Functional and Structural Analysis of the Visual System in the Rhesus Monkey Model of Optic Nerve Head Ischemia

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PURPOSE. A redistribution of neurochemicals has been identified in the visual cortex of monkeys with laser-induced glaucoma. Examined were functional, structural, and neurochemical changes to the retina, optic nerve, and central visual system in a nonhuman primate model of optic nerve head (ONH) ischemia caused by sustained unilateral administration of endothelin (ET)-1 to the optic nerve.

METHOD. ET-1 or sham control solution was delivered by osmotic minipump to the retrolaminar region of one optic nerve of rhesus monkeys (Macaca mulatta) for 1.5 years. ONH topography and blood flow velocity were serially studied with scanning laser tomography and laser Doppler flowmetry, respectively. Retinal and cortical electrophysiologic measurements from pattern-derived stimuli were obtained quarterly. Immunohistochemistry was used to identify the distribution of calbindin (CB) and c-Fos labeled neurons in the visual cortex areas V1 and V2, and lateral geniculate nucleus (LGN). Retinal ganglion cell counts and optic nerve axon density were determined by light microscopy.

RESULTS. No significant changes in retinal and ONH morphology, ONH blood flow velocity, and retinal and cortical pattern-derived functional activity were detected. Measurement of CB-positive cell density in V1 and V2 showed a significant decrease in CB labeling to the contralateral side of the ET-1-treated eye (P < 0.04). CB-positive cells were present in the magnocellular layers of the LGN with no differences noticed between the ET-1- and sham-treated eyes. c-Fos-labeled neurons were found in striate area V1 and extrastriate V2 of both groups. No c-Fos labeling was observed in the LGN.

CONCLUSIONS. Administering ET-1 to the orbital optic nerve alters neuronal metabolic activity in the visual cortex in rhesus monkeys. Metabolic activity reductions in the visual cortex precede the ability to detect functional and structural alterations in the retina, ONH, and visual cortex in this animal model. (Invest Ophthalmol Vis Sci. 2004;45:1830–1840) DOI: 10.1167/iovs.03-09590

Glaucoma is a neurodegenerative disease as it results in the death of retinal ganglion cells (RGCs).1–3 Retinal ganglion cell apoptotic degeneration and subsequent optic neuropathy in glaucoma occurs from optic nerve axoplasmic flow obstruction, depletion of the neurotrophic factors necessary for RGC survival, excess intraocular endothelin (ET)-1, retinal and ONH accumulation of nitric oxide and oxygen free radicals, and amino acid excitotoxicity and loss of intraneuronal calcium homeostasis at the ONH.4–6 Mechanical deformation of the scleral lamina cribrosa by elevated intraocular pressure (IOP) and hypoperfusion-induced ischemia at the ONH from dysregulation of the ocular microcirculation are pathogenic mechanisms that, alone and in combination, contribute to the visual deficits in glaucomatous optic neuropathy.3–4 Laser-induced photococagulation of the nonhuman primate trabecular meshwork produces a sustained elevation in IOP that models the mechanically induced functional and structural alterations at the ONH present in the human disease.7–9 RGC death, loss of optic nerve axons, and visual field defects are present in this animal model.1,8 Sustained ET-1 administration to the periorbital optic nerve of animals may cause ONH ischemia, and induce alterations to the retina and optic nerve that model the presumed microcirculatory dysfunction noted in the human disease.4,10–15 Optic nerve vessels supplying the ONH in rabbits were significantly constricted by ET-1 delivered by osmotic minipump in rabbits.13 The optic nerve circulation was reduced by ~38% and the optic nerve axon density reduced by 17% in the ET-1 nonhuman primate ONH ischemia animal model.10–12

The RGC death in glaucomatous optic neuropathy is accompanied by transsynaptic degradation of neurons in the lateral geniculate nucleus (LGN) and visual cortex, independent of elevated IOP.14–16 Activity of neurons in the glaucomatous LGN may be so reduced that these cells undergo shrinkage because they are unable to maintain the neurotrophic connections to the visual cortex that are necessary for their survival.15,16 The LGN of the nonhuman primate laser-induced glaucoma model displays reduced axoplasmic flow, peroxynitrite accumulation (Luthra A, et al. IOVS 2001;42:ARVO Abstract 2225), and a reduction in the metabolic activity marker cytochrome oxidase (CO).15,17–19 CO levels are also reduced in the visual cortex of this animal model.17,20 Long-term reduction in visual sensory input imposes substantial calcium buffering requirements on degenerating cortical neurons.21,22 The calcium binding protein calbindin (CB) labels distinct neuronal subgroups, maps local neural activity in the nonhuman primate visual pathway,23–25 and is altered in neuronal degeneration caused by loss of sensory input.22

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The cellular immediate early gene c-Fos is involved in survival, apoptosis, and regeneration of RGCs.26 Neurons expressing c-Fos protein have been used to map polysynaptic neuronal pathway functions activated by visual stimulation in the visual system.27,28 c-Fos levels were reduced in the LGN and visual cortex of cats with central retinal lesions.27 CB and c-Fos colocalize in inhibitory neurons of the feline visual cortex.29 We used immunocytochemistry to examine the CB and c-Fos distributions in the Rhesus monkey LGN and visual cortex areas V1 and V2 after prolonged unilateral administration of ET-1 to the orbital optic nerve. The number of CB-stained neurons and changes in c-Fos staining in the rhesus visual cortex that represent ipsilateral and contralateral retinal input of the ET-1-treated and sham-treated groups were compared. Pattern-evoked retinal and cortical electrophysiologic potentials, confocal scanning laser Doppler flowmetry of the ONH capillary microcirculation, and in vivo morphologic analysis of ONH morphology with confocal scanning laser tomography were measured to evaluate retinal and cortical electrophysiologic activity, ONH blood flow velocity, and ONH topography, respectively.

