Nitric oxide dilates rat retinal blood vessels by cyclooxygenase-dependent mechanisms

(Running head: NO dilates retinal vessels by COX-dependent mechanisms)

Naoto Ogawa, Asami Mori, Masami Hasebe, Maya Hoshino, Maki Saito, Kenji Sakamoto, Tsutomu Nakahara and Kunio Ishii

Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Address correspondence to: Tsutomu Nakahara, Department of Molecular Pharmacology, Kitasato University School of Pharmaceutical Sciences, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan. Telephone: +81-3-3444-6205; Fax: +81-3-3444-6205. E-mail: nakaharat@pharm.kitasato-u.ac.jp

Copyright © 2009 by the American Physiological Society.
Abstract

It has been suggested that nitric oxide (NO) stimulates the cycloxygenase (COX)-dependent mechanisms in the ocular vasculature; however, the importance of the pathway in regulating retinal circulation in vivo remains to be elucidated. Therefore, we investigated the role of COX-dependent mechanisms in NO-induced vasodilation of retinal blood vessels in thiobutabarbitral-anesthetized rats with and without neuronal blockade (tetrodotoxin treatment). Fundus images were captured with a digital camera that was equipped a special objective lens. The retinal vascular response was assessed by measuring changes in diameter of retinal blood vessel. The localization of COX and soluble guanylyl cyclase in rat retina was examined using immunohistochemistry. The NO donors (sodium nitroprusside and NOR3) increased diameter of retinal blood vessels but decreased systemic blood pressure in a dose-dependent manner. Treatment of rats with indomethacin, a non-selective COX inhibitor, or SC-560, a selective COX-1 inhibitor, markedly attenuated the vasodilation of retinal arterioles, but not depressor response, to the NO donors. However, both the vascular responses to NO donors were unaffected by the selective COX-2 inhibitors NS-398 and nimesulide. Indomethacin did not change the retinal vascular and depressor responses to hydralazine, 8-(4-chlorophenylthio)-guanosine-3’, 5’-cyclic monophosphate (a membrane permeable cGMP analogue) and 8-(4-chlorophenylthio)-adenosine-3’, 5’-cyclic monophosphate (a membrane permeable cAMP analogue). Treatment with SQ 22536, an adenyl cyclase inhibitor, but not ODQ, a soluble guanylyl cyclase inhibitor, significantly attenuated the NOR3-induced vasodilation of retinal arterioles. The COX-1 immunoreactivity was found in retinal blood vessels. The retinal blood vessel was faintly stained for soluble guanylyl cyclase, although the apparent immunoreactivities on mesenteric and choroidal blood vessels were observed. These results suggest that NO exerts a substantial part of its
dilatory effect via a mechanism which involves COX-1-dependent pathway in rat retinal vasculature.

**Keywords:** Cyclooxygenases; Nitric oxide (NO); Prostaglandins; Retinal blood vessel
Introduction

Nitric oxide (NO) is a potent vasodilator that activates soluble guanylyl cyclase (sGC) resulting in the elevation of intracellular cGMP in vascular smooth muscle cells, thereby dilating blood vessels in many organs (18). However, there is the evidence showing that cGMP-independent alternative/additional pathways are also involved. For example, NO directly activates the Ca^{2+}-dependent K^+ (Kca) channels in vascular smooth muscle cells, which leads to cause the vasodilator response (2, 16). In another case, NO stimulates production of prostaglandins by interacting with cyclooxygenase (COX) (17, 26-28).

In the ocular circulation, NO is indeed an important regulator of the vascular tone (14). A previous in vitro study on isolated porcine ocular vasculatures has demonstrated that the vasodilator responses to NO donors were significantly reduced by inhibition of COX and largely dependent on the presence of endothelium (10). Stimulation of prostacyclin synthesis in the endothelial cells has been proposed as a possible mechanism (10). Thus, NO appears to stimulate the COX-dependent mechanisms in the ocular vasculature; however, the importance of the pathway in regulating retinal circulation in vivo remains to be completely elucidated.

Recently, we found that the vasodilator responses to nicorandil that acts as an NO donor (8, 15, 30) were prevented by treatment with indomethacin (22). Similar results were obtained when the NO donor sodium nitroprusside (SNP) was used instead of nicorandil (22). Therefore, it is likely that the vasodilator effects of NO on retinal blood vessels are mediated through a COX-dependent pathway in rats in vivo. This study was designed to expand the previous study and in this process we investigated the effects of several vasodilators and the COX isozyme selective inhibitors. We also examined localization of COXs and sGC in rat retina.
Materials and Methods

This study was performed in accordance with the Guidelines for Animal Experiments in Kitasato University adopted by the Committee on the Care and Use of Laboratory Animals of Kitasato University and tenets of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

General preparation

Male Wistar rats (8 to 10-week-old) were maintained in a room with constant temperature (22 ± 2 °C), constant humidity (55 ± 5 %) and 12-hour light/dark cycle, and allowed free access to regular rat chow and tap water. Rats were anesthetized with thiobutabarbitral (120 mg/kg, i.p.). After disappearance of the corneal reflex, each animal was placed on a heating pad. A tracheotomy was performed and catheters were inserted into the femoral and jugular veins for administration of drugs. The left femoral artery was cannulated for measurement of systemic blood pressure, which was recorded on a thermal pen recorder (WT-645G, Nihon Kohden, Tokyo, Japan), via a pressure transducer (DX-360, Nihon Kohden) and a preamplifier (AP-610G, Nihon Kohden). Heart rate (HR) was measured with a cardiotachometer (AT-601G, Nihon Kohden) triggered by the blood pressure pulse. Arterial pressure and HR were digitized at 1 Hz using SCIENCE LINK II (Keisoku Giken, Utsunomiya, Japan) and stored on the hard disk of a personal computer (PowerBook 165C, Apple Japan, Tokyo, Japan).

