Role of Nitric Oxide in Regulation of Retinal Blood Flow in Response to Hyperoxia in Cats

Naohiro Izumi, Taiji Nagaoka, Eiichi Sato, Kenji Sogawa, Hiroyuki Kagokawa, Atsushi Takahashi, Atsushi Kawahara, and Akitoshi Yoshida

Purpose. To investigate whether nitric oxide (NO) regulates retinal circulation during and after induction of hyperoxia in cats.

Methods. Hyperoxia was induced for 10 minutes with 100% oxygen. The vessel diameter and blood velocity were measured simultaneously in second-order retinal arterioles by laser Doppler velocimetry; the retinal blood flow (RBF) and wall shear rate (WSR) were calculated during and after hyperoxia. PBS, L-NAME, D-NAME, BQ-123, BQ-788, and 7-nitroindazole (7-NI) were administered before induction of hyperoxia.

Results. In the PBS group, vessel diameter, blood velocity, and RBF decreased during hyperoxia and returned to baseline within 10 minutes after hyperoxia ended. WSR decreased transiently and then returned to baseline by the delayed constriction of retinal arterioles during hyperoxia. In the L-NAME and BQ-788 groups, the decreases in RBF during hyperoxia did not differ from those in the PBS group. However, the recovery of RBF after hyperoxia ended was attenuated significantly until 20 minutes after hyperoxia ended in both groups compared with the PBS group ($P < 0.05$). In the BQ-123 group, the intravitreous injection of BQ-123 caused less reduction of blood velocity and RBF during hyperoxia compared with that in the PBS group, whereas the RBF immediately returned to baseline after hyperoxia. D-NAME and 7-NI did not affect RBF in response to hyperoxia.

Conclusions. The current results indicate that NO contributes to RBF recovery after hyperoxia, probably through the action of endothelial NOS via the ETB receptor in the vascular endothelium of the retinal arterioles, suggesting that the RBF response to hyperoxia may be used to evaluate the endothelial function of the retinal arterioles. (*Invest Ophthalmol Vis Sci.* 2008;49:4595–4603) DOI:10.1167/iovs.07-1667

It is well-known that hyperoxia constricts the retinal arterioles and venules and decreases retinal blood flow (RBF) by various methods in humans[1–4] and animals.[5,6] Hyperoxia has been induced frequently to provoke vascular reactivity in the retinal circulation, and impaired vascular reactivity to hyperoxia has been implicated in the pathogenesis of diabetic retinopathy.[7–10] The previous studies revealed that endothelin (ET)-1 plays a major role in hyperoxia-induced vasoconstriction in humans[1,11] and animals.[5,6,12] In addition, an in vitro study showed that hyperoxia stimulates release of ET-1 from bovine retinal endothelial cells.[13] Taken together, ET-1 can cause vasoconstriction[14,15] and decreased RBF[6] likely by binding to the high-affinity ET type A (ETA) receptor in retinal vascular smooth muscle cells and pericytes.[16,17]

However, the ET type B (ETB) receptor, which is mainly present on endothelial cells, mediates vasodilation by a process that includes release of nitric oxide (NO).[18] ET-3, which interacts primarily with the endothelial cell ETB receptor, increased RBF in nondiabetic rats by the ET-3/ETB receptor interaction and NO synthase (NOS) action,[19] suggesting that the ETB receptor is present in the retinal endothelium and dilates retinal vessels by NO production from the endothelium. However, no study was conducted to investigate the role of the ETB receptor in the change in retinal blood flow in response to hyperoxia.

There is a biphasic response to exogenous ET-1 on choroidal vessels, suggesting that endogenous ET-1 preferentially elicits vasodilation via the ETB receptor, most likely by stimulating endothelial NO release.[20] In addition, an in vivo feline study reported that intravenously administered ET-1 increased, but an intravitreous injection of ET-1 decreased, tissue blood flow in the optic nerve head in cats.[21] If these results obtained from choroidal and optic nerve circulation are the case in the retinal circulation, the possible increase in the retinal concentration of ET-1 during hyperoxia may cause vasoconstriction via the ETA receptor in smooth muscle and vasodilation via ETB in the vascular endothelium. NO is synthesized enzymatically by NOS from L-arginine and molecular oxygen as a substrate and is a highly diffusible gas with potent vasodilatory action.[22] We previously reported that NO contributes to increased RBF during hypoxia through a flow-induced mechanism[23] and during hypercapnia mainly through neuronal (n)NOS in the retina.[24] However, no study has been undertaken to examine the role of NO in the changes in RBF in response to hyperoxia. Therefore, in the present study, we tested the hypothesis that NO contributes to the regulation of retinal circulation in response to hyperoxia via the interaction of endothelin receptors.

Materials and Methods

Animal Preparation

Protocols describing the use of cats were approved by the Animal Care Committee of Asahikawa Medical College and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Forty adult cats of either sex (weight, 1.9–4.0 kg) were used. Anesthesia was induced with enflurane, oxygen, and nitrous oxide in a closed box, followed by intraperitoneal injection of atropine (0.04 mg/kg). The animals were tracheostomized and mechanically ventilated with 1.5% to 2.0% enflurane and room air. End-tidal carbon dioxide was maintained at a constant level. Catheters were placed in the femoral arteries and veins. Pancuronium bromide (0.1 mg/kg/hr;

From the Department of Ophthalmology, Asahikawa Medical College, Asahikawa, Japan.

Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Tokyo, Japan: A-19689035 (TN) and C-18591904 (AY).

Submitted for publication December 28, 2007; revised May 6, 2008; accepted August 20, 2008.

Disclosure: **N. Izumi**, None; **T. Nagaoka**, None; **E. Sato**, None; **K. Sogawa**, None; **H. Kagokawa**, None; **A. Takahashi**, None; **A. Kawahara**, None; **A. Yoshida**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Taiji Nagaoka, Department of Ophthalmology, Asahikawa Medical College, Asahikawa, Hokkaido, 078-8510, Japan; nagaoka@asahikawa-med.ac.jp.

TABLE 1. Blood Gas Analysis, MABP, and HR before and during Hyperoxia

	PBS Group (n = 7)		L-NAME Group (n = 6)		D-NAME Group (n = 7)		BQ-123 Group (n = 7)		BQ-788 Group (n = 6)	
	Before	Hyperoxia	Before	Hyperoxia	Before	Hyperoxia	Before	Hyperoxia	Before	Hyperoxia
pH	7.37 ± 0.21	7.38 ± 0.18	7.39 ± 0.23	7.40 ± 0.23	7.40 ± 0.22	7.41 ± 0.21	7.38 ± 0.19	7.39 ± 0.18	7.35 ± 0.16	7.37 ± 0.16
$PaCO_2$ (mm Hg)	30.3 ± 1.6	29.3 ± 1.6	31.0 ± 1.7	29.8 ± 1.9	30.0 ± 1.5	29.1 ± 1.4	31.3 ± 1.5	30.0 ± 1.5	31.3 ± 1.4	30.2 ± 1.3
PaO_2 (mm Hg)	119.7 ± 4.4	697.1 ± 7.5*	128.8 ± 5.0	702.5 ± 8.2*	134.3 ± 6.0	677.0 ± 6.5*	131.7 ± 4.5	689.6 ± 8.3*	133.3 ± 4.6	722.8 ± 6.6*
MABP (mm Hg)	93.4 ± 3.7	93.4 ± 3.7	86.1 ± 4.4	85.6 ± 4.3	86.9 ± 3.9	86.2 ± 4.0	91.2 ± 3.3	90.7 ± 3.1	92.5 ± 3.1	93.6 ± 3.3
HR (beats/min)	150.0 ± 4.3	152.6 ± 4.2	141.0 ± 5.0	141.0 ± 4.8	139.7 ± 4.8	142.3 ± 4.6	158.6 ± 4.4	158.6 ± 4.1	159.0 ± 3.9	157.0 ± 4.2

Data are the mean ± SE. Before, before induction of hyperoxia; hyperoxia, at the end of hyperoxia.
* $P < 0.05$ versus prehyperoxic values by paired Student's t-test.

