Time-Dependent Effects of Elevated Intraocular Pressure on Optic Nerve Head Axonal Transport and Cytoskeleton Proteins


PURPOSE. To study the time-dependent effects of elevated intraocular pressure (IOP) on axonal transport and cytoskeleton proteins in the porcine optic nerve head.

METHODS. Fifteen pigs were used for this study. Rhodamine-β-isothiocyanate was injected into the vitreous of each eye to study axonal transport. IOP in the left eye was elevated to 40 to 45 mm Hg, and IOP in the right eye was maintained between 10 and 15 mm Hg. Cerebrospinal fluid pressure was also continually monitored. IOP was elevated for 3 hours (n = 7) or 12 hours (n = 8) before animal euthanatization. Antibodies to phosphorylated neurofilament heavy (NFHp), phosphorylation-independent neurofilament heavy (NFH), neurofilament light, neurofilament medium (NFM), microtubule, and microtubule-associated protein (MAP) were used to study the axonal cytoskeleton. Confocal microscopy was used to compare axonal transport and cytoskeleton change between control and high IOP eyes in different laminar regions and quadrants of the optic nerve head. Results from these experiments were also compared with 6-hour elevated IOP data from an earlier study.

RESULTS. Three hours of IOP elevation caused a decrease in NFH, NFHp, and NFM within laminar regions, with no demonstrable change in axonal transport. Changes to MAP and microtubules were only seen after 12 hours of IOP elevation. Axonal transport change occurred in a time-dependent manner with peripheral nerve bundle changes occurring earlier and being greater than central nerve bundle changes.

CONCLUSIONS. Time-dependent changes in axonal transport and cytoskeletal structure in the optic nerve head provide further pathogenic evidence of axonal damage caused by elevated IOP.

Elevated intraocular pressure (IOP) is the greatest known risk factor for glaucoma, the second most common cause of blindness worldwide. A rise in IOP leads to an elevated pressure gradient across the lamina cribrosa, predisposing the retinal ganglion cell (RGC) axons to damage at this site. Previous studies have demonstrated axonal transport abnormalities in the lamina cribrosa region after a rise in IOP, with some authors observing peripheral nerve changes to be greater than central nerve changes. This suggests that a rise in IOP has a nonhomogeneous effect on the optic nerve head.

The RGC axonal cytoskeleton is composed mostly of neurofilaments, microtubules, and microtubule-associated proteins (MAPs) and provides the scaffolding along which motor proteins involved in axonal transport mobilize. In addition to providing a conduit for the axonal transport process, changes to the RGC cytoskeleton can potentially act as surrogate markers for axonal injury and degeneration. Primate models of ocular hypertension and glaucoma have shown a reduction in neurofilament subtypes in regions of optic nerve injury. In the rat optic nerve, the process of Wallerian degeneration is also characterized by a time-dependent change in cytoskeletal structures within the RGC axons.

Using techniques with a reliable degree of reproducibility, we have previously reported simultaneous changes to axonal transport and the RGC cytoskeleton in the porcine optic nerve head after 6 hours of IOP elevation. An IOP of 40 to 45 mm Hg for 6 hours caused a heterogeneous reduction in axonal transport within nerve bundles, with an uneven degree of transport inhibition in the different laminar regions and quadrants of the optic nerve head. These axonal transport changes were associated with a homogenous and selective change to neurofilament subtypes, with changes to the cytoskeleton similar in all regions of the optic nerve head. Experimental models of optic neuritis have demonstrated a time-dependent change to neurofilaments and microtubules in regions of demyelination and neural injury, but this has not previously been demonstrated in an elevated IOP model.

The purpose of this study was to determine whether axonal transport and cytoskeleton changes induced by elevated IOP were dependent on the duration of the IOP insult. Using an experimental model with continuous IOP, cerebrospinal fluid pressure (CSFp), and blood pressure monitoring, we studied axonal transport and cytoskeleton changes within the optic nerve head after brief (3 hours) and prolonged (12 hours) periods of IOP elevation at 40 to 45 mm Hg. We studied changes within the different laminar regions and quadrants of the optic nerve head. The results of these experiments, taken together with our previous study on the effects of 6 hours of IOP elevation, have allowed us to describe the temporal sequence of axonal transport and cytoskeleton change in the optic nerve after a rise in IOP. Changes to the RGC cytoskeleton may contribute to axonal transport abnormalities occurring in glaucoma; alternatively, cytoskeletal change may be an independent process that simply identifies regions of axonal injury. In this study, we investigated axonal transport vulnerability within optic nerve head regions after variable periods of IOP rise. We also investigated the ability of different cytoskeleton components to tolerate an acute IOP rise and defined the early and late changes to cytoskeletal subunits within the optic nerve head. The concurrent study of axonal transport and...
cytoskeletal change has also allowed us to address their relationship after various periods of IOP rise.

**MATERIALS AND METHODS**

**General**

Much of the methodology used in this work was identical to that in our previous report. Fifteen animals were used for this work. These experiments were performed on 2 separate groups. The first group consisted of seven animals whose IOP was elevated for 3 hours before euthanatization. The second group consisted of eight animals whose IOP was elevated for 12 hours before euthanatization. Female White Landrace pigs weighing between 20 and 30 kilograms were used for all experiments. All experiments were conducted and all laboratory animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the University of Western Australia Animal Ethics Committee.

**Anesthesia**

For the 3- and the 12-hour experiments, the anesthetic protocol was the same. Anesthesia was administered by a veterinary anesthetist. Food was withheld for 12 hours and water for 0.5 hours before the experiment. Anesthesia was induced with an intramuscular injection of tiletamine/zolazepam 4.4 mg/kg (Zoletil 100 mg/mL; Virbac, Peakhurst, NSW, Australia) and xylazine 2.2 mg/kg (Xylazil 100 mg/mL; Troy Laboratories, Smithfield, NSW, Australia). For our previous study, we used isoflurane for maintenance anesthesia. Preliminary work in our laboratory revealed that prolonged anesthetic time with isoflurane resulted in unacceptably high CSF pressure after 6 hours of anesthesia. For this reason we altered our maintenance anesthetic protocol. Anesthesia for 3- and 12-hour IOP experiments was maintained with constant-rate infusion of propofol 12 mg/kg/h (Fresofol 1%; Pharmatel Fresenius Kabi Pty Ltd, Hornsby, NSW, Australia) and fentanyl citrate 30 μg/kg/h (Fentanyl Injection 50 μg/mL, Mayne Pharma Pty Ltd, Mulgrave, VIC, Australia).

