Nitric Oxide Controls Arteriolar Tone in the Retina of the Miniature Pig

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**Purpose.** Experimental evidence indicates that the retinal microcirculation is mainly controlled by factors released from the tissue surrounding the arterioles. This study explores whether nitric oxide (NO), a possible factor, is released in the retina and controls the arteriolar tone.

**Methods.** Using a NO microprobe, the authors measured [NO] in the preretinal vitreous of miniature pigs as a function of distance from the retinal surface. Additionally, the NO-synthase inhibitor nitro-L-arginine was pressure injected. Finally, the retinal pool size of arginine and its biosynthesis from U-14C-glucose were biochemically assessed on retinal tissue and acutely isolated Müller cells.

**Results.** At the retinal surface, [NO] measured 6 to 9 μM, and, in the vitreous, it fell to zero approximately 180 μm away from the retina. Therefore, NO is degraded faster in the vitreous (65 to 80 μM·minute⁻¹) than in aqueous solution. Light flicker stimulation of the dark-adapted retina induced a reversible increase of [NO] (≈1.6 μM). Preretinal juxta-arteriolar microinjections of nitro-L-arginine (0.6 mM) induced a segmental and reversible arteriolar vasoconstriction of 45%; in contrast, intravenous infusion of nitro-L-arginine had no measurable effect on arteriolar diameter. The retinal pool size of arginine was small (≈200 μM), but there was an important rate of arginine biosynthesis in Müller cells.

**Conclusions.** These results strongly suggest that cells in the retina, other than endothelial cells, produce and release NO, which in turn controls the basal dilating arteriolar tone in the inner retina. Invest Ophthalmol Vis Sci. 1995;36:2228-2237.

Experimental evidence indicates that the retinal microcirculation is mainly controlled by factors released from the tissue surrounding arterioles. Most of this evidence has been provided by experiments performed in anesthetized animals1-4 and in humans5 in which strong vasomotor effects were recorded either on altering blood gases (PO2 and PCO2), and thereby the metabolism of the tissue, or on injecting metabolites close to the vitreal side of the arteriolar wall. For example, acute hypercapnia and hypoxia have been shown to induce important arteriolar vasoconstriction in conjunction with interstitial acidification of the inner retina.3,4,6-8 The hypercapnia-induced vasodilation was suppressed by the cyclo-oxygenase inhibitor indomethacin, and juxta-arteriolar microinjections of prostaglandin E1 (PGE1) caused segmental vasodilation of retinal arterioles.3,4 In contrast, the hypoxia-induced vasodilation was mimicked by local periarteriolar microinjections of L-lactate and involved a prostaglandin-independent pathway.9 The question was, therefore, whether in the retina there is a unique predominant metabolic process controlling arteriolar tone or, on the contrary, several interacting metabolic factors.

Nitric oxide (NO) could be one of these factors because a metabolic process, i.e., the transformation of L-arginine to citrulline, is necessary for its biosynthesis.10,11 It has been shown that NO is produced by endothelial cells of peripheral arteries, where it accounts for the biologic properties of the endothelium-derived relaxing factor.10,12-14 The current hypothesis is that NO plays a role in the control of basal dilating vascular tone and that an increase in NO production causes vasodilation. Only scarce and indirect
evidence suggests that NO plays a similar role in the control of retinal blood flow. First, the dilation of isolated rings of ophthalmic arteries induced by acetylcholine or bradykinin was markedly reduced in the presence of the NO-synthase (NOS) inhibitor, nitro-L-arginine. Second, injection of nitro-L-arginine methyl ester in isolated perfused porcine eye induced a decrease in ocular blood flow. However, the intravenous injection of nitro-L-arginine caused a 40% decrease of choroidal blood flow, without any modification of the retinal blood flow in anesthetized dogs.

There is evidence that in the central nervous system the endothelial cell is not the sole site for NO production. Indeed, both neurons and astrocytes have apparently the machinery for the production and release of NO. In the mammalian retina, NOS has been found in photoreceptor cells, amacrine, horizontal and ganglion cells, and Müller glial cells.

In this article, we present evidence showing that NO released from the retina of an intact animal.