Daiichi Sankyo Co., Tokyo, Japan) was infused continuously. Arterial pH, arterial partial CO_2 tension ($PaCO_2$), and arterial partial oxygen tension (PaO_2) were measured intermittently with a blood gas analyzer (model ABL5; Radiometer, Copenhagen, Denmark). The mean arterial blood pressure (MABP) and heart rate (HR) were monitored continuously. The rectal temperature was maintained between 37°C and 38°C with a heated blanket.

The pupils were dilated with 0.5% tropicamide (Santen Pharmaceutical Co., Osaka, Japan). A 0-D contact lens was placed on the cornea, and a drop of sodium hyaluronate (Healon; Advanced Medical Optics, Inc., Santa Ana, CA) was instilled.

RBF Measurement

We measured RBF with a laser Doppler velocimetry system (Laser Blood Flowmeter, model CLBF 100; Canon, Inc., Tokyo, Japan) customized for feline use.[23,24] The instrument measures vessel diameter and blood velocity simultaneously in retinal vessels and calculates the RBF.[25,26] The laser Doppler velocimetry system has been described elsewhere.[23,24] Briefly, the blood velocity was measured by bidirectional laser Doppler velocimetry, which provides absolute measurements of the speed of the red blood cell (RBCs) flowing at discrete, selected sites in the retinal vessel, assuming Poiseuille's flow.[25,26] The signals from the two photomultiplier tube detectors undergo computer-controlled spectrum analysis and sequential measurement of the maximum speed (V_{max}) at the center of the vessel. In this system, each pair of spectra was recorded, and the V_{max} was calculated automatically every 5 ms for 1 second during each measurement. The V was defined as the averaged V_{max} during one cardiac cycle.

The retinal vessel diameter was determined automatically by computer analysis of the signal produced by the vessel image. The value of the vessel diameter was defined as the average of the values determined at each time point.

The RBF was calculated from the formula $RBF = S \times V_{mean}$, where S is the cross-sectional area of the retinal artery at the laser Doppler measurement site, assuming a circular cross-section, and V_{mean} is the mean blood velocity calculated as $V_{mean} = V_{max}/2$.[27] The wall shear rate (WSR), an indicator of wall shear stress, was calculated from the vessel diameter and blood velocity data assuming a parabolic flow profile.[28] WSR was calculated as $WSR = 8 \times V_{mean}/D$.[29]

Laser Doppler measurements were obtained from a temporal retinal artery in one eye of each animal. The second-order arteries chosen for measurement had relatively straight segments that were sufficiently distant from the adjacent vessels.

Intravitreous Injection of L-NAME and Induction of Hyperoxia

We used L-NAME as the nonselective NOS inhibitor and N^G-nitro-D-arginine-methylester (D-NAME) as the inactive stereoisomer. The intravitreous microinjection technique was performed with a 30-gauge needle placed into the vitreous 3 mm posterior to the limbus. The injection was performed with a 100-μL syringe (Hamilton, Reno, NV) with care taken not to injure the lens or retina. The head of the needle was positioned over the optic disc. Given that the volume of the feline vitreous is approximately 2.5 mL, 50 μL of L-NAME (100 mM; n = 6) or D-NAME (100 mM; n = 7) dissolved in phosphate-buffered saline (PBS) was injected into the vitreous for an extracellular concentration of 2.0×10^{-3} M near the retinal vessels.

This concentration may be sufficient for the half-maximum inhibitory concentration of L-NAME and is the same as the dose used in previous feline studies.[23,24] As a vehicle, 50 μL of PBS (n = 7) was injected into another cat in the same manner as L-NAME.

Hyperoxia was induced 120 minutes after the injection of PBS, L-NAME, or D-NAME into each cat, by inhalation of 100% oxygen for 10 minutes. The RBF measurements started 10 minutes before induction of hyperoxia. An average of five measurements taken at 2-minute intervals was defined as the baseline before induction of hyperoxia. During and after induction of hyperoxia, RBF measurements were

TABLE 2. Changes in Systemic Parameters during Hyperoxia with and without 7-NI

	Without 7-NI (n = 7)		With 7-NI (n = 7)	
	Before	**Hyperoxia**	**Before**	**Hyperoxia**
pH	7.38 ± 0.23	7.40 ± 0.26	7.40 ± 0.19	7.41 ± 0.21
$PaCO_2$ (mm Hg)	32.0 ± 1.4	29.1 ± 1.8	30.1 ± 1.8	29.0 ± 1.8
PaO_2 (mm Hg)	130.7 ± 5.4	668.0 ± 8.2*	141.6 ± 5.4	688.7 ± 9.0*
MABP (mm Hg)	88.8 ± 2.9	89.5 ± 3.0	97.9 ± 3.0	98.1 ± 3.0
HR (beats/min)	145.7 ± 4.9	145.7 ± 4.8	143.1 ± 5.3	144.9 ± 5.4

Data are the mean ± SE. Before, before induction of hyperoxia; hyperoxia, at the end of hyperoxia.
* $P < 0.05$ versus prehyperoxic values by paired Student's t-test.

performed every 2 minutes. At each time point, three successive measurements at 20-second intervals were recorded, and the average of the three measurements was used. Blood gas analysis was performed before the induction of hyperoxia and at the end of hyperoxia.

Intravitreous Injection of BQ-123 and -788 and Induction of Hyperoxia

The intravitreous microinjection technique was the same as that used for L-NAME; 50 μL of the specific ETA antagonist BQ-123 (1 mM; n = 7) and 50 μL of the specific ETB antagonist BQ-788 (1 mM; n = 6) were injected into the vitreous for an extracellular concentration of 2.0×10^{-5} M near the retinal vessels. These concentrations in BQ-123[12] and -788[19] ensure a maximum retinal hemodynamic response. Hyperoxia was induced 60 minutes after BQ-123 and -788 were injected. The RBF measurements were similar to those in L-NAME during and after hyperoxia.