After induction, pigs were intubated and ventilated. Ventilator settings were adjusted to maintain end tidal carbon dioxide (Paco₂) tensions between 30 and 45 mm Hg, and oxygen and nitrogen flow rates were adjusted to maintain arterial oxygen tensions between 80 and 105 mm Hg. Arterial blood gas samples were collected every 120 minutes for measurement of carbon dioxide, oxygen, and pH levels. Normal body temperature was maintained with a thermal blanket placed beneath and above the pig. Plastic bubble wrap was also used at extremities to minimize heat loss.

Heart rate and blood pressure were continually monitored with an electrocardiograph and a femoral arterial line, respectively. To maintain diastolic pressure above 60 mm Hg, balanced isotonic fluid (Hartmann; Baxter Healthcare Pty Ltd, Toongabbie, NSW, Australia) was administered at 10 mL/kg/h, supplemented when necessary with dobutamine 1 to 5 μg/kg/h (dobutamine injection 12.5 mg/mL; Mayne Pharma Pty Ltd, Mulgrave VIC, Australia). Intravenous fluids and drugs were administered through a 22-gauge, 1-inch catheter in the auricular vein. Pancuronium (pancuronium injection BP 2 mg/mL; AstraZeneca, North Ryde, NSW, Australia) was given at 0.2 mg/kg intravenously, followed by a constant rate infusion of 0.3 mg/kg/h intravenously to create muscle relaxation.

**Surgical Procedures**

Two cannulae were placed in the lateral ventricles of the brain using stereotaxic coordinates for positioning. One cannula was attached to a chart recorder through a pressure transducer so that CSF pressure could be continually monitored and recorded. The second cannula was attached to an infusion of Ringer’s lactate so that IOP could be continually monitored and recorded. The second cannula was connected to a variable height infusion of Ringer’s lactate so that IOP could be adjusted. The right eye was used as the control eye with the IOP maintained between 10 to 15 mm Hg, and the left eye was designed as a high IOP model, with the IOP between 40 and 45 mm Hg for the 3-hour and the 12-hour experiments. All pressure transducers (P23 ID Gould Statham; Gould, Cleveland, OH) were calibrated before the experiment and held at eye level. They were connected through a conditioning module (Analog Devices, Norwood, MA) to a chart recorder (LR810; Yokogawa, Tokyo, Japan) to allow continuous recording of IOP, CSF pressure, and blood pressure.

Pupils were then dilated, and 100 μL freshly prepared 3% rhodamine-β-isothiocyanate (RTIC) was injected into the vitreous humor of each eye for the study of axonal transport. Animals in the first group were humanely killed by intravenous infusion of pentobarbitone 3 hours after RTIC injection. Animals in the second group were humanely killed through an identical technique 12 hours after RTIC injection.

**Tissue Preparation and Immunohistochemistry**

After euthanatization, eyes and optic nerves were immediately enucleated. Before sectioning, the dura was removed from enucleated tissue to expose the optic nerve. The retina and proximal 8 mm of optic nerve were then embedded in OCT and sectioned on a cryotome set at −30°C. To limit the potential tilting of sections, the exposed optic nerve was aligned parallel to the blade on the cryotome before cutting. Previously described measures were adopted to orientate sections and to ensure that the levels of the sections used for comparison of axonal transport and cytoskeleton studies between control and high IOP eyes were reliably matched. Specimens for axonal transport studies were stored immediately after sectioning and viewed under the confocal scanning laser microscope the following day. Remaining sections were either stained with Van Gieson stain to determine prelaminar and lamina cribrosa thickness or they were incubated with primary antibodies for the cytoskeleton study. Primary antibodies used to study the cytoskeleton were polyclonal antibody NF-L directed against the neurofilament light subunit (1:500, AHP286; Serotec, Oxford, United Kingdom), monoclonal antibody NF-M directed against the neurofilament medium subunit (1:200, N5264, clone N118; Sigma-Aldrich, St. Louis, MO), monoclonal antibody NF-H directed against the phosphorylated and the nonphosphorylated neurofilament heavy subunit (1:400, N0142, clone N52; Sigma-Aldrich), monoclonal antibody NF-Hp directed against the phosphorylated neurofilament heavy subunit (1:200, N5389, clone NE14; Sigma-Aldrich), monoclonal antibody Tub directed against isotypes I and II of β-tubulin (1:200, T8553, clone D3R; Sigma-Aldrich), and anti-MAP antibody (1:200, M7273; Sigma-Aldrich). After primary antibody incubation, slides were washed and incubated with either a goat anti–mouse IgG (1:400, Alexa Fluor 488; A11001; Molecular Probes, Portland, OR) or a goat anti–rabbit IgG (1:400, Alexa Fluor 488; A11008; Molecular Probes) secondary antibody. After this, sections were washed and mounted in glycerol before study under the microscope.

**Microscopy**

Gray-scale digital images of RTIC-labeled and cytoskeletal antibody-labeled tissue were captured with a confocal laser scanning microscope. An oil-immersion lens (PlanoApo 60× NA 1.4; Nikon, Tokyo, Japan) was used to view slides labeled with cytoskeletal antibodies, whereas a dry lens (20× NA 0.4; Nikon) was used to view RTIC-labeled tissue. With the aid of a motorized stage, a series of z-stacked images (7 slices/stack) was captured for each slide, beginning in the prelaminar region and extending through the postlaminar tissue. Individual z stacks were then stitched together to create a montage using Image J.
Quantitation of Cytoskeletal and Axonal Transport Differences

Quantitation of all axonal transport and cytoskeletal images was performed on a computer (Image Pro Plus, version 5.1; Media Cybernetics, Silver Spring, MD) in a randomized manner such that the observer was masked to control and high IOP images and to the time point analyzed.

Cytoskeleton

The methodology for quantitating cytoskeletal change was similar to that used in our previous report. In brief, individual slices from each montaged z-stack were analyzed separately. Neural tissue from each slice was first divided into three regions for analysis: the prelaminar region, the lamina cribrosa, and the proximal 200 μm of postlaminar tissue. For these experiments, we also divided each of the laminar regions into nasal, central, and temporal regions. Thus, a total of nine regions were analyzed for each slide, allowing us to study regional variations in cytoskeletal change. With the use of a quantitative histogram function, the mean pixel intensity in each of these regions was calculated and used for statistical analysis.

Axonal Transport

Axonal transport analysis was identical with that in our previous report. In brief, nerve bundles in each eye were followed from the prelaminar region to 3 mm behind the lamina cribrosa. Nerve bundles were divided into nasal, central, and temporal nerve bundles during sampling to allow analysis of regional differences in axonal transport. Average pixel intensity was calculated at specific points along each nerve bundle and expressed as percentage intensity of the prelaminar region.

