Neural Nitric Oxide Mediates Edinger-Westphal Nucleus Evoked Increase in Choroidal Blood Flow in the Pigeon

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Purpose. Nitric oxide (NO) has been identified as a putative neurotransmitter in choroidal perivascular nerve fibers originating parasympathetically. Although constitutively produced NO has been implicated in the regulation of the choroidal circulation, the specific role of neurally derived NO in choroidal vasodilation has not been determined. This study examined the role of neurally derived NO in the control of the choroidal blood flow (ChBF) in vivo.

Methods. Resting ChBF and an increase in ChBF elicited by electrical stimulation of the nucleus of Edinger-Westphal (EW) were measured transclerally by laser Doppler flowmetry in anesthetized pigeons before and after administration of a selective inhibitor of neural NO synthase, 7-Nitroindazole (7NI; 50 mg/kg given intraperitoneally); a nonselective NO synthase inhibitor, N\textsuperscript{\textsun}o-nitro-L-arginine methyl ester (L-NAME; 30 mg/kg given intravenously); L-arginine (300 mg/kg given intravenously) followed by 7NI (50 mg/kg given intraperitoneally); or vehicle.

Results. The 7NI and L-NAME, but not the vehicle, attenuated the EW-evoked response (maximally by 78% and 83%, respectively), and this effect lasted for at least 1 hour. Pretreatment with L-arginine abolished this effect of 7NI. Resting ChBF was reduced and systemic blood pressure was increased after L-NAME administration, but both were unchanged after 7NI or vehicle were administered.

Conclusions. Neurally derived NO is responsible for a major component of the ChBF increase caused by EW stimulation in pigeons. This represents the first demonstration in vivo that neurally produced NO is an important factor in the control of ChBF by the parasympathetic nervous system. In particular, neurally produced NO appears to play a role in rapid upregulation of ChBF in the pigeon, whereas endothelially produced NO plays a major role in control of resting ChBF. Invest Ophthalmol Vis Sci. 1996; 37:666-672.
regulation, and these arginine analogues inhibit both endothelial and neuronal NOS. Recently, a selective competitive inhibitor of the neuronal isoform of NOS, namely 7-Nitroindazole (7NI), has been reported,\textsuperscript{12} and its selective inhibitory effect on neural NO production has been confirmed.\textsuperscript{13} This study's goal was to investigate the effects of NOS inhibition by 7NI on resting ChBF and on vasodilation in the choroid elicited by parasympathetic activation, using electrical stimulation of the preganglionic input to the choroidal neurons of the ciliary ganglion (that is, the medial part of the nucleus of Edinger-Westphal [EW]) in pigeons. Previously, we showed that electrical stimulation of the medial part of the nucleus of EW of the oculomotor nuclear complex, which projects to the choroidal neurons of ciliary ganglion with boutonal endings (Fig. 1), increases ChBF.\textsuperscript{14} For comparison, we also studied the effects of N\textsuperscript{5}-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, on resting ChBF and on the increase in ChBF elicited by EW electrical stimulation.

MATERIALS AND METHODS

Animal Preparation

White Carneaux pigeons (n = 26) were deeply anesthetized with ketamine (100 mg/kg given intramuscularly) and xylazine (50 mg/kg given intramuscularly) and positioned in a stereotaxic device. Supplemental doses of anesthetic were administered every hour to maintain deep anesthesia. Body temperature was maintained at 38°C with a Harvard heating blanket and rectal thermoprobe. Skin, fascia, and bone were removed to expose the brain. An insulated stainless steel stimulating electrode was placed in or near EW by using stereotaxic atlas coordinates, and EW was stimulated using a Grass S48 stimulator and Grass (Quincy, MA) SIU 6D stimulus isolation unit (50 to 100 Hz, 0.5 msec anodal current pulses of 100 to 300 μA for 5 to 10 seconds). Accurate electrode placement was confirmed by low-threshold miosis (with 50 μA pulses) in the ipsilateral eye during electrical stimulation. Bone and fascia overlying the superior pole of the ipsilateral eye were removed and the superior rectus muscle was retracted. The tip of the probe of a laser Doppler monitor was positioned with a micro-manipulator above the sclera. A small amount of Aquasonic 100 gel (Parker Laboratories, Orange, NJ) was used in the interface between the probe tip and the sclera to prevent tissue drying during the experiment. Arterial blood pressure was monitored by a blood pressure analyzer (BPA-100 Micro-Med, Louisville, KY) via a cannula inserted in the brachial artery. The brachial vein was cannulated for L-arginine (300 mg/kg) and L-NAME (30 mg/kg) infusion in some birds.