Material and Methods

Subjects

One eye of each of five rhesus monkeys received ET-1 (Peptide Institute Inc., Osaka, Japan) by osmotic minipumps (Alzet, Palo Alto, CA) to the nasal perineural region of the right optic nerve (0.6 μg/d) for 1.5 years. Four monkeys (control group) received the ET-1 vehicle or sham solution (0.1% acetic acid, diluted 1:4 in 0.9% saline) to the right optic nerve for the same period. Minipumps were surgically implanted under isoflurane anesthesia in a subcutaneous space superior to the right orbital rim. A polyethylene delivery tube was run subcutaneously to the bulbar conjunctiva, the end directed toward the nasal optic nerve under the superior rectus muscle, and the tube sutured to the sclera. The pump reservoirs were changed at 6-week intervals.

Animal care and experiments were approved by the University of Florida Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Electrophysiology, circulatory, and morphologic measurements were obtained before minipump implantation (baseline) and quarterly thereafter.

Anesthesia

The monkeys were sedated with ketamine hydrochloride (10 mg/kg intramuscularly [IM]) and placed in ventral recumbency on a special padded, heated examination table. An intravenous (IV) catheter was placed in the saphenous vein, and IV lactated Ringer’s solution administered (10 mL/kg per hour). Xylocaine hydrochloride (0.35 mg/kg IV) was given to aid intubation. The animal was connected to a respirator and ventilated with 33% oxygen and 66% nitrous oxide (respiration volume 10–20 mL/kg, individually adjusted). The anesthesia was maintained with 1% isoflurane, and then the animal was paralyzed with pancuronium bromide (0.05 mg/kg IV) to prevent globe movement. The following parameters were closely monitored: body temperature, pulse rate, indirect mean arterial blood pressures (MAP; Dinamap Veterinary Blood Pressure Monitor 8300; Critikon, Inc., Tampa, FL), mucus membrane color, and end-expiratory CO2 partial pressures (Vet/Cap 7000 Capnometer; SDI Sensor Devices, Inc., Waukesha, WI). Systolic and diastolic blood pressures were maintained at levels of 70 to 100 and 38 to 55 mm Hg, respectively, with a mean blood pressure of 60 to 75 mm Hg. End-expiratory CO2 partial pressures were kept between 39 and 42 mm Hg by adjusting the respiration rate. The pancuronium bromide was administered (0.025 mg/kg IV) every 45 minutes until the end of the procedure.

At completion of the testing, the animals were recovered with neostigmine bromide (0.05 mg/kg intramuscularly [IM]) to reverse the neuromuscular blockade, and yohimbine hydrochloride (0.11 mg/kg IV) to reverse the remaining xylazine effects. As soon as spontaneous respiration resumed, each animal was extubated, placed back into its cage, and monitored until conscious.

Tonometry

Tonometry of both eyes was performed with an application tonometer (Tonopen XL; Medtronic Ophthalmics, Jacksonville, FL) with the monkeys sedated with ketamine hydrochloride (10 mg/kg IM).

Electrophysiology

The animals were positioned on their chests with their heads in a custom-designed holder to minimize head movement during the procedure. The pupils were dilated with 1% tropicamide ophthalmic solution. A hard contact lens (6 mm radius) was placed in both eyes with 2.5% hydroxypropyl methylcellulose to protect the corneas and preserve optical properties.

The fundus of the monkey was visualized through a highly modified ophthalmoscope (Bausch & Lomb Optical Company, Rochester, NY) with a second optical channel. Through this channel, a 30 visual degree diameter, computer-generated video grating pattern of alternating light and dark, vertically oriented bars was projected onto the macula in a Maxwellian view. A 50% duty cycle with a mean luminance of 500 (bright-field) and 0.82 (dark-field) cd/m2 and a contrast of 82% were used. For a few eyes, corrective lenses (Silor, St. Petersburg, FL) had to be placed in front of the ophthalmoscope to guarantee a sharp projection of the grating patterns onto the fundus. The grating width of the pattern was progressively increased. Eight different grating sizes numbered 1 to 8 were used from 5 to 0.04 cycl/deg (5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 cycl/deg). Gratings were reversed in a square-wave fashion at a temporal frequency of 6 Hz.

Retinal and cortical evoked potentials were recorded simultaneously after 5 minutes (for bright-field stimulus: PH-RE-photopic retina; PH-CO-photopic cortex) and 15 minutes (for dark-field stimulus: SC-RE-scotopic retina; SC-CO-scotopic cortex) of dark adaptation. Retinal evoked potentials were recorded with a custom-built corneal contact lens electrode and a reference lid hook electrode at the upper eyelid. Cortical evoked potentials were recorded with Ag-AgCl electrodes (Biopotential skin electrodes: In Vivo Metric, Healdsburg, CA) that were placed on the skin surface with electrode washers and saline electrode gel (Signa Gel; In Vivo Metric) 2 cm to the right of the median plane and 2 cm caudal of the interaural plane over the lateral operculum (active electrode) and on the right ear (reference electrode). A metal plate placed in the right vestibule of the oral cavity served as ground electrode. Electrode impedances to ground were kept below 10 kΩ. Signals were amplified with a gain of 10,000 and a 0.1- to 100-Hz band-pass (Iso-Dam; World Precision Instruments, Sarasota, FL). We averaged 175 responses of 170 ms duration each, time locked to the shifting of the stimulus, and calculated the root mean square (rms) amplitude for each spatial frequency (LabView, ver. 5.0; National Instruments, Austin, TX).