Previous axonal transport study using radioactive tracer demonstrated a logarithmic decrease in tracer along the distal course of the axon. Mathematical models of axonal transport have also shown that a change in tracer quantity follows a logarithmic decrease down the axon, with an exponential decay function the most appropriate formula in calculating tracer distribution along the nerve. This implies that a relatively constant proportion of tracer is transported from one segment to the subsequent segment along the nerve. Hence, we calculated the proportional change in RITC intensity in each subsequent segment compared with that in the prior segment to identify segments with nonhomogeneous axonal transport. We calculated this by dividing the difference in RITC intensity between the sample point and that immediately preceding it by the distance separating these two points in millimeters. This result is expressed relative to the mean RITC intensity of these two points. This is represented in the formula below. We used the proportional change in RITC intensity as an index of segmental axonal transport:

$$\left| \frac{I_b - I_a}{D_b - D_a} \right| \left( \frac{I_a + I_b}{2} \right)$$

where $I_b$ is RITC intensity at distal point, $I_a$ is RITC intensity at proximal point, $D_b$ is distance of distal point from inner limiting membrane (ILM) (mm), and $D_a$ is distance of the proximal point from ILM (mm).

Statistical Analysis

All statistical analyses were performed with commercial software (SigmaStat, version 3.1; SPSS, Chicago, IL). Before analysis, Kolmogorov-Smirnov testing was used to determine whether data were normally distributed. Normally distributed data were analyzed using ANOVA with post hoc factor comparison performed using a paired Student’s t-test with Bonferroni correction. Non-normally distributed data were analyzed using ANOVA on ranks, with the Tukey test for post hoc paired analysis. Previously published data from 6-hour experiments were also used to make statistical comparisons among 3-, 6-, and 12-hour time points.

Physiological Variables and Optic Nerve Head Measurements

Factorial ANOVA was used to assess the effect of IOP (control or high), time of IOP elevation (3, 6, and 12 hours) and disc location (temporal, central, and nasal) on the parameters prelaminar thickness and lamina cribrosa thickness and the physiological variables blood pressure, control IOP, high IOP, CSFp, pCO2, pO2, and pH. This allowed us to determine whether there were significant differences in optic nerve head measurements and physiological parameters among the three groups.

Axonal Transport and Cytoskeleton

For the 3-hour and 12-hour experiments, factorial ANOVA was used to assess the effects of IOP (control or high), individual pig, nerve bundle location, (temporal, central and nasal), and position along the nerve (prelaminar, lamina cribrosa, postlaminar, or distance along the nerve in millimeters) on the parameters RITC intensity, proportional change in RITC intensity and cytoskeletal antibody intensity (a separate analysis was performed for each antibody). When comparing axonal transport data between 3-, 6-, and 12-hour groups, the control and high IOP data were analyzed separately. Peripheral (temporal and nasal data combined) and central nerve bundle data within the control and high IOP groups were analyzed independently. Factorial ANOVA was used to assess the effects of time of IOP elevation (3, 6, or 12 hours) and position along the nerve on the parameters RITC intensity and proportional change in RITC intensity.

RESULTS

Animal Physiology

Mean systolic blood pressure for all 15 pigs was $87.1 \pm 1.7$ mm Hg. Average arterial $pO_2$ was $100.8 \pm 2.8$ mm Hg, $pCO_2$ was $38.4 \pm 1.1$ mm Hg, and pH was $7.5 \pm 0.0$ on blood gas analysis. Mean CSFp was $4.2 \pm 1.5$ mm Hg. Average left- and right eye IOP was $42.6 \pm 0.4$ and $13.0 \pm 0.4$ mm Hg, respectively. Average differences between the IOP and CSFp in the left and right eyes were $38.4 \pm 1.1$ and $8.8 \pm 1.2$ mm Hg, respectively. Mean experimental data for individual pigs in the 3-hour and 12-hour group are presented in Table 1. Results from ANOVA testing revealed no statistical differences in blood pressure, CSFp, left eye IOP, right eye IOP, $pO_2$, $pCO_2$, and pH among the 3-, 6-, and 12-hour groups (one-way ANOVA; all $P > 0.169$).

Optic Nerve Head Measurements

The mean prelaminar thickness for all 15 pigs was $260.7 \pm 8.3$ μm. The mean temporal, central, and nasal lamina cribrosa thicknesses from all eyes were $366.2 \pm 8.5$ μm, $399.6 \pm 9.2$ μm, and $373.4 \pm 9.6$ μm, respectively. Mean lamina cribrosa thickness measurements in the nasal, central, and temporal regions in the control and high IOP eyes for each pig in the 3-hour and 12-hour groups are presented in Table 2. There was no significant difference in the prelaminar thickness and the lamina cribrosa thickness between the control and high IOP eyes in the 3-hour and 12-hour groups (two-way ANOVA; all $P > 0.110$).

Mean lamina cribrosa thickness in the control and high IOP eyes of 12-hour experiments was significantly higher than the 3- and 6-hour group (three-way ANOVA; $P < 0.001$). There was
Table 1. Physiology Data for Individual Pigs