MATERIALS AND METHODS

Experiments were performed on the intact eye of miniature pigs (10 to 12 kg body weight; Arare Animal Facility, Geneva, Switzerland) whose retina closely resembles the human retina in both neuroanatomic and vascular aspects. All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Preparation

Miniature pigs were prepared for experiments as follows: After intramuscular injection of azaperone (Stresnil, 5 mg; Cilag Chemie AG, Buchs, Switzerland), anesthesia was induced with metamidate hydrochloride (Hypnodil, 25 to 30 mg; Cilag Chemie AG) injected into the ear vein. After arterial, venous, and bladder catheterization, each animal was curarized (d-tubocurarine chloride, 1 mg/10 kg; Sigma Chemical), intubated, and artificially ventilated. During the experiment, anesthesia was maintained by continuous perfusion of Hypnodil, 100 mg/hour, and tubocurarine, 0.1 mg/hour. The animal was ventilated at approximately 18 strokes/minute, with a continuous flow of 20% O2 and 80% N2O using a variable volume respirator (Monaghan, model 300 PIM Sandoz SA, Basel, Switzerland). Systolic and diastolic blood pressures were monitored via the femoral artery using a transducer (Mingograph, Siemens–Elema). PaCO2, PaO2, and pH were measured intermittently from the same artery with a blood gas analyzer (AVL, Roswell, GA) and controlled by adjusting ventilatory rate, stroke volume, and composition of the inspired gas.

Surgical preparation of the animal was performed as follows: The head was fixed to a head-holder to avoid respiratory movements; upper and lower eyelids were removed, as was a rectangular area of skin surrounding the eye; the bulbar conjunctiva was detached, the sclera was carefully cleaned to 5 mm from the limbus, the superficial scleral vessels were thermo-cauterized, and an incision was made at the pars plana.

Electrochemical Nitric Oxide Microprobe

Our NO microprobe is a miniaturized version of that developed by Shibuki and it made use of our technology previously developed for the construction of O2-sensitive microelectrodes. The tip of a Pt-wire (Pt 90%, Ir 10%) was etched by electrolysis so as to fit into a Pb-glass micropipette, which was made with low heat and strong pull and broken with a diamond cutter to obtain a tip diameter of approximately 50 μm. The Pt-wire was advanced through the micropipette by means of a micromanipulator until its etched tip protruded from the opening of the micropipette. A spiral heating element (Pt 50% + Ir 50%) was positioned over the tip. Low heat was applied so that the Pb-glass melted and coated the Pt-wire. Finally, the protruding tip of the Pt-wire was etched by electrolysis until the Pt was flush with the glass. Then another micropipette was pulled from borosilicate glass capillary (GC 150-15; Clark Electromedical Instruments, Reading, UK) using low heat and was broken to a final tip diameter of 100 μm. It was dipped in a Nafion solution (Nafion perfluorinated membrane reinforced with teflon from Aldrich, Buchs, Switzerland). After evaporation, a membrane approximately 40 μm thick was obtained. Finally, the Pt-microelectrode was introduced into the borosilicate-glass micropipette using a micromanipulator and positioned as closely as possible to the Nafion membrane and fixed at this position with wax. The borosilicate-glass pipette was filled with NaCl solution (30 mM, pH 3.5), and an AgCl wire (reference electrode) was introduced there and also fixed with wax.

The Pt-microelectrode and the reference electrode of the microprobe were connected to a current-voltage converter of the type used for measuring Po2. Nitric oxide is oxidized at the surface of the Pt-microelectrode polarized at +0.9 V, and the resultant current is measured. The Nafion membrane provides selectivity against other NO derivatives.

The NO-microprobe and puffer micropipettes filled with the chemicals to be tested were introduced into the eye through a scleral hole and then advanced with a novel micromanipulator through the vitreous until positioned either close to a retinal arteriole.
were positioned near a retinal arteriole, red-free light stimulation was passed through neutral density and infrared filters and an electronic shutter before being focused onto an optic fiber (500-μm opening) whose other end was placed in the vitreous approximately 500 μm away from the retina. The light flux at the level of the retina was 1.73 × 10^15 photons·mm⁻²·second⁻¹, as measured with a photodiode (Centronic, Croydon, UK) placed 500 μm in front of the optic fiber tip.

Measurement of the Arteriolar Diameter

Once the micropipette puffer and the NO-microprobe were positioned near a retinal arteriole, red-free light pictures of the fundus were taken using a modified Zeiss fundus camera. Experiments were documented by photographs taken before and then 1 minute, 2 minutes, and 5 minutes after the injection.