Experimental protocols

Protocol 1: effects of the non-selective COX inhibitor indomethacin on the responses to several vasodilators in anesthetized rats. Rats were treated with either
indomethacin (5 mg/kg, i.v.) or the vehicle. Immediately after the treatment, the animals were administered N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) (50 mg/kg, i.v.) to minimize the influence of endogenous NO (32). After hemodynamic parameters reached a stable (approx. 20 min later), SNP (1-10 µg/kg/min), NOR3 (±(E)-ethyl-2-[(E)-hydroxyamino]-5-nitro-3-hexenamide) (1-30 µg/kg/min), 8-cpt-cAMP (8-(4-chlorophenylthio)adenosine-3’, 5’-cyclic monophosphate) (14 µmol/kg), 8-cpt-cGMP (8-(4-chlorophenylthio)guanosine-3’, 5’-cyclic monophosphate) (14 µmol/kg) or hydralazine (0.4 mg/kg) was injected into the femoral vein by means of a syringe pump (Model 1140-001 Harvard Apparatus, South Natick, MA).

**Protocol 2:** effects of COX inhibitors (indomethacin, SC-560, NS-398 and nimesulide) on the vascular responses to NO in the absence of baroreceptor reflexes. To eliminate nerve activity totally, rats were treated with tetrodotoxin (TTX, 50 µg/kg, i.v.) under artificial ventilation with room air (the stroke volume, 10 mL/kg; the frequency, 80 strokes/min) using a rodent respirator (SN-480-7, Sinano, Tokyo, Japan). Because TTX decreased blood pressure and HR, a mixture solution of norepinephrine (NE) and epinephrine (Epi) (NE: Epi=1:9) was continuously injected into the right jugular vein at a constant rate by means of a syringe pump (Model 1140-001, Harvard Apparatus) to maintain systemic blood pressure and HR at the control level (19, 20). This procedure also abolished the pressor response to spinal cord stimulation and the acetylcholine-induced reflex tachycardia observed in anaesthetized rats for several hours (4). In this protocol, the responses to NOR3 (30 µg/kg/min, i.v.) determined before and after treatment with indomethacin (5 mg/kg, i.v.), SC-560 (5 mg/kg, i.v.), NS-398 (5 mg/kg, i.p.) or nimesulide (1 mg/kg, i.v.) were compared. The doses of COX inhibitors were chosen on the basis of previous reports (1, 7, 31).

**Protocol 3:** effects of COX inhibitors (indomethacin, SC-560 and NS-398) given by
intravitreal injection on the vascular responses to NO. Indomethacin (10 nmol), SC-560 (10 nmol), NS-398 (10 nmol), or the vehicle (10% DMSO), in a total volume of 10 µL, was injected into the vitreous cavity of the left eye before surgical procedures and TTX treatment described above. Infusion of NOR3 (30 µg/kg/min, i.v.) was started 70-80 min after the injection. Intravitreal injection was performed under a microscope using a 32-gauge needle connected to a microsyringe and the needle was inserted approximately 1 mm behind the corneal limbus.

Protocol 4: effects of the adenylyl cyclase inhibitor SQ 22536 and the soluble guanylyl cyclase inhibitor ODQ on the vascular responses to NO. In TTX-treated rats, the vasodilator responses to NOR3 (30 µg/kg/min, i.v.) of retinal arterioles were examined after intravitreal injection of SQ 22536 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine) (100 nmol/eye) (11), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) (10 nmol/eye)(9) or the vehicle (10% DMSO), as described in “Protocol 3”. In some experiments, effects of intravitreal injection of SQ 22536 on the L-type Ca²⁺ channel blocker nicardipine (30 µg/kg, i.v.)-induced responses were examined.

Measurement of diameter of retinal blood vessels

The pupils were dilated with one drop of 1% atropine sulfate (Nihon Tenganyaku Institute, Nagoya, Japan). To protect the eye, 0.3% sodium hyalurate (Santen Pharmaceutical, Osaka, Japan) was dropped onto the cornea. The optic disc was centered and focused in the field of view. Sodium fluorescein (10% solution, 0.8 mL/kg, i.v.) and brilliant blue 6B (5% solution, 0.8 mL/kg, i.v.) were injected into the right femoral vein to enhance vessel contrast. Fundus images were captured with a digital camera (D1x, Nikon, Tokyo, Japan) that was equipped with the bore scope-type objective lens for small animals (Model 01, Magnification x20; Scalar, Tokyo, Japan) and stored on the hard disk of a
laboratory computer system (Power Macintosh G3-300DT, Apple Japan, Tokyo, Japan). The region (120 x 240 μm) containing a retinal arteriole or a retinal venule in the fundus image (2,624 x 4,000 μm) was selected. The diameter of blood vessel in the region was measured throughout the experiment as described previously (20, 22).