<table>
<thead>
<tr>
<th>Pig</th>
<th>BP</th>
<th>Left</th>
<th>Right</th>
<th>CSFp</th>
<th>pO₂</th>
<th>pCO₂</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>88.0 ± 2.3</td>
<td>44.1 ± 0.6</td>
<td>13.0 ± 0.6</td>
<td>-0.1 ± 0.8</td>
<td>81.4 ± 11.6</td>
<td>36.2 ± 0.5</td>
<td>7.5 ± 0.0</td>
</tr>
<tr>
<td>3b</td>
<td>97.5 ± 2.3</td>
<td>40.8 ± 1.1</td>
<td>14.2 ± 0.6</td>
<td>9.7 ± 0.1</td>
<td>107.9 ± 18.5</td>
<td>37.5 ± 4.4</td>
<td>7.5 ± 0.0</td>
</tr>
<tr>
<td>3c</td>
<td>87.6 ± 1.4</td>
<td>41.9 ± 0.7</td>
<td>13.5 ± 0.2</td>
<td>0.8 ± 0.8</td>
<td>95.8 ± 2.5</td>
<td>37.7 ± 1.2</td>
<td>7.5 ± 0.0</td>
</tr>
<tr>
<td>3d</td>
<td>76.4 ± 1.8</td>
<td>40.2 ± 0.3</td>
<td>14.8 ± 0.2</td>
<td>-0.8 ± 1.1</td>
<td>116.1 ± 25.0</td>
<td>39.5 ± 6.6</td>
<td>7.5 ± 0.0</td>
</tr>
<tr>
<td>3e</td>
<td>95.1 ± 2.7</td>
<td>40.8 ± 0.5</td>
<td>13.1 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>106.6 ± 0.2</td>
<td>45.2 ± 3.1</td>
<td>7.4 ± 0.0</td>
</tr>
<tr>
<td>3f</td>
<td>92.1 ± 1.4</td>
<td>42.1 ± 0.4</td>
<td>10.8 ± 0.9</td>
<td>2.9 ± 0.3</td>
<td>103.5 ± 7.2</td>
<td>42.4 ± 1.9</td>
<td>7.4 ± 0.0</td>
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<tr>
<td>3g</td>
<td>92.3 ± 1.5</td>
<td>41.0 ± 0.8</td>
<td>13.1 ± 0.3</td>
<td>-0.6 ± 0.4</td>
<td>117.0 ± 10.8</td>
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<td>7.5 ± 0.0</td>
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<tr>
<td>12a</td>
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<td>87.0 ± 2.5</td>
<td>28.2 ± 1.1</td>
<td>7.5 ± 0.0</td>
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<tr>
<td>12b</td>
<td>79.5 ± 1.8</td>
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<td>86.9 ± 5.7</td>
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<td>12c</td>
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<td>102.0 ± 5.2</td>
<td>38.5 ± 1.2</td>
<td>7.5 ± 0.0</td>
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<tr>
<td>12d</td>
<td>91.2 ± 1.6</td>
<td>44.2 ± 0.3</td>
<td>14.2 ± 0.5</td>
<td>9.6 ± 1.1</td>
<td>110.4 ± 6.0</td>
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<td>12e</td>
<td>88.5 ± 1.1</td>
<td>42.8 ± 0.3</td>
<td>12.8 ± 0.2</td>
<td>4.6 ± 0.5</td>
<td>106.8 ± 4.6</td>
<td>37.2 ± 0.6</td>
<td>7.5 ± 0.0</td>
</tr>
<tr>
<td>12f</td>
<td>92.4 ± 1.1</td>
<td>44.6 ± 0.5</td>
<td>15.5 ± 0.5</td>
<td>9.8 ± 1.1</td>
<td>105.5 ± 10.7</td>
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<td>7.5 ± 0.0</td>
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<td>12g</td>
<td>80.7 ± 1.5</td>
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<td>10.0 ± 0.6</td>
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<td>88.2 ± 5.5</td>
<td>43.3 ± 1.5</td>
<td>7.4 ± 0.0</td>
</tr>
<tr>
<td>12h</td>
<td>85.6 ± 1.2</td>
<td>42.7 ± 0.2</td>
<td>11.3 ± 0.3</td>
<td>-5.4 ± 1.2</td>
<td>97.2 ± 3.5</td>
<td>42.2 ± 1.5</td>
<td>7.4 ± 0.0</td>
</tr>
</tbody>
</table>

no difference in mean lamina cribrosa thickness between the 3- and 6-hour animals (three-way ANOVA; P = 0.590). The difference in lamina cribrosa thickness between the control and high IOP eyes in the three groups was not significantly different (three-way ANOVA; P = 0.160). There was no statistically significant difference in the prelaminal thickness among the three groups (three-way ANOVA; P = 0.540).

Axonal Transport in 3- and 12-Hour Experiments. Figures 1 and 2 are representative images taken 3 hours and 12 hours, respectively, after RITC injection into the vitreous cavity. After 3 hours of RITC injection, there was little difference in the pattern of RITC stain between the control and high IOP eyes. Twelve hours after injection, RITC traveled past the lamina cribrosa into distal postlaminar tissue in the normal IOP eye. In the high IOP eye of the 12-hour animals, the intensity of RITC was observed to be greatest in the prelaminar and lamina cribrosa regions. Although some tracer was evident in the postlaminar tissue of high IOP eyes, RITC was seen to travel much further distally into the postlaminar tissue of control IOP eyes in the 12-hour group.

Axonal Transport within Control IOP Eyes. Figures 3 and 4 illustrate the change in RITC intensity in the temporal, central, and nasal nerve bundles at different points along the control IOP eyes in the 3-hour and 12-hour groups, respectively. In the 3-hour group, the change in RITC intensity in the lamina cribrosa of nasal and temporal nerve bundles was significantly less than that in central nerve bundles (three-way ANOVA; P < 0.001). In the 12-hour group, there was no significant difference in the change in RITC intensity among the three nerve bundles at any point along the nerve (three-way ANOVA; P = 0.805). In comparison with the prelaminar region, the change in RITC intensity in the lamina cribrosa was decreased in all nerve bundles in the normal pressure eye of the 3-hour and 12-hour groups (three-way ANOVA; all P < 0.019).

Axonal Transport within High IOP Eyes. Figures 3 and 4 illustrate the change in RITC intensity in the temporal, central, and nasal nerve bundles at different points along the high IOP eye in the 3-hour and 12-hour groups, respectively. In the 3-hour group, the changes in RITC intensity in the lamina cribrosa of temporal and nasal nerve bundles were significantly less than in the central nerve bundles (three-way ANOVA; P < 0.003). In the 12-hour group, there was no difference in the change in RITC intensity among the three nerve bundles at any point along the optic nerve (three-way ANOVA; P = 0.398) All nerve bundles in the 12-hour group and the nasal and temporal

Table 2. Laminar Thickness Measurements for Individual Pigs

<table>
<thead>
<tr>
<th>Pig</th>
<th>Nasal</th>
<th>Central</th>
<th>Temporal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control IOP</td>
<td>High IOP</td>
<td>Control IOP</td>
</tr>
<tr>
<td>3a</td>
<td>335.4</td>
<td>293.9</td>
<td>413.1</td>
</tr>
<tr>
<td>3b</td>
<td>428.1</td>
<td>437.6</td>
<td>421.3</td>
</tr>
<tr>
<td>3c</td>
<td>344.3</td>
<td>350.7</td>
<td>359.5</td>
</tr>
<tr>
<td>3d</td>
<td>391.3</td>
<td>414.6</td>
<td>399.9</td>
</tr>
<tr>
<td>3e</td>
<td>361.3</td>
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<td>3f</td>
<td>303.6</td>
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<td>3g</td>
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<tr>
<td>12c</td>
<td>328.3</td>
<td>338.2</td>
<td>355.3</td>
</tr>
<tr>
<td>12d</td>
<td>395.3</td>
<td>414.1</td>
<td>416.8</td>
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<tr>
<td>12e</td>
<td>435.9</td>
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<tr>
<td>12h</td>
<td>360.1</td>
<td>379.2</td>
<td>410.1</td>
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</table>
nerve bundles in the 3-hour group showed a significant difference in the change in RITC intensity among the prelaminar, lamina cribrosa, and postlaminar regions (three-way ANOVA; all \( P < 0.001 \)). Post hoc testing revealed that the change in RITC intensity was most reduced in the lamina cribrosa region of all nerve bundles in the 3-hour and 12-hour experimental groups (both \( P < 0.001 \)).