At the end of each experiment, an electrolytic lesion (produced by applying 800 μA pulses for 30 seconds) was made in EW, and each animal was perfused transcardially with 0.75% saline followed by a fixative of 4% paraformaldehyde in a 0.1 M lysine–0.01 M periodate in 0.1 M phosphate buffer (pH 7.4). The brain was removed, cryoprotected with 20% sucrose–10% glyc erin in 0.1 M sodium phosphate buffer and sectioned frozen at 40 μm on a sliding microtome. Electrode placement was confirmed histologically from cresyl violet-stained sections. All procedures in this study adhered to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Choroidal Blood Flow Measurements and Experimental Protocol

Choroidal blood flow was measured using a laser Doppler flow probe using a TSI LASERFLO blood perfusion monitor (model BPM 403 A; St. Paul, MN). Although laser Doppler flowmetry does not yield absolute measures of blood flow, it provides valuable relative measures of blood flow and has been used to monitor blood flow changes in the choroid.\textsuperscript{2,14–16} Thus we refer to our measure as being expressed in blood flow units. A MacLab data acquisition system (AD Instruments, Milford, MA) or a Stoelting (Wooddale, IL)
Baseline ChBF was determined as the average measurement taken in the 1-minute interval immediately before each EW stimulation. The magnitude of the ChBF increase elicited by EW stimulation was measured from the baseline to the highest point during the stimulation. In general, this peak was achieved shortly after stimulus onset and maintained during the rest of the stimulation period. Baseline ChBF and peak response to EW stimulation were recorded over three or four trials separated by 5-minute interstimulation intervals to determine a mean baseline ChBF and mean response to EW stimulation before any vehicle or drug injection. The 7-NI (Lancaster Synthesis, Windham, NH) was dissolved in peanut oil (3 ml per dose) using an ultrasonic water bath heated to 70°C. A 50 mg/kg dose of 7NI was injected intraperitoneally in one group of birds after the pre-7NI measures were completed. This dose of 7NI has been shown to provide maximum inhibition of neural NOS 15 minutes after administration. A group of birds receiving vehicle were given an equimolar intraperitoneal injection of peanut oil after prevehicle measurements were completed. A third group of birds received intravenous infusions of L-NAME (30 mg/kg in 0.1 ml saline). After drug or vehicle injection, EW stimulation was repeated every 5 minutes for 1 hour, and ChBF baseline and EW-evoked responses were recorded. In the last group of birds, L-arginine (300 mg/kg) was infused intravenously. After post-L-arginine baseline ChBF and EW-evoked responses were recorded, these birds received intraperitoneal injections of 50 mg/kg 7NI 15 to 20 minutes after the L-arginine infusion. Edinger-Westphal stimulation was then repeated every 5 minutes for 1 hour, and ChBF baseline and EW-evoked responses were recorded for these birds as well. This group was used to verify the involvement of the L-arginine-NO pathway in 7NI's observed effect.

**Statistical Analysis**

Data are presented as the means ± standard error of the mean. A repeated-measures, two-way analysis of variance with preplanned comparisons was used to test statistical significance of the effects. One-way analysis of variance was used to evaluate differences between groups of birds. A P < 0.05 was considered significant.