Intraperitoneal Injection of 7-NI and Induction of Hyperoxia

To examine the role of nNOS in RBF regulation after hyperoxia, we used 7-NI, which is selective for nNOS.[30] 7-NI (50 mg/kg in 10 mL peanut oil; n = 7) was injected intraperitoneally, because in our preliminary study the intravitreous injection of 7-NI made it impossible to measure the RBF due to the hazy vitreous. This dose of 7-NI maximally inhibits nNOS[31] and is the same as used in a previous feline study.[24] The RBF response to 10 minutes of hyperoxia was measured 90 minutes after injection, as previously published.[24]

All drugs were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

All data are expressed as the mean ± the SEM (SE). For statistical analysis, we used analysis of variance (ANOVA) for repeated measurements followed by post hoc comparison with the Dunnett procedure versus baseline. For comparisons with the PBS group at each time point, one-way ANOVA was used, and significance was assessed using post hoc comparison with the Dunnett procedure. Differences between the means in the circulatory changes between groups with and without 7-NI and between means in systemic parameters before and during hyperoxia were assessed with the Student's paired t-test. $P < 0.05$ was considered significant.

RESULTS

Systemic Changes in Response to Hyperoxia

Intravitreous injections of PBS, L-NAME, D-NAME, BQ-123, and BQ-788 (Table 1) and intraperitoneal injection of 7-NI (Table 2) did not alter the pH, $PaCO_2$, PaO_2, MABP, or HR. During hyperoxia, no significant differences in the pH $PaCO_2$ MABP, or HR occurred, whereas PaO_2 increased significantly ($P < 0.05$) in all groups.

Changes in Retinal Circulation by Hyperoxia

In the PBS group (n = 7), vessel diameter and blood velocity significantly decreased during hyperoxia compared with baseline (repeated-measures ANOVA) followed by the Dunnett procedure (Fig. 1). Two minutes after the onset of hyperoxia, the vessel diameter was unchanged, but blood velocity decreased significantly ($P < 0.05$) by −15.4% ± 3.3%, resulting in decreased RBF (Fig. 1). The WSR decreased significantly by −12.9% ± 3.4% at 2 minutes of hyperoxia. At 4 minutes, the vessel diameter started to decrease to a significant level, and the WSR recovered to the baseline level. At 10 minutes, the percentage decreases from the baseline values were −18.3% ± 2.1% in diameter, −23.4% ± 3.3% in velocity, and −48.4% ± 2.6% in RBF, respectively. After hyperoxia, the vessel diameter, blood velocity, and RBF began to return to the baseline values. The blood velocity increased to the baseline level 2 minutes after the end of hyperoxia, whereas the vessel diameter remained decreased compared with baseline. The WSR significantly increased 2 minutes after hyperoxia. At 6 minutes, the vessel diameter increased to a significant level, and the WSR returned to baseline. Ten minutes after the end of hyperoxia, all parameters recovered to baseline.

Effect of L-NAME on Hyperoxia-Induced Changes in Retinal Circulation

Two hours after the intravitreous injection of L-NAME, there were significant decreases in vessel diameter (−6.5% ± 1.6%; from 87.6 ± 2.9 to 82.0 ± 2.4 μm; $P < 0.05$), blood velocity (−13.3% ± 2.8%; from 34.7 ± 2.5 to 30.1 ± 1.7 mm/s; $P < 0.05$), and RBF (−25.5% ± 3.2%; from 6.5 ± 1.5 to 4.8 ± 1.1 μL/min; $P < 0.05$) compared with baseline (preinjection; Table 3). In contrast, 2 hours after intravitreous injection of D-NAME, the values did not differ significantly from baseline (Table 3).

In the L-NAME group (n = 6), the decreases in vessel diameter, blood velocity, and RBF were similar to that in the PBS group during hyperoxia (Fig. 2). However, the recovered RBF decreased significantly compared with the PBS group after hyperoxia. All parameters returned to the prehyperoxic levels approximately 30 minutes after the end of hyperoxia (data not shown). The recoveries of decreased vessel diameter and RBF were significantly attenuated compared with the PBS group at the same times (Fig. 2). In the D-NAME group (n = 7), there were no significant differences in the decreases in any parameters compared with the PBS group during and after hyperoxia (Fig. 2).

Effects of BQ-123 and -788 during Hyperoxia

One hour after injection of BQ-123 and -788, there were no significant differences in vessel diameter, blood velocity, and