**Axonal Transport Comparisons between Control and High IOP Eyes.** Figure 3 illustrates the RITC intensity and the change in RITC intensity in the temporal, central, and nasal nerve bundles at different points along the control IOP and high IOP eyes in the 3-hour group. A comparison in the intensity of RITC between the control and high IOP eyes revealed no significant difference in any of the three nerve bundles (three-way ANOVA; all \( P > 0.134 \); Figs. 3A–C). There was also no difference in the change in RITC intensity between control and high IOP eyes in any of the three nerve bundles in this group (three-way ANOVA; all \( P > 0.168 \); Figs. 3D–F).

Figure 4 illustrates the RITC intensity and the change in RITC intensity in the temporal, central, and nasal nerve bundles at different points along the control IOP and high IOP eyes in the 12-hour group. The intensity of RITC was significantly different between the two eyes in all three nerve bundles (three-way ANOVA; all \( P < 0.001 \)). Post hoc analysis revealed that RITC intensity was lower at all points distal to the lamina cribrosa in the high IOP eyes (all \( P < 0.017 \)), except for the 0.2-mm postlaminar segment of temporal nerve bundles (\( P = 0.201 \)). At the lamina cribrosa, the intensity of RITC was greater in the temporal and central nerve bundles of high IOP eyes (both \( P < 0.019 \)), suggesting that the axonal transport decrease in the lamina cribrosa of the high IOP eye was causing the accumulation of tracer in this region.

Figures 4D, 4E, and 4F represent the change in RITC intensity in the temporal, central, and nasal nerve bundles of 12-hour animals. Change in RITC intensity was significantly lower in the lamina cribrosa and proximal 0.4 mm of postlaminar tissue in all three nerve bundles of the high IOP eyes compared with control (three-way ANOVA; all \( P < 0.022 \)). In the central and temporal nerve bundles, change in RITC intensity was also significantly lower in the 0.6-mm and 0.8-mm postlaminar segments, respectively, of the high IOP eye (both \( P < 0.007 \)).

**Axonal Transport Comparisons among 3-, 6-, and 12-Hour Experiments**

**Central Nerve Bundles.** In the control eyes, there was a significant difference in the intensity of RITC among the 3-, 6-, and 12-hour experiments (three-way ANOVA; all \( P < 0.001 \)). In the high IOP eyes, the tracer was mostly localized to the prelaminar and lamina cribrosa regions, with some tracer seen in the proximal region of postlaminar tissue. Change in RITC intensity was significantly lower in the lamina cribrosa and proximal 0.4 mm of postlaminar tissue in all three nerve bundles of the high IOP eyes compared with control (three-way ANOVA; all \( P < 0.022 \)). In the central and temporal nerve bundles, change in RITC intensity was also significantly lower in the 0.6-mm and 0.8-mm postlaminar segments, respectively, of the high IOP eye (both \( P < 0.007 \)).

**Figure 1.** Axonal transport in the control and high IOP eyes from a 3-hour experiment. (A) Confocal images of RITC transport after 3 hours of raised IOP. (left) Control eye. (right) High IOP eye. RITC was mostly seen in the prelaminar regions of both eyes, with some tracer evident in the lamina cribrosa. Very little tracer was seen in the postlaminar region. There was no remarkable difference between the two eyes. (B) Van Gieson-stained sections taken from similar regions of the RITC-labeled optic nerve are provided for reference. (left) Control eye. (right) High IOP eye. (dotted lines) Lamina cribrosa region in each image. Scale bar, 400 μm.

**Figure 2.** Axonal transport in the control and high IOP eyes from a 12-hour experiment. (A) Confocal images of RITC transport after 12 hours of raised IOP. (left) Control eye. (right) High IOP eye. RITC was seen to travel well past the lamina cribrosa in the control eye. In the high IOP eye, the tracer was mostly localized to the prelaminar and lamina cribrosa regions, with some tracer seen in the proximal region of postlaminar tissue. (B) Van Gieson-stained sections taken from similar regions of the RITC-labeled optic nerve are provided for reference. (dotted lines) Lamina cribrosa region in each image. Scale bar, 400 μm.
and 12-hour time points (two-way ANOVA; all \( P < 0.001 \)). The intensity of RITC increased at each point along the nerve as the duration of the experiment increased. Post hoc testing revealed that differences among the three groups occurred at the lamina cribrosa and at all points in the first 2 mm of postlaminar tissue (all \( P < 0.022 \)). In the high IOP eyes, the difference in the intensity of RITC was only significant between the 3-hour and the 12-hour animals and between the 6-hour and the 12-hour animals (two-way ANOVA; \( P < 0.001 \)). Post hoc testing revealed that these differences were only significant in.

**FIGURE 3.** Three-hour experiments. Relationship between averaged RITC intensity and distance from the prelaminar region along the optic nerve in the temporal (A), central (B), and nasal (C) nerve bundles. RITC intensity is normalized and expressed as a percentage intensity of the prelaminar value. There was no difference in RITC intensity between the normal and high IOP eyes at any point along the nerve (\( P > 0.05 \); three-way ANOVA). Proportional changes in RITC intensity, an index of regional axonal transport, within different segments of the temporal (D), central (E), and nasal (F) nerve bundles. There were no differences in the change in RITC intensity between the control and high IOP eyes at any segment of the nerve (\( P > 0.05 \); three-way ANOVA). The prelaminar point is denoted as 0 \( \mu m \) from the ILM, and the midpoint of the lamina cribrosa is 442 \( \mu m \) from the ILM.
The lamina cribrosa and the first 0.2 mm of postlaminar tissue (both $P/H_{11021} 0.021$). There was no difference between the 3-hour and 6-hour experiments (two-way ANOVA; $P/H_{11005} 0.171$).

There was no significant difference in the change in RITC intensity along the central nerve bundles among the 3-, 6-, and 12-hour groups in control and high IOP eyes after allowing for

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**FIGURE 4.** Twelve-hour experiments. Relationship between averaged RITC intensity and distance from the prelaminar region along the optic nerve in the temporal (A), central (B) and nasal (C), nerve bundles. RITC intensity is normalized and expressed as a percentage intensity of the prelaminar value. RITC intensity was significantly higher in the lamina cribrosa region of the temporal and central nerve bundles in high IOP eyes than in control ($P < 0.017$; three-way ANOVA). Distal to the lamina cribrosa, the intensity of RITC was lower in the high IOP eye at most points along the nerve in all nerve bundles. Proportional changes in RITC intensity, an index of regional axonal transport, within different segments of the temporal (D), central (E), and nasal (F) nerve bundles are also represented. Change in RITC intensity was significantly reduced in the high IOP eye in the posterior region of the lamina cribrosa and the first 0.4 mm of postlaminar tissue in temporal, central, and nasal nerve bundles (all $P < 0.022$; three-way ANOVA). In the central nerve bundle, change in RITC intensity was also reduced in the high IOP eye in the 0.6 mm postlaminar segment ($P < 0.007$; three-way ANOVA). *Significant difference as determined by three-way ANOVA with post hoc testing. The prelaminar point is denoted as 0 µm from the ILM, and the midpoint of the lamina cribrosa is 458 µm from the ILM.
the effects of differences at various positions along the nerve (two-way ANOVA; both P > 0.167).

