Regional Choroidal Blood Flow and Multifocal Electroretinography in Experimental Glaucoma in Rhesus Macaques

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PURPOSE. To test a hypothesis of regional variation in the effect of experimental glaucoma on choroidal blood flow (ChBF) and retinal function.

METHODS. Five rhesus macaques underwent laser trabecular destruction (LTD) to induce elevated intraocular pressure (IOP). Intraocular pressures were elevated for 56 to 57 weeks. Multifocal electroretinographic (mfERG) and multifocal visual evoked cortical potential (mfVEP) testing were performed at regular intervals before and during the period of IOP elevation. At euthanasia, the IOP was manometrically controlled at 35 (experimentally glaucomatous eye) and 15 (fellow control eye) mm Hg. Fluorescent microspheres were injected into the left ventricle. Regional ChBF was determined.

RESULTS. All of the experimentally glaucomatous eyes exhibited supranormal first-order kernel (K1) root mean square (RMS) early portions of the mfERG waveforms and decreased amplitudes of the late waveforms. The supranormality was somewhat greater in the central macula. Second-order kernel, first slice (K2.1) RMS mfVEP response was inversely correlated ($R^2 = 0.97$) with axonal loss. Total ChBF was reduced in the experimentally glaucomatous eyes. The mean blood flow was 893 ± 123 and 481 ± 37 $\mu\text{L}/\text{min}$ in the control and glaucomatous eyes, respectively. The ChBF showed regional variability with the greatest proportional decrement most often found in the central macula.

CONCLUSIONS. This is the first demonstration of globally reduced ChBF in chronic experimental glaucoma in the nonhuman primate. Both the alteration of mfERG waveform components associated with outer retinal function and the reduction in ChBF were greatest in the macula, suggesting that there may be a spatial colocalization between ChBF and some outer retinal effects in glaucoma.

Keywords: experimental glaucoma, choroidal blood flow, multifocal electroretinography

Blindness from primary open-angle glaucoma results from what is typically a gradual loss of retinal ganglion cells (RGCs) that may extend over years or even decades. It is generally thought that this may be due to direct injury to the optic nerve, caused either by decreased blood flow or by mechanical injury from deformation of the lamina cribrosa or a combination of the two. Such a proposed mechanism would not predict outer retinal injury. Consistent with this, some histopathologic studies of the outer retina have not found marked loss of photoreceptors.^{1,2} However, numerous other studies have shown ischemic-like effects on the outer retina, such as cone photoreceptor swelling and focal loss,³ decreased cone opsin,⁴ and neuroglobin⁵ production. Fundus reflectometry has been used in glaucoma patients to show loss of integrity of the foveal cone outer segments.⁶ Multiphoton confocal microscopy has demonstrated neuronal loss in the outer nuclear layer in postmortem eyes from patients with glaucoma.^{7,8} Recent work with high-resolution optical coherence tomography (OCT) and adaptive optics has shown loss of cone optical signal in chronic human glaucoma.^{9,10} Optical coherence tomography has also been used to show thickening of the foveal outer nuclear layer,¹¹ which is consistent with an

earlier histologic observation of cone perikaryal swelling.³ Similarly, electroretinographic (ERG) evidence for functional impairment in outer retinal responses has been observed in humans with glaucoma in the form of decreased full-field ERG a- and b-waves,^{12,13} as well as multifocal electroretinography (mfERG) early waveform (cone and/or off bipolar cell generated) decrement in the macular region of patients with open-angle glaucoma.¹⁴ Increased mfERG N1 and P1 early waveform features (also an indicator of outer retinal stress) have been observed in experimentally glaucomatous nonhuman primates.¹⁵ Outer retinal injury has also been found in rodents in both the episcleral venous destruction¹⁶⁻²¹ and the spontaneous DBA/2NNia²² and DBA/2J^{23,24} models. (It should be noted that, unlike the laser trabecular destruction [LTD] nonhuman primate model of chronic experimental glaucoma, there is marked photoreceptor loss in the rodents.) Retinal ganglion cell loss without elevated IOP does not result in similar outer retinal effects in simian,²⁵ porcine,²⁶ or rodent^{20,27} models of axotomy.

In addition to indirect evidence for ischemia, decreased choroidal and retinal PO_2 levels^{28,29} as well as increased ERG c-wave and decreased b-wave responses have been found

TABLE 1. IOP History and Axon Loss in Experimental Animals

Animal	Age, y	Weight, kg	Mean IOP, mm Hg		Duration*	IOP × Days†	Axon Loss
			OD ± SD	OS ± SD			
Rh1	14.0	6.2	47.8 ± 10.5	15.5 ± 2.5	397	13,173	100%
Rh2	12.9	6.1	48.0 ± 11.0	17.8 ± 2.7	407	13,397	81%
Rh3	12.7	6.3	37.7 ± 9.4	14.5 ± 2.6	397	9,045	98%
Rh4	11.5	7.8	44.3 ± 8.3	17.7 ± 3.1	393	10,689	10%
Rh5	11.5	8.6	34.5 ± 9.3	15.2 ± 2.8	406	7,770	61%

* Days after IOP first reached 25 mm Hg.