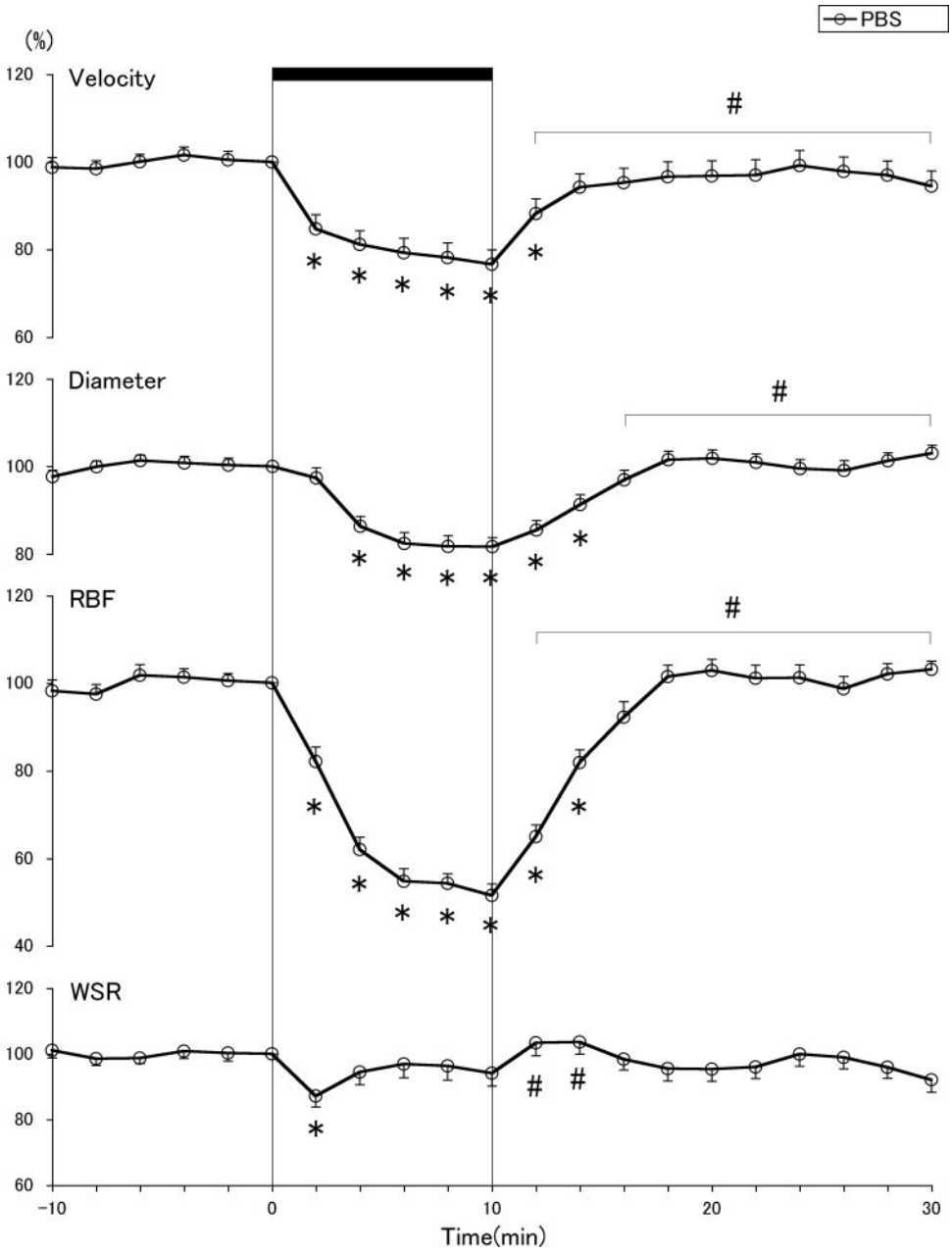

FIGURE 1. Time course of changes in retinal circulation in response to hyperoxia in the control (PBS) group ($n = 7$). Data are expressed as the mean percentage ± SE of the prehyperoxic levels. *Solid bar*: period of hyperoxia. $^{*}P < 0.05$ in a comparison with the prehyperoxic values and $^{\#}P < 0.05$ in a comparison with hyperoxic values at 10 minutes by repeated-measures ANOVA followed by the Dunnett procedure.

RBF compared with the preinjection levels. The absolutes values for the hemodynamic parameters are listed in Table 3.

In the BQ-123 group ($n = 7$), the intravitreous injection of BQ-123 caused significantly ($P < 0.05$) less reduction of blood velocity and RBF during hyperoxia compared with that in the PBS group (Fig. 3). After hyperoxia, the diameter, velocity, and RBF immediately returned to baseline. In the BQ-788 group ($n = 6$), the decreases in vessel diameter, blood velocity, and RBF were unaffected by intravitreous BQ-788 and comparable to those in the PBS group (Fig. 3). After hyperoxia, the recoveries of the vessel diameter and RBF were attenuated significantly by intravitreous injection of BQ-788 compared with the PBS group. The diminished recovery of RBF after hyperoxia was similar to that in the L-NAME group (Fig. 2).

Effect of 7-NI during Hyperoxia

Ninety minutes after intraperitoneal injection of 7-NI, there were no significant changes in any retinal circulatory parameters compared with the preinjection levels (Table 3).

The vessel diameter, blood velocity, and RBF significantly decreased during hyperoxia with and without 7-NI compared with the prehyperoxic levels (Fig. 4). There were also no significant differences in the changes in systemic parameters (Table 2) during hyperoxia between the groups that received 7-NI and the one that did not.

DISCUSSION

In the present study, the RBF significantly decreased (48.4%) after initiation of hyperoxia in the PBS control group (Fig. 1). Hyperoxia has been reported to induce a pronounced reduction in RBF in animals[5,6] and humans.[1–4,7,8,32] Although there may be differences among species and with different measurement techniques, the magnitude and time course of the decrease in RBF during hyperoxia in the present study were comparable with previously reported findings.

The current data showed a trend for changes in blood velocity to occur before the changes in diameter just after the

TABLE 3. Retinal Circulation during Normoxia

	PBS Group (n = 7)		L-NAME Group (n = 6)		D-NAME Group (n = 7)		BQ-123 Group (n = 7)		BQ-788 Group (n = 6)		Without 7-NI (n = 7)		With 7-NI (n = 7)	
	Pre-injection	Pre-hyperoxia	Pre-injection	Pre-hyperoxia	Pre-injection	Pre-hyperoxia	Pre-injection	Pre-hyperoxia	Pre-injection	Pre-hyperoxia	Pre-injection	Pre-hyperoxia	Pre-injection	Pre-hyperoxia
Velocity (mm/s)	32.0 ± 1.9	33.8 ± 2.0	34.7 ± 2.5	30.1 ± 1.7*	36.6 ± 2.6	36.1 ± 2.5	37.0 ± 2.7	36.9 ± 2.3	33.7 ± 2.5	33.2 ± 2.5	34.1 ± 2.1	33.5 ± 1.8	31.9 ± 1.7	33.1 ± 2.3
Diameter (μm)	84.6 ± 2.8	84.0 ± 2.4	87.6 ± 2.9	82.0 ± 2.4*	88.1 ± 2.3	87.6 ± 2.4	90.7 ± 2.7	91.8 ± 2.9	88.2 ± 2.6	85.8 ± 2.5	90.0 ± 2.7	92.1 ± 3.1	90.5 ± 3.3	91.9 ± 3.4
RBF (μL/min)	5.4 ± 1.0	5.6 ± 0.9	6.5 ± 1.5	4.8 ± 1.1*	6.8 ± 1.4	6.7 ± 1.4	7.3 ± 1.4	7.5 ± 1.6	6.3 ± 1.4	5.9 ± 1.4	6.6 ± 1.3	6.8 ± 1.4	6.3 ± 1.4	6.9 ± 1.6
WSR (/s)	1527 ± 15	1617 ± 15	1579 ± 14	1470 ± 10	1653 ± 16	1652 ± 17	1630 ± 11	1604 ± 11	1520 ± 13	1539 ± 14	1515 ± 13	1459 ± 11	1412 ± 9	1438 ± 9

Data are the mean ± SE.
* $P < 0.05$ versus preinjection values by paired Student's t-test.

initiation of hyperoxia in the PBS group (Fig. 1). Our results, that the blood velocity decreased but the vessel diameter did not change significantly 2 minutes after the beginning of hyperoxia, suggested that the downstream vessels, which were more peripheral than the points measured by laser Doppler velocimetry, contract just after the onset of hyperoxia. These rapid changes in velocity with no changes in diameter also were observed during systemic hypoxia,[23] suggesting that the downstream vessels react to changes in oxygen tension. Gilmore et al.[4] reported that the response times during hyperoxia did not differ between diameter and velocity in the retinal arterioles in healthy humans. This finding differed from ours and may reflect differences in the vessel sizes measured in the previous study (average diameter, 85 μm in the present study and 111 μm in the previous study). The vessel size in the arterioles is considered important to data interpretation, because three different vasoregulatory mechanisms (metabolic, myogenic, and flow-induced mechanisms) coordinate the overall microvascular response from the downstream arterioles to the upstream arteries in the microcirculation.[33] Moreover, preliminary data reported by Rosa et al. (*IOVS* 2005;46:ARVO E-Abstract 3900) indicated that second-order arterioles are more sensitive to the flow-induced dilation than first-order arterioles. Although Gilmore et al. did not report if they measured the first- or second-order retinal arterioles in their human study, the discrepancy may have been the result of our measuring second-order arterioles in the present study and their measuring larger arterioles.

