Ultrastructural Changes and Immunocytochemical Localization of Microtubule-Associated Protein 1 in Guinea Pig Optic Nerves after Acute Increase in Intraocular Pressure

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PURPOSE. To investigate ultrastructural changes and localization of microtubule-associated protein 1 (MAP 1) in optic nerves of guinea pigs in conditions of acute increase in intraocular pressure (IOP).

METHODS. Intraocular pressure was increased acutely in 40 guinea pig eyes, and 40 eyes were examined in 20 normal control animals. Analytic methods included quick-freezing and deep-etching (QF-DE) and conventional fixation. Microtubule-associated protein 1 immunocytochemistry was performed.

RESULTS. In conventional ultrathin sections, an alteration of the axoplasmic ultrastructure was seen only at the level of the lamina cribrosa sclerae. The number of microtubules was significantly decreased in the axoplasm that exhibited accumulations of membranous organelles. Cross-bridged filaments on microtubules were significantly reduced in number when determined by the QF-DE method. The immunostaining of tubulin or MAP 1 was less positive than in other samples with normal IOP, revealed by light microscopic immunocytochemistry. Immunoelectron microscopy demonstrated that MAP 1 was significantly decreased in the axoplasm that showed accumulations of membranous organelles.

CONCLUSIONS. The acute alteration of axoplasmic ultrastructures in some parts of the optic nerves at the level of the lamina cribrosa involves a decrease in the amount of MAP 1 and a decrease in the number of axonal cross-bridges on microtubules in a condition of acutely increased IOP, which could be related to microtubule instability and abnormal organelle transport in the axoplasm.

FIGURE 1. Conventional electron micrographs of optic nerve axons in normal intraocular pressure conditions. In (a) longitudinal and (b) cross sections cut through a level of the lamina cribrosa, the axoplasm is composed of microtubules (large arrow) and neurofilaments (small arrows) with scattered membranous organelles, such as mitochondria (M) and vesicles (V). Inset: Wispy structures (arrowheads) can be seen among microtubules and neurofilaments. Scale bars, 0.2 μM; G, glia cell.

It is known that membranous organelles within ganglion cell axons are accumulated at the lamina cribrosa in the optic nerve, after an increase in IOP. However, it is unclear how the increase in IOP leads to the cessation of axonal organelle transport at the lamina cribrosa. A fast axonal transport of the axonal organelles depends on the presence of microtubules and transport motor proteins. Some observations of organelle movements in isolated axoplasm, seen with video-enhanced light microscopy, have demonstrated that a bidirectional organelle translocation occurs along cytoskeletal filaments that correspond to microtubules. In crude extracts of squid axoplasm, purified kinesin enhanced movement of axonal organelles along microtubules. It is also thought that Tau proteins play a role as short cross-linking structures involved in making bundles of microtubules, whereas MAPI works to form channels in microtubules for the transportation of membranous organelles. Because axonal cross-bridges could be involved in axonal transport and microtubule stability, they may be morphologically changed in optic nerve axons of animal eyes in which IOP is increased, in addition to the disorganization of microtubules and neurofilaments. In the present study, ultrastructural changes of axoplasm in guinea pig optic nerves were examined after an acute increase in IOP. First, we examined crosscut nerve specimens using conventional electron microscopy to determine damaged regions and the extent of involvement of the axons. Second, we used the quick-freezing and deep-etching (QF-DE) method to observe whether cross-bridges between microtubules in the affected axon are damaged. Third, the localization of MAP 1 was quantitatively examined by electron microscopic immunocytochemistry using colloidal gold-labeled secondary antibody.

MATERIALS AND METHODS

All experimental animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Guinea pigs were used in the present study because the axonal ultrastructure in a normal optic nerve has been studied by the present or other investigators. Both eyes in 20 animals in which IOP had not been increased were studied as normal control specimens. The remaining 20 animals were included in an increased IOP group. The IOP of one eye in each of 20 guinea pigs was artificially increased in the following way: After sodium pentobarbital anesthesia, IOP was controlled by a saline manometer connected to a 25-gauge needle, which was inserted into the anterior chamber of both eyes. The IOP was raised by changing the height of the manometer reservoir. In 40 control eyes, the pressure was maintained at 15 mm Hg for 4 hours. In 40 experimental eyes, the pressure of 60 mm Hg was kept constant for 4 hours. After 4 hours, all animals were immediately perfused through their hearts with 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) containing 10 μM taxol (Sigma Chemical, St. Louis, MO). After perfusion, the optic nerves were dissected and prepared in various ways, as will be described later. Ten control and 10 experimental optic nerves were used in each of the following four methods.