† Area under right IOP graph – left IOP graph (mm Hg × Days).

following acute IOP elevation in the cat. An obvious candidate for these ischemic-like effects is decreased choroidal blood flow (ChBF). Using nonrecirculating radiolabeled microspheres, it has been shown that even moderately acute elevations of IOP in monkeys³⁰ and cats^{3,31} can result in marked decreases in ChBF. However, no similar studies have been carried out in the nonhuman primate model of pressure-induced chronic experimental glaucoma. Even so, there is evidence for decreased foveal choroidal perfusion^{32,33} as well as histopathologic evidence for choroidal thinning³⁴ (although not confirmed by OCT³⁵) in chronic human glaucoma. Decreased ChBF has also been observed in the DBA/2J mouse.³⁶

The purpose of this study was to determine if the decrease in ChBF observed following acute IOP elevation becomes permanent in chronic ocular hypertension in the LTD monkey model. In addition, with the use of a newly developed method of quantitatively measuring regional ChBF,³⁷ we sought to determine whether the ChBF decrement, if any, is uniform throughout the eye or if there is regional variation. Further, we wanted to know if there were also regional differences in functional (mfERG) effects of elevated IOP and, if so, whether the pattern of deficit was similar to any regional ChBF effects.

METHODS

Animals

Five female rhesus macaques (*Macaca mulatta*) were used for the collection of data in this study. All of the experimental methods and techniques adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Wisconsin-Madison's Animal Care and Use Committee. Before experiments began, all five animals were determined to be ocularly normal and healthy. The ages and weights of the animals at the time of euthanasia are shown in Table 1.

Experimental Glaucoma

Ocular hypertension was induced in all five animals by laser trabecular meshwork destruction (LTD).^{38–40} After topical application of 0.5% proparacaine hydrochloride ophthalmic solution to the cornea of the right eye, a 532-nm diode laser and slit-lamp delivery system (OcuLight GL; Iridex Corp., Mountain View, CA, USA) were employed to deliver laser light through a Kaufman-Wallow single-mirror monkey gonioscopy contact lens (Ocular Instruments, Inc., Bellevue, WA, USA). Ketamine hydrochloride (10–15 mg/kg intramuscular) and medetomidine hydrochloride (40–75 µg/kg intramuscular) were used for anesthesia. Initially, approximately 130 confluent laser spots were delivered to the inferior 270° of the trabecular meshwork of the eye. The spots were 75 µm in

diameter, 1.0 W in strength, and 0.5 s in duration and were directed to the anterior (nonpigmented) portion of the meshwork. Intraocular pressures were then checked at least weekly with a handheld digital tonometer (Tono-Pen XL; Mentor O & O, Norwell, MA, USA). (This device underestimates the IOP at higher pressures in the cynomolgus monkey.⁴¹ However, a similar study has not been done for the rhesus monkey.) If the IOP was not elevated in the treated eye after 3 weeks, then a second laser treatment was performed that was also 270° in extent and included the previously untreated superior trabecular meshwork. None of the animals required more than two treatments. If an IOP was found to be greater than 70 mm Hg at the weekly check, the animal was treated daily with either topical antiglaucoma drops and/or systemic acetazolamide until the IOP dropped to below 70 mm Hg.

Multifocal Electroretinography

The mfERG testing procedure was performed as previously described.⁴² Briefly, the animals were preanesthetized with ketamine hydrochloride (15 mg/kg, intramuscular). Propofol was used to induce each animal into a deeper level of anesthesia. The induction dose of propofol was 2 to 5 mg/kg intravenous (IV) and was maintained with a continuous IV infusion rate of 6 to 24 mg/kg/h, as needed, in order to restrict eye movements. The IOPs for each eye were measured with the handheld digital tonometer prior to and following induction with propofol to document anesthetic effects, if any, on IOP determinations. Heart rate and blood oxygen saturation were monitored continuously with a pulse oximeter, and with respiration rate were recorded every 15 minutes. Core body temperature was monitored and recorded intermittently and maintained at 37°C to 39°C with the use of a water-circulating heating pad. Topical 1% tropicamide and 2.5% phenylephrine hydrochloride were used to induce pupillary mydriasis and cycloplegia. Proparacaine hydrochloride (0.5%) was applied topically, and wire specula were used for lid retraction. Electroretinography-jet contact lens electrodes were placed on the corneas with 2.5% hydroxypropyl methylcellulose. Reference electrodes for each corneal contact lens electrode consisted of subdermal needles that were inserted at the ipsilateral outer canthus. The VERIS Science 4.9 system (Electro-Diagnostic Imaging, Inc., San Mateo, CA, USA) was applied for generating stimuli, collecting data, and preliminary analyses. The luminance of the stimulation monitor was calibrated by the VERIS autocalibration system at the outset of the mfERG studies. The visual stimulus consisted of 241 unstretched hexagonal elements that were displayed on a Philips model MGD403 monochromatic monitor (Koninklijke Philips Electronics NV, Eindhoven, The Netherlands). The VERIS fast sequence (binary maximum-length sequence cycle of 2¹⁵–1) was used with a frame rate of 75 Hz (13.3 ms/frame). Maximum and minimum luminances of the

display were 200 and ~ 1 cd/m², with a mean luminance of ~ 100 cd/m². The sampling rate of the signal was 1200 Hz (0.83 ms). The low- and high-frequency filter settings were 3 Hz and 300 Hz, respectively. The eyes were refracted for the 20-cm viewing distance. A refracting lens (5 diopters of positive sphere) was positioned in front of the tested eye, and an opaque occluder was employed to obstruct the untested eye. A reversing ophthalmoscope with corner cube was used to align the stimulus display with the visual axis. The visual angle subtended by the entire stimulus was $\sim 80^\circ$, and each hexagonal element subtended $\sim 5.2^\circ$. Testing order of the eyes was counterbalanced; that is, the eye tested first in a given session was the one that had been tested second in the previous session. Counterbalancing was required since the amplitudes of the early mfERG waveforms were somewhat lower on days on which either the control or treated eyes were tested after the first eye. Multifocal ERG recordings were collected at intervals separated by at least 1 week. A total of 9 to 11 baseline recordings and 16 to 19 post LTD recordings were obtained for each animal.