Immunohistochemistry

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The chest was opened rapidly and the vasculature was perfused for 5 min at a pressure of 120 mmHg with fixative (1% paraformaldehyde in phosphate-buffered saline, pH 7.4) from an 18-gauge cannula inserted into the aorta via an incision in the left ventricle. The right atrium was incised to create a route for the fixative to exit. After the perfusion, tissues were removed and stored in fixative for 1 hr at 4°C. Specimens were rinsed several times with phosphate-buffered saline (PBS) and infiltrated overnight with 30% sucrose in PBS at 4°C, embedded in OCT compound (Sakura Finetek, Torrance, CA), and frozen at -80°C.

Tissue sections were cut with a cryostat at a thickness of 20 μm and dried on glass slides. Sections were rinsed of OCT compound and then incubated in blocking solution (5% normal goat serum or 5% normal hamster serum) in PBS containing 0.3% Triton X-100 (PBS/0.3% Triton X-100) for 0.5 to 1 hr at room temperature.

To determine whether COX is expressed in retinal blood vessels, COX-1 or COX-2 staining was performed in combination with anti-rat endothelial cell antigen (RECA)-1 antibody (endothelial cell marker). Sections of retina were incubated for 12-15 hr in a combination of rabbit polyclonal anti-COX-1 antibody (1:500, Cayman, Ann Arbor, MI) or rabbit polyclonal anti-COX-2 antibody (1: 500, Cayman) and mouse monoclonal anti-RECA-1 antibody (1:100, Serotec, Oxford, UK). After several rinses with PBS/0.3% Triton X-100, sections were further incubated for 4 hr with a combination of two
fluorescently labeled, species-specific secondary antibodies against anti-COX-1 antibody (anti-rabbit) or anti-COX-2 antibody (anti-rabbit) and anti-RECA-1 antibody (anti-mouse). Sections were rinsed in PBS/0.3% Triton X-100 and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Control sections incubated in the absence of primary antibodies were also processed and evaluated for specificity or background levels of staining.

To determine whether sGC is expressed in vascular smooth muscle of retinal blood vessels, sGC staining was performed in combination with α-smooth muscle actin (SMA) antibody (smooth muscle marker). For comparison, the expression of sGC in the vascular smooth muscle of mesenteric blood vessels was examined. Sections of eye or intestine and mesentery with mesenteric vessels were incubated for 12-15 hr in a combination of rabbit polyclonal anti-sGC β1 subunit antibody (1:500, Cayman) and mouse monoclonal anti-αSMA antibody (1:500, Sigma-Aldrich, St. Louis, MO). As described above, after several rinses with PBS/0.3% Triton X-100, sections were further incubated for 4 hr with FITC- or Cy3-labeled, species-specific secondary antibodies against anti-sGC antibody (anti-rabbit) and anti-αSMA antibody (anti-mouse). Sections were rinsed in PBS/0.3% Triton X-100 and mounted with Vectashield. In negative controls, the primary antibodies were replaced in PBS and background staining levels were evaluated.

Images were taken by using a fluorescent microscope system BZ-9000 (Keyence, Osaka, Japan) or a confocal laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany).

*Induction of retinal ischemia*

It has been shown that COX-2 is significantly up-regulated in rat retina at 6 hr after a transient ischemia (13). To test whether the anti-COX-2 antibody works well under
our experimental conditions, we examined the immunoreactivity of COX-2 in rat retina at 6 hr after induction of retinal ischemia. Retinal ischemia was induced by raising the intraocular pressure as reported previously (25). In brief, rats were anesthetized and the anterior chamber of the one eye, the pupil of which had been dilated with 1% atropine sulfate (Nihon Tenganyaku), was cannulated with a 27-gauge needle connected to a bottle filled with saline. Retinal ischemia was induced by raising intraocular pressure to 130 mmHg by lifting the bottle for 60 min. The opposite eye of each animal, which served as a non-ischemic control, was cannulated without raising its intraocular pressure. At 6 hr after the ischemia, rats were systemically perfused with fixative as described above.

**Drugs**

The following drugs were used: 8-cpt-cAMP, 8-cpt-cGMP, (-) epinephrine (+) bitartrate, hydralazine, indomethacin, nicardipine hydrochloride, (-)-norepinephrine bitartrate, L-NMMA, ODQ, sodium fluorescein, SNP, SQ 22536 (Sigma-Aldrich); NOR3 (Dojin, Kumamoto, Japan); brilliant blue 6B (Tokyo Kasei, Tokyo, Japan); TTX (Nacalai Tesque, Kyoto, Japan); nimesulide, NS-398, SC-560 (Cayman).