**Peripheral Nerve Bundles.** Comparisons of RITC intensity in the peripheral nerve bundles of control eyes revealed a significant difference among the 3-, 6-, and 12-hour time points (two-way ANOVA; all P < 0.001). Post hoc testing revealed that the difference in intensity was significant at the lamina cribrosa and at all points up to the first 2 mm of postlaminar tissue (all P < 0.056). The intensity of RITC increased at each point along the nerve as the duration of the experiment increased. In the high IOP eyes, the intensity of RITC was only different between the 3- and 12-hour animals and between the 6- and 12-hour animals (two-way ANOVA; P < 0.001). Post hoc testing revealed that these differences were significant in the lamina cribrosa and the first 0.6 mm of postlaminar tissue (all P < 0.025). The intensity of RITC was greater at each of these points in the 12-hour experiments. There was no difference between the 3- and 6-hour experiments (two-way ANOVA; P = 0.085).

There was no significant difference in the change in RITC intensity along the peripheral nerve bundles among the 3-, 6-, and 12-hour groups in the control eyes after allowing for the effects of differences at various positions along the nerve (two-way ANOVA; P = 0.135). In the high IOP eyes, there was a significant difference in the change in RITC intensity along the peripheral nerve bundles among the three time points (two-way ANOVA; P < 0.001). These differences were significant between the 3- and the 6-hour (P = 0.006) and between the 3- and the 12-hour groups (P < 0.001). Post hoc testing revealed that these differences were significant in the prelaminar region and the proximal 0.8 mm of postlaminar tissue (all P < 0.028). The change in the intensity of RITC in the high IOP eyes of the 6-hour and 12-hour groups was lower. The difference between the 6- and the 12-hour groups did not reach significance (P = 0.949). There was no difference in the change in the intensity of RITC at the lamina cribrosa among the three groups (all P > 0.380).

**Cytoskeleton**

Staining of neurofilament subtypes in the optic nerve head of control eyes demonstrated a homogeneous stain pattern in most animals. In some animals we observed a patchy and scattered stain pattern within nerve bundles, but this appearance was consistent throughout the entire optic nerve head, with no differences in morphology among temporal, central, and nasal nerve bundles (three-way ANOVA; all P > 0.050). For the neurofilament light (NFL) subtype, we observed the intensity of stain to be less in the postlaminar region of control eyes than in the prelaminar and lamina cribrosa regions (three-way ANOVA; P < 0.001). Unlike neurofilament proteins, MAP proteins showed similarities in sequence morphology with other structures in the central nervous system, particularly collagen proteins. Consequently, antibodies against MAP proteins demonstrated some cross-reactivity to the epitopes of collagen proteins present in the same tissue. This accounted for a slight degree of variability in the appearance of MAP stain in the optic nerve head of control eyes. We found some MAP control sections to have a more punctate stain pattern than others, and this was most likely attributable to the difference in collagen content caused by variability in animal size and age. As was found with neurofilament proteins, there was no difference in stain patterns among temporal, central, and nasal nerve bundles for MAP-stained sections (three-way ANOVA; P > 0.050).

When cytoskeletal proteins changed after a rise in IOP, most proteins demonstrated a homogenous and diffuse decrease in stain pattern in the nerve bundles of the high IOP eyes. Neurofilament medium (NFM) protein initially demonstrated a patchy and heterogeneous decrease in stain intensity that became more diffuse and homogeneous after 12 hours of IOP elevation. We have described below the effects of 3 and 12 hours of IOP elevation on each cytoskeletal protein. Figures 5 and 6 illustrate the quantitative change to cytoskeletal proteins in each region of the optic nerve head after 3 and 12 hours, respectively, of IOP rise.

**NFHp Staining.** Figures 7A and 8A show the pattern of phosphorylated neurofilament heavy (NFHp) staining in the porcine optic nerve in the 3- and 12-hour experiments, respectively. In the high IOP eye of both groups, less stain was visible in the prelaminar, laminar, and postlaminar regions of the temporal, nasal, and central nerve bundles compared with the control eye. Three-way ANOVA with post hoc testing revealed a significant decrease in intensity of NFHp stain in the high IOP eye in the temporal, central, and nasal nerve bundles of both groups (three-way ANOVA; all P < 0.001; Figs. 5, 6). Subanalysis revealed significantly less stain in the prelaminar, laminar, and postlaminar regions of these nerve bundles in the high IOP eye of both groups (three-way ANOVA; all P < 0.001).

**NFH Staining.** The pattern of phosphorylation-independent neurofilament heavy (NFH) staining in the porcine optic nerve in the 3-hour and 12-hour experiments is illustrated in Figures 7B and 8B, respectively. In the high IOP eye, less stain was seen in the prelaminar, lamina cribrosa, and postlaminar regions of the temporal, central, and nasal nerve bundles. Three-way ANOVA with post hoc testing demonstrated a statistically significant decrease in intensity of NFH stain in the high IOP eye in the temporal, central, and nasal nerve bundles of both groups (three-way ANOVA; all P < 0.001; Figs. 5, 6). Subanalysis revealed significantly less stain in the prelaminar, laminar, and postlaminar regions of these nerve bundles in the high IOP eye of both groups (three-way ANOVA; all P < 0.002).

**NFM Staining.** Figures 7C and 8C show the pattern of NFM staining in the porcine optic nerve in the 3-hour and 12-hour experiments, respectively. Three-way ANOVA with post hoc testing demonstrated a statistically significant decrease in the intensity of the NFM stain in the high IOP eye in the temporal, central, and nasal nerve bundles of the 12-hour group (three-way ANOVA; all P < 0.001; Fig. 6). In the 3-hour group, the intensity of NFM was only decreased in the temporal nerve bundles of the high IOP eyes (three-way ANOVA; P < 0.010; Fig. 5). In the 3- and the 12-hour groups, when significant changes occurred within temporal, central, and nasal nerve bundles, subanalysis revealed that these changes were significant in the prelaminar, laminar, and postlaminar regions of these nerve bundles (three-way ANOVA; all P < 0.002).