HRT/HRF Protocols

The optic nerve head (ONH) topography and ONH circulation of the monkeys were examined with the Heidelberg Retina Tomograph/ Flowmeter (HRT/HRF; Heidelberg Engineering, Vista, CA) under the same anesthetic protocols.

Heidelberg Retina Tomograph. The HRT is a confocal laser scanning microscope designed for the acquisition and analysis of three-dimensional images of the retina and ONH. The HRT uses a diode laser (670-nm wavelength) and confocal optics to scan 32 consecutive and equidistant optical section reflectance images (each consisting of 256 × 256 pixels) and produce a layer-by-layer, three-dimensional image. HRT values were obtained first to determine the proper focal plane settings for the HRF.
The eyelids were elevated with a lid speculum and the scanning laser device positioned in front of the animal. The HRT laser beam entered the pupil and was focused on the superficial retina. Both eyes were examined, the normal eye serving as a control. A minimum of three $10^3 \times 10^9$ scan images were obtained after centering the optic disc in the image frame. Mean reflectivity and topography images were obtained for each session. Baseline images were taken for each eye, and follow-up images were taken at 4-month intervals.

A circular contour line was drawn along the inner margin of Elschnig’s ring to define the optic disc. HRT proprietary software (ver. 2.01; Heidelberg Engineering) was applied with the standard reference plane to calculate cup area, rim area, cup-to-disc area ratio, rim volume, cup volume, retinal nerve fiber layer (RNFL) height, and RNFL cross-sectional area.

Heidelberg Retinal Flowmeter. ONH blood flow velocity measurements were obtained while the animals were ventilated with 100% oxygen, and with carbogen (5% CO$_2$ and 95% O$_2$). Baseline measurements were obtained under 100% oxygen. The inhaled gas was then switched to carbogen. After 7 minutes of exposure to the new gas, another series of blood flow measurements was performed. End-tidal CO$_2$ was measured with a capnometer throughout the procedure.

The HRF was used to determine blood flow velocity of the capillaries of the nerve fiber layer of the ONH for the last 6 months of the study. The HRF combines confocal scanning technology and laser Doppler flowmetry to measure the amount and the velocity of moving red blood cells. Using an infrared diode laser with a wavelength of 780 nm, confocal scanning optics and a photodetector, the HRF scans along 64 lines of 256 points (pixels) each (total pixels = 16,384) in a field of $10^3 \times 2.5^2$ (2.7 x 0.7 mm) in 2.04 seconds. Each of the 256 measurement points in a scanned line has 128 discrete recordings of the intensity of the back-scattered light versus time per pixel which is used to obtain a power spectrum of the optical Doppler shift frequencies for each pixel. This frequency shift is proportional to the velocity of the moving cells relative to the speed of the wave. Matrices of the $256 \times 64$ perfusion measurements show the HRF perfusion parameters: volume, flow, and velocity in two-dimensional, high-spatial-resolution, color-coded, perfusion maps. The brightness the pixel color in the perfusion maps, the higher the HRF perfusion parameters. Capillary networks and larger vessels can be resolved in the perfusion images.

The eyelids were elevated with a lid speculum, and the scanning laser device positioned in front of the animal. The HRT laser beam entered the pupil, was focused on the superficial retina, and then was centered to scan the ONH. After generation of the perfusion map, a $10 \times 10$-pixel window measurement frame (which corresponds to an area of 100 $\mu m^2$) was placed away from visible vessels on the temporal and nasal neuroretinal rim (NRR) of the ONH to obtain HRF blood flow velocity parameters (average of five measurements). The measured parameters were the relative velocity of all moving erythrocytes (flow, FLW), the mean velocity of erythrocytes (velocity, VEL), and the relative volume of moving erythrocytes (volume, VOL). The units of the parameters are arbitrary units (AU). Statistical comparisons were made with t-tests and linear regression.

Immunochemical Procedures

Three ET-1- and three sham-treated animals were available for this procedure. The monkeys were anesthetized with ketamine (15 mg/kg IM) and then euthanized with an overdose of pentobarbitonal. Immediately after death, the animals were intracardially perfused with 0.4 M phosphate buffered saline (PBS, pH = 7.4) followed by 4% paraformaldehyde in PBS at room temperature. The brain and globes were removed and postfixed in 4% paraformaldehyde for 1 to 2 days. The primary visual area and LGN were blocked and placed in 30% sucrose in PBS at 4°C overnight before the blocks were cut coronally at 50 $\mu m$. Serial sections were taken as follows throughout each tissue block: section 1 was mounted on a slide for Nissl fiber staining; section 2 was placed in a microwell containing PBS to be processed for CB; and section 3 was placed in a separate large well containing PBS for c-fos immunocytochemistry. The Nissl fiber-stained sections were used for orientation while examining the CB and c-fos sections and to determine loss of neuronal elements. Ten-micrometer-thick retinal sections were embedded in paraffin and examined by light microscopy for staining intensity. The number of peripheral RGCs was counted at 400X. Optic nerve sections were embedded in plastic and sectioned with an ultramicrotome. The sections were photographed at 1000X (Labophot-2 microscope; Nikon, Melville, NY), digitized (Radeon 8500DV; ATi Technologies Inc., Markham, Ontario, Canada(b)), and the mean axon density determined from the digitized images using image analysis software (SigmaScan Pro, ver. 5; SPSS Inc., Chicago, IL).