Arteriolar diameter was measured with a digital display slide caliper on 13/18 fundus photographs, a method with a mean intraobserver variability of 7%. The measurements were performed near the site of microinjection. The results are expressed as the mean percent retinal arteriolar diameter change ± SEM. Statistical analysis was performed using paired t-tests.

Chemicals

Nitro-L-arginine was purchased from Sigma Chemical (Buchs, Switzerland); 1 M stock solutions were prepared in NaCl 0.9% (37°C, pH 7.4). Reagents used for retinal dissociations and biochemical analysis are those reported in references 34 and 35. D[U-14C]-glucose (specific activity 292 to 320 mCi/mmol) was obtained from Amersham (Hans Rahn, Zurich, Switzerland) and Dupont (Boston, MA).

Cell Preparation

Solitary Müller glial cells were isolated from juvenile guinea pig and miniature pig retinas as previously described, with the following modification: Density gradient centrifugation was performed at 8497 g (10 minutes at 4°C), and the relative proportions cell suspension:solution A containing Mg²⁺:Percoll (100%) were 1:2.4:1.6. After removal from the gradient, cell preparations were washed free of Percoll and then either frozen immediately in liquid nitrogen or used for metabolic experiments as described below. For comparison, whole retinas also were frozen and stored in liquid nitrogen for subsequent biochemical analysis. Those obtained from guinea pig were dissected from the eye in chilled bicarbonate-buffered Ringer’s solution (containing 5 mM glucose) and frozen within 15 minutes of decapitation. Those from miniature pigs (normoxic and normocapnic) were dissected after the completion of NO measurements.

Cell Incubation

Suspensions of Müller cells from guinea pig (≈13 mg of protein/ml) were incubated in darkness for 30 minutes at 37°C in bicarbonate-buffered Ringer’s solution (in mM: NaCl 124; KCl 5; CaCl₂ 2; KH₂PO₄ 1.25; NaHCO₃ 20; MgSO₄ 4; pH 7.4; 95% O₂ 5% CO₂) carrying 5.1 to 5.3 mM D[U-14C]-glucose substrate. As described previously, the uptake and phosphorylation of radiolabeled glucose were stopped by immersion of the cell chamber in melting ice, and the cells were separated from the incubation medium by centrifugation. Before freezing, cells were washed in substrate-free Ringer’s solution to remove nonphosphorylated [U-14C]-glucose. The corresponding incubation medium was frozen immediately in liquid nitrogen.

High-Pressure Liquid Chromatography Analysis

The preparation of frozen samples for high-pressure liquid chromatography analysis is fully described elsewhere. Biochemical analysis of amino acids was performed using ultraviolet detection (Chrom-A-Scope UV detector; Bar Spec, Rehovot, Israel) at 254 nm and reverse-phase chromatography (Nucleosil C18 column from Macherey Nagel, Oensingen, Switzerland) coupled to an off-line fraction collector (Helicar 2212; Pharmacia LKB, Uppsala, Sweden). The detection limits of amino acid standards were satisfactory, down to 10 μg. Hence, given the amount of Müller cells in the suspension, we estimated the lower limit of reported intracellular values to be 80 μM.

Fumarate was measured using refractive index detection (Erma ERC-7115A detector; Erma, Tokyo, Japan) and ion-exchange chromatography (2 Aminex HPX 87H+ in series; Bio-Rad Laboratories, Richmond, CA) coupled to an off-line fraction collector, as described.

RESULTS

Calibration of the Nitric Oxide Probe

The concentration of NO was calculated by taking a solubility coefficient of 45 ml·1-atm⁻¹ in water at 22°C. The NO was mixed with N₂ using a thin layer gas exchanger (Dunnschicht Dialysator; L. Eschweiler and Co., Kiel, Germany) equipped with nylon membranes. The calibration curve presented in Figure 1 shows that the response of the NO-probe was linear at micromolar concentrations of NO. The residual current measured (no NO in the mixture) was close to zero, probably because only NO diffuses through the Nafion membrane rapidly enough and is oxidized at the surface of the Pt-microelectrode. Calibration at
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**FIGURE 1.** Response of the nitric oxide (NO) microprobe to μM concentrations of NO. Typical calibration curve of a Nafion membrane-based, NO-sensitive microprobe obtained at 22°C. Current responses were linear down to μM concentrations of NO, and residual current was close to zero. Tip diameters were ~100 μm.

