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

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

**METHODS.** N^δ^-nitro-o-arginine-methylester (L-NAME; n = 8), a NOS inhibitor; N^δ^-nitro-l-arginine-methylester (L-NAME; n = 6), the inactive isomer; or phosphate-buffered saline (PBS; n = 8) was injected intravitreally into the cat’s eye. A selective neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7-NI; n = 6), was injected intraperitonetically. 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. n-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, N^δ^-monomethyl-o-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 N^δ^-nitro-o-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 intraperitoneal 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 dried 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 vein. 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% phenyl-ephrine 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.3-16 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.3-15-20 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 Poussile’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-photomultiplier tube detectors undergo computer-controlled spectrum analysis and sequential measurement of the maximum speed (V_{max}) at the center of the vessel. In the laser Doppler velocimetry 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_{max} was defined as the averaged V_{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 × 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}/2.20 The ocular perfusion pressure (OPP) was calculated as 2/3MABP – IOP.21 From the OPP and RBF, the retinal arterial vascular resistance (RVR) was determined by RVR = OPP/RBF. The WSR was used as an indicator of wall shear stress.22,23

**Intravitreal Injection of t-NAME and Induction of Hypercapnia**

To examine the role of nNOS in RBF during hypercapnia, we used 7-NI (Sigma-Aldrich), which is selective for nNOS.24 The first RBF response to 10 minutes of hypercapnia was measured before 7-NI injection (n = 6). The time interval between tests ranged from 30 to 60 minutes to allow 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.25 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.26 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 PaCO₂ or pH. There were no significant changes in MABP and HR before and during hypercapnia (Table 1). Although the IOP increased slightly during hyper-
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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 i-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. 1). 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

capnia in both groups, the OPP (2/3MABP − IOP) did not change significantly.

Two hours after the intravitreal injection of i-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 i-NAME, the values did not differ significantly from the preinjection level (data not shown). The intravitreal injections of PBS, i-NAME, and i-NAME did not alter the pH, PaCO₂, PaO₂, Ht, MABP, or HR. Because vessel diameter, blood velocity, and RBF 2 hours after the injection of i-NAME were stable, hypercapnia was induced 2 hours after the injection.

In the i-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 i-NAME groups.

In the i-NAME 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 i-NAME group, whereas the average increase in RBF was 9.3% ± 6.1% in the i-NAME group. The average increase in RBF (37.2% ± 3.7%) observed in the PBS group was significantly reduced by the intravitreal injection of i-NAME but was not reduced by the intravitreal injection of i-NAME (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 i-NAME group, this negative correlation disappeared (Fig. 2).

Figure 1. Time course of the changes in retinal circulation and MABP in response to hypercapnia in the PBS (n = 8), i-NAME (n = 8), and i-NAME (n = 6) groups. PBS and i-NAME data are presented as a mean percentage ± SE of the prehypercapnia levels for clarity. Symbols and error bars represent the mean ± SE. Error bars for i-NAME are excluded because the error bars are similar in magnitude to those of PBS. Solid rectangle: period of hypercapnia. *P < 0.05 compared with prehypercapnia values, by repeated-measures ANOVA, followed by the Dunnett procedure.

Figure 2. Relation between the increase in vessel diameter (ΔD) and the increase in blood velocity (ΔV) during hypercapnia. The ΔD and ΔV are averages obtained from 7 to 10 minutes into the period of hypercapnia. In the PBS group, a negative correlation was found between ΔD and ΔV (r = -0.75, P = 0.03). In the i-NAME group, a statistically significant correlation was not found between ΔD and ΔV (r = -0.038, P = 0.93). (○) PBS; (●) i-NAME.

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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.6–9,28 Using fluorescein angiography, Tsacopoulos and David6 reported that, in monkeys, a PaCO₂ increase of 1 mm Hg induces a 3% increase in RBF and a 1% increase in vessel diameter during hypercapnia. In humans, Harris et al.,7 using fluorescein angiography, reported that hypercapnia-induced increases in blood velocity averaged 1% per mm Hg PaCO₂. In the present study, a PaCO₂ increase of 1 mm Hg induced a 2.8% increase in RBF, a 0.7% increase in vessel diameter, and a 1.2% increase in blood velocity during hypercapnia in the PBS group (Fig. 1). Although there may be differences among species and with different measurement techniques, the degree of increase of RBF in our study seems to agree with previous findings.