CB. In all monkeys, alternate 50-$\mu m$ sections containing the left and right occipital lobe, and left and right LGN metathalamic area were incubated for 15 minutes in 3% H$_2$O$_2$ in PBS to block endogenous peroxidase-like activity. This was followed by placing the sections for 30 minutes in 5% normal goat serum (NGS) in PBS containing 0.2% Triton X-100, to block nonspecific protein-immunoglobulin binding. Sections were then labeled with the primary antibody 1:500 monoclonal anti-CB D-28K (Sigma-Aldrich, St. Louis, MO) dissolved in PBS with 1% NGS containing 0.1% Triton X-100 at room temp for 8 to 16 hours. The sections were rinsed three times (10 minutes each) in PBS and then incubated for 8 to 16 hours at room temperature in the secondary antibody 1:5000 anti-mouse IgG (Sigma-Aldrich) dissolved in PBS with 1% NGS containing 0.1% Triton X-100. The tissue was again rinsed three times and placed in a solution of 1:1000 Extravidin-Peroxidase in PBS for 8 to 16 hours at room temperature. The sections were rinsed three times and reacted for 3 to 5 minutes in a solution of 3,3' dianimobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) dissolved at 5 mg per 10 mL PBS with 10 $\mu l$ of 3% H$_2$O$_2$ added per 10 mL just before use. The sections were rinsed several times in PBS to stop the reaction, mounted on slides, and air dried overnight. Sections were then dehydrated through alcohol to xylenes and coverslipped (Permount, Fisher Scientific, Pittsburgh, PA).

c-Fos. Frozen coronal sections of the left and right occipital lobe and left and right LGN metathalamic area were cut at 50 $\mu m$ and placed in PBS solution in a 6 x 6 multiwell tissue culture plate for the c-fos immunocytochemical procedure. The blocking solution was applied for 1 hour (1:30 NGS in PBS+Triton X-100), followed by the primary antibody, 1:500 anti-c-Fos Ab (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) applied at dilution of 1:1000 for 16 to 18 hours.

The sections were then rinsed in 1% NGS and processed using biotin for 3 hours at a dilution of 1:500. After rinsing in 1% NGS an ABC Kit (Vector Laboratories, Inc., Burlingame, CA) solution was applied for 30 minutes, rinsed in PBS, placed in DAB solution for 5 to 6 minutes, rinsed very well in PBS, and mounted. The mounted sections were air dried overnight, dehydrated, and coverslipped.

Nissl. Nissl stains neural cytoarchitecture and degenenrating neurons. The Nissl stain used in the histology for the monkeys was a one-to-one combination of 1% cresyl violet (1 g cresyl violet dissolved in 100 mL dl-H$_2$O, filtered) and 1% thionin (1 g thionin dissolved in 100 mL dl-H$_2$O, filtered). Glacial acetic acid was added a drop at time until a pH of 4.5 was achieved. The stain was placed on a thermal stir plate and the solution warmed to approximately 37°C before the tissue was placed in the stain. The optimal staining time for 10- to 50-$\mu m$ tissue sections was 2 minutes. The stained tissue showed neuron Nissl substance to be a dark purple-blue color with high definition of cellular elements possible at low and high magnification.

Image Analysis

In most experiments, alternate sections were labeled for either CB or c-Fos, examined with a microscope (Axioskop; Carl Zeiss Meditec, Thornwood, NY), the image digitized (AIS; Imaging Research Inc., St. Catharines, Ontario, Canada), and then saved on disc. The counting of CB-staining cells in cortical areas V1 (striate) and V2 (extrastriate) were made from digital images acquired at an objective magnification of 20X and presented on a video monitor. Only cells that were clearly labeled,
as determined by the dark reaction product (nuclear staining for c-Fos, and cell body staining for CB), and clarity of the cell boundary were included in the CB or c-Fos measurements. The counting frame was 100 μm wide, and the section thickness was 50 μm. Investigators were masked to the treatment status of the images during measurements. An atlas of the rhesus brain was used to aid determination of the regions of interest of the visual cortex.30

Statistical Methodology
Statistical comparison of the electrophysiology and HRT measurements of the right (OD) and left (OS) eyes of the two treatment groups (ET-1 and sham) were made with a two-sample t-test at pretreatment (last measurement before administration, PRE), posttreatment (last post-treatment measurement, POST), adjusted OD (OD-OS), and the CHANGE in values from pretreatment to posttreatment at the end of the study (POST-PRE). All analyses were conducted on computer (SAS System software, ver. 8.2; SAS Institute, Cary, NC). Comparisons were deemed statistically significant when \( P < 0.05 \) for two-sided tests. The statistical power for each measure was also calculated.

Electrophysiology. Thirty-degree patterns in a range of grating sizes were made of photopic cortical (PH-CO-30), scotopic cortical (SC-CO-30), photopic retinal (PH-RE-30), and scotopic retinal (SC-RE-30) measurements. The summed rms values for pattern gratings 1 to 8 (Sum[PH-CO30-1 to PH-CO30-8]) between the treatment groups were compared for treatment group, ONH, time period, and all two- and three-way interactions. The time points were baseline and the last 6 months of ET-1 administration.

Table 1. IOP Measurements in the Treatment Groups (ET-1 vs. Sham)

<table>
<thead>
<tr>
<th>Eye</th>
<th>Treatment Group</th>
<th>Pre IOP (mmHg, Mean ± SD)</th>
<th>Post IOP (mmHg, Mean ± SD)</th>
<th>Within-Group ( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td>ET-1</td>
<td>16.1 ± 1.7</td>
<td>15.5 ± 1.0</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>17.6 ± 1.2</td>
<td>15.5 ± 2.2</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>Between-group ( P )</td>
<td>0.016</td>
<td>0.959</td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td>ET-1</td>
<td>17.7 ± 1.8</td>
<td>15.4 ± 1.4</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>17.6 ± 1.4</td>
<td>16.0 ± 2.0</td>
<td>0.226</td>
</tr>
<tr>
<td></td>
<td>Between-group ( P )</td>
<td>0.821</td>
<td>0.369</td>
<td></td>
</tr>
</tbody>
</table>

IOPs from pretreatment to posttreatment at the end of the study for the two groups of animals.

