Role of Nitric Oxide in Regulation of Retinal Blood Flow during Hypercapnia in Cats

Eiichi Sato,1 Takashi Sakamoto,2 Taiji Nagaoka,1 Fumibiko Mori,1 Kaoru Takakusaki,2 and Akitosbi Yoshida1

PURPOSE. To investigate whether nitric oxide (NO) contributes to the regulation of retinal circulation during hypercapnia in cats.

METHODS. N⁴-nitro-l-arginine-methylester (l-NAME; n = 8), a NOS inhibitor; N⁴-nitro-l-arginine-methylester (l-NAME; n = 6), the inactive isomer; or phosphate-buffered saline (PBS; n = 8) was injected intravenously into the cat’s eye. A selective neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7-NI; n = 6), was injected intraperitoneally. Hypercapnia was induced for 10 minutes by inhalation of 5% carbon dioxide with 21% oxygen and 74% nitrogen. The vessel diameter and blood velocity were measured simultaneously in large retinal arterioles in cats by laser Doppler velocimetry and the retinal blood flow (RBF) calculated. Retinal vascular resistance (RVR) was also estimated.

RESULTS. In the PBS group, the vessel diameter (9.5% ± 2.7%, P < 0.05), blood velocity (15.6% ± 4.4%, P < 0.05), and RBF (37.2% ± 3.7%, P < 0.05) increased, and the RVR decreased (−26.0% ± 2.7%, P < 0.05) during hypercapnia. In the l-NAME group, those changes were greatly suppressed in response to hypercapnia. l-NAME was inactive with regard to RBF during hypercapnia. The RBF responses to hypercapnia after the 7-NI injection were significantly attenuated compared with those before 7-NI injection (P < 0.05).

CONCLUSIONS. These results indicate that NO contributes to the increase in RBF during hypercapnia. Furthermore, the NO synthesized by the action of nNOS may participate in regulation of RBF during hypercapnia. (Invest Ophthalmol Vis Sci. 2003;44:4947–4953) DOI:10.1167/iovs.03-0284

The autoregulatory mechanism of blood flow in the vascular tissue beds is defined as the ability of the tissue to adapt blood flow to tissue oxygen demands or metabolic demands when tissue oxygen levels decrease or metabolic activity facilitates. Ischemia leads to reduction in tissue oxygen tension and accumulation of carbon dioxide in the tissue. Both hypoxia and hypercapnia caused by retinal ischemia contribute to increased retinal blood flow (RBF) and the return of the rate of blood flow toward the control level as the result of the autoregulatory mechanism. Nitric oxide (NO) is synthesized enzymatically by NO synthase (NOS) from l-arginine and molecular oxygen as a substrate and is a highly diffusible gas with a potent vasodilator action. We have shown in experimental animal models that NO contributes to the increase in RBF during hypoxia through a flow-induced mechanism. A flow-induced mechanism has been shown to be the adaptive response of the vessels to the change in blood flow that maintains a constant level of shear stress on the vessel wall. The wall shear rate (WSR), used as an indicator of shear stress on the vessel wall, is calculated from the vessel diameter and blood velocity, which were measured simultaneously. Hypercapnia, as well as hypoxia, increases RBF, as shown in previous studies using various methods in various species. However, it is unclear how the retinal circulation is regulated during hypercapnia. To the best of our knowledge, only two studies have examined the role of NO in retinal circulation during hypercapnia, and these have reported that hypercapnic vasodilation in the retina is independent of NO production, although, in the human choroid, there is evidence for blunting of hypercapnic vasodilation by the NOS inhibitor, N⁴-monomethyl-l-arginine (l-NMMA). There is little clear evidence of a significant role for NO in the autoregulatory blood flow mechanism in ocular circulation.

In the present study, to investigate whether NO contributes to the regulation of retinal circulation during hypercapnia, we injected N⁴-nitro-l-arginine-methylester (l-NAME), a nonselective NOS inhibitor, and 7-nitroindazole (7-NI), a neuronal NOS (nNOS) inhibitor, into cats and studied how RBF changes during hypercapnia.