**RESULTS**

**Effect of NOS Inhibition on Increase in ChBF Elicited by Electrical Stimulation of the Nucleus of Edinger-Westphal**

Electrical stimulation of EW yielded a rapid increase in ChBF, with a return to baseline when stimulation was stopped. The peak response was typically achieved within 3 to 5 seconds after current onset and was maintained during the stimulation period. Choroidal blood flow returned to baseline 5 to 15 seconds after current was stopped. The ChBF response, however, sometimes began to decline before the stimulus was stopped if the stimulation period was longer than 5 seconds. The magnitude of the peak response was directly related to current amplitude. In initial studies (in animals not used in the four groups reported here), we found that the peak response to EW stimulation was not stable over time when 300 μA (or higher) current pulses were used, presumably because of the tendency of such high current amplitudes to cumulatively produce tissue damage with repeated stimulation. We also discovered in initial studies (in animals not used in the four groups reported here) that the peak response was not stable if the magnitude of the evoked response was less than 30% above baseline ChBF. Because stable responses with repeated EW stimulation were essential to our experimental design, the current parameters for EW stimulation were adjusted for each bird to produce a peak response that was 30% to 100% of baseline ChBF, before any drug injections or infu-
Neural NO and Choroidal Blood Flow

**FIGURE 3.** Effect of nitric oxide synthase inhibition by 7-Nitroindazole (7NI) and Nω-nitro-L-arginine methyl ester (L-NAME) on the increase in choroidal blood flow (ChBF) after electrical activation of Edinger-Westphal nucleus (EW). The effect of 7NI was abolished by pretreatment with L-arginine (L-arg + 7NI). The 0 time point shows the mean EW-evoked increase in ChBF, which was obtained before drug or vehicle administration (time zero). This mean EW-evoked response was designated as 100%, and all EW responses subsequent to drug or vehicle administration are presented as a percentage of this zero time point. The symbols * and # indicate a significant difference from the zero time point within group. Bars represent standard errors of the mean.

**Effect of NOS Inhibition on Resting Choroidal Blood Flow**

Neither resting ChBF nor arterial blood pressure changed significantly after 7NI administration, although sometimes a slight decrease in systemic blood pressure was observed in some animals by the end of an experiment (Fig. 2A). Neither resting ChBF nor systemic blood pressure changed after vehicle injection. No significant changes in resting ChBF and arterial pressure were observed after 7NI administration after L-arginine administration. In contrast, resting ChBF decreased (mean maximal reduction, 33%) and blood pressure increased (maximal increase, 17 mm Hg) after administration of L-NAME (Fig. 2B). Figure 4 and Table 1 summarize the data.

**DISCUSSION**

This study shows that the increase in ChBF elicited by electrical stimulation of EW was attenuated after neuronal NOS blockade by 7NI, whereas baseline ChBF was unchanged. As reported for mammals, no significant changes in systemic blood pressure after 7NI administration were observed, which suggests that
7NI has no significant effect on endothelial NOS in pigeons given the anesthesia used in this study. Because the effect of 7NI on EW-elicited increases in ChBF in the pigeon could be reversed by pretreatment with L-arginine, 7NI appears to have acted by blocking production of NO (or a related compound) through neuronal NOS. Because we stimulated the preganglionic input (EW), in principle 7NI could have had this action at two points in the circuit: the synapses between the terminals of EW neurons and choroidal neurons of the ciliary ganglion, and the terminals of choroidal neurons within the choroid. Because there is no evidence of NO formation by the EW terminals (that is, the boutonal endings) on choroidal neurons or of an action of NO at this synapse, it is unlikely that the action of 7NI in this study was mediated within the ciliary ganglion. It is also unlikely that 7NI could have acted presynaptically on ciliary ganglion terminals in the choroid (for example to diminish acetylcholine release) to alter the responses to EW stimulation. Thus this study indicates that NO (or a related compound synthesized from L-arginine) released by ciliary ganglion terminals in the choroid and acting on choroidal blood vessels is responsible in vivo for most of the ChBF increase caused by EW stimulation in the pigeon. The residual response to EW stimulation after 7NI may reflect incomplete block of neuronal NOS or the minor involvement of a mechanism independent of neuronal NO (such as through acetylcholine-evoked release of NO from the endothelium). This study is the first in which central parasympathetic stimulation and inhibition of neuronal NOS were used to show that neuronally produced NO plays a major role in ChBF control by the parasympathetic nervous system. Previous studies examining the NO-mediated parasympathetic control of uveal blood flow used either in vitro preparations of the posterior ciliary artery or nonspecific inhibitors of NOS.