Intravenous administration of l-NAME, the NOS inhibitor, increases systemic blood pressure,29–31 resulting in a change in the tissue perfusion pressure and blood flow. In the present study, the NOS inhibitor was administered using an intravitreal injection technique that resulted in topical application of NOS primarily in the retina. Our results (i.e., that MABP did not significantly change after injection of l-NAME) indicate that this technique seems to minimize the systemic effects of the NOS inhibitor (Table 1). Furthermore, the fact that MABP did not significantly change during hypercapnia indicates that the changes in vessel diameter, blood velocity, and RBF were caused mainly by a retinal vascular response.

In the present study, we provided new evidence that intravitreal injection of l-NAME markedly reduced the increase in RBF during hypercapnia (Fig. 1). Inhibition of NOS as a mechanism for the effects of l-NAME is supported by the fact that d-NAME had no effect (Fig. 1). These results indicate that NO production in the retina may contribute to the increased RBF in response to hypercapnia. However, Gidday and Zhu,12 who measured the vessel diameter in newborn pigs, reported 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 their 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 l-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

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<td>Before</td>
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<tr>
<td>pH</td>
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<td>7.24 ± 0.02*</td>
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<td>PaCO₂ (mm Hg)</td>
<td>31.6 ± 0.7</td>
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<td>PaO₂ (mm Hg)</td>
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<td>Ht (%)</td>
<td>29.3 ± 1.7</td>
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<td>End-tidal CO₂ (mm Hg)</td>
<td>33.0 ± 1.1</td>
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<td>MABP (mm Hg)</td>
<td>104.6 ± 5.4</td>
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<td>57.5 ± 2.5</td>
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<td>HR (beats/min)</td>
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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.
guanylate cyclase to increase production of cyclic guanylic acid. 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, and Müller 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 participate in the vasodilation of the retinal arterioles. In the present study, we did not perform any control experiments in which cats were injected only with the vehicle (10 mL peanut oil). Teppema et al. reported that the infusion of the peanut oil vehicle of 7-NI did not result in measurable circulatory and respiratory changes in cats. That result suggested that peanut oil has little effect on the systemic parameters. Other reports have indicated that 7-NI had no effect on MABP, HR, and cardiac output. Consistent with the findings of the previous studies, we did not observe any increase in MABP and HR after administration of 7-NI.

In the cerebral cortex, NO released from nNOS-containing neurons directly regulates cerebral blood flow. The presence of nicotinamide adenine dinucleotide phosphate diaphorase-positive/NOS-immunoreactive cells and processes near the retinal capillaries and larger vessels was reported. This may indicate that NO from a neuronal source plays a role in linking RBF with metabolism, as suggested in the brain. This could be of particular importance in the retina, where arterioles and capillaries are not subject to autonomic innervation. A flow-induced mechanism has been shown to be an adaptive response of the vessels to the change in blood flow that maintains a constant level of shear stress on the vessel wall. The flow-induced mechanism exerts dominant effects on the upstream large arterioles (~100–150 μm). The diameters of the measured retinal arterioles in our study, which ranged from 65 to 95 μm, correspond approximately to those of the upstream large arterioles that have a dominant flow-induced mechanism. The flow-induced vasodilatation, which probably results from the release of relaxing factors such as NO by the endothelium, can be evaluated by measuring the changes in WSR as an index of stress. The flow-induced vasodilation is characterized by a delay between the increase in blood flow and the vasodilatory response. Thus, an increase in WSR preceding an increase in vessel diameter suggests that an increase in shear stress is the stimulus for the flow-induced vasodilator response. In the present study, however, no latency period was detected between blood velocity and vessel diameter increases (Fig. 1). Moreover, in the present study, the WSR did not increase significantly, even in the PBS groups (Fig. 1), although, during hypoxia in the previous study, flow-induced vasodilation was caused by increased WSR. The flow-induced mechanism may have contributed little to the increase in RBF during hypercapnia. The response of the retinal circulation to hypercapnia may be different from the response to hypoxia, because there may be a difference of the degree of increase in blood flow. Although flow-induced vasodilation was caused by increased WSR during hypoxia in the previous study, these responses were induced by a very large blood flow. The 13.7% dilation in response to a 39.5% increase in blood velocity was induced by hypoxia. The 39.5% increase in blood velocity during hypoxia was much greater than the 15.5% increase during hypercapnia in the present study. We speculate that the small increase in blood flow during hypercapnia did not cause the WSR to increase significantly and the flow-induced vasodilation to be activated.

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.

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