RESULTS
The osmotic minipumps were well tolerated by the monkeys for the length of the experiment. The ET-1 made the sub-Tenon’s tissue quite friable and collagenolytic. Ophthalmic anterior segment and fundic examinations were normal throughout the study. The mean IOP before perineural ET-1 and sham vehicle administration and after ET-1 or sham treatment at the end of the study are listed in Table 1. There was a significant difference in the pretreatment IOPs of the right eye of the two groups. A significant decline in IOP (~13%) in the untreated left eye, but no change in the IOP of the treated right eye of the ET-1 group, was present.

Electrophysiology
Results are summarized in Table 2. There was no significant difference between ET-1 and sham groups in the measurements of PH-CO-30 for grating sizes 1 to 8 with respect to CHANGE in OD (\( P = 0.657 \)), CHANGE in OS (\( P = 0.800 \)), and ADJUSTED OD (\( P = 0.549 \)). There was no significant difference between ET-1 and sham groups in the measurement of SC-CO-30 for grating sizes 1 to 8 with respect to CHANGE in OD (\( P = 0.613 \)), CHANGE in OS (\( P = 0.865 \)), and ADJUSTED OD (\( P = 0.668 \)). There was no significant difference between ET-1 and sham groups in the measurement of PH-RE-30 for grating sizes 1 to 8 with respect to CHANGE in OD (\( P = 0.313 \)), CHANGE in OS (\( P = 0.077 \)), and ADJUSTED OD (\( P = 0.933 \)). There was no significant difference between ET-1 and sham groups in the measurement of SC-RE-30 for grating sizes 1 to 8 with respect to CHANGE in OD (\( P = 0.313 \)), CHANGE in OS (\( P = 0.077 \)), and ADJUSTED OD (\( P = 0.933 \)). There was no significant difference between ET-1 and sham groups in the measurement of SC-RE-30 for grating sizes 1 to 8 with respect to CHANGE in OD (\( P = 0.313 \)), CHANGE in OS (\( P = 0.077 \)), and ADJUSTED OD (\( P = 0.933 \)).

Table 2. Electrophysiology Analysis Summary between Treatment Groups (ET-1 vs. Sham)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment</th>
<th>Change from Pre- to Posttreatment OD (Power %)</th>
<th>Change from Pre- to Posttreatment OS (Power %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-CO-30</td>
<td>ET-1</td>
<td>(-3.69 ± 6.20) (9)</td>
<td>(-3.22 ± 2.89) (35)</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>(-2.06 ± 3.95) (8)</td>
<td>(-4.02 ± 6.74) (7)</td>
</tr>
<tr>
<td>PH-RE-30</td>
<td>ET-1</td>
<td>(-1.39 ± 2.21) (9)</td>
<td>(-0.95 ± 1.09) (28)</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>(-0.09 ± 1.13) (10)</td>
<td>(0.25 ± 0.47) (33)</td>
</tr>
<tr>
<td>SC-CO-30</td>
<td>ET-1</td>
<td>(-1.95 ± 4.12) (9)</td>
<td>(-1.95 ± 3.44) (16)</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>(-0.63 ± 3.18) (8)</td>
<td>(-2.62 ± 3.95) (8)</td>
</tr>
<tr>
<td>SC-RE-30</td>
<td>ET-1</td>
<td>(-0.76 ± 1.74) (9)</td>
<td>(-0.46 ± 0.99) (22)</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>(-0.42 ± 1.60) (9)</td>
<td>(-0.24 ± 0.56) (43)</td>
</tr>
</tbody>
</table>

Change in values from pre- to posttreatment at the end of the study for photopic cortical (PH-CO-30), photopic retinal (PH-RE-30), scotopic cortical (SC-CO-30), and scotopic retinal (SC-RE-30) measurements (mean microvolts ± SD) of the right and left eyes of the two treatment groups (ET-1 and sham). The statistical power to detect a 20% change from pretreatment values (two-sided test at 5% significance level) is shown.
Selected HRT results are summarized in Table 3. There was no significant difference between ET-1 and sham with respect to PRE, POST, or CHANGE in OD, CHANGE in OS (P = 0.690), and ADJUSTED OD (P = 0.934). The power to detect a 20% change in pretreatment to levels varied from 9% to 10% in the OD and 8% to 43% in the OS.

### Heidelberg Retinal Tomograph

Selected HRT results are summarized in Table 3. There was no significant difference between ET-1 and sham with respect to PRE, POST, or CHANGE in OD, CHANGE in OS, and ADJUSTED OD for cup-to-disc area ratio, cup area, maximum cup depth, mean cup depth, cup shape, cup volume, disc area, height variation, rim volume, mean RNFL thickness, rim area, and RNFL cross-sectional area. The power to detect a 20% change in pretreatment to levels varied from 6% to 53% in the OD and 9% to 99% in the OS.