In addition, we continuously observed changes in the retinal circulation for 20 minutes after hyperoxia because we focused on changes in the retinal circulation not only during but also after hyperoxia. The decreased RBF returned to baseline almost 10 minutes after hyperoxia and the baseline value was maintained until the end of the examination in the PBS group (Fig. 1). The recovery of the retinal circulation is comparable to that in previous human studies.[4,34] The current data also indicated that the blood velocity returned to the baseline level 4 minutes after hyperoxia, whereas the vessel diameter returned to the baseline level 6 minutes after hyperoxia (Fig. 1), suggesting that blood velocity recovers faster than the vessel diameter. A previous human study reported that the time course of the response to hyperoxia did not differ between the diameter and velocity both during and after hyperoxia.[4] As discussed previously, this discrepancy may have resulted from the difference in species or vessel size of the measured retinal arterioles.

The strength of the present study was the use of laser Doppler velocimetry, which enables simultaneous measurement of the vessel diameter and blood velocity. Using these two independent retinal circulatory parameters, we can evaluate changes in the WSR in the retinal circulation.[35] Most previous studies[1,2,32] did not report WSR data, which is an index of wall shear stress,[28] because the vessel diameter and blood velocity were not measured simultaneously as a result of methodologic difficulties. Only one study reported decreased WSR in the retinal arterioles in response to systemic hyperoxia.[8] In the present study, the WSR decreased significantly at 2 minutes of hyperoxia (Fig. 1), whereas the WSR returned to the baseline level at 4 minutes of hyperoxia because of decreased vessel diameter, suggesting that the WSR remains constant in response to hyperoxia. Although inconsistent with results in a previous study,[8] this finding seems reasonable because shear stress should remain constant under physiologic conditions.[36] As stated previously, this may be caused by differences in vessel sizes between our observations and a previous human study.[4] If the WSR remains constant by the flow-induced mechanism in response to hyperoxia, hyperoxia-induced changes in the retinal circulation may be good

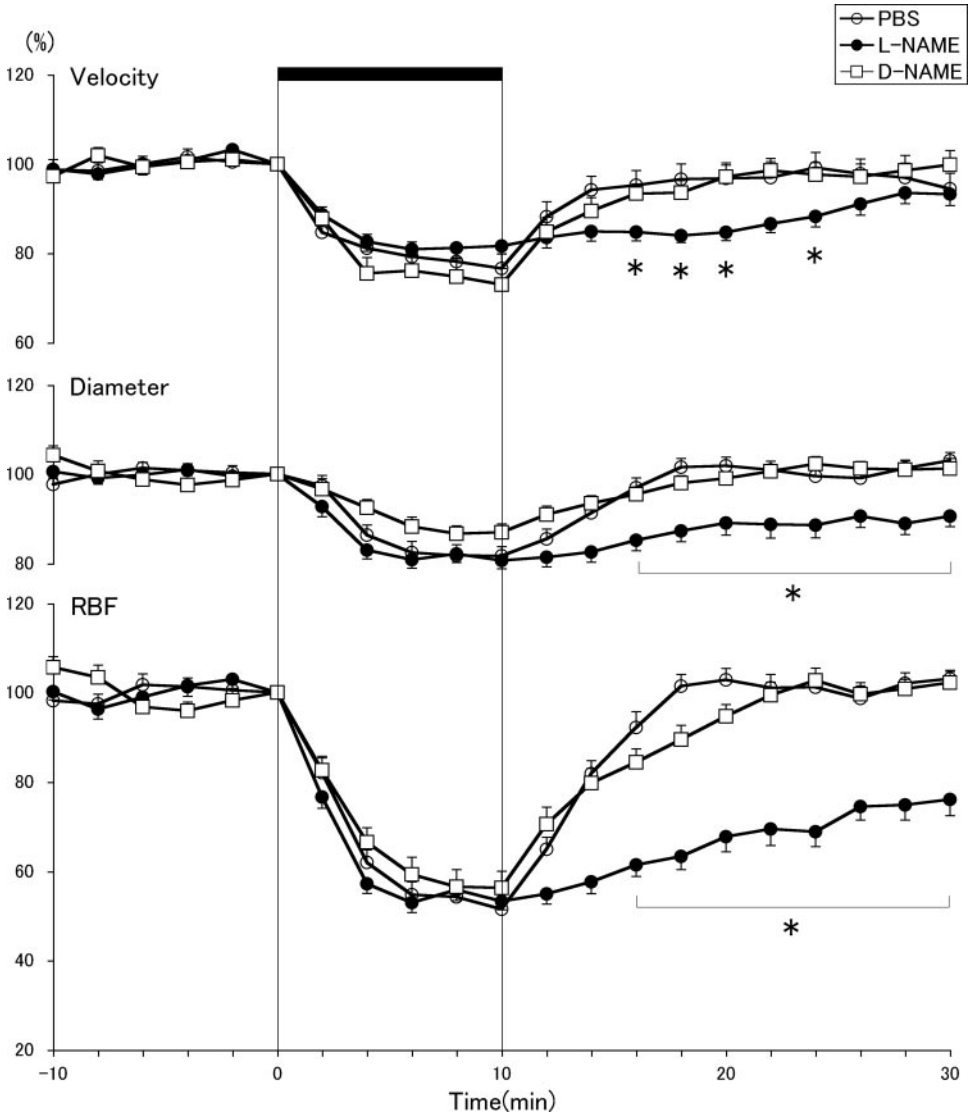

FIGURE 2. Time course of the changes in retinal circulation in response to hyperoxia in the PBS ($n = 7$), L-NAME ($n = 6$), and D-NAME ($n = 7$) groups. Data are expressed as a mean percentage ± SE of the prehyperoxic levels. *Solid bar*: indicates the period of hyperoxia. *Significant differences compared with the PBS group at each time point ($P < 0.05$).

indicators of endothelial function, because the flow-induced mechanism can be used to evaluate endothelial function.

We have provided new evidence that intravitreous injection of L-NAME markedly inhibits recovery of the decreased RBF to baseline after hyperoxia ends, whereas there was no difference in the decrease in RBF during hyperoxia between the PBS and L-NAME groups (Fig. 2). Inhibition of NOS as a mechanism of the effects of L-NAME is supported by the fact that D-NAME did not affect RBF in response to hyperoxia (Fig. 2). To our best knowledge, this is the first study to show that NO is associated with the response of the RBF to hyperoxia.

Because inducible NOS activity was thought to be minimal in the acute response to hyperoxia, we evaluated which constitutive NOS was involved in the changes in RBF in response to hyperoxia. We examined the effect of the selective inhibitor of nNOS, one of two constitutive NOS isoforms, on the regulation of RBF in response to hyperoxia. There was no significant difference in any retinal or systemic parameters with and without (vehicle only) 7-NI (Fig. 4), suggesting that retinal nNOS may not be associated with increased RBF in response to hyperoxia. Moreover, although we did not use a specific eNOS inhibitor, our findings indicated that eNOS in the vascular endothelium may be involved in the recovery of RBF after hyperoxia.

ET is considered crucial in vascular control because there is increasing evidence that ET-1 also plays an important role in ocular blood flow control.[6,14,15,37] In the present study, BQ-123 blunted the decrease in RBF during hyperoxia (Fig. 2). This result seems to agree with those in previous studies in which enhanced ET-1 activity played a primary role in regulating the retinal hemodynamic during hyperoxia.[1,5,6,11,12] However, in the present study, intravitreous injections of BQ-123 and -788 did not have a substantial effect on retinal circulatory parameters before the induction of hyperoxia, suggesting that ET-1 does not play an important role in regulating retinal circulation under basal condition (normoxia). Polak et al.[11] reported that intravenous injection of BQ-123 does not affect the retinal hemodynamic parameters, which supports our findings.