L-NMMA, SNP, hydralazine, 8-cpt-cAMP, and 8-cpt-cGMP were dissolved in saline just before use. NOR3 was dissolved in DMSO and further diluted in saline. The final concentration of DMSO in the solution was 0.2% and infusion of the concentration of DMSO did not show any detectable effect in rats *in vivo*. Indomethacin and SC-560 were dissolved in 0.24% Na$_2$CO$_3$ solution and in polyethylene glycol 400, respectively. NS-398 was dissolved in DMSO and further diluted in saline and nimesulide was dissolved in 60% polyethylene glycol 400, 5% ethanol, and 35% saline. For intravitreal injection, indomethacin, SC-560, NS-398, SQ 22536 and ODQ were dissolved in 10% DMSO.
Data analyses

The diameter of retinal blood vessel was expressed as percentage of the baseline value just before infusion of the vasodilator. The significance of the difference between mean values was evaluated with GraphPad Prism™ (San Diego, CA) by Student’s paired *t*-test or repeated measures of ANOVA followed by the Bonferroni correction. When comparing the responses to vasodilators between groups, two-way analysis of variance (Two-way ANOVA) was used. A *P* value smaller than 0.05 was considered to be statistically significant. All values are presented as means ± SEM.

Results

Baseline values of retinal blood vessel diameter, mean arterial pressure (MAP) and HR of anesthetized rats

Baseline values of retinal arteriolar diameter, retinal venular diameter, mean arterial pressure (MAP) and HR of anesthetized rats were 57.7 ± 1.0 μm, 78.9 ± 1.7 μm, 97 ± 2 mmHg and 351 ± 6 beats/min, respectively (n=50 rats).

Treatment of rats with L-NMMA decreased the diameter of retinal blood vessels, increased MAP and decreased HR (Table 1). Similar changes were observed after the combined treatment with L-NMMA plus indomethacin. These changes were not significantly different between L-NMMA alone and L-NMMA plus indomethacin.

Effects of indomethacin on responses to NO donors and hydralazine in anesthetized rats

To determine whether NO dilates retinal blood vessels through a COX-dependent mechanism, we examined the effect of indomethacin on changes in diameter of retinal blood vessels induced by SNP and NOR3. SNP (1-10 μg/kg/min, i.v.) increased the diameter of retinal blood vessels in a dose-dependent manner (Fig. 1A and B). The
vasodilator effects of SNP on the retinal arterioles were greater than those on retinal venules (at 10 μg/kg/min, changes in arteriolar diameter, 10.7 ±1.0%, n=4 vs. changes in venular diameter, 5.1 ±1.8%, n=4 \( P<0.05 \)). Indomethacin significantly prevented the vasodilator responses to SNP of retinal arterioles, whereas it had no significant effect on changes in MAP and HR induced by SNP (Fig. 1C and D). Similar phenomenon was observed when NOR3 was tested instead of SNP (Fig. 2).

We examined the effect of indomethacin on vasodilator responses to hydralazine, a nonspecific vasodilator, to determine whether the COX inhibitor prevents the vasodilator effect on retinal blood vessels in a non-selective manner. As shown in Fig. 3, indomethacin had no significant effect on the responses to hydralazine (0.4 mg/kg, i.v.). Therefore, it is unlikely that indomethacin non-selectively attenuates the retinal vascular response.

*Distribution of COXs in the rat retina*

We next determined the distribution of COX-1 and COX-2 in the rat retina using immunohistochemistry. As reported previously (12), the COX-1 immunoreactivities were present in cells of the ganglion cell layer and the inner nuclear layer. In addition, the strong COX-1-immunoreactivities were found in the outer plexiform layer (Fig. 4A). The double immunostaining with COX-1 and RECA, a marker of endothelial cells, demonstrated that COX-1 was expressed in retinal blood vessels (Fig. 4A and B). The arterioles and venules could be distinguished morphologically (i.e., wall thicknesses). The staining for COX-1 was more intense in retinal arterioles (Fig. 4A). Confocal microscopic imaging confirmed that the COX-1 immunoreactivities were present in retinal arterioles (Fig. 4C and D) and venules (Fig. 4E and F). The COX-1 immunoreactivities were partly localized to endothelial cells labeled with anti-RECA antibody, whereas they were also
observed outside the endothelial cell layer (Fig. 4D and F). The immunoreactivities of COX-2 were very weak in the normal retina (Fig. 4G). However, at 6 hr after a brief ischemia for 60 min, immunoreactivities of COX-2 in cells of the ganglion cell layer and the inner nuclear layer were increased (Fig. 4H).

*Expression of sGC in the rat retina*

To determine whether sGC is expressed in vascular smooth muscle cells of rat retinal blood vessels, we performed multiple labeling of sGC with αSMA on the sections of eye and mesenteric blood vessels. The sGC staining was strong in the inner plexiform and nuclear layers but staining was barely detectable in outer nuclear layer (Fig. 5A). The staining for sGC was more intense in smooth muscle of choroidal blood vessels than in that of retinal blood vessels (Fig. 5A). The strong immunoreactivities of sGC were observed in smooth muscle of mesenteric blood vessels (Fig. 5B)

*Effects of indomethacin on responses to analogue of cGMP or cAMP in anesthetized rats*