Each 241-element first-order kernel (K1) trace array was visually inspected to locate the hexagonal element with the largest N1-P1 response density amplitude (referred to subsequently as "amplitude"). The elements were grouped into six concentric rings with the first ring consisting of only the single hexagonal element with the largest N1-P1 response (VERIS Science 4.1). The processed data (from the VERIS Science 4.1 software) were exported for further analysis to a task-specific routine using MATLAB (The MathWorks, Inc., Natick, MA, USA). K1 root mean square (RMS) determinations were made for the 9- to 35-ms epoch of the central hexagonal element (ring 1) and for the concentrically positioned rings 2 through 6. Root mean square values were then averaged across all study animals for each of the six rings, and compared as a ratio between eyes for the baseline and high IOP experimental periods using paired *t*-tests, with $P < 0.05$ considered to be statistically significant. The amplitude for the P2 component of K1 was derived from the same rings as for the RMS values. P2 amplitude was determined by a MATLAB routine that defined it as the difference between the peak of the 38- to 51-ms epoch and the trough of the 26- to 38-ms epoch.

Multifocal Visual Evoked Cortical Potential (mfVEP)

The mfVEP signals were recorded during the same testing session as the mfERG from two subdermal needle electrodes, each inserted approximately 1 cm lateral to the midline and just superior to the occipital ridge in order to place the electrode approximately overlying cortical area V1. The reference electrodes were inserted just lateral to the midline at the apex of the head, approximating the Cz placement in the international 10–20 system. The mfERG was recorded first in the first eye tested (either right or left depending on the scheduled counterbalancing) followed by the mfVEP from the same eye. The sequence was the same for the second eye tested.

The multifocal stimulus consisted of a seven-element stretched hexagonal array, each $\sim 20^\circ$, displayed across the $\pm 40^\circ$ of the central visual field. The horizontal diameter of the stimulus image was 28.1 cm and the longest diagonal was 29.4 cm. Because the viewing distance was 20 cm, this gives an uncorrected total viewing angle of 70.2° for the horizontal dimension and 72.6° for the greatest diameter. The low- and high-frequency filter settings were 1 Hz and 100 Hz, respectively. Further details of the visual stimulation procedure were given in a previous publication from this laboratory.⁴³ For the data analysis, the second-order kernel, first slice (K 2.1)

RMS amplitude of the 0- to 240-ms epoch of the central hexagon (from the experimentally glaucomatous eye) of the final mfVEP prior to euthanasia was plotted against the percentage of axon loss for each animal. The central element of the K2.1 mfVEP was chosen because it was found to have the most robust response with respect to signal-to-noise ratio in a previous study.⁴³

Microsphere Injection

Following an average of 400 days of elevated IOP (Table 1), regional ChBF was determined at the time of euthanasia. Approximately 10 million fluorescent polystyrene spheres (15.5 ± 0.42 μ m, mean $\pm$ standard deviation of diameter) (Invitrogen, Carlsbad, CA, USA) were injected. The process by which this was accomplished is described in a previous paper.³⁷ Briefly, the animals underwent preanesthesia with ketamine (10–15 mg/kg, intramuscular [IM]) before being intubated and anesthetized with an oxygen/isoflurane mix (3%–5% isoflurane for induction, 1%–3% isoflurane, or to effect, for maintenance) in order to sustain a deeper level of anesthesia during the procedure. Buprenorphine (0.01–0.03 mg/kg IM also was administered soon after isoflurane induction in advance of incisional procedures. A bilateral cut down of the femoral arteries was performed to place two intra-arterial catheters; one catheter was used to collect reference blood samples, while the other was connected to a continuous intra-arterial blood pressure monitor and used to collect samples for analyzing blood chemistry in assessing physiological stability. Arterial blood gas and chemistry samples were collected immediately after the intra-arterial catheters were placed, after the chest cavity was opened, and after the completion of the fluorescent microsphere injection but before euthanasia. All arterial blood gas and chemistry parameters were determined using an i-Stat blood gas analyzer (Abbott Point of Care, Princeton, NJ, USA). An incision was made along the ventral midline of the animal from approximately the area of the sternum toward the level of the diaphragm. A rib spreader was used to retract the rib cage to permit visualization of the heart. A direct injection of the microspheres into the left ventricle of the heart was carried out over a period of approximately 40 seconds. During the injection and for 2 minutes immediately following it, an arterial reference blood sample (1 mL/min) was collected. The animals were immediately euthanized with an overdose of pentobarbital sodium (>50 mg/kg) injected directly into the heart or intra-arterially. One animal (Rh1) underwent perfusion fixation by flushing of phosphate-buffered saline through the left ventricle followed by perfusion with 2% glutaraldehyde and 2.5% paraformaldehyde (because the tissue was subsequently used for electron microscopic studies). The eyes were then enucleated and stored in the same fixative at 4°C and then transferred to 0.1 M sodium phosphate buffer after 1 day for storage at 4°C . The remaining four animals also underwent flushing with phosphate-buffered saline through the left ventricle, but the eyes were enucleated prior to perfusion with fixative. They were promptly immersed in 4% paraformaldehyde for 1 day at 4°C and then stored at 4°C in 0.1 M sodium phosphate buffer.

There was concern that the anesthetic dosage required to perform the terminal procedure (thoracotomy and injection of microspheres) could affect the IOP. Also, IOP in the nonhuman primate chronic experimental glaucoma model may be highly variable from week to week. Therefore, IOP was set manometrically to 15 mm Hg in the left control eyes and to 35 mm Hg in the experimentally glaucomatous right eyes. Once the animal was anesthetized, a 25-gauge cannula was inserted using a trocar into the anterior chamber of each eye.