Based on our experiments, the contribution of ETB receptors was excluded in hyperoxia-induced vasoconstriction, because there was no difference between the PBS group and BQ-788 group during hyperoxia. In contrast, BQ-788 suppressed RBF recovery after hyperoxia (Fig. 3), which was comparable to that in the L-NAME group. Therefore, NO production in the retinal vascular endothelium may contribute to RBF recovery after hyperoxia via ETB receptor activation. Haefliger et al.[38] reported that ET-1 induced potent contrac-

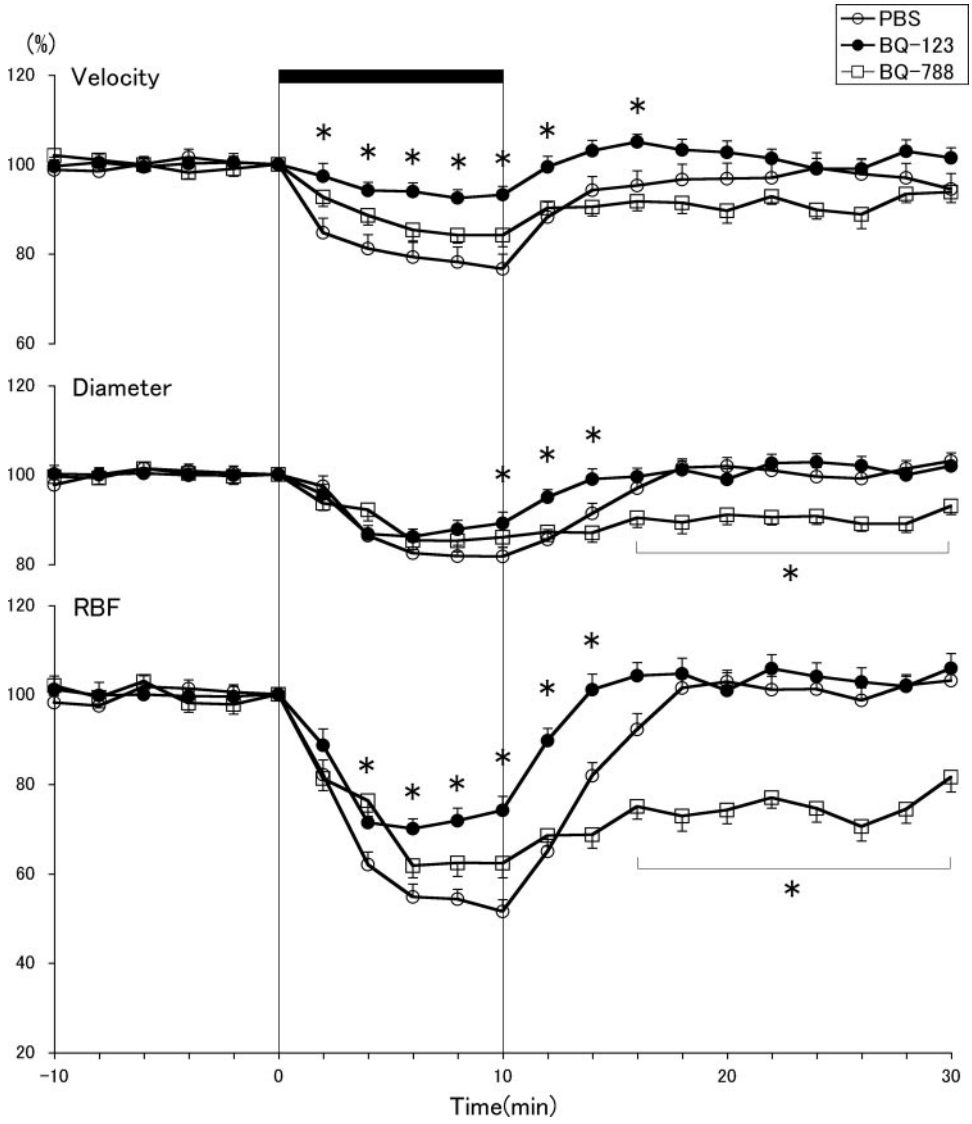

FIGURE 3. Time course of the changes in retinal circulation in response to hyperoxia in the PBS ($n = 7$), BQ-123 ($n = 7$), and BQ-788 ($n = 6$) groups. Data are expressed as the mean percentage ± SE of the prehyperoxic levels. *Solid bar*: period of hyperoxia. *Significant differences compared with the PBS group at each time point ($P < 0.05$).

tions that were more pronounced in the ciliary artery than in the ophthalmic artery, suggesting that the effect of ET may depend on vessel size and increase with decreasing vascular diameter. Therefore, the differences in vessel size may be associated with differences in the time course of the diameter and velocity changes between the findings of Gilmore et al.[4] and ours, as discussed previously. The ability of BQ-123 to reduce the velocity and flow but not to alter the diameter of second-order vessels (Fig. 3) further supports the idea that the smaller downstream arterioles may have greater sensitivity to ET-1 and initiate constriction in response to hyperoxia.

ET-1 induces an initial biphasic action (brief vasodilation followed by prolonged constriction) on systemic blood pressure.[39] ET-1 first stimulates the endothelial ETB receptors, occupies all the receptors, and then diffuses into the media to act on receptors on the smooth muscle.[40] If this biphasic effect of ET-1 occurs in the retinal circulation, high-dose ET-1 during hyperoxia should constrict the retinal arterioles via the ETA receptors, whereas a reduction of the increase in ET-1 after the end of hyperoxia may cause vasodilation via the ETB receptors.

The present study had some limitations. First, we did not measure ET-1 and NO concentrations in the retina or retinal vessels. Knowledge of the ET-1 concentration after hyperoxia may be essential when considering a balance between the vasodilatory actions of ETB in the endothelium and the vasoconstrictive actions of ETA in the smooth muscle in the retinal arterioles. Further studies including those measurements are crucial to an understanding of the mechanism in the retinal circulation after hyperoxia. Second, we did not measure the intraocular pressure (IOP), which is important in retinal circulation. In our preliminary study, there was no substantial difference in the IOP changes between the PBS group and the other groups. We also confirmed that hyperoxia did not affect the IOP during and after hyperoxia ($n = 4$, data not shown) and that the IOP transiently increased but returned to the preinjection level within 5 minutes ($n = 4$, data not shown). Taken together, we believe that the IOP had little effect on our results. Third, we did not examine the role of other vasoactive factors on the response to hyperoxia. Zhu et al.[5] reported that thromboxane and 20-hydroxyeicosetetraenoic acid also are involved in the hyperoxia-induced decrease in RBF in newborn pigs. Further study is needed to determine whether mechanisms other than ET-1 are associated with hyperoxia-induced reduction of the RBF. Fourth, we could not exclude the possible role of ETB receptors and eNOS in the endothelium and neural/glial cells in this in vivo study, because the ETB receptors