MATERIALS AND METHODS

Animal Preparation

Protocols describing the use of cats were approved by the Animal Care Committee of Asahikawa Medical College and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-eight adult cats of either sex (weight, 2.5–4.2 kg) were used in the study. Each cat underwent induction of anesthesia with enflurane, oxygen, and nitrous oxide in a closed box followed by intratracheal injection of atropine (0.04 mg/kg). The animals were then tracheostomized and mechanically ventilated with 1.5% to 2.0% enflurane and room air. Concentrations of doped carbon dioxide were monitored continuously with a carbon dioxide analyzer (Respina H126; NEC San-ei Instruments, Ltd., Tokyo, Japan). End-tidal carbon dioxide was maintained at a constant level throughout the experiment, except when the animals inhaled gases containing carbon dioxide. Catheters were placed in the femoral arteries and veins. Pancuronium bromide (0.1 mg/kg per h; Sankyo Pharmaceutical Co., Tokyo, Japan) was infused continuously. The animal was placed prone, and the head was fixed in the stereotaxic instrument. Arterial pH, arterial partial carbon dioxide tension (PaCO₂), arterial partial oxygen tension (PaO₂), and hematocrit (Ht) were measured intermittently with a blood gas analyzer (Chiba Corning Co., Tokyo, Japan). The mean arterial blood pressure (MAP) and heart rate (HR) were monitored continuously. Rectal temperature was maintained between 37°C and 38°C with a heated blanket.

The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine sulfate (Santen Pharmaceutical Co., Osaka, Japan). A 0-D con-
tact lens was placed on the cornea, which was protected with a drop of sodium hyaluronate (Healon; Pharmacia & Upjohn, Inc., Peapack, NJ). A 26-gauge butterfly needle was inserted into the anterior chamber and connected to a pressure transducer to monitor the intraocular pressure (IOP).

**RBF Measurement**

We measured RBF using a laser Doppler velocimetry system (Laser Blood Flowmeter, model CLBF 100; Canon, Inc., Tokyo, Japan) that was customized for use in cats. The instrument, which is similar to a fundus camera, is designed to measure vessel diameter and blood velocity simultaneously in retinal vessels and to calculate the RBF.4,15,16 Diode (wavelength, 670 nm) and helium-neon lasers (wavelength, 543 nm) were used to measure the blood velocity and the vessel diameter, respectively. The diode laser was focused on the center of a vessel, and the helium-neon laser was positioned vertical to the vessel by direct visualization.

The principles of the laser Doppler velocimetry system have been described in detail elsewhere.4,15-16 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.17,18 The diode laser illuminates a retinal vessel at the same position. The Doppler-shifted light scattered from the RBCs flowing in the retinal vessel is detected simultaneously in two directions separated by a fixed angle. The signals from the two-photon multiplier tube detectors undergo computer-controlled spectrum analysis and sequential measurement of the maximum speed ($V_{\text{max}}$) at the center of the vessel. In the laser Doppler velocimetry system, each pair of spectra was recorded, and the $V_{\text{max}}$ was calculated automatically every 5 ms for 1 second during each measurement. The $V$ was defined as the averaged $V_{\text{max}}$ during one cardiac cycle.

The diameter of the retinal vessel is determined automatically by computer analysis of the signal produced by the image of the vessel. The vessel images were captured every 4 ms for 60 ms, just before and after the measurement of blood velocity. The captured images were analyzed to obtain the vessel diameter by using the half height of the transmittance profile to define the vessel edge, using microdensitometry.19 The value of the vessel diameter was defined as the average of the values determined at each time point.

The RBF was calculated from $RBF = S \times V_{\text{mean}}$, where $S$ is the cross-sectional area of the retinal artery at the laser Doppler measurement site, assuming a circular cross section, and $V_{\text{mean}}$ is the mean blood velocity calculated as $V_{\text{mean}} = V_{\text{max}}/2$.20 The ocular perfusion pressure (OPP) was calculated as $2/3\text{MAP} - \text{IOP}$.21 From the OPP and RBF, the retinal arterial vascular resistance (RVR) was determined by $\text{RVR} = \text{OPP}/\text{RBF}$. The WSR was used as an indicator of wall shear stress measured by bidirectional laser Doppler velocimetry, which provides a well-defined baseline to reestablish. From 30 to 60 minutes after the first trial, 7-NI (50 mg/kg in 10 mL peanut oil) was injected intraperitoneally. This dose of 7-NI maximally inhibits nNOS.26 The RBF response to 10 minutes of hypercapnia was measured 90 minutes after injection.

**Statistical Analysis**

All data are expressed as the mean ± SE. For statistical analysis, we used analysis of variance (ANOVA) for repeated measurements, followed by post hoc comparison with the Dunnett procedure.27 Differences between means in systemic parameters before and during hypercapnia were assessed with Student’s paired $t$-test. For multiple comparisons, one-way ANOVA was used for statistical comparison, and significance was assessed using the Tukey-Kramer post hoc test. The relations among the changes in vessel diameter and the changes in blood velocity were examined by linear least-squares regression analysis. The Wilcoxon matched-pairs signed ranks test was used to analyze the changes between means in parameters during hypercapnia, with and without 7-NI at each time point. In all cases, a $P < 0.05$ was considered statistically significant.