The cannula/trocar system is from a small-gauge vitrectomy pack (Alcon, Fort Worth, TX, USA). Each cannula is connected to IV tubing and an open-topped 20-mL syringe affixed to a ring stand. The syringe is partially filled with balanced salt solution and the height of the fluid level adjusted such that the IOP is at the desired level. The cannulae remain in both eyes and the IOPs are maintained at a constant level until at least 2 minutes after the microspheres are injected.

Choroidal Blood Flow Measurement

Although we and others refer to ChBF, the microspheres all come to rest in capillary beds. Therefore, this procedure actually measures blood flow in the choriocapillaris.

The total number of microspheres in the reference samples was calculated by manually counting the spheres in 40- μ L aliquot samples from the well-agitated (by means of sonication and vortexing) collection tube. The aliquots were placed on large microscope slides and coverslipped. An epifluorescence microscope (Olympus BH2; Olympus America, Inc., Melville, NY, USA) was used to visualize the spheres. A sufficient number of aliquots were used that at least 400 microspheres were counted per sample. Assuming that the liquid in the collection tube was 1 mL/g, the total number of microspheres in the reference sample (S_r) was calculated as $S_r = S_a(V_t/V_a)$, where S_a is the total number of microspheres in the aliquots, V_t is the volume of liquid in the collection tube, and V_a is the total volume of all of the aliquots used for counting.³⁷

The anterior segment of the globe was removed and the retina carefully dissected away from the choroid and retinal pigment epithelial layer. Potassium permanganate and oxalic acid were used to bleach the choroidal and RPE melanin.³⁷ Six radial incisions were made through the peripheral choroid and sclera, and the tissue was coated with 2.5% hydroxypropyl methylcellulose before flattening between two glass slides. An epifluorescence microscope was used to obtain digital images. The x - and y -coordinates of each sphere were obtained using NIH ImageJ (National Institutes of Health, Bethesda, MD, USA). The total number of microspheres in each eye was used to derive the total ChBF according to the formula $ChBF = R(S_i/S_r)$, where R is the rate at which blood was withdrawn for the reference sample, in microliters per minute; S_i is the total number of spheres in the eye, and S_r is the total number of spheres in the reference sample.³⁷ This was compared to ocular perfusion pressure at the time of microsphere injection, which was defined as $2/3$ of the mean arterial pressure (MAP) minus IOP where $MAP = [(2 \times \text{diastolic}) + \text{systolic}]/3$.

Regional blood flow was determined by mathematically dividing the choroidal surface into 0.25-mm (0.0625 mm²) bins using a task-specific routine written in MATLAB (The MathWorks, Inc.). The formula was similar that used for total ChBF: $ChBF = R(S_b/S_r)$, where S_b is the number of spheres in each bin. Three-dimensional plots of regional ChBF were created. A three-dimensional smoothing function was applied (locally weighted scatter plot smooth [LOESS], fourth-order polynomial) to produce a contour graph using Sigma Plot 11.0 (Systat Software, Inc., San Jose, CA, USA). Because blood flow is being measured in the choriocapillaris and not the entire thickness of the choroid, the flow units are expressed per unit area and not volume (μ L/min/mm²), which can be thought of as flux, that is, the blood flowing through a given area of tissue over time.

To compare the regional variations between the ChBF of the control left eyes and experimentally glaucomatous right eyes, the number of microspheres in each bin of the right eye was multiplied by the ratio of total spheres in the left eye to the total spheres in the right eye. Doing so provided a linearly scaled (normalized) plot of the right eyes that when subtracted

from the left eyes would give a meaningful picture of the regional differences beyond the often-marked overall change in ChBF. Prior to plot subtraction, the plot of the right eyes was rotated and flipped such that the optic nerves and the fovea of the right eyes could be superimposed on the left eyes.²⁵

The number of microspheres injected was chosen based on our past experience³⁷ with this model, to strike a balance between the possible inhibitory effect of the first microspheres passing through the choriocapillaris blocking subsequent microspheres and having enough microspheres in the eyes to determine regional differences. If this phenomenon were important, it would affect the control eyes (with the larger number of expected microspheres) more than the experimentally glaucomatous eyes and, thus, reduce any apparent differences between the control and experimental eyes. Therefore, any differences we do see should be at least as great as or greater than the true differences in blood flow.

Axon Counting in Optic Nerve Cross Sections

Segments of the optic nerves of each eye were cut 2 mm posterior to the back of the globe. Each segment was approximately 2 mm in length. Shallow radial orientation cuts were made—a single cut at the 12 o'clock meridian and two parallel cuts on the nasal side. The optic nerve segments were then postfixed in glutaraldehyde and osmium tetroxide and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, USA). Coronal sections of the nerves were then cut 1 μ m thick, mounted on glass slides, and stained with paraphenylenediamine. The optic nerve cross sections were examined by light microscopy. A semiquantitative grading scheme based on that described by Chauhan et al.⁴⁴ as modified for nonhuman primate nerves¹⁵ was used for estimating axonal loss.

This method of axon counting has several advantages. As with the other measurements in this study, we are interested in relative differences, not absolute numbers. The number of axons can vary by a factor of 2 $\times$ between individuals. Counting all of the axons (whether by hand or computerized image analysis) is prone to error. Sampling is not valid in glaucoma models because the axonal loss is so patchy. We chose the modified Chauhan method because it is rapid, takes into account the entire nerve, and directly compares right (experimental) and left (control) eyes.