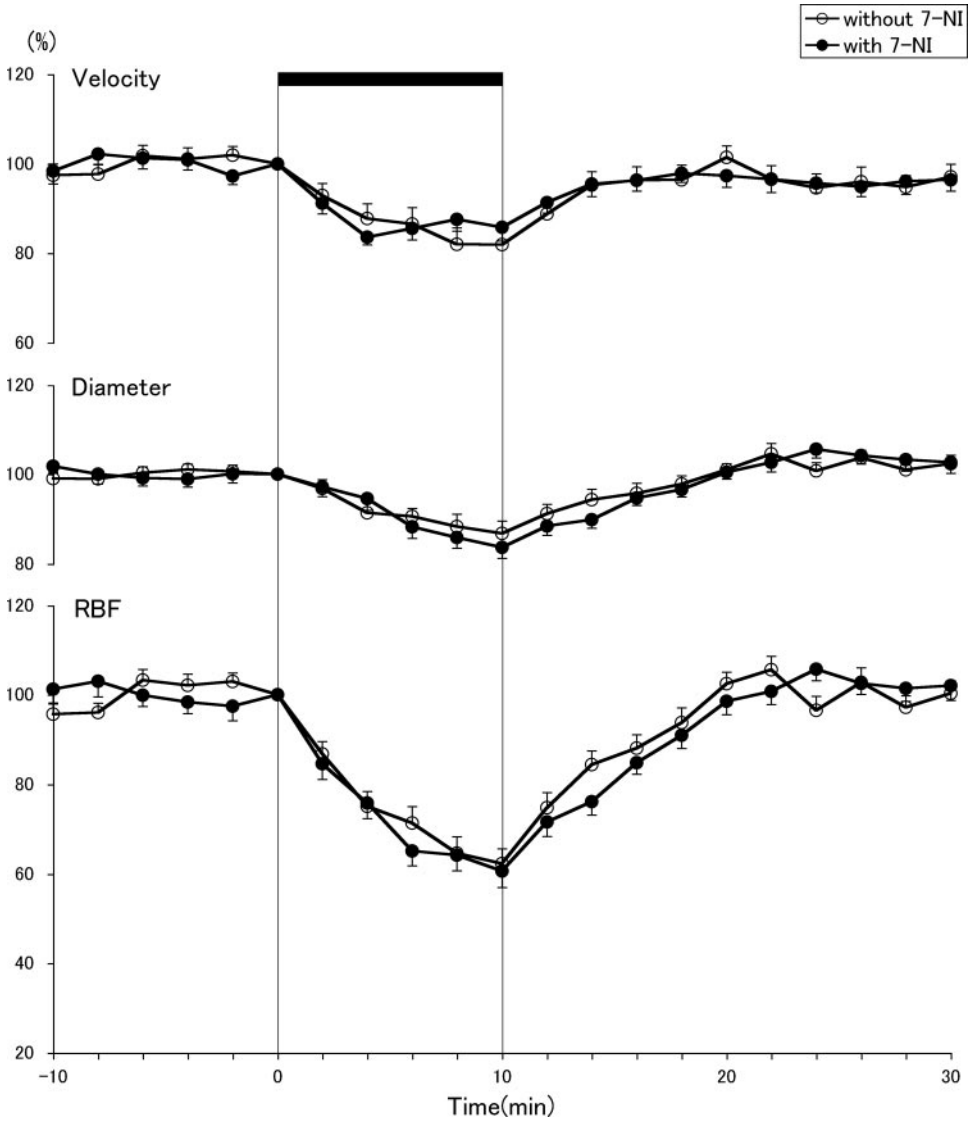

FIGURE 4. Time course of the changes in retinal circulation in response to hyperoxia with 7-NI (n = 7) and without 7-NI (n = 7). Data are expressed as the mean percentage ± SE of the prehyperoxic levels. *Solid bar*: period of hyperoxia.

have been localized in the optic nerve head[41] and retinal ganglion cell layer[42] and eNOS has been identified in retinal Müller cells[43] and ganglion cells.[44] Further histologic study may be needed to determine the location of ETB receptors and eNOS in the feline retina.

We believe that the current results have great potential for future clinical investigation. Because NO production via eNOS diminishes in the presence of endothelial dysfunction, measuring the vascular reaction after hyperoxia may allow evaluation of the retinal endothelial function and the reduced magnitude of retinal vascular reactivity to hyperoxia in patients with diabetes, as reported by numerous clinical studies.[7–10] Future study of the retinal circulation after hyperoxia is needed to compare healthy subjects and subjects with diabetes, in the latter of whom the retinal vessels have endothelial dysfunction, such as in diabetic retinopathy.

In summary, the present study showed that NO contributes to RBF recovery after hyperoxia and that eNOS in the vascular endothelium may be involved in the reaction. We concluded that NO plays a major role in the regulatory mechanism of RBF after hyperoxia. ET-1, probably produced from the endothelium during hyperoxia, may be involved in NO production in the endothelium via activation of the ETB receptor in the retinal endothelium when the RBF begins to return to baseline after hyperoxia.

References

1. Dallinger S, Dorner GT, Wenzel R, et al. Endothelin-1 contributes to hyperoxia-induced vasoconstriction in the human retina. *Invest Ophthalmol Vis Sci*. 2000;41:864–869.
2. Wimpissinger B, Resch H, Berisha F, Weigert G, Schmetterer L, Polak K. Response of retinal blood flow to systemic hyperoxia in smokers and nonsmokers. *Graefes Arch Clin Exp Ophthalmol*. 2005;243:646–652.
3. Luksch A, Garhofer G, Imhof A, et al. Effect of inhalation of different mixtures of O(2) and CO(2) on retinal blood flow. *Br J Ophthalmol*. 2002;86:1143–1147.
4. Gilmore ED, Hudson C, Preiss D, Fisher J. Retinal arteriolar diameter, blood velocity, and blood flow response to an isocapnic hyperoxic provocation. *Am J Physiol Heart Circ Physiol*. 2005; 288:H2912–H2917.
5. Zhu Y, Park TS, Gidday JM. Mechanisms of hyperoxia-induced reductions in retinal blood flow in newborn pig. *Exp Eye Res*. 1998;67:357–369.
6. Takagi C, King GL, Takagi H, Lin YW, Clermont AC, Bursell SE. Endothelin-1 action via endothelin receptors is a primary mechanism modulating retinal circulatory response to hyperoxia. *Invest Ophthalmol Vis Sci*. 1996;37:2099–2109.