**RESULTS**

**Changes in Retinal Circulation during Hypercapnia**

In the PBS group, vessel diameter and blood velocity significantly increased during hypercapnia compared with prehypercapnia levels by repeated-measures ANOVA, followed by the Dunnett procedure (Fig. 1). The maximum percentage increase above the prehypercapnia vessel diameter was 11.4% ± 2.4% and above baseline blood velocity was 20.0% ± 4.1%. The maximum percentage increase in RBF was 44.9% ± 4.3% for 7 to 10 minutes after the initiation of hypercapnia, and that level was maintained until the end of hypercapnia. The maximum percentage decrease in RVR was −28.6% ± 5.2%. Because the decrease level of RVR was stable from approximately 7 minutes after the initiation of hypercapnia and remained the same until the end of hypercapnia (Fig. 1), we used an averaged value of each parameter from 7 to 10 minutes after induction of hypercapnia for statistical analysis (Fig. 2).

The averaged (from 7 to 10 minutes) increases in diameter were 9.5% ± 2.7%; in blood velocity, 15.6% ± 4.4%; and in RBF, 37.2% ± 3.7%. At the end of hypercapnia, there were no significant differences in PaCO$_2$ or pH. There were no significant changes in MABP and HR before and during hypercapnia (Table 1). Although the IOP increased slightly during hyper-
capnia in both groups, the OPP (2/3MABP − IOP) did not change significantly.

Two hours after the intravitreal injection of L-NAME, the vessel diameter decreased to −8.4% ± 2.3% (from 86.9 ± 2.9 to 79.2 ± 2.7 μm), blood velocity to −14.7% ± 5.3%, and RBF to −28.6% ± 5.6% and RVR increased to 37.5% ± 10.6% compared with the preinjection level (data not shown). In contrast, 2 hours after intravitreal injection of PBS or ω-NNAME, the values did not differ significantly from the preinjection level (data not shown). The intravitreal injections of PBS, L-NAME, and ω-NNAME did not alter the pH, PaCO₂, PaO₂, Ht, MABP, or HR. Because vessel diameter, blood velocity, and RBF 2 hours after the injection of L-NAME were stable, hypercapnia was induced 2 hours after the injection.

In the L-NAME group, the vessel diameter, blood velocity, and RBF did not significantly increase in response to hypercapnia (Fig. 1) without significant changes in MABP and HR. The averaged increases in diameter were 3.5% ± 2.2%, velocity 1.6% ± 3.5%, and RBF 9.3% ± 6.1%. The decrease in RVR was −4.7% ± 6.3%, which was minimal. There was no significant increase in WSR in the PBS and L-NAME groups.

In the ω-NNAME group, the time course of the change in response to hypercapnia was similar to that in the PBS group (Fig. 1). The vessel diameter, blood velocity, and RBF significantly increased during hypercapnia compared with prehypercapnia levels, determined by repeated-measures ANOVA followed by the Dunnett procedure (Fig. 1). During hypercapnia, the average increase in RBF was 49.7% ± 14.1% in the ω-NNAME group, whereas the average increase in RBF was 9.3% ± 6.1% in the L-NAME group. The average increase in RBF (37.2% ± 3.7%) observed in the PBS group was significantly reduced by the intravitreal injection of ω-NNAME (Turkey-Kramer post hoc test).

Relation between Increased Vessel Diameter and Increased Blood Velocity

In the PBS group, the smaller the increase in vessel diameter, the larger the increase in blood velocity. There was a significant negative correlation between increased diameter (ΔD) and increased velocity (ΔV) (r = −0.55, P = 0.012). However, in the L-NAME group, this negative correlation disappeared (Fig. 2).

Effect of 7-NI during Hypercapnia

The intraperitoneal injection of 7-NI did not alter the pH, PaCO₂, PaO₂, Ht, MABP, or HR. During hypercapnia, PaCO₂ increased to 47 mm Hg, with no significant differences observed when comparing hypercapnic episodes (Table 2). The vessel diameter, blood velocity, and RBF significantly increased in response to hypercapnia both with and without 7-NI compared with each prehypercapnia level, determined by repeated-measures ANOVA followed by the Dunnett procedure (Fig. 3). During hypercapnia without 7-NI, the average increases in diameter were 5.6% ± 2.3%, velocity 17.8% ± 9.7%, and RBF 30.4% ± 14.5%. During hypercapnia with 7-NI, the average increases in diameter were 10.6% ± 6.3%, velocity 21.6% ± 10.6%, and RBF 48.4% ± 21.2%. The increase in vessel diameter with 7-NI was significantly attenuated compared with that
TABLE 1. Blood Gas Analysis, End-Tidal Carbon Dioxide, Mean Arterial Blood Pressure, Intraocular Pressure, Ocular Perfusion Pressure, and Heart Rate