22°C and at 37°C showed less than 10% difference in the NO signal at micromolar concentrations (results not shown).

**Preretinal Nitric Oxide Profiles**

To see whether there was a flux of NO produced by the retina, we measured, under stable physiological conditions, the concentration of NO ([NO]) in the preretinal vitreous as a function of distance from either the surface of an arteriole (juxta-arteriolar profile) or the surface of the retina, at least 200 μm away from visible arterioles (intervascular profile). Under continuous illumination of the eye, the NO microprobe was advanced toward the retina at a constant speed of 150 μm/minute. When the tip of the NO-microprobe touched the retina, progression was stopped and the position was maintained for 1 to 2 minutes. Thereafter, the NO-microprobe was withdrawn 200 μm, at the same speed as before. The oscillations superimposed on the recorded NO current (Figs. 2a, 3a) were probably caused by respiratory movements. In 21 measurements, we found that [NO] decreased from the retinal surface toward the vitreous, at both sites (Figs. 2, 3). At the surface of the retina, [NO] was on average 6 μM in an intervascular region, and near an arteriole, it was 9 μM. At 200 μm from the retinal surface, at both sites, [NO] was nearly zero (the current measured was not different from the residual current). Statistical analysis using various tests (unpaired t-test of the [NO] values at a given distance from the retinal surface; unpaired t-test of the sum of these values; paired t-test of the average profiles obtained on each animal) showed that the difference between the values of [NO] in the juxta-arteriolar zone (Fig. 3) and those in the intervascular zone (Fig. 2) is barely significant (0.05 ≤ P ≤ 0.1). This indicates that our measurements detect only a small contribution from the arteriolar endothelium in the overall production of NO in the inner retina.

**Degradation Rate of Nitric Oxide in the Vitreous**

The finding that the sustained efflux of NO from the retina did not cause any gradual increase of [NO] in the vitreous indicates that NO is degraded there. Nitric oxide is known to be unstable in the presence of O₂, and the kinetics of their reaction in aqueous solution is described by the rate law: 

\[
-d[NO]/dt = 4 k_{\text{ini}} [NO]^2 [O]^2,
\]

with \( k_{\text{ini}} = 2 \times 10^6 \text{ M}^{-2} \text{ s}^{-1} \) at 25°C (see ref. 38). Using this rate law and the mathematics of diffusion (see ref. 39), we calculated that the expected...
Juxta-arteriolar, preretinal [NO] profile. (A) Continuous NO recording obtained as the NO microprobe was advanced toward an arteriole (rising phase) and then was retracted 200 μm into the vitreous (falling phase). Current was maximal at the arteriolar wall, that portion of recording between the top arrows. (B) [NO] plotted as a function of distance between the arteriolar wall (0 μm) and 200 μm out into the vitreous. Values are means ± SEM of 12 recordings of the type shown in A. NO = nitric oxide.

decrease of [NO] in the vitreous as a function of distance (d) from the retina should be described by the equation: [NO] = [NO]₀ (A d + 1)^{-5}, where A stands for (2 k_{eq} [O₂], [NO])/3 D^{1/2}, [NO]₀ is the value of [NO] at the surface of the retina, [O₂] is the value of [O₂] in the vitreous, and D is the diffusion coefficient of NO in the vitreous. For our numerical calculations, we considered that [O₂] is 36 μM (about 20 mm Hg) close to intervascular regions of the retina, and 90 μM (about 50 mm Hg) near arterioles; k_{eq} increases by a factor of 2 between 25°C and 37°C (which is probably an overestimation); and D is 3.3 × 10^{-5} cm² seconds^{-1} at 37°C. With these numerical values, we calculated that, 200 μm away from the retina, [NO] should reach approximately 7 μM in a juxta-arteriolar region and 5 μM in an intervascular region. Because our measurements showed that [NO] is actually undetectable at this distance from the retina, we conclude that NO is degraded faster in the vitreous than in aqueous solution.