**NFL Staining.** The pattern of NFL staining in the porcine optic nerve in the 3- and 12-hour experiments is illustrated in Figures 7D and 8D, respectively. There was no visible difference in staining between the control and high IOP eyes in either group. Three-way ANOVA with post hoc testing demonstrated no statistically significant difference in the intensity of the NFL stain between the high IOP and control eyes in the temporal, central, or nasal nerve bundles of both groups (three-way ANOVA; all P > 0.122; Fig. 5 and 6).

**Tubulin Staining.** Figure 7E shows the pattern of tubulin staining in the porcine optic nerve in the 3-hour experiments. No difference in staining was visible between the control and high IOP eyes. Three-way ANOVA with post hoc testing demonstrated no statistically significant difference in the intensity of tubulin stain between the high IOP and the control eye in the temporal, central, or nasal nerve bundles (three-way ANOVA; all P = 0.926; Fig. 5). After 12 hours of IOP elevation, less tubulin stain was visible in the high IOP eye (Fig. 8E). Three-
way ANOVA with post hoc testing demonstrated a statistically significant decrease in the temporal, central, and nasal nerve bundles (three-way ANOVA; all $P < 0.001$; Fig. 6). Subanalysis revealed significantly less stain in the prelaminar, laminar, and postlaminar regions of these nerve bundles in the high IOP eye (three-way ANOVA; all $P < 0.001$).

**Microtubule-Associated Protein Staining.** Figure 7F shows the pattern of MAP staining in the porcine optic nerve in the 3-hour experiments. No difference in staining was visible between the control and the high IOP eyes. Three-way ANOVA with post hoc testing demonstrated no statistically significant difference in intensity of MAP stain between the high IOP and control eyes in the temporal, central, or nasal nerve bundles (three-way ANOVA; all $P = 0.437$; Fig. 5). After 12 hours of IOP elevation, less MAP stain was visible in the high IOP eye (Fig. 8F). Three-way ANOVA with post hoc testing demonstrated a statistically significant decrease in the temporal, central, and nasal nerve bundles (three-way ANOVA; all $P < 0.001$; Fig. 6). Subanalysis revealed significantly less stain in the prelaminar, laminar, and postlaminar regions of these nerve bundles in the high IOP eye (three-way ANOVA; all $P < 0.001$).

**DISCUSSION**

The temporal sequence of axonal transport and cytoskeleton change in the optic nerve head after an acute elevation of IOP has been described in this report. We performed experiments with continuous IOP, CSFp, and blood pressure monitoring and examined optic nerve head changes after 3 and 12 hours of IOP elevation. We compared the results from these experiments with those of our previous report to determine the time-dependent sequence of change. The major findings from this study are as follows: (1) Axonal transport change in the laminar regions of the optic nerve head occurs in a time-dependent and a nonhomogeneous pattern after an elevation of IOP. (2) Cytoskeletal proteins in the laminar regions of the optic nerve head are affected in a time-dependent manner after an elevation of IOP. (3) Changes to neurofilament proteins can precede axonal transport impairment, as evidenced by the decrease in neurofilament subunits despite no measurable change in axonal transport after 3 hours of IOP elevation. IOP and CSFp are important determinants of axonal transport in the optic nerve head. Although previous investiga-
tors have studied axonal transport change after a rise in IOP, few of these experiments have included continual monitoring of IOP, CSFp, or blood pressure as part of the experimental methodology. This might have been because of the surgical and anesthetic difficulties inherent with such an experimental design. The availability of a veterinary anesthetist to perform all anesthesia for this work, together with our previous surgical experience in CSF and intraocular tissue pressure measurements, allowed us to conduct lengthy experiments involving IOP elevation while ensuring that animal physiology was maintained within the normal range.

Although stringent measures were adopted to prevent tilting of specimens during sectioning, a small degree of angulation was still observed in some slides. This was largely attributed to the nonlinear path taken by RGC axons as they traveled from the prelaminar region to postlaminar tissue, experiencing some degree of tortuosity in the region of the lamina cribrosa. This has been previously described in human eyes. Consequently, tilting of sections was most commonly evident in the form of obliquely cut nerve fiber bundles in the region of the lamina cribrosa. Occasionally, we also observed nerve bundles to be cut obliquely in proximal postlaminar tissue. By cutting the sections at a thickness of 12 μm and by acquiring a z stack consisting of seven images for each slide, we minimized the impact of tilting by analyzing the individual slices and average projections of each stack for cytoskeleton and axonal transport studies.

We did not find a significant difference in axonal transport between control and high IOP eyes after 3 hours of IOP elevation. However, after 12 hours of IOP elevation, axonal transport in the lamina cribrosa and proximal postlaminar region of the high IOP eye was significantly reduced in the temporal, central, and nasal nerve bundles. These findings, taken together with our previous report, suggest that measurable axonal transport retardation in the porcine optic nerve occurs after 3 to 6 hours of IOP elevation, with this retardation sustained when IOP is kept elevated. This is consistent with previous work performed by Chihara et al., who reported no difference in axonal transport between control and high IOP eyes in rabbit optic nerves after 3 hours of IOP elevation but who found significant differences at 6 hours.

Axonal transport rates were most reduced in the lamina cribrosa region of control and high IOP eyes in all experimental groups, implying that this region is an anatomic choke point for axonal transport even in normal physiological conditions. This has been previously described. Although the greatest decrease in axonal transport after IOP elevation took place in the lamina cribrosa, after 12 hours of IOP elevation we found that axonal transport in the postlaminar tissue was also reduced in a manner comparable to that described in our previ-

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** Comparison of average intensities of cytoskeletal proteins in each region of the optic nerve for the 12-hour experiments. (A) Prelaminar-temporal region. (B) Prelaminar-central region. (C) Prelaminar-nasal region. (D) Laminar-temporal region. (E) Laminar-central region. (F) Laminar-nasal region. (G) Postlaminar-temporal region. (H) Postlaminar-central region. (I) Postlaminar-nasal region. There is a significant reduction in NFHp, NFH, NFM, tubulin, and MAP across all nine regions in the high IOP eye. **Significant difference with P < 0.001, as determined by three-way ANOVA with post hoc testing.**
ous report. The important difference was that after 12 hours of IOP elevation, the reduction in postlaminar transport was seen in central and peripheral nerve bundles, whereas 6 hours of IOP elevation only caused a reduction in the peripheral nerve bundles. This implies that some functional optic nerve alteration in peripheral postlaminar tissue occurs before changes to the central nerve.