RESULTS

Variable Retinal Ganglion Cell Axon Loss

Despite the similar IOP profiles for the five animals, there was marked inconsistency in the number of surviving RGC axons. The percentage loss varied from 100% to 10% (Table 1; Fig. 1). Rh4, in particular, seemed to be an outlier with only a 10% RGC loss but an IOP history that was not noticeably different from that of the other animals. Figure 2 (top) shows graphically the poor correlation between IOP $\times$ Days (i.e., the area under the IOP curve) and the amount of axonal loss. By contrast, the anatomy and function (as determined by mfVEP) were highly correlated (Fig. 2, bottom).

Retinal Electrophysiology

As was the case in our previous study with a different set of animals,¹⁵ we found a supranormal response in the early K1 mfERG waveforms (N1 and P1) but a decrement in the later P2 amplitude (Fig. 3). The unfiltered traces were from the central stimulus hexagon (ring 1) of Rh3 of the control left eye and the experimentally glaucomatous right eye. During this session, the right eye was tested first. There was likely a small decrease

FIGURE 1. Comparison of optic nerve cross sections and intraocular pressure (IOP) profiles from the control left eye of animal Rh2 and the right eyes that had undergone laser trabecular destruction (LTD). In the graphs, the *white circles* indicate the IOP of the control left eye, the *black circles* the IOP of the experimentally glaucomatous right eye. Despite the similarity in the IOP profiles in terms of total days and mean IOP levels, there is marked variability in the number of surviving axons. Epon sections (1 μ m) stained with paraffin-phenylenediamine (PPD). Scale bars: 500 μ m (bottom left frame); 25 μ m (bottom center frame).

FIGURE 2. *Top:* IOP $\times$ Days versus percentage of axonal loss for a particular animal ($n = 5$). IOP $\times$ Days (i.e., area under the curve, Fig. 1, *right*) is not predictive of the amount of axon loss in the optic nerves of the experimentally glaucomatous eyes. *Bottom:* K2.1 RMS amplitudes for the 0- to 240-ms epoch of the central hexagon of a seven-element mfVEP versus percentage of axonal loss for the same animal's data plotted in the *top graph*. The mfVEP measurements were the final determinations prior to euthanasia. This functional measurement is highly correlated with anatomic loss of axons in the optic nerves of the experimentally glaucomatous eyes. The *left inset* shows the waveform of the control left eye (*light line*) and glaucomatous right eye (*heavy line*) of Rh4 (10% axon loss, *left arrow*) and Rh1 (100% axon loss, *right arrow*).

FIGURE 3. Representative unfiltered individual K1 mfERG traces from the central ring at one time point (52 days) following LTD in one animal (Rh3). Comparing the control (OS) eye with the experimental glaucoma (right) eye shows that, in experimental glaucoma, the N1 and P1 waveform features are exaggerated or supranormal, and the P2 amplitude is reduced. The 9- to 35-ms epoch used for the N1-P1 RMS calculations is illustrated by the *heavy line*.

FIGURE 4. *Top:* Mean K1 RMS amplitudes for the 9- to 35-ms epoch for all six rings of each study animal versus percentage of axonal loss for that particular animal. The data are shown as a ratio of the right and left eyes during the period of high IOP (denoted by the subscript "h") and the baseline ratio (denoted by "b"). The means of all available data are included along with the ± 1 standard error of the mean (SEM). Note that all of the animals show a supranormal response to high pressure but that there is no correlation with the amount of axon loss. *Bottom:* Mean P2 amplitudes (K1) for all six rings of each study animal versus percentage of axonal loss for that particular animal. The data are shown as a ratio of the right and left eyes during the period of high IOP (denoted by the subscript "h") and the baseline ratio (denoted by "b"). The means of all available data are included along with ± 1 SEM. Note that all of the animals exhibited decreased P2 amplitudes. Although the P2 ratio and axon loss are highly correlated, the regression line does not pass through unity for no axon loss (unlike the mfVEP, Fig. 2). For example, the eye with only a 10% loss showed a marked reduction in the P2 ratio.

in overall signal amplitude by the time the left eye was tested, which is the rationale for counterbalancing the testing. That is, the first eye to be tested was alternated with each session.

The mean 9- to 35-ms K1 RMS of all six rings from all of the testing sessions during the period of elevated IOP was compared to the baseline measurements (Fig. 4, top). (The 9- to 35-ms K1 RMS is fairly stable during the period of elevated IOP as was demonstrated in our previous study.¹⁵) The ratio of the right to left responses after LTD was compared to the ratio of responses at baseline. Similar to what we found in an earlier study with a different set of animals,¹⁵ all of the animals showed a supranormal response in these early waveforms. However, there did not seem to be a correlation between the degree of RGC loss and level of supranormality.

A similar analysis comparing the ratio of K1 amplitudes of P2 versus axonal loss (Fig. 4, bottom) showed marked decrement in the P2 waveform. Although there is a high correlation between P2 ratio and % axon loss, the regression

FIGURE 5. *Top:* Mean K1 RMS amplitudes for the 9- to 35-ms epoch for all of the study animals versus mfERG ring (ring 1 being the central stimulus hexagon). The data are shown as a ratio of the right and left eyes during the periods of high IOP (denoted by the subscript “h”) and baseline IOP (denoted by “b”). The means of all available data are included along with ± 1 SEM. A supranormal response is evident in all of the rings, with a statistically greater ratio in rings 1 and 2 compared to the more peripheral rings ($P < 0.007$). *Bottom:* Mean P2 (K1) amplitudes for all of the study animals versus mfERG ring (ring 1 being the central stimulus hexagon). The data are shown as a ratio of the right and left eyes during the periods of high IOP (denoted by the subscript “h”) and baseline IOP (denoted by “b”). The means of all available data are included along with ± 1 SEM. A marked reduction in P2 relative to the control eyes is seen in all of the rings.

line does not intersect with the origin (i.e., 1.0 ratio at 0% axon loss). For example, R4, the animal with only a 10% axon loss, had a greater reduction in P2 than might be expected if P2 reflected only axon loss.