To estimate the actual degradation rate of NO in the vitreous, we compared our measured profiles of [NO] with the profiles predicted by two models: one model in which NO is degraded with zero-order kinetics (i.e., the degradation rate is constant, independent of [NO]), and the other in which NO is degraded with first-order kinetics (i.e., the degradation rate is proportional to [NO]). For the first model, the predicted profile of [NO] is a parabola (for calculations, see for example ref. 30), whereas for the second model it is an exponential (see ref. 39). The model with zero-order kinetics gave a much better fit of our data, and the degradation rates that we deduced in this way were 80 μM minute^{-1} in the juxta-arteriolar region and 65 μM minute^{-1} in the intervascular region. Thus, if the production of NO in the retina was abruptly stopped, [NO] would fall by half in approximately 3 seconds in the vitreous, a time of the same order as that found in other biologic systems.

Effect of Light Stimulation on Preretinal [NO]

In these experiments, the retina was stimulated by flickering light (16 Hz) because this induces an increase of both retinal and optic nerve blood flow. Because the measured NO currents are small, we have taken precautions to avoid any direct effect of light on the NO microprobe. Indeed, the heat produced by the light source could affect the current measured with the microprobe. This was shown by placing the microprobe and a micro-thermistor (Cr/Ni wires coupled by soldering) in an artificial eye filled with aqueous humor and then introducing and positioning the optic fiber normally used for stimulation. Flicker or continuous light stimulation for 60 seconds induced a transient increase of the NO current (N = 3) and, in parallel, a reversible increase in temperature. This increase was suppressed by passing the light beam through an infrared filter (Fig. 4A, third response). Therefore, the following electrophysiological experiments were performed under these optical and stimulation conditions.

Flicker light stimulation of the dark-adapted retina induced a reversible increase of [NO] in the preretinal vitreous (about 50 μm from the retina in an intervascular region) (Fig. 4B). The mean increase was 1.59 μM ± 0.135 (N = 45). In some experiments (see Fig. 4B), the increase elicited by flicker stimulation was preceded by a transient decrease in the NO signal; the origin of this decrease is unclear. Strong light adaptation of the retina by exposure to continuous light for 10 minutes caused suppression of the flicker-induced increase in [NO] (N = 5) (Fig. 4C). When the retina was dark adapted again for 60 minutes, the flicker-induced increase recovered (not shown).
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Vasomotor Effect of Nitro-L-Arginine

Preretinal, periarteriolar, pressure microinjection of approximately 1 μl of the NOS inhibitor NLA (0.6 mM in the puffer pipette), during systemic normoxia–normocapnia, caused a decrease in the preretinal [NO] measured with the NO microprobe positioned near the site of microinjection (not shown) and, in parallel, a segmental and reversible arteriolar vasoconstriction (Fig. 5). The peak of this vasoconstriction (approximately 45%) occurred 1 minute after puffing the arginine analogue nitro-L-arginine (NLA) and was almost completely reversed 5 minutes later (Fig. 5). Microinjections of the solvent (NaCl 0.9%, pH 7.4) had no measurable effects on either the arteriolar diameter or the NO current (three animals, data not shown).

Moreover, in the steady state (normoxia–normocapnia, stable NO current), intravenous infusion of NLA (20 mg kg⁻¹ for 10 minutes) had no measurable effect on vascular diameter (three animals) during the next 60 minutes. Taken that nitro-L-arginine is only slowly metabolized,¹⁷ we calculated that in the steady state, the final concentration in the arterial blood was approximately the same as that injected in the vitreous. In these experiments, however, the retinal arterioles were reactive because hypercapnia induced the expected vasodilation, indicating that the animals were in good condition (see ref. 6).

Arginine Biosynthesis

A large part of the biochemical experiments aiming to explore the ability of retinal cells to produce NO were performed on the guinea pig retina. This is because we have developed a method to obtain purified...
TABLE 1. Concentration of Selected Amino Acids in Müller Cells and in Retina