We next sought to determine whether cGMP per se dilates the retinal blood vessels and, if so, how the COX inhibition affects the response. For this purpose, we examined effects of the membrane permeable analogue of cGMP (8-cpt-cGMP) in rats with or without indomethacin treatment. Fig. 6 shows that 8-cpt-cGMP (14 μmol/kg, i.v.) increased the diameter of retinal blood vessels but decreased MAP. These responses were unaffected by indomethacin. For comparison, the effects of the membrane permeable analogue of cAMP (8-cpt-cAMP) were examined (Fig. 7). The vascular and HR responses to 8-cpt-cAMP (14 μmol/kg, i.v.) were not affected by inhibition of COX. Interestingly, 8-cpt-cAMP and 8-cpt-cGMP produced comparable vasodilator responses of retinal arterioles (8-cpt-cAMP, 5.8 ± 3.4%, n=5 vs. 8-cpt-cGMP, 3.0 ± 0.9%, n=6), while
the depressor responses to 8-cpt-cAMP (12.8 ± 2.4%, n=5) were less than those to 8-cpt-cGMP (27.1 ± 4.1%, n=6, P<0.01). The magnitude of vasodilation induced by analogue of cAMP or cGMP was not different between retinal arterioles and venules (8-cpt-cAMP, arterioles 5.8 ± 3.4% vs. venules 4.1 ± 2.2%; 8-cpt-cGMP, arterioles 3.0 ± 0.9% vs. venules 2.8 ± 1.6%).

**Effects of COX inhibitors on responses to NOR3 in TTX-treated rats**

To ascertain the inhibitory effects of COX inhibitors on the responses to NOR3 in the absence of baroreceptor reflexes, experiments were conducted under treatment with TTX. After TTX treatment, baseline values of MAP and HR were adjusted to the same ranges between animals by changing infusion rate of a mixture solution of NE and Epi (NE:Epi=1:9) (MAP: 117±4 mmHg, n=20; HR: 443±8 beats/min, n=20). The diameter of retinal arterioles were 41.0±1.4 μm (n=20). Both indomethacin (5 mg/kg, i.v.) and the COX-1 selective inhibitor SC-560 (5 mg/kg, i.v.) significantly decreased the diameter of retinal arterioles without affecting MAP and HR (Table 2). They reduced the vasodilation of retinal arterioles, but not depressor response, to NOR3 (30 μg/kg/min, i.v.) (Fig. 8 A and B). The COX-2 selective inhibitor NS-398 (5 mg/kg, i.p.) did not affect baseline values of MAP, HR and retinal arteriolar diameter (Table 2). The responses to NOR3 (30 μg/kg/min, i.v.) were unaffected by NS-398 (Fig. 8C). Similarly, nimesulide (1 mg/kg, i.v.) had no significant effect on the baseline conditions (data not shown) and NOR3 (30 μg/kg/min, i.v.)-induced responses (e.g., increase in arteriolar diameter: before, 16.4 ±5.4% vs. after nimesulide, 16.0 ±7.2%, n=5; decrease in MAP: before, 56.3 ±2.9% vs. after nimesulide, 57.5±3.1%, n=5). Despite the depressor effects, NOR3 had no effect on HR (baseline, 443±8 beats/min vs. NOR3, 445 ± 8 beats/min, n=20).
Effects of intravitreal injection of COX inhibitors on responses to NOR3 in TTX-treated rats

To determine whether local application of COX inhibitors exerts the inhibitory effects on the vascular responses to NO, the effects of NOR3 (30 μg/kg/min, i.v.) on retinal arterioles were examined after intravitreal injection of indomethacin (10 nmol/eye), SC-560 (10 nmol/eye), NS-398 (10 nmol/eye) or the vehicle (10% DMSO) (increase in arteriolar diameter: vehicle, 29.4 ±3.1%, n=5; indomethacin, 11.9 ±1.3%, n=5, P<0.05; SC-560, 7.4 ±2.7%, n=5, P<0.05). NS-398 had no significant effect on the responses to NOR3 (30.2 ±2.4%, n=5).

Effects of intravitreal injection of SQ 22536 and ODQ on responses to NOR3 in TTX-treated rats

The results from studies described above suggest that NO preferentially stimulates the COX-1-dependent pathway, rather than the sGC/cGMP signaling pathway, in the rat retinal vasculature. The vasodilator actions of COX-1-derived prostanoids, such as prostacyclin and prostaglandin E2, are elicited by stimulation of Gs protein-coupled receptors that activates adenyl cyclase with subsequent increased formation of cAMP (5, 21, 29). Therefore, we sought to determine the role of adenyl cyclase in NOR3 (30 μg/kg/min, i.v.)-induced vasodilation of retinal arterioles. Intravitreal injection of SQ 22536 (100 nmol/eye) significantly reduced the vasodilator responses to NOR3 (Fig. 9), whereas it did not affect the L-type Ca²⁺ channel blocker nicardipine (30 μg/kg, i.v.)-induced responses (increase in arteriolar diameter: vehicle, 22.7 ±3.0%, n=5 vs. SQ 22536, 20.7 ±2.0%, n=5). The vasodilator responses to NOR3 were unaffected by intravitreal injection of ODQ (10 nmol/eye)(Fig. 9).
**Discussion**

The present study demonstrates that the non-selective COX inhibitor indomethacin attenuates the vasodilation of retinal arterioles to NO donors without affecting the responses to analogue of cAMP or cGMP in rats. The vasodilator effects of NOR3 on retinal arterioles were significantly prevented by the COX-1 selective inhibitor SC-560 and the adenylyl cyclase inhibitor SQ 22536. These results suggest that, in rat retinal arterioles, COX-1 contributes to the vasodilator responses to NO and NO exerts a substantial part of its dilatory effect via a mechanism which involves cAMP-mediated pathway. On the other hand, the vasodilator effects of NO on peripheral resistance vessels are mediated by a COX-independent mechanism, possibly a cGMP signaling pathway, because the decreases in MAP elicited by NO donors were unaffected by the non-selective inhibition of COX.