Averaging the individual rings from all of the animals and plotting them against the interocular response ratios of the elevated and baseline IOP periods (Fig. 5, top) shows that the K1 N1-P1 supranormal effect was similar throughout the stimulated areas of the retinae. A significantly greater effect was present in rings 1 and 2, that is, the rings corresponding to the macula, compared to rings 3 to 6 ($P < 0.007$). For P2 amplitude (Fig. 5, bottom), the interocular response ratios were markedly decreased everywhere with no obvious regional effect with retinal eccentricity (“ring” analysis), given the somewhat more variable nature of the later waveforms (as indicated on the graph by the larger standard errors of the mean [SEM]) compared to the N1-P1 RMS (Fig. 5, top).

FIGURE 6. Comparison of total choroidal blood flow as a function of ocular perfusion pressure. Each line represents a single animal. The left (control) eyes are shown as open symbols, and the right (experimental glaucoma) eyes are indicated by the solid symbols. The mean and ± 1 SEM are shown as the red line and red symbols. The data are consistent except for the control eye of Rh1 (see text).

Choroidal Blood Flow

Total ChBF was, on average, markedly decreased in the right eyes with experimental glaucoma when compared to the left control eyes (Fig. 6). One exception was Rh1, the animal with the greatest loss of RGC axons. Rh1 had a similar blood flow in both eyes. However, the outlier was not the eye with experimental glaucoma, within which ChBF was similar to that of the glaucomatous eyes of the other animals, but the control left eye, which had the lowest total ChBF of all of the control eyes.

Regional ChBF analysis showed that the decreased ChBF in the experimentally glaucomatous eyes (the four eyes that had such a decrease) was widespread (Fig. 7, top frames). To determine if the decrease was everywhere uniform, the regional ChBF plots of the right eye of each animal were linearly scaled (see Methods) to equal the total flow of the left eye. The plots were then rotated, flipped, and superimposed. As can be seen in Figure 7 (bottom frames), the X and Y projections of the Z-axes for the two eyes were not exactly the same. To better illustrate this, the left eye plot was subtracted from the linearly scaled right eye plot, and the difference was shown on a two-dimensional plot with color indicating the regional differences (Fig. 8, top five frames). Despite the normalization, the interocular differences in some locations were up to 20% of the ChBF for that region. There was also considerable variability within an animal and between animals. In four of the five animals, there was a relatively greater decrease in regional ChBF in the macula; but in Rh4 (the animal with the least RGC axon loss), there was a relative increase in macular ChBF (note that there is everywhere a decrease in this animal, but the “increase” is seen after being linearly scaled to equalize total ChBF between the eyes).

Even with the Rh4 outlier, averaging the plots from all five animals showed a relatively greater decrement in mean ChBF of the maculae (Fig. 8, bottom left frame).

DISCUSSION

Variable Response to Elevated IOP

Although the pressure profiles of the five animals in this study were similar in terms of duration, mean IOP elevation, and area under the curve (Fig. 1), there was an unexpected markedly

FIGURE 7. *Top frames:* Three-dimensional (3D) contour map of regional choroidal blood flow (of animal Rh2) in the left (control) and right (experimentally glaucomatous) eyes. The IOPs at the time of euthanasia were manometrically set at 15 and 35 mm Hg, respectively. Note that the blood flow is markedly reduced everywhere in the glaucomatous eye. *Bottom frames:* *Left graph:* 3D contour maps of regional choroidal blood flow of the two eyes shown in the *top frames* in which the graph of the right eye has been flipped, rotated, and superimposed on that of the left eye. The two-dimensional (2D) projections show a markedly reduced flow in the right (*blue curve*) eye compared to the left (*red curve*). *Right graph:* Similar to *left graph* except that the total blood flow of the right (glaucomatous) eye has been linearly scaled to match that of the left (control) eye. Even though the total normalized flow is equal in the two eyes, the 2D projections do not align perfectly, showing that reduction in blood flow in experimental glaucoma is not proportional throughout the eye. (The *peak of each curve* corresponds to the location of the fovea, and the *shoulder (small plateau to the left of the peak)* corresponds to the optic nerve.

uneven response in terms of optic nerve axonal loss—varying from only 10% to 100% (Fig. 2, top). We considered the possibility that some of this variation might be due to a dissociation between axon number and function. For example, in the animal with only a 10% percent axonal loss, it is possible that the remaining 90% of axons, although visible histologically, were non- or partially functional. To test this hypothesis, we analyzed the central hexagon of the seven-element mfVEP⁴² that was recorded at the same time as the mfERG. Since we were comparing this with postmortem anatomy, we looked at the final mfVEP obtained prior to euthanasia. As is evident from Figure 2 (bottom), the anatomic measure (axon loss) was indeed highly correlated with function as measured by the mfVEP. It is still not clear why the animals should have highly variable rates of axon loss in response to IOP elevation.

Global Effect on mfERG

An exaggerated or supranormal N1-P1 (9–35 ms, K1 RMS) mfERG response was observed in all five animals, similar to what was observed with a different set of animals in a previous study.¹⁵ However, the supranormal response was not dependent on the amount of axonal loss (Fig. 4, top). This is to be expected since the supranormal response was observed to return to normal in the earlier study following IOP lowering by trabeculectomy.¹⁵ Furthermore, a supranormal N1-P1 response was not observed in a previous study following physical removal of the RGCs by endodiatheirmy axotomy.²⁵ It is likely that these early waveforms are driven by the

photoreceptor and bipolar cell responses⁴⁵ and thus would be largely independent of the presence or absence of RGCs.