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<tr>
<td>Guinea pig</td>
<td>intracellular</td>
<td>659 ± 120 (16)</td>
<td>494 ± 116 (3)</td>
<td>400 ± 295 (3)</td>
<td>ND (3)</td>
<td>130 ± 105.7 (13)</td>
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<tr>
<td>Guinea pig</td>
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<td>1.66 ± 0.35 (3)</td>
<td>12.62 ± 2.23 (3)</td>
<td>—</td>
<td>11.62 ± 11.25 (14)</td>
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<tr>
<td>Miniature pig</td>
<td>intracellular</td>
<td>—</td>
<td>2073 ± 191 (17)</td>
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<td>&lt;30 (1)</td>
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<td></td>
<td></td>
<td></td>
<td>400 ± 295 (3)</td>
<td>ND (3)</td>
<td>130 ± 105.7 (13)</td>
<td>191 ± 16.2 (24)</td>
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Values are mean ± standard error of the mean. ND = not detectable.

suspensions of Müller cells without vascular contamination, which at present is not possible in the miniature pig retina. In the intact guinea pig retina, the amount of arginine was below the detection limit of the arginine standard (i.e., less than 10 µM). The miniature pig retina, obtained at the end of the experiments described in the previous sections, contained approximately 200 µM of arginine (Table 1). When freshly isolated Müller cells from the guinea pig retina were incubated with 5 mM 14C(U)-glucose for 30 minutes, we found at most 200 µM of arginine in the cells and 20 µM in the bathing medium. We measured much larger cellular concentrations of aspartate and fumarate (see Table 1), two key amino acids of the urea cycle through which arginine is normally synthesized. The histogram presented in Figure 6 shows that 14C-arginine, 14C-aspartate and 14C-fumarate are synthesized from 14C(U)-glucose in the Müller cells and largely released into the bathing medium.

DISCUSSION

In this article, we provide experimental evidence showing that intact retinal tissue produces and releases NO, which constitutes a chemical factor controlling the basal, dilating, arteriolar tone in the inner retina. Indeed, using a NO microprobe, we found that there is a NO gradient from the vitreal surface of the retina toward the vitreous and that the NO concentration comes close to zero at a distance of approximately 200 µm from the retinal surface. We have confidence that the measured NO values are precise enough. Indeed, the very small currents recorded with our NO microsensors strongly indicate that the relatively thick Nafion membrane mostly excluded the passage of organic molecules abundantly found in nervous tissues, like ascorbic acid or amines, which could be oxidized at +900 mV and generate NO-independent currents. Using a carbon fiber coated with porphyrin and Nafion, Malinski et al32 found that this sensor was approximately three orders of magnitude more sensitive to NO than to catecholamines. Note, however, that the NO currents measured with their sensor were several orders of magnitude higher than with ours. In addition, our sensor showed only a slow and small response after an application of 10 µM catecholamines.

The vitreous body near the retinal surface is practically stagnant. Therefore, on the basis of Fick's law, the results demonstrate that there is a source of NO, the retina (including the endothelial cells of the vascular wall), and an extracellular compartment, the vitreous, in which NO is degraded. Our NO measurements and theoretical considerations showed that there was a faster degradation rate of NO in the vitreous than in aqueous solution. This indicates that NO is not reacting solely with O2 but that enzymes may also be involved in this degradation. For example, it is known that the half-life of NO is prolonged by superoxide dismutase because the reaction NO + O2~ -> NO3~ does not occur because O2~ is unavailable and it is shortened by Fe3+. This suggests that in some vascular retinal diseases such as diabetic retinopathy and retinal ischemia, the degradation of NO could be accelerated or inhibited, affecting consequently the vascular tone in the inner retina (see ref. 48).

Our results demonstrate a continuous production of NO in the inner retina, which could be controlled by other vasoactive factors.

FIGURE 6. Acutely isolated Müller glial cells produce arginine from [14C(U)-glucose. Radioactivity incorporated into arginine (Arg), aspartate (Asp), and fumarate (Fum) was measured inside the cells (intra) and in the corresponding baths (extra). Results are expressed as means ± SEM of 18 experiments.
of NO by the retinal tissue. It is established that the substrate for this production is arginine, which is transformed to citrulline by the enzyme NO synthase. Taking the stoichiometry of 1 mole of arginine per mole of NO, it appears that the retina should consume 34.4 nmoles of arginine per milliliter of retinal tissue per minute. Because we recorded a NO gradient far from visible arterioles, we would expect the retina to produce the calculated amount of arginine. In other words, cells in the retina, other than endothelial cells, may produce NO.