### Heidelberg Retinal Flowmeter

There were no significant effects of MAP, carbogen, or pulse on HRF values in ET-1 and sham control groups. HRF data are reported in Table 4 for FLWM only, because all HRF parameters are interrelated. There was no detectable difference in HRF parameters between the ET-1 and sham groups. The FLWM of the OD nasal ONH was significantly less than the temporal ONH of the OD (P < 0.05) with no group differences. The FLWM of the ET-1 OS nasal ONH was less than

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment</th>
<th>Change from Pre- to Posttreatment OD (Power %)</th>
<th>Change from Pre- to Posttreatment OS (Power %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD area ratio</td>
<td>ET-1</td>
<td>-0.0105 ± 0.1115 (10)</td>
<td>-0.1033 ± 0.0859 (27)</td>
</tr>
<tr>
<td>Height VAR (mm)</td>
<td>Sham</td>
<td>-0.0075 ± 0.1937 (6)</td>
<td>-0.0845 ± 0.1544 (9)</td>
</tr>
<tr>
<td>Mean RNFL (mm)</td>
<td>ET-1</td>
<td>-0.0067 ± 0.0733 (55)</td>
<td>-0.2110 ± 0.0839 (76)</td>
</tr>
<tr>
<td>Rim area (mm²)</td>
<td>Sham</td>
<td>-0.0313 ± 0.0955 (21)</td>
<td>-0.1028 ± 0.0427 (65)</td>
</tr>
<tr>
<td>RNFL Cross section (mm²)</td>
<td>ET-1</td>
<td>-0.0475 ± 0.0639 (26)</td>
<td>-0.0845 ± 0.0529 (43)</td>
</tr>
</tbody>
</table>

HRT cup-to-disc area ratio, height variation (mm), mean RNFL thickness (mm), neuroretinal rim area (mm²), and RNFL cross-sectional area (mm²) of the right (OD) and left eyes (OS) of the two treatment groups (ET-1 and sham). Data are expressed as the mean ± SD. The statistical power to detect a 20% change from pretreatment values (two-sided test at 5% significance level) is shown.

### Table 4. HRF Analysis Summary between Treatment Groups (ET-1 vs. Sham)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Eye</th>
<th>Main Effect</th>
<th>Level</th>
<th>LS Mean</th>
<th>SE</th>
</tr>
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<tbody>
<tr>
<td>FLWM</td>
<td>OS</td>
<td>Period</td>
<td>Carbogen</td>
<td>263.7109</td>
<td>22.59602</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Oxygen</td>
<td>274.4939</td>
<td>22.59602</td>
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<td></td>
<td></td>
<td></td>
<td>Power (%)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group*ONH</td>
<td>ET-1*Nasal</td>
<td>265.0398</td>
<td>26.09164</td>
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<td></td>
<td>ET-1*Temporal</td>
<td>267.3855</td>
<td>26.09164</td>
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<td>Sham*Nasal</td>
<td>362.2730</td>
<td>36.89915</td>
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<td></td>
<td>Sham*Temporal</td>
<td>181.7113</td>
<td>36.89915</td>
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<td>OD</td>
<td>Group</td>
<td>ET-1</td>
<td>263.0617</td>
<td>34.18018</td>
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<td>Sham</td>
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<td>Power (%)</td>
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<td>ONH</td>
<td>Nasal</td>
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<td>Temporal</td>
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<td></td>
<td>Power (%)</td>
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<tr>
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<td>Period</td>
<td>Carbogen</td>
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<td>41.86200</td>
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<td>Oxygen</td>
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<td>41.86200</td>
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<td></td>
<td>Power (%)</td>
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<td></td>
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<tr>
<td>Adjusted OD</td>
<td>Group</td>
<td>ET-1</td>
<td>-3.1510</td>
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<td></td>
<td></td>
<td>Power (%)</td>
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<tr>
<td></td>
<td></td>
<td>ONH</td>
<td>Nasal</td>
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<td>41.43406</td>
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<td></td>
<td></td>
<td></td>
<td>Temporal</td>
<td>86.0838</td>
<td>41.43406</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Power (%)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Period</td>
<td>Carbogen</td>
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<td>Oxygen</td>
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<td>41.43406</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Power (%)</td>
<td>10.0000</td>
<td></td>
</tr>
</tbody>
</table>

Data are the least square (LS) means (± SE) of the HRF Flow (FLWM) measurements of the right and left eyes and the difference between the OD and OS (Adjusted OD). The measurements for the two treatment groups (ET-1 and Sham), the regions of the ONH (Nasal and Temporal), and period groups (Oxygen and Carbogen) are included. Measures are expressed in arbitrary units (AU). The statistical power to detect a 20% change from pretreatment values (two-sided test at 5% significance level) is shown.
the ET-1 OS temporal ONH, which was less than the OD. The VELM of the OD nasal ONH was greater than the OS temporal ONH. The VOLUM of the OD nasal ONH was significantly less than the OD temporal ONH. The ADJUSTED OD FLWM, VELM, and VOLUM were less in the nasal ONH than the temporal ONH. The power to detect a 20% change in pretreatment to levels varied from 18% to 20% in the OD to 55% in the OS.

Lateral Geniculate Nucleus
Examination of CB-stained sections found a distinct distribution in the LGN. Numerous CB-positive cells were located in layer 1 (magnocellular), and a few CB-positive cells were present in layers 5 and 6 (parvocellular). No differences were observed between ET-1- and sham-treated eyes.

Examination of c-Fos stained sections of LGN did not reveal any stained cells in any layer of the LGN in either group. Nissl-stained sections reveal a laminated LGN nucleus with six distinct cell layers and interlaminar zones. The largest layer was cytoarchitectural layer 1 (magnocellular).

Primary Visual Cortex
CB-stained sections of striate V1 and extrastriate V2 cortex showed a high density of labeled cells in layers 2 and 3 with no clear boundary between the two. Layer III contained the most intense labeling of CB fibers. Layers 4, 5, and 6 showed light CB staining. A few CB-stained cells were found in deep layers in the experimental and control groups. The CB-positive neurons in V1 and V2 generally showed organizational uniformity, but “bare” areas of nearly absent CB positive neurons alternating with areas with CB positive neurons were also present (Figs. 1, 2). The CB-staining cortical neurons in areas V1 and V2 were similar in shape and probably belonged to the stellate and basket cell classes.