It is generally accepted that both cAMP- and cGMP-mediated signaling pathways contribute to the regulation of vascular tone, however, our results suggest that NO preferentially stimulates the COX-1/cAMP-mediated pathway in rat retinal arterioles. One possible explanation is that the NO/sGC/cGMP signaling pathway plays a minor role in regulating retinal vascular tone. Indeed, in a previous study, it has been shown that vascular smooth muscle cells of retinal blood vessels were faintly stained for sGC, while the apparent immunoreactivities of sGC were detected in inner retina (6). This pattern of sGC staining was observed in our immunohistochemical study. In addition, our study demonstrated that the sGC inhibitor ODQ fails to prevent the vasodilator responses to NOR3 of retinal arterioles. The results with 8-cpt-cGMP (a membrane permeable analogue of cGMP) indicate that the downstream pathway from cGMP synthesis exists in retinal blood vessels. However, the depressor responses to 8-cpt-cGMP were greater than
those to the same dose of 8-cpt-cAMP, though they produced the comparable vasodilator responses of retinal blood vessels. These findings suggest that the NO/sGC/cGMP signaling pathway appears to be less important in retinal blood vessels than in peripheral resistance vessels.

The vasodilator effects of NO donors on retinal arterioles were greater than those on retinal venules, while no significant difference in the vasodilator response to analogue of cAMP or cGMP was observed between arterioles and venules. Therefore, it is likely that the NO/COX-1/cAMP signaling pathway seems to be more important in retinal arterioles than in retinal venules. The immunoohistochemical data indicate that the expression level of COX-1 in retinal arterioles is slightly higher than that in retinal venules. This difference of expression levels may explain the difference in magnitude of NO-induced vasodilation was observed in between retinal arterioles and venules.

In the present study, we evaluated changes in diameter of retinal blood vessels induced by the drugs administered systematically because we intended to compare their vasodilator effects on retinal blood vessels with those on peripheral resistance vessels. However, there were two concerns that should be addressed. First, it is well known that a change in the retinal arteriolar pressure leads to a compensatory change in the vascular diameter, ensuring a constant capillary perfusion (i.e., pressure autoregulation)(23, 24). Prostaglandins may contribute to autoregulation of retinal circulation (3). Therefore, changes in blood pressure induced by intravenously infused NO donors might lead to the compensatory increases in diameters of retinal arterioles by stimulating production of vasodilatory prostaglandins. If this is the case, indomethacin non-selectively prevents the vasodilation of retinal arterioles induced by depressor agents. However, we found that indomethacin had no significant effect on the vasodilator responses of retinal arteriole to other vasodilators (hydralazine, 8-cpt-cAMP and 8-cpt-cGMP). These results strongly
suggest that abolition of an autoregulatory mechanism by the COX inhibitor cannot explain the attenuation of NO donors-induced vasodilation of retinal arterioles. Second, decrease in blood pressure induced by the drugs would produce a reflex tachycardia under physiological conditions. The changes in systemic hemodynamics may affect the retinal vascular response. However, the results with COX inhibitors obtained from anesthetized rats (in the presence of baroreceptor reflexes) were practically identical to those obtained from TTX-treated animals (in the absence of baroreceptor reflexes). These results support the idea that effects on retinal blood vessel and peripheral resistance vessel could be evaluated by determining changes in diameter of retinal blood vessel and systemic blood pressure induced by systemic administration of the vasodilators. This procedure allows us to conclude that the COX-dependent pathway is more important in retinal blood vessels than in peripheral resistance vessels.

The present data indicating that L-NMMA, an inhibitor of NOS, decreased the diameter of retinal blood vessels suggest an important role for NO in maintaining basal vascular tone in the retinal vasculature. As compared with L-NMMA alone, the combination of L-NMMA plus indomethacin did not produce a further reduction in diameter of retinal blood vessel. However, in a previous study, we found that indomethacin per se decreased diameter of retinal blood vessels (22). In addition, the present study shows both indomethacin and the COX-1 inhibitor decrease the basal retinal arteriolar diameter even in the absence of neuronal influences. Therefore, the basally released NO also appears to exert the vasodilator effect through production of vasodilatory prostanoids in retinal vasculature.

Previous studies demonstrated the immunoreactivities of COX-2 in the normal retina (12, 13). In the present study, the COX-2 immunoreactivities in cells of the ganglion cell layer and the inner nuclear layer were found in the retina after a brief
ischemia, whereas those in the normal retina were very weak. The differences between our results and those of others regarding the level of COX-2 immunoreactivity in normal retina may be related to the difference in staining protocol. The present \textit{in vivo} study shows that the COX-2 inhibition failed to affect the diameter of retinal arteriole and the retinal vascular response to NOR3. Therefore, it is unlikely that COX-2 plays an important role in regulating the retinal circulation under experimental conditions adopted in this study.

The immunoreactivities of COX-1 were found in not only vascular cells but also non-vascular cells, such as ganglion cells and microglia. The functional role of prostaglandins produced by these cells is not clear at present. The cells expressing COX-1, especially those located around blood vessels, may affect retinal blood flow by producing prostanoids via the NO-dependent mechanism.