We have shown that purified suspensions of freshly isolated Müller cells from a mammalian retina synthesize substantial amounts of 14C-arginine and, even more important, 14C-fumarate, from 14C(U)-D-glucose. The concentration of arginine in the retina is very small probably because arginine is continuously consumed and not stored. Our result indicates an important flux of arginine biosynthesis in mammalian Müller cells, at least compatible with the rate of NO production that we measured. One possibility is that arginine synthesized in Müller cells could be used for the production of NO there, but also could be transported into endothelial cells when they are unable to produce enough arginine (see ref. 48). It is of interest that cultured rat Müller cells contain an inducible isozyme of NO synthase (25). Hence Müller cells have the metabolic machinery to produce NO. This is consistent with previous findings showing that brain astrocytes synthesize and release an NO-like substance (29) and that L-arginine is predominantly found in astrocytes contacting blood vessels (49). Thus, our results raise the possibility that, in the inner retina, the endothelial cell of the vascular wall is not the sole site of NO production.

Flicker stimulation of the retina induced a transient rise of NO concentration as recorded in the preretinal vitreous, indicating either an increase of NO release from the synaptic terminals of retinal neurons, similar to other neuronal networks (18), or an increase in the production of NO (or NO precursors) by glial Müller cells. Indeed, it has been shown that there are ionic and metabolic interactions between Müller cells and retinal neurons (50) modulated by photostimulation through K+ and glutamate uptake (51).

**Nitric Oxide as a Factor Controlling the Dilating Arteriolar Tone in the Inner Retina**

The arginine analogue NLA is a competitive inhibitor of the enzyme NO synthase and therefore inhibits the cellular production of NO. Consequently, to inhibit NO production, NLA has to be transported through the cellular membrane, possibly using a Na+-independent and competitive system because its uptake into endothelial cells is strongly inhibited by leucine (52,53). Therefore, when NLA was puffed in the preretinal vitreous, we expected that it was transported first into the astrocytes and Müller cells because the membranes of these cells form the vitreoretinal interface and because the extracellular space of the retina is not directly accessible to the vitreous fluid (54). Because of the predominant positive charge at neutral pH of this amino acid, it appears unlikely that it could be pumped out from the glial cells and accumulated secondarily in other cells of the retina, inhibiting NO synthase there. Our idea, therefore, is that NLA inhibits predominantly NO synthase in retinal glial cells. The absence of effect of intravenous infusion of NLA is not completely understood: One possibility is that the inhibitor had not reached a sufficient concentration in the endothelial cells to inhibit completely their NO synthase. It is of interest that Seligsohn and Bill (55), using more than 10 times higher concentrations in rabbit, obtained an approximately 50% reduction of retinal blood flow in parallel with a rise of the blood pressure; however, their experiments were performed on rabbits, and there may be species differences.

The NLA-induced segmental vasoconstriction unambiguously demonstrates that a continuous production of NO is necessary to maintain a dilating arteriolar tone in the inner retina. It is striking that under similar experimental conditions, inhibition of prostaglandin production by indomethacin induces, also in the steady state, arteriolar vasoconstriction (9). However, synergistic interactions between prostaglandin and endothelium-derived relaxing factor (which is NO) occur in regard to inhibition of platelet aggregation (56). Closer to our experimental model, Shimokawa et al. (57) showed that prostacyclin exerted three different relaxant effects in the porcine coronary artery: direct relaxation of the smooth muscle, synergistic interactions with NO, and stimulated release of NO. Even though there are major differences between aortic and retinal arteries, and though PGE1 or prostacyclin rather than PGF2 exert vasodilation in the retina (58), in the absence of available biochemical data concerning the retinal arteries, we tentatively propose that in the inner retina prostaglandins also potentiate relaxation caused by NO. A close interaction between the prostaglandin and the NO systems could explain why, despite a continuing inhibition of either cyclo-oxygenase by indomethacin (see Fig. 5 of ref. 9) or NO synthase by NLA (this article), the vasoconstriction induced by each inhibition was not maintained. This suggests that when one system is inhibited, the other can rapidly compensate the deficient system and maintain a normal vascular tone. If this suggestion is confirmed, the interplay of two metabolic systems would constitute a powerful and subtle control of vasomotion in the inner retina.

**Key Words**

flicker sensitivity, Müller cells, nitric oxide, retinal blood flow, retinal metabolism, vascular regulation
Acknowledgments

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