Previous investigators have observed axonal transport to be unequally affected in the optic nerve head regions after a rise in IOP. Several reports have described axonal transport to be
more affected in the peripheries\textsuperscript{4-5,20}; changes in the temporal regions were greater than those in the nasal regions.\textsuperscript{19,25} This has been demonstrated in monkeys and rabbits. When we compared axonal transport rates at different time points within central and peripheral nerve bundles of control and high IOP eyes, the only groups to show a statistically significant decrease in axonal transport over time were the peripheral nerve bundles of the high IOP eyes. This supports previous findings and suggests that a rise in IOP has a greater effect on axonal transport in the peripheral nerve bundles.

Because of our experimental design, we have only been able to study the effects of an acute rise in IOP on axonal transport...
transport in the anterograde direction. The human RGC axon travels approximately 50 mm from the cell soma to its target synapse and has only a rudimentary capacity to synthesize its own proteins. Consequently, neuronal axons are dependent on the cell body for the synthesis of essential neurotrophins and growth factors required for axonal survival and synaptic function. Inhibition of anterograde axonal transport can therefore result in the deprivation in distal axons and synapses of vital trophic factors such as NT-3 and brain-derived neurotrophic factor. In the CNS and the developing visual system, a decrease in both these growth factors resulting from an alteration in anterograde trophic support has been thought to contribute to neuronal degeneration. Through a similar mechanism, the inhibition of anterograde axonal transport in the laminar regions may play a role in the pathogenesis of RGC damage seen in diseases with elevated IOP.

Previous work has demonstrated that the sequence of change to cytoskeletal proteins after axonal injury is variable, with the progression of change dependent on the mode of neuronal insult. In experimental optic neuritis there is a time-dependent decrease in neurofilaments and microtubules in regions of demyelination, with the earliest changes to both these proteins observed after 1 day. In contrast, stretch injury to guinea pig optic nerves causes a dramatic and early decrease in the number of microtubules in the nodal blebs, with no significant change to the number of neurofilaments. A study of Wallerian degeneration in the rat optic nerve demonstrated a significant reduction in NFL and nonphosphorylated NFH subunits 48 hours after injury, with no change to NFM or NFHp subunits. Changes to cytoskeletal proteins, and the order in which they change, may therefore be useful markers of axonal injury.

Previous investigators have described the effects of acute and chronic IOP elevation on the optic nerve cytoskeleton, however, as far as we are aware, this is the first report to examine the temporal sequence of cytoskeletal change after a rise in IOP. After 3 hours of IOP elevation, we observed a dramatic decrease in phosphorylated NFH in all laminar regions of the high IOP eyes. Phosphorylation of NFH protects it from proteolysis. Therefore, it was not surprising that we also observed a decrease in total NFH where NFHp changes occurred. We also found a decrease in NFM in the temporal nerve bundles after 3 hours of IOP elevation. In our previous study, we found that 6 hours of IOP elevation caused a decrease in NFH, NFHp, and NFM in all regions of the optic nerve head. Twelve hours of IOP elevation resulted in a reduction of NFH, NFHp, NFM, tubulin, and MAPs in all laminar regions. The temporal course of cytoskeletal change after a rise in IOP appears to involve the neurofilament subtypes initially, with microtubules and MAPs becoming affected after prolonged periods of IOP elevation. Changes to microtubules and MAPs were observed only after significant changes to neurofilaments. Neurofilaments have a role in controlling tubulin polymerization, with a modification of this regulatory ability when neurofilaments are dephosphorylated. This may explain why a reduction in microtubules and MAPs only occurred after a significant change to neurofilaments had taken place.

Structurally, the neurofilament protein is a triplet heteropolymer composed of a core filament backbone with sidearms that extend perpendicularly from the core filament. The hypervariable carboxyl-terminal tail domains of NFM and NFH are responsible for forming the neurofilament sidearms, whereas the core filaments are composed of the amino-terminal head and conserved helical domains of all neurofilament subtypes, in particular NFL. The neurofilament protein structure confers some degree of intrinsic protection to the NFL subtype, with NFM and NFHp subtypes more vulnerable to the activity of kinase and phosphatase enzymes in conditions of altered cellular physiology. This may explain why we did not observe changes to NFL in any of our experiments despite marked changes to NFH and NFM. A reduction in NFH subunits in the optic nerve has, however, been previously reported in the monkey ocular hypertension model.

Detailed studies have previously shown that motor proteins and their associated cargo travel along the surface of microtubules and MAPs. Although the overexpression and deletion of neurofilament subtypes can result in variable forms of neurologic disease, the role of neurofilaments in the axonal transport process is still undetermined. After 3 hours of IOP elevation, despite a dramatic decrease in neurofilament subtypes in the optic nerve head, we did not detect a change in axonal transport. This implies that neurofilaments are affected early after a rise in IOP, before axonal transport changes occur, and could be a useful marker of RGC axonal injury. Six hours of IOP elevation caused simultaneous changes to neurofilaments and axonal transport, so it is possible that in addition to being surrogate markers for early axonal injury, neurofilaments may play a role in the axonal transport process.

Increased IOP is a major risk factor for glaucomatous optic nerve axonal damage. Studying the time-dependent changes in axonal transport and structure resulting from elevated IOP provides a better understanding of the pathogenic role of increased IOP in RGC axonal damage. Such time dependence also suggests that the period of IOP elevation is critical for the severity of axonal damage. One of the limitations of this study is that the longest period of IOP elevation was 12 hours. Although we have been able to report the effects of increased IOP on axonal transport and cytoskeleton change, the concurrent study of both these processes in established rodent and primate models of ocular hypertension will further improve our understanding regarding the contribution of these changes to glaucomatous optic neuropathy.

Understanding the sequence of neurofilament change that precedes the nonhomogeneous alteration in axonal transport could also allow further exploration of the interaction between increased IOP and induced changes in axonal transport and neurofilaments. Another limitation of this work is that a porcine model of acute IOP elevation was used. The histomorphometry of the porcine optic disc exhibits some differences to the human eye. The lamina cribrosa in the pig is elliptically shaped along the horizontal meridian, without any significant variation in laminar pore size. This is in contrast to the human lamina cribrosa, which is elliptically shaped along the vertical meridian and has larger laminar pores within the superior and inferior regions. The optic nerve is affected asymmetry in the early stages of human glaucoma, with the ganglion cell axons in the superior and inferior poles the most vulnerable to premature injury. Because of these regional differences in laminar pore size, the effects of an acute rise in IOP may result in a more selective and localized change to the RGC cytoskeleton in human eyes, contrasting with the diffuse cytoskeletal loss seen in the porcine optic nerve head. Again, this limitation could be addressed by using primate models of ocular hypertension, where the lamina cribrosa structure is known to be more similar to the human structure.

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