\textit{Perspectives and Significance}

The sGC/cGMP signaling pathway plays an important role in the vasodilator action of NO in various types of vascular beds. In the present study, we provide evidence that the vasodilator effects of NO on retinal blood vessels are mediated at least in part through the COX-1/cAMP signaling pathway in rats \textit{in vivo}. The interaction between NO and COX-dependent pathway may play a role in regulating retinal hemodynamics under physiological conditions. However, the role of the interaction in the pathogenesis of retinal diseases, such as diabetic retinopathy and glaucoma, remains to be elucidated. These issues should be addressed in the future studies.
Grants

This study was partly supported by Kitasato University Research Grant for Young Researchers (A.M., M.S., T.N.), Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Nos. 10672051, 12672116, 17790070 and 20590090), and the Uehara Memorial Foundation.
References


and selective inhibition of nitric oxide-sensitive guanylyl cyclase by


27. Salvemini D, Currie MG, Mollace V. Nitric oxide-mediated cyclooxygenase activation. A key event in the antiplatelet effects of nitrovasodilators. *J Clin Invest* 97: 2562-2568,
1996.


Figure legends

Figure 1. Changes in retinal arteriolar diameter (AD)(A), retinal venular diameter (VD)(B), mean arterial pressure (MAP)(C) and heart rate (HR)(D) induced by intravenous infusion of sodium nitroprusside (1-10 μg/kg/min) in anesthetized rats treated with vehicle (Cont) or indomethacin (5 mg/kg, i.v., Indo) under conditions of NO synthase blockade with ω-monomethyl-L-arginine (L-NMMA; 50 mg/kg, i.v.). Data are expressed as percentage of the control level (baseline values measured before starting infusion of sodium nitroprusside). Each point with a vertical bar represents mean ± SEM of four animals. *P<0.05 vs. control values (Time 0), #P<0.05.

Figure 2. Changes in retinal arteriolar diameter (AD)(A), retinal venular diameter (VD)(B), mean arterial pressure (MAP)(C) and heart rate (HR)(D) induced by intravenous infusion of NOR3 (1-30 μg/kg/min) in anesthetized rats treated with vehicle (Cont) or indomethacin (5 mg/kg, i.v., Indo) under conditions of NO synthase blockade with ω-monomethyl-L-arginine (L-NMMA; 50 mg/kg, i.v.). Data are expressed as percentage of the control level (baseline values measured before starting infusion of NOR3). Each point with a vertical bar represents mean ± SEM of five animals. *P<0.05 vs. control values (Time 0), #P<0.05.

Figure 3. Changes in retinal arteriolar diameter (AD)(A), retinal venular diameter (VD)(B), mean arterial pressure (MAP)(C) and heart rate (HR)(D) induced by intravenous injection of hydralazine (0.4 mg/kg) in anesthetized rats treated with vehicle (Cont) or indomethacin (5 mg/kg, i.v., Indo) under conditions of NO synthase blockade with ω-monomethyl-L-arginine (L-NMMA; 50 mg/kg, i.v.). Data are expressed as percentage of the control level (baseline values measured before injection of hydralazine). Each point
with a vertical bar represents mean ± SEM of five animals. *P<0.05 vs. control values (Time 0).

Figure 4. Cellular localization of COX-1 and COX-2 in the rat retina. Panel A and B show the COX-1 and RECA immunostaining in the same retinal section. The COX-1 immunoreactivities were present in cells of the ganglion cell layer (GCL) and the inner nuclear layer (INL). In addition, the strong COX-1-immunoreactivities were found in the outer plexiform layer (OPL)(A). Double immunostaining with COX-1 and RECA, a marker of endothelial cells, demonstrated that COX-1 was expressed in the retinal arteriole (arrowhead) and venule (arrow). The staining for COX-1 was more intense in retinal arteriole. Confocal microscopic images show that the COX-1 immunoreactivities were present in the retinal arteriole (C and D) and venule (E and F). The COX-1 immunoreactivities were partly localized to endothelial cells visualized with anti-RECA antibody, whereas they were also observed outside the endothelial cell layer (D and F). The immunoreactivities of COX-2 were very weak in the normal retina (G). However, at 6 hr after ischemia, the levels of expression of COX-2 in cells of the ganglion cell layer and the inner nuclear layer were elevated (H). Bar length: 40 µm (A, B, G, H), 13 µm (C-F).

IPL: inner plexiform layer, ONL: outer nuclear layer

Figure 5. Multiple labeling of sGC with αSMA on the section of eye and mesenteric blood vessels. The sGC staining was strong in the inner plexiform and nuclear layers but staining was barely detectable in outer nuclear layer (A). In A, nuclei were stained with DAPI (blue). The staining for sGC was more intense in smooth muscle of choroidal blood vessels (arrowheads) than in that of retinal blood vessels (arrows) (A). The strong immunoreactivities of sGC in smooth muscle of mesenteric blood vessels were observed
(B). Bar length: 25.5 µm (A), 51 µm (B). IPL: inner plexiform layer, INL: inner nuclear layer, ONL: outer nuclear layer

Figure 6. Changes in retinal arteriolar diameter (AD)(A), retinal venular diameter (VD)(B), mean arterial pressure (MAP)(C) and heart rate (HR)(D) induced by intravenous injection of 8-cpt-cGMP (14 µmol/kg) in anesthetized rats treated with vehicle (Cont) or indomethacin (5 mg/kg, i.v., Indo) under conditions of NO synthase blockade with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 50 mg/kg, i.v.). Data are expressed as percentage of the control level (baseline values measured before injection of 8-cpt-cGMP). Each point with a vertical bar represents mean ± SEM of five to six animals.