7. Grunwald JE, Riva CE, Brucker AJ, Sinclair SH, Petrig BL. Altered retinal vascular response to 100% oxygen breathing in diabetes mellitus. *Ophthalmology*. 1984;91:1447–1452.
8. Gilmore ED, Hudson C, Nrusimhadevara RK, et al. Retinal arteriolar diameter, blood velocity, and blood flow response to an isocapnic hyperoxic provocation in early sight-threatening diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2007;48:1744–1750.
9. Kohner EM, Patel V, Rassam SM. Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. *Diabetes*. 1995;44:603–607.
10. Patel V, Rassam SM, Chen HC, Kohner EM. Oxygen reactivity in diabetes mellitus: effect of hypertension and hyperglycaemia. *Clin Sci* (*Lond*). 1994;86:689–695.
11. Polak K, Luksch A, Frank B, Jandrasits K, Polska E, Schmetterer L. Regulation of human retinal blood flow by endothelin-1. *Exp Eye Res*. 2003;76:633–640.
12. Takagi C, Bursell SE, Lin YW, et al. Regulation of retinal hemodynamics in diabetic rats by increased expression and action of endothelin-1. *Invest Ophthalmol Vis Sci*. 1996;37:2504–2518.
13. Higgins RD, Hendricks-Munoz KD, Caines VV, Gerrets RP, Rifkin DB. Hyperoxia stimulates endothelin-1 secretion from endothelial cells; modulation by captopril and nifedipine. *Curr Eye Res*. 1998; 17:487–493.
14. Meyer P, Flammer J, Luscher TF. Endothelium-dependent regulation of the ophthalmic microcirculation in the perfused porcine eye: role of nitric oxide and endothelins. *Invest Ophthalmol Vis Sci*. 1993;34:3614–3621.
15. Chakravarthy U, Gardiner TA, Anderson P, Archer DB, Trimble ER. The effect of endothelin 1 on the retinal microvascular pericyte. *Microvasc Res*. 1992;43:241–254.
16. Ihara M, Noguchi K, Saeki T, et al. Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. *Life Sci*. 1992;50:247–255.
17. Eguchi S, Hirata Y, Ihara M, Yano M, Marumo F. A novel ETA antagonist (BQ-123) inhibits endothelin-1-induced phosphoinositide breakdown and DNA synthesis in rat vascular smooth muscle cells. *FEBS Lett*. 1992;302:243–246.
18. Inoue A, Yanagisawa M, Kimura S, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A*. 1989;86:2863–2867.
19. Mori F, King GL, Clermont AC, Bursell DK, Bursell SE. Endothelin-3 regulation of retinal hemodynamics in nondiabetic and diabetic rats. *Invest Ophthalmol Vis Sci*. 2000;41:3955–3962.
20. Kiel JW. Endothelin modulation of choroidal blood flow in the rabbit. *Exp Eye Res*. 2000;71:543–550.
21. Nishimura K, Riva CE, Harino S, Reinach P, Cranstoun SD, Mita S. Effects of endothelin-1 on optic nerve head blood flow in cats. *J Ocul Pharmacol Ther*. 1996;12:75–83.
22. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J*. 1992;6:3051–3064.
23. Nagaoka T, Sakamoto T, Mori F, Sato E, Yoshida A. The effect of nitric oxide on retinal blood flow during hypoxia in cats. *Invest Ophthalmol Vis Sci*. 2002;43:3037–3044.
24. Sato E, Sakamoto T, Nagaoka T, Mori F, Takakusaki K, Yoshida A. Role of nitric oxide in regulation of retinal blood flow during hypercapnia in cats. *Invest Ophthalmol Vis Sci*. 2003;44:4947–4953.
25. Feke GT, Goger DG, Tagawa H, Delori FC. Laser Doppler technique for absolute measurement of blood speed in retinal vessels. *IEEE Trans Biomed Eng*. 1987;34:673–680.
26. Feke GT, Riva CE. Laser Doppler measurements of blood velocity in human retinal vessels. *J Opt Soc Am*. 1978;68:526–531.
27. Baker M, Wayland H. On-line volume flow rate and velocity profile measurement for blood in microvessels. *Microvasc Res*. 1974;7: 131–143.
28. Nagaoka T, Yoshida A. Noninvasive evaluation of wall shear stress on retinal microcirculation in humans. *Invest Ophthalmol Vis Sci*. 2006;47:1113–1119.
29. Cabel M, Smiesko V, Johnson PC. Attenuation of blood flow-induced dilation in arterioles after muscle contraction. *Am J Physiol*. 1994;266:H2114–H2121.
30. Moore PK, Wallace P, Gaffen Z, Hart SL, Babbedge RC. Characterization of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociceptive and cardiovascular effects. *Br J Pharmacol*. 1993;110:219–224.
31. Yamamoto R, Bredt DS, Snyder SH, Stone RA. The localization of nitric oxide synthase in the rat eye and related cranial ganglia. *Neuroscience*. 1993;54:189–200.
32. Grunwald JE, Riva CE, Petrig BL, Sinclair SH, Brucker AJ. Effect of pure O2-breathing on retinal blood flow in normals and in patients with background diabetic retinopathy. *Curr Eye Res*. 1984;3:239–241.
33. Kuo L, Davis MJ, Chilian WM. Endothelial modulation of arteriolar tone. *News Physiol Sci*. 1992;7:5–9.
34. Jean-Louis S, Lovasik JV, Kergoat H. Systemic hyperoxia and retinal vasomotor responses. *Invest Ophthalmol Vis Sci*. 2005;46:1714–1720.
35. Nagaoka T, Ishii Y, Takeuchi T, Takahashi A, Sato E, Yoshida A. Relationship between the parameters of retinal circulation measured by laser Doppler velocimetry and a marker of early systemic atherosclerosis. *Invest Ophthalmol Vis Sci*. 2005;46:720–725.
36. Kamiya A, Togawa T. Adaptive regulation of wall shear stress to flow change in the canine carotid artery. *Am J Physiol*. 1980;239: H14–H21.
37. Bursell SE, Clermont AC, Oren B, King GL. The in vivo effect of endothelins on retinal circulation in nondiabetic and diabetic rats. *Invest Ophthalmol Vis Sci*. 1995;36:596–607.
38. Haefliger IO, Flammer J, Luscher TF. Heterogeneity of endothelium-dependent regulation in ophthalmic and ciliary arteries. *Invest Ophthalmol Vis Sci*. 1993;34:1722–1730.
39. Douglas SA, Elliott JD, Ohlstein EH. Regional vasodilation to endothelin-1 is mediated by a non-ETA receptor subtype in the anaesthetized rat: effect of BQ-123 on systemic haemodynamic responses. *Eur J Pharmacol*. 1992;221:315–324.
40. Frelin C, Guedin D. Why are circulating concentrations of endothelin-1 so low? *Cardiovasc Res.* 1994;28:1613–1622.
41. Prasanna G, Krishnamoorthy R, Clark AF, Wordinger RJ, Yorio T. Human optic nerve head astrocytes as a target for endothelin-1. *Invest Ophthalmol Vis Sci*. 2002;43:2704–2713.
42. Stitt AW, Chakravarthy U, Gardiner TA, Archer DB. Endothelin-like immunoreactivity and receptor binding in the choroid and retina. *Curr Eye Res*. 1996;15:111–117.
43. Haverkamp S, Kolb H, Cuenca N. Endothelial nitric oxide synthase (eNOS) is localized to Muller cells in all vertebrate retinas. *Vision Res*. 1999;39:2299–2303.
44. Tsumamoto Y, Yamashita K, Takumida M, et al. In situ localization of nitric oxide synthase and direct evidence of NO production in rat retinal ganglion cells. *Brain Res*. 2002;933:118–129.