<table>
<thead>
<tr>
<th></th>
<th>PBS Group (n = 8) Before</th>
<th>Hypercapnia</th>
<th>t-NAME Group (n = 8) Before</th>
<th>Hypercapnia</th>
<th>t-NAME Group (n = 6) Before</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.36 ± 0.02</td>
<td>7.24 ± 0.01*</td>
<td>7.38 ± 0.01</td>
<td>7.25 ± 0.01*</td>
<td>7.37 ± 0.01</td>
<td>7.25 ± 0.01*</td>
</tr>
<tr>
<td>PacO2 (mm Hg)</td>
<td>32.1 ± 0.7</td>
<td>48.1 ± 0.9*</td>
<td>35.1 ± 0.7</td>
<td>48.0 ± 0.9*</td>
<td>32.8 ± 0.6</td>
<td>47.2 ± 1.1*</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>111.4 ± 3.4</td>
<td>99.9 ± 2.0</td>
<td>101.2 ± 1.5</td>
<td>96.4 ± 1.6</td>
<td>104.2 ± 2.5</td>
<td>100.6 ± 2.5</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>32.2 ± 2.9</td>
<td>34.0 ± 2.4</td>
<td>29.0 ± 1.3</td>
<td>30.7 ± 1.4</td>
<td>31.8 ± 1.5</td>
<td>32.5 ± 1.5</td>
</tr>
<tr>
<td>End-tidal CO2 (mm Hg)</td>
<td>34.0 ± 0.7</td>
<td>57.5 ± 2.2</td>
<td>33.0 ± 0.7</td>
<td>54.1 ± 0.7*</td>
<td>32.7 ± 0.2</td>
<td>52.7 ± 0.9*</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>96.5 ± 4.8</td>
<td>96.3 ± 4.2</td>
<td>85.4 ± 5.2</td>
<td>83.9 ± 3.8</td>
<td>90.2 ± 1.7</td>
<td>83.9 ± 3.8</td>
</tr>
<tr>
<td>IOP (mm Hg)</td>
<td>5.6 ± 1.1</td>
<td>10.1 ± 2.0</td>
<td>9.2 ± 1.2</td>
<td>9.11 ± 0.8*</td>
<td>12.6 ± 0.9</td>
<td>12.6 ± 0.9</td>
</tr>
<tr>
<td>OPP (mm Hg)</td>
<td>54.1 ± 2.9</td>
<td>54.1 ± 2.9</td>
<td>46.5 ± 2.6</td>
<td>46.1 ± 2.7</td>
<td>47.4 ± 1.4</td>
<td>46.8 ± 1.4</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>145.9 ± 4.3</td>
<td>144.9 ± 5.1</td>
<td>133.6 ± 9.9</td>
<td>133.9 ± 9.2</td>
<td>155.9 ± 4.4</td>
<td>153.7 ± 4.2</td>
</tr>
</tbody>
</table>

Data are means ± SE. Before, before induction of hypercapnia; Hypercapnia, from 7 to 10 minutes of hypercapnia.

* P < 0.05 versus prehypercapnia values by paired Student’s t-test.

without 7-NI from 8 to 10 minutes at the same time points, and the increase in RBF with 7-NI was significantly attenuated from 8 to 10 minutes (Wilcoxon signed ranked test; Fig. 3). However, there was no significant difference in the increase in blood velocity during hypercapnia between the group that received 7-NI and the one that did not.

DISCUSSION

In the present study, the RBF increased by 37% during hypercapnia (5% carbon dioxide with 21% oxygen and 74% nitrogen) in the PBS group (Fig. 1). There have been reports about changes in RBF during hypercapnia.6 In the present study, the RBF increased by 37% during hypercapnia during hypercapnia in the brain.32,33 Carbon dioxide or acidosis-associated hypercapnia may stimulate NO production in response to hypercapnia in the brain.32,33 Inhibition of NOS as a mechanism for the effects of hypercapnia is supported by the fact that the increase in the diameter of the retinal arterioles occurred in response to hypercapnia in animals pretreated with L-NMMA, another nonselective NOS inhibitor. Those investigators concluded that NO does not contribute to hypercapnia-induced vasodilatation. Their findings do not agree with ours, possibly because of differences between our study and ours in species, methods of measurement, NOS inhibitors administered, and the age of the animals. Another possible explanation is the time course from the injection of the NOS inhibitor to the induction of hypercapnia. L-NMMA was administered 20 minutes before the induction of hypercapnia in their study, but t-NAME was administered 2 hours before the initiation of hypercapnia in our study.