The mean number of CB-labeled neurons in the striate regions of the right and left V1 of the ET-1-treated eyes was 25.9 ± 5.8 and 14.8 ± 4.6, respectively; in the extrastriate regions of the right and left V2 of the ET-1-treated eyes was 33.6 ± 7.9 and 22.5 ± 6.3, respectively; in the striate regions of the right and left V1 of the sham-treated eyes was 38.9 ± 4.9 and 43.4 ± 7.9, respectively; and in the extrastriate regions of the right and left V2 of the sham-treated eyes was 36.9 ± 2.1 and 36.7 ± 5.8, respectively.

Retina
CB staining was strong in all retinal cell layers in sham-treated monkeys, but was more diffuse with only a few cells strongly stained in the retina of the ET-1-treated monkeys. Nissl fiber-stained sections of ET-1-treated monkeys had a slight reduction in retinal fiber staining intensity (Figs. 6, 7). There was no significant difference in the number of RGCs between ET-1 (16.0 ± 10.3 RGC/HPF) and sham-treated (14.3 ± 9.3 RGC/HPF; P = 0.809) eyes.

Optic Nerve
There was no significant difference in the axon density of the ET-1-treated eyes (231,006 ± 4,057 axons/mm²) and the control eyes (223,389 ± 15,845 axons/mm²; P = 0.464).

Discussion
Transsynaptic neural cell death causing focal to generalized neuronal atrophy and loss of sensory function in the LGN and
Neurons in the visual cortex are grouped into columns of cells according to ocular dominance. Vertical columns of neurons that respond preferentially to the right eye alternate with vertical columns of neurons that preferentially respond to the left eye. A notable feature of the suprastriate cortex in our ET-1–treated monkeys was CB-bare, nuclear-free areas that we interpret to be groups of neurons receiving input from neurons in the ET-1–treated right nasal retina. The ET-1 administration to the right nasal optic nerve reduced CB staining in cortical areas that receive input from the right nasal retina or optic nerve. These areas may reflect functional alterations as well. Adjacent columns containing CB-labeled nuclei may receive input from the left eye. Denervated ocular dominance columns have been identified by a CO-labeled in layer 4c of the primary visual cortex of monkeys with laser-induced glaucoma.

The endothelium-derived vasoconstrictive factor ET-1 may have a prominent role in the pathogenesis of some types of glaucoma and has been used in animal models to induce...
some degree of ONH hypoperfusion-ischemia. Excess functional ET-1 may induce IOP regulatory instability, cause circulatory hypoperfusion and ischemia in the ONH, promote ONH astrocyte proliferation, reduce optic nerve axoplasmic flow, and increase ONH nitric oxide levels.

The effects of ET on a tissue depend on the ET-1 concentration and the expression and distribution of the ET receptors in that tissue. ET causes vasodilatation at low tissue concentrations and marked and sustained contractions at high concentrations. The vasodilator response to ET involves activation of endothelial receptors (ET_α-type) linked to NO and/or prostacyclin release by endothelial cells. The vasoconstrictive response involves the activation by ET of specific membrane receptors (i.e., ET_β-receptors) on smooth muscle cells. Such effects on vasoconstriction could be exacerbated with increasing concentrations of ET.

Repeated exposure of the isolated human ophthalmic artery to ET-1 in vitro demonstrated a marked tachyphylaxis that may...
have been caused by a downregulation or internalization of ET receptors.11,42 In contrast, the ET-induced elevation in systemic blood pressure or pressor response did not undergo tachyphylaxis in cats in vivo.42,43 The turnover rate of the ET-receptors responsible for the pressor response may be relatively high, so that these receptors are promptly synthesized after internalization. Downregulation of ET receptors is functionally linked to a decreased ET-induced response.44 ET-1 receptors may become refractory on repeated or maintained exposure to ET.15

Vasoconstriction of the anterior ONH microvasculature in a rabbit model of constant perineural ET-1 perfusion for 3 days was highly correlated to the dose of ET-1.3–5 µg/d had 30% constriction with 0.001 µg/d inducing ~15% vasoconstriction.15

A nonhuman primate model of “chronic” ONH vascular insufficiency and “ischemia” used ET-1 at dose of 0.1 µg ET-1/d for 7 days in three treated and three control monkeys. A 35.7% ± 9.1% decrease in ONH blood flow in ET-1-treated versus normal eyes and a 0.7% ± 5.5% decrease in balanced salt solution controls versus normal eyes were present.19 No reduction in response or tachyphylaxis was noted in the ET-1-induced decrease in ONH blood flow in the three ET-1-treated monkeys after 1 week of the experiment. This suggests the in vivo turnover rate of the ET receptors responsible for the pressor response is high at least in the short term.

To increase the ability to detect retinal and cortical changes in function and structure changes that might be associated with ONH “ischemia,” we used a higher ET-1 dose in our monkeys than the previous nonhuman primate study10 to attempt to enhance the degree of ONH microcirculatory vasoconstriction and maximize the presumed hypoperfusion induced “ischemic effect” to the ONH. We also used the acidic vehicle for our controls rather than balanced salt solution as in the other study to make sure that it was not the vehicle inducing optic nerve change. It is our belief that the earlier 1-week nonhuman primate study was more of an acute study than chronic.10 Tachyphylaxis related to their blood flow studies was not observed, because of the lower ET-1 dose and short study length. Preliminary data in our laboratory had indicated no detectable effect on the functional or structural measures at doses of 0.1 and 0.3 µg ET-1/d. A longer exposure time was used to mimic more closely the prolonged human glaucomatous condition. Our 1.5-year study correlated to 5.25 human years, as 1 year in the life of a rhesus monkey is thought to represent 3.5 human years.44 The study length in the prior nonhuman primate study was 7 monkey days or 24.5 human days.

