This is a first trial of comparisons in the responses of intraocular (in vivo) and extraocular (in vitro) retinal central arteries to vasoactive agents. The findings shown in the present study suggest that pharmacological, detailed analyses of the mechanism of drug actions can be made by the combined use of these methods.

Key Words

substance P, retinal central artery, retinal arterioles, endothelium, nitric oxide

FIGURE 7. Modification by N<sup>6</sup>-nitro-L-arginine (L-NA, 10<sup>−6</sup> and 10<sup>−5</sup> M) and 10<sup>−5</sup> M L-NA plus L-arginine (L-arg, 10<sup>−3</sup> M) of the response to substance P (10<sup>−8</sup> M) in dog retinal artery strips with intact endothelium. The strips were treated with 10<sup>−6</sup> M indomethacin and partially contracted with PGF<sub>2α</sub> (3 × 10<sup>−7</sup> M). Relaxations induced by 10<sup>−4</sup> M papaverine were taken as 100%; mean absolute values in control strips (C, open column) and those treated with 10<sup>−6</sup> M L-NA (hatched), 10<sup>−5</sup> M L-NA (solid) and 10<sup>−5</sup> M L-NA + 10<sup>−3</sup> M L-arg (dotted) were 259 ± 17 (n = 7), 260 ± 15 (n = 7), 246 ± 12 (n = 7) and 216 ± 17 mg (n = 7), respectively. Significantly different from control, <i>P</i> < 0.01; <i>P</i> < 0.05. Significantly different from the value with 10<sup>−5</sup> M L-NA, <i>P</i> < 0.01 (Tukey's method). Vertical bars represent SEM.
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References