Many hypotheses have been forwarded to explain increased NO production in response to hypercapnia in the brain.32,33 Carbon dioxide or acidosis-associated hypercapnia may stimulate NOS enzyme activity35 to produce NO, which activates

TABLE 2. Changes in Systemic and Ocular Parameters during Hypercapnia, with and without 7-NI

<table>
<thead>
<tr>
<th></th>
<th>Without 7-NI Before</th>
<th>Hypercapnia</th>
<th>With 7-NI Before</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.36 ± 0.02</td>
<td>7.24 ± 0.02*</td>
<td>7.38 ± 0.03</td>
<td>7.26 ± 0.03*</td>
</tr>
<tr>
<td>PacO2 (mm Hg)</td>
<td>31.6 ± 0.7</td>
<td>47.2 ± 1.4*</td>
<td>34.5 ± 0.5</td>
<td>47.1 ± 1.1*</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>104.7 ± 4.4</td>
<td>99.9 ± 4.2</td>
<td>103.0 ± 8.1</td>
<td>93.8 ± 4.4</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>29.3 ± 1.7</td>
<td>31.0 ± 1.9</td>
<td>30.0 ± 1.8</td>
<td>30.2 ± 1.8</td>
</tr>
<tr>
<td>End-tidal CO2 (mm Hg)</td>
<td>33.0 ± 1.1</td>
<td>50.0 ± 1.8*</td>
<td>33.0 ± 0.6</td>
<td>50.7 ± 1.6*</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>104.6 ± 5.4</td>
<td>103.2 ± 4.8</td>
<td>103.9 ± 6.0</td>
<td>106.9 ± 6.3</td>
</tr>
<tr>
<td>IOP (mm Hg)</td>
<td>12.2 ± 2.6</td>
<td>12.4 ± 2.2</td>
<td>9.7 ± 0.7</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>OPP (mm Hg)</td>
<td>57.5 ± 2.5</td>
<td>56.4 ± 1.7</td>
<td>59.6 ± 3.4</td>
<td>61.2 ± 3.7</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>154.5 ± 5.6</td>
<td>157.3 ± 8.8</td>
<td>146.2 ± 6.6</td>
<td>150.4 ± 6.2</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 6). Before, before induction of hypercapnia; Hypercapnia, 10 minutes of hypercapnia.

* P < 0.05 versus prehypercapnia values by paired Student’s t-test.
The nNOS activity increases as pH decrease, whereas endothelial NOS (eNOS) activity appears to increase as the pH increases in the brain. In mammalian retina, NOS has been found in photoreceptor cells, amacrine, horizontal and ganglion cells. In the cerebral circulation, intracellular acidification produced by carbon dioxide may trigger NO production. However, l-NAME is a nonselective NOS inhibitor, and we could not determine whether these effects were caused by inhibition of endothelial or neuronal NOS, or both.

In the present study, we further examined the effect of the selective nNOS inhibitor 7-NI on the regulation of RBF during hypercapnia. Our result (i.e., that the 7-NI significantly attenuated the increase in RBF during hypercapnia, although it did not completely abolish the increase; Fig. 3) suggests that nNOS in the retina is partially involved in the increase in RBF during hypercapnia. The fact that 7-NI significantly attenuated the increase in vessel diameter (Fig. 3) raises the possibility that NO released from nNOS in the retina during hypercapnia may contribute to the increase in vessel diameter.

In the PBS group, there was a significant negative correlation between the increase in vessel diameter and the increase in blood velocity (Fig. 3)—namely, there was a tendency that the smaller the increase in vessel diameter, the larger the increase in blood velocity. This may reflect a well-coordinated vascular response to adapt blood flow to tissue demands so that the decrease of the RVR is adequate. However, in the L-NAME group, this negative correlation disappeared (Fig. 2). These results suggest that NO plays a major role in the autoregulatory mechanism of the RBF during hypercapnia.

In summary, the present study demonstrated that NO contributes to the increase in RBF during hypercapnia and further-
more that nNOS in the retina is involved in the increase. We conclude that NO plays a major role in the autoregulatory mechanism of RBF during hypercapnia.

Acknowledgments
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References

43. Cholot N, Seylaz J, Lacombe P, Bonvento G. Local uncoupling of the cerebrovascular and metabolic responses to somatosensory