The underlying mechanism for a supranormal mfERG response is unknown. Figure 3 appears to show decreased high-frequency components (oscillatory potentials [OPs]) in the experimentally glaucomatous eye. However, in our previous study, the high-frequency components (80–120 Hz) were filtered out, yet the supranormal response persisted.¹⁵ Therefore, the supranormality cannot be explained solely by decreased OPs.

It should be noted that Hood et al.^{45,46} injected tetrodotoxin (TTX) into the vitreous cavity of otherwise normal rhesus eyes and found an increase in amplitude of the early mfERG waveform components. Why TTX but not surgical axotomy should produce a supranormal response is not clear. Perhaps the TTX has effects on the retina that go beyond simply stopping action potentials.

The later P2 waveform was markedly reduced in all of the experimentally glaucomatous eyes (Fig. 4, bottom). Although P2 reduction is highly correlated with axon loss, this correlation does not appear to be completely linear in that only a mild loss of axons (as with animal Rh4) results in a marked reduction in P2. This contrasts with the mfVEP/axon relationship (Fig. 2, bottom). Decreased P2 and P3 amplitudes have been observed following optic nerve transection in the pig⁴⁷ and decreased P2 after endodiatheirmy hemiaxotomy in cynomolgus monkeys.²⁵ Hare et al.⁴⁸ reported that (in cynomolgus monkeys) K1 mfERG macular amplitudes of N1, N1-P1, and P1-N2 are greater for the ocular hypertensive eye, although the amplitude of N2-P2 is smaller. And although

FIGURE 8. *Top five frames:* After linear scaling (normalization) of blood flow to be equal in the right and left eyes and flipping/rotating the graphs to align (see Fig. 7), the plot of the left (control) eye is subtracted from the normalized plot of the glaucomatous eye. The result shown here is then plotted, with the *vertical (color) scale* indicating flow differences. The *ovals* indicate the location of the optic nerves, and the *plus signs* denote the location of the foveae. Marked regional variation in the normalized choroidal blood flow is evident both within each animal and among animals. In four of the five animals, there is a relatively greater decrease in flow in the macula, but in Rh4 there is an increase in the normalized macular blood flow. *Bottom two frames:* Mean and standard deviation plots of the five animals shown in the *top five frames*. On average, there is a moderately greater relative decrease in macular ChBF in the glaucomatous eyes after being linearly scaled to make total blood flow in the right and left eyes equal. However, the actual blood flow is markedly decreased in the glaucomatous eyes (see Fig. 7, *top*). The *ovals* show the location of the optic nerves, and the *plus signs* indicate the location of the fovea.

specific mention of P2 reduction is not made, review of the figures in publications by Hood et al.,⁴⁶ Frishman et al.,⁴⁹ and Raz et al.^{50,51} suggest P2 decrements between the experimentally glaucomatous and fellow control monkey eyes.

Regional Differences in mfERG Response

Supranormal N1-P1 responses were present throughout the retina (Fig. 5, top). However, the effect was significantly greater in the central (macular) two rings compared to the peripheral four rings ($P < 0.007$). This greater supranormality in the central two rings could not be explained by cone density since the analysis was done using ring ratios. One possible explanation is that the central macular cones are relatively more affected than those in the periphery, which might be due to the relatively greater decrease in ChBF in the maculae of most of the animals in this study (see discussion in “Regional Differences in ChBF”).

P2 amplitude was markedly decreased in all of the rings with no obvious pattern with respect to eccentricity (Fig. 5, bottom). As noted in the previous Discussion section, P2 amplitude likely is related to RGC loss, but the correlation is not strong and there is considerable variability.

Global Reduction in ChBF

Four of the five animals showed a marked reduction in total ChBF in the experimentally glaucomatous eyes when compared to the fellow control eyes (Fig. 6). Our results are similar to those of earlier studies by Alm and Bill^{30,52} using radioactive nonrecirculating microspheres in a model of acutely (manometrically) elevated IOP in normal cat and cynomolgus monkey³⁰ eyes. In addition to being a study of acutely elevated IOP, their study also differed from ours in that they looked at the same eye at both high and low IOP using microspheres with two different radionuclides. As in our experiment, they also had an outlier, that is, a single animal that did not have a change in ChBF despite a marked reduction in ChBF with increased IOP in all of their other subjects. The reason why one of our animals did not respond as the others is unknown. Uneven mixing of the microspheres seems unlikely since the distribution of the spheres within both the control and experimental eyes was similar for all of the other eyes in the study. Perhaps the blood flow in the two eyes was different at baseline, but there would be no way to determine that using microspheres because it is a nonsurvival procedure.

When designing this experiment, a decision was made to manometrically control the IOP in the eyes at the time of

TABLE 2. Analysis of Total and Peripapillary ChBF

Animal	Total ChBF		PP ChBF		Total OD/OS	PP OD/OS	T PP OD/OS	N PP OD/OS
	OD	OS	OD	OS				
Rh1	457	437	56	70	1.05	0.80	0.82	0.78
Rh2	500	1104	84	198	0.45	0.42	0.40	0.45
Rh3	613	908	78	144	0.68	0.54	0.58	0.49
Rh4	425	902	79	155	0.47	0.51	0.51	0.51
Rh5	408	1116	48	187	0.37	0.26	0.25	0.26
Mean	481	893	69	151	0.60	0.51	0.52	0.50
SEM	41	137	8	25	0.14	0.10	0.11	0.09
<i>P</i>	<0.016		<0.009		0.12		0.12*	0.14*

ChBF, choroidal blood flow; PP, peripapillary; T, temporal; N, nasal.