Previously we noted that EW in pigeon is part of a circuit that may control ChBF, in part as a function of retinal illumination, and that stimulation of EW yields a dramatic increase in ChBF. The present study indicates that this increase is mediated primarily by NO release from the terminals of this circuit within the choroid. Because of the acetylcholine-rich nature of the ciliary ganglion input to the choroid, it was previously assumed that cholinergic mechanisms were responsible for the EW-induced choroidal vasodilation. A prominent NO-mediated role in ChBF control in birds is consistent with the abundant nitrinergic innervation of the avian choroid. Because the blockade of neuronal NO synthesis did not affect resting
TABLE 1. Effect of 7NI on Resting ChBF (BFU) and Arterial Blood Pressure (mm Hg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Resting ChBF</th>
<th>Blood pressure</th>
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<tr>
<td></td>
<td></td>
<td>Before</td>
<td>Before</td>
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<tr>
<td>7NI</td>
<td>9</td>
<td>55 ± 8</td>
<td>83 ± 5</td>
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<tr>
<td>Vehicle</td>
<td>6</td>
<td>50 ± 11</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>L-Arginine + 7NI</td>
<td>6</td>
<td>37 ± 16</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>L-NAME</td>
<td>5</td>
<td>44 ± 10</td>
<td>78 ± 4</td>
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Because maximal inhibition of NOS was observed 30 minutes after intraperitoneal administration of 7NI,17 mean CBF at 30 minutes after treatment is shown in the table (column "After"). Measurements of blood pressure were made by averaging data within the 10-minute interval before treatment and within the 10-minute interval commencing 20 minutes after treatment. Data represent mean ± SEM.

* Resting ChBF was significantly lower after L-NAME administration than before L-NAME administration (P < 0.05).
† Blood pressure was significantly higher after L-NAME administration than before L-NAME administration (P < 0.005).

ChBF, our results suggest that NO released by this nitrinergic innervation may not contribute substantially to the tonic control of choroidal vessels in the pigeon under resting physiologic conditions. In contrast, inhibition of both endothelial and neural NOS by L-NAME substantially decreases resting ChBF, and causes a substantial loss of the EW-evoked response. Thus neurally derived NO may be involved in rapid changes in the choroidal circulation in response to centrally transmitted neural signals reflective of changes in retinal needs, whereas endothelially derived NO may be more involved in long-term maintenance of choroidal vascular tone.

The ciliary ganglion is a major source of nitrinergic innervation in birds and may be a minor source in mammals.5,6,8 In addition, the pterygopalatine ganglion is an important source of perivascular nitrinergic vasodilatory innervation in mammals, and a lesser source in birds.5,9-11 Studies in mammals suggest that NO mediates uveal vasodilation in response to activation of the preganglionic axons (that is, facial nerve) or the postganglionic axons of the pterygopalatine ganglion.19,20 Our findings for the ciliary ganglion raise the possibility that NO released by the perivascular terminals of the pterygopalatine circuit may be involved in rapid adjustments of ChBF to retinal need. The vasodilatory action of neurally produced NO may be particularly important in the fovea region of humans, where the retina is supplied only by the choroidal vasculature.11 Available data from studies in mammals are consistent with the suggestion that NO produced by pterygopalatine terminals may be involved in rapid increases in ChBF in mammals, whereas choroidal endothelial NO may be more involved in maintaining basal ChBF.2,5,19,20 The intrinsic NOS containing ganglion cell plexus in the fovea region may be an additional source of neural NO in the human choroid. Nitric oxide synthase-containing ganglion cells that may serve a similar function have also been observed in the avian choroid.2,6

Key Words
choroidal blood flow, neural regulation, nitric oxide

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