CB and c-Fos label different neuron populations in the monkey visual pathway. The prolonged administration of ET-1 to the optic nerve in our monkey study altered neuronal metabolic activity in the rhesus monkey’s visual cortex, but not the LGN. The prolonged administration of ET-1 to the retrolaminar region of the optic nerve affected transsynaptic transmission of signals to the visual cortex so that a reduction of CB activity in V1 and V2 occurred. The weak retinal CB immunoreactivity in the ET-1 monkeys suggests a slight decrease in activity and function, but any retrograde injury to the photoreceptors, bipolar, amacrine, and ganglion cells appears to have been minimal. c-Fos cell labeling was reduced in the LGN and V1 cortex of rats with hypertensive glaucoma (Imamura K, et al. IOVS 2001;42:ARVO Abstract 4446) and cats with retinal disease,27 but was not notably different from normal in our monkeys with chronic ET-1 administration to the optic nerve.

Visual function testing to detect early functional glaucoma damage appears to be problematic because it does not appear to be sufficiently sensitive. Ophthalmoscopically observed structural changes in the retina and ONH can be detected clinically before the functional changes from glaucoma can be detected.45–47 A substantial loss of RGC and optic nerve axons must occur before the standard visual field is affected.45,47 As the remaining population of RGCs declines in advancing glaucoma, reduction in functional redundancy of RGCs causes deficits in vision to become more pronounced, to progress more rapidly, and to be more easily detectable.

No “effects” of chronic ET-1 administration were detected in ONH topography, ONH blood flow velocity parameters, or retinal and cortical electrophysiology despite the evidence of altered cortical neuronal activity. The HRB blood flow velocity parameters were reduced in the nasal ONH of the treated eye compared with that in the control eye and increased in the temporal ONH of the treated eye compared with that of the control eye in both the ET-1- and sham-treatment groups. The level of ONH hypoperfusion or degree of ischemia associated with these alterations in HRB parameters is not known. The electrophysiologic changes in the eyes and visual cortex of our monkeys may have been too focal to be detected, even after 1.5 years of ET-1 exposure.

It appears we may have overwhelmed the ET-1 receptors in the treated eyes. Too much ET-1 may have downregulated the ET-1 receptors so that no functional defect in the classic ET-1 sense occurred and tachyphylaxis was thus observed. This is in contrast to the rapidly progressive glaucoma in dogs, in which decreased ET, receptors and sustained levels of elevated intraocular ET-1 are associated with detectable changes in retinal and cortical function.54,55 The absence of CB staining in the regions of the VC served by the ET-1-treated nasal OD compared with the vehicle-treated nasal OD indicate some slight effect or “tickling” of the visual system, although the statistical power was too low to differentiate ET-1-induced functional and structural effects from the large inherent test variability.

The purpose of sample size and power calculations is to show that the sample size in a study is sufficient to have a reasonable chance of finding statistical significance if such a treatment actually induces a biologically meaningful effect. High levels of α, larger sample sizes, low variability in the data, and larger effect size increase the power of statistical studies. The power or probability of detecting a 20% change from pretreatment levels, given that the sample size and amount of variability in the data in this study was generally low. The 20% change is somewhat arbitrary, but changes in the measured parameters probably have little or no biological meaning unless they are at least 20% or 50% or more. Given the large amount of observed variability in the electrophysiologic, tomographic, and blood flow data collected in our small number of animals, it appears likely that the probability of detecting a 20% change in the pretreatment-to-posttreatment condition for several of our measures in our monkeys is of a rather low degree of statistical power. In that light, our conclusions concerning functional and structural changes must be considered with extreme caution.

Animal models of RGC death caused by elevated IOP induce greater retinal injury than models of RGC death induced by vascular occlusion and ischemia.49 Injury to the RGC and optic nerve axons in the elevated IOP laser-induced glaucoma monkey model extends beyond ischemia alone. Pattern ERG (PERG) amplitude was reduced in monkeys with laser-induced glaucoma before ophthalmoscopic evidence of ONH cupping, with the severity of the PERG reduction progressive and associated with the degree of optic nerve cupping and elevation of IOP.8 The pattern VEP (PVEP) may have poor sensitivity, as glaucoma affects the peripheral retina first, whereas the central retina which produces the VEP response is more preserved.40 Our 30° field of pattern stimulation should have evaluated more than the immediate macular region. We trust our technical capabilities, as our equipment had been used successfully
in parallel studies of retinal and cortical changes in monkeys with laser-induced glaucoma and we therefore were assured that it could detect subtle retinal and cortical change (Komaromy A, et al. IOVS 2000;41: ARVO Abstract 451).54
Vascular hypoperfusion and “ischemia” of the ONH due to dysregulation of the anterior optic nerve microcirculation have been hypothesized as potential causative factors in the development of RGC apoptosis and glaucomatous optic neuropathy.52,53 ONH “ischemia” in the glaucomas can be intermittent or persistent and moderate or severe depending on the associated level of IOP.54 Although “ischemia” itself has never been directly demonstrated to cause or contribute to glaucomatous optic neuropathy, moderate to severe ONH hypoperfusion due to vascular insufficiency and dysregulation may induce persistent levels of sustained low-grade “ischemia” in the ONH to interfere with RGC function in normotensive glaucoma. Episodic spikes of elevated IOP induce more severe, but intermittent, optic nerve “ischemia” from severe reductions in ONH perfusion pressure, and are hypothesized to occur in glaucomas with elevated IOP. Abrupt IOP spiking may be particularly damaging, because the microcirculation of the ONH may not be able to autoregulate or compensate quickly.54 The ET-1 nonhuman primate model of ONH “ischemia” possesses subtle pathologic changes in the visual cortex that appear to progress only slowly to involve the retina. This suggests that chronic low-grade ONH ischemia from ET-1 administration has transsynaptic, neural effects in the visual cortex.

References