* *P* values are comparisons to total OD/OS.

microsphere injection. This was because we were most interested in global as well as in regional ChBF of these experimentally glaucomatous eyes in an environment similar to that experienced for the previous 400 days. Without the manometers, the IOP could have been unusually high or low due to daily fluctuations and/or anesthetic effects. A previous study by Alm et al.⁵³ showed that if the IOP was manometrically lowered to normal levels in cynomolgus monkey eyes with a history of chronic LTD-induced glaucoma prior to injecting the microspheres, then the total blood flow was similar to that in the control eyes. That is, the effects of chronic IOP elevation on the global ChBF were reversible. Our results show that the global ChBF does not return to normal in chronic experimental glaucoma despite the elevated IOP. That is, there does not seem to be a compensatory mechanism that permits the choroid to overcome the effects of chronic glaucoma. However, we have not ruled out the possibility that it may do so for periods of IOP elevation longer than 400 days.

Our results are similar to those of the acute studies showing decreased ChBF when the IOP is manometrically elevated in both cat³¹ and monkey eyes.³⁰ However, under these same experimental conditions, there is little change in retinal blood flow, which led investigators to conclude that the retinal circulation can autoregulate but the choroidal circulation does not. More recent work by Kiel et al.^{54–56} has shown that the choroid does have the ability to autoregulate (at least in rabbits) when the MAP is controlled and the IOP is held constant. Another possible influence on ChBF might be hypoxia. As the ChBF decreases, the oxygen demands of the retina would increase PaCO₂. Even so, studies by Wang et al.⁵⁷ in rats and Milley et al.⁵⁸ in newborn lambs suggest that the ChBF is relatively stable as a function of PaCO₂ levels. Likewise, Friedman and Chandra⁵⁹ observed only a slight increase in ChBF when cats were made hypercapnic. In our study, the MAP was the same in both the control and experimental eyes since they were in the same animal. Although, to our knowledge, PaCO₂ variation has not been estimated in monkeys, it seems reasonable to conclude that the primary factor affecting ChBF in monkeys is the decreased perfusion pressure caused by elevated IOP.

Peripapillary ChBF

Detailed histomorphometry by Yang et al.⁶⁰ and subsequent in vivo high-resolution OCT measurements by Strouthidis et al.⁶¹ have documented posterior deformation and thinning of the peripapillary sclera in cynomolgus and rhesus monkeys, respectively. We were interested in learning if such morphologic changes affect ChBF in the peripapillary to a greater or lesser degree than in the eye as a whole. However, comparison of the right-to-left ratios of total ChBF with peripapillary ChBF

did not reveal a statistically significant difference (Table 2), although there was a trend toward a greater reduction of ChBF in the peripapillary region. Perhaps a larger number of animals would reveal a mildly greater peripapillary effect. Similarly, dividing the peripapillary zone into nasal and temporal halves did not show that either area had a statistically significant relative decrease compared to the total ChBF.

Regional Differences in ChBF

As noted above, four of the five animals had a marked global decrease in the experimentally glaucomatous eyes compared to the fellow eyes. However, this decrease was not everywhere uniform. When the total blood flow in the experimentally glaucomatous right eye was linearly scaled to equal the control left eye of each animal and then rotated and subtracted (control subtracted from experimental glaucoma eye), distinct regional variations were noted in all of the animals (Fig. 8, top five frames). Considering that the IOP must be everywhere the same in the eye, it seems logical that the regional variation in blood flow is likely due to regional vessel compliance, which differs among the subjects.

Although the pattern of regional differences was quite variable, four of the five animals had relatively greater decreases in ChBF in the macular region. The one exception was Rh4, in which there was relatively less decrease in the macular region (Fig. 8, lower left of top five frames). Rh4 was also an outlier in that this animal had markedly less axonal loss than the others. Whether these two points are related cannot be determined with such a small sample size.

As is the case with global ChBF, it is not possible from this study to determine if the variation in regional ChBF is fully reversible, that is, whether the regional changes we are seeing after 400 or so days of ocular hypertension would disappear if the IOP were lowered to a normal level. Likewise, we do not know if similar regional effects would be present in a model of acutely elevated IOP.

CONCLUSIONS

To our knowledge, this is the first demonstration of globally reduced ChBF in chronic experimental glaucoma in the nonhuman primate. Both the supranormality of the early mfERG waveforms and the reduction in ChBF tended to be relatively greater in the macula, suggesting that there may be a causal link between ChBF and outer retinal effects in glaucoma. Earlier studies have shown that RGC loss in both human glaucoma^{62–65} and experimental glaucoma in the nonhuman primate^{66,67} occurs throughout the eye, but perhaps to an even greater degree in the macula, despite the

fact that the earliest visual field defects in humans are in the arcuate and peripapillary regions.⁶⁸

As discussed above, the pressure-induced ChBF⁵³ reductions and supranormal mfERG¹⁵ are both reversible, even in advanced stages of experimental glaucoma in nonhuman primates. Although this suggests that they are independent of the loss of RGCs, it does not rule out the possibility that decreased ChBF and outer retinal ischemia create a noxious environment for RGCs in glaucoma. One possibility is a scenario wherein hypoxic photoreceptors fail to reuptake glutamate or the Müller cells inadequately process toxic glutamate into nontoxic glutamine that results in RGCs, via their N-methyl-D-aspartate (NMDA) receptors, undergoing excitotoxic stress (e.g., Russo et al.⁶⁹). Many additional potential pathways for RGC toxicity in glaucoma have been proposed in what is most likely a multifactorial disease (see Osborne⁷⁰). Given the dramatic changes in ChBF resulting from increased IOPs observed in the present study, it is likely that such indirect choroidal/outer retinal mechanisms also play a role in exacerbating glaucomatous injury.

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