Figure 7. Changes in retinal arteriolar diameter (AD)(A), retinal venular diameter (VD)(B), mean arterial pressure (MAP)(C) and heart rate (HR)(D) induced by intravenous injection of 8-cpt-cAMP (14 µmol/kg) in anesthetized rats treated with vehicle (Cont) or indomethacin (5 mg/kg, i.v., Indo) under conditions of NO synthase blockade with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 50 mg/kg, i.v.). Data are expressed as percentage of the control level (baseline values measured before injection of 8-cpt-cAMP). Each point with a vertical bar represents mean ± SEM of five to six animals.

Figure 8. Effects of indomethacin (5 mg/kg, i.v., Indo)(A), SC-560 (5 mg/kg, i.v.)(B) and NS-398 (5 mg/kg, i.p.)(C) on changes in retinal arteriolar diameter (AD) and mean arterial pressure (MAP) induced by intravenous infusion of NOR3 (30 µg/kg/min) in anesthetized rats treated with tetrodotoxin (50 µg/kg, i.v.) under artificial ventilation. Data are expressed as percentage of the control level (baseline values measured before injection of NOR3). Each point with a vertical bar represents mean ± SEM of four to six animals.
Figure 9. Effects of SQ 22536 (100 nmol/eye) and ODQ (10 nmol/eye) on changes in retinal arteriolar diameter (AD) induced by intravenous infusion of NOR3 (30 μg/kg/min) in anesthetized rats treated with tetrodotoxin (50 μg/kg, i.v.) under artificial ventilation. Data are expressed as percentage of the control level (baseline values measured before injection of NOR3). Each point with a vertical bar represents mean ± SEM of five animals.
Table 1. Diameters of retinal arteriole (AD), diameters of retinal venule (VD), mean arterial pressure (MAP) and heart rate (HR) before and after treatment with \(N^G\)-monomethyl-L-arginine (L-NMMA; 50 mg/kg, i.v.) or the combination of L-NMMA plus indomethacin (5 mg/kg, i.v.) in anesthetized rats.

<table>
<thead>
<tr>
<th></th>
<th>AD ((\mu)m)</th>
<th>VD ((\mu)m)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NMMA (n=25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>57.3 ± 1.3</td>
<td>79.0 ± 2.2</td>
<td>99 ± 3</td>
<td>348 ± 8</td>
</tr>
<tr>
<td>After</td>
<td>51.8 ± 1.3*</td>
<td>74.6 ± 1.8*</td>
<td>117 ± 3*</td>
<td>317 ± 7*</td>
</tr>
<tr>
<td>L-NMMA plus indomethacin (n=25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>58.0 ± 1.5</td>
<td>78.9 ± 2.6</td>
<td>96 ± 2</td>
<td>354 ± 8</td>
</tr>
<tr>
<td>After</td>
<td>54.1 ± 1.5*</td>
<td>75.0 ± 2.4*</td>
<td>114 ± 2*</td>
<td>323 ± 8*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *P<0.05 vs. before treatment.
Table 2. Diameters of retinal arteriole (AD), mean arterial pressure (MAP) and heart rate (HR) before and after treatment with indomethacin (5 mg/kg, i.v.), SC-560 (5 mg/kg, i.v.) NS-398 (5 mg/kg, i.p.) in tetrodotoxin-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AD (μm)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indomethacin (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>41.5 ± 3.3</td>
<td>110 ± 5</td>
<td>436 ± 14</td>
</tr>
<tr>
<td>After</td>
<td>37.9 ± 3.1*</td>
<td>113 ± 12</td>
<td>438 ± 14</td>
</tr>
<tr>
<td><strong>SC-560 (n=4)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>40.7 ± 2.8</td>
<td>125 ± 6</td>
<td>481 ± 19</td>
</tr>
<tr>
<td>After</td>
<td>34.3 ± 3.8*</td>
<td>124 ± 6</td>
<td>484 ± 17</td>
</tr>
<tr>
<td><strong>NS-398 (n=5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>41.3 ± 2.6</td>
<td>113 ± 10</td>
<td>423 ± 13</td>
</tr>
<tr>
<td>After</td>
<td>42.3 ± 2.8</td>
<td>103 ± 13</td>
<td>433 ± 14</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *P<0.05 vs. before treatment
Sodium nitroprusside (μg/kg/min, i.v.)

Fig. 1. Ogawa et al.
Fig. 2. Ogawa et al.
Hydralazine (0.4 mg/kg, i.v.)

Fig. 3. Ogawa et al.
8-cpt-cGMP (14 μmol/kg, i.v.)

Fig. 6. Ogawa et al.
8-cpt-cAMP (14 μmol/kg, i.v.)

![Graph showing the effects of 8-cpt-cAMP on AD, VD, MAP, and HR.](image)

Fig. 7. Ogawa et al.
Fig. 8. Ogawa et al.
Fig. 9. Ogawa et al.