Conventional Electron Microscopy

The optic nerves were further fixed with 2.5% glutaraldehyde in PB then with 1% osmium tetroxide in PB, each for 2 hours at room temperature. The specimens were routinely dehydrated in a graded series of ethanol baths and were embedded in Epon 812. Ultrathin sections were serially crosscut from the
Microtubule-Associated Protein 1 Localization in Optic Nerve

FIGURE 2. Conventional electron micrographs of optic nerve axons with normal (a, c, e) or increased (b, d, f) intraocular pressure. In prelaminar portions, the ultrastructural appearance of b is similar to that of a. In laminar portions (c, d), an ultrastructural difference of axoplasmic appearance is observed only in d. In postlaminar portions, the ultrastructural appearance of f is similar to that of e. Scale bars, 0.5 μM.

prelaminar to the postlaminar level, stained with uranyl acetate and lead citrate, and observed by an electron microscope (H-500 or H-600; Hitachi, Tokyo, Japan).

Quick-Freezing and Deep-Etching Method
Replica membranes were prepared by the QF-DE method, as previously reported. 26,27 Briefly, the optic nerves were cut longitudinally into halves, which were immersed in 2% paraformaldehyde and 0.05% glutaraldehyde in PB containing 10 μM taxol to stabilize microtubules; they remained in the same fixative for 30 minutes. These tissues were then treated with 0.5% saponin in PB containing 10 μM taxol for 30 minutes and were postfixed with 0.25% glutaraldehyde in PB for 30 minutes. They were infiltrated with 10% methyl alcohol, frozen using a quick-freezing apparatus (JFD-RFA; JEOL, Tokyo, Japan), and cooled in liquid nitrogen, as reported previously. 26,27 The frozen tissue surfaces were manually freeze-fractured by using a scalpel in liquid nitrogen. The specimens were transferred into a freeze-etching device (FD-3AS; Eiko Engineering, Ibaragi, Japan) and were deeply etched under vacuum conditions of 1 × 10⁻⁷ to 4 × 10⁻⁷ Torr at a temperature of -95°C for 20 to 30 minutes. They were rotary-shadowed with platinum and carbon. The replica membranes were treated in household bleach, washed in distilled water and mounted on polyvinyl-coated (Formvar; Nisshin EM, Tokyo, Japan) copper grids. All replica membranes were observed under an electron microscope (H-500 or H-600; Hitachi) at an accelerating voltage of 75 kV. Electron micrographs were printed from the inverted negative films.

Immunofluorescence Microscopy
The optic nerves were further fixed for 1 hour in a mixture of 2% paraformaldehyde and 0.05% glutaraldehyde in PB containing 10 μM taxol. They were then sequentially incubated in PB containing 5%, 10%, or 20% sucrose for 3 hours. The samples were buried in ornithine carbamoyltransferase (OCT; Miles, Elkhart, IN) compound and were frozen at -80°C. Using a cryostat machine, 10-μm sections were crosscut from the optic nerves at the level of the lamina cribrosa. The sections were mounted on gelatin-coated glass slides. After treatment with 0.5% saponin for 30 minutes, they were pretreated with 1% bovine serum albumin in phosphate-buffered saline for 30 minutes. They were immunostained with a mixture of 1:20 rabbit polyclonal antitubulin (Biomedical Technologies, Stoughton, MA) and 1:10 mouse monoclonal anti-MAP 1 (Chemicon, Temecula, CA) antibodies for double-labeling experiments for 1 hour at room temperature. They were immunostained with a combination of goat antirabbit IgG antibody coupled with fluorescein isothiocyanate (Cappel, West Chester, PA) and 1:100 goat antimouse IgG antibody coupled with Texas red (Cappel) for 30 minutes at room temperature. The glass slides were overlaid with 50% glycerol and glass coverslips and were observed by confocal laser scanning microscopy (TCS 4D; Leica, Deerfield, IL). For immunococontrol specimens, some sections were incubated with PBS or nonimmune serum instead of the primary antibodies and were prepared in the same way.

Electron Microscopic Immunocytochemistry
The optic nerves were further immersed for 1 hour in 2% paraformaldehyde mixed with 0.05% glutaraldehyde in PB containing 10 μM taxol. They were dehydrated by using a methanol series at -20°C, transferred to a mixture of methanol and London Resin Gold (LR Gold; Polysciences, Warrington, PA), and finally embedded in pure LR Gold. The LR Gold was polymerized at -20°C with ultraviolet irradiation. Ultrathin sections were crosscut at the lamina cribrosa and picked up on 200-mesh grids. They were incubated with 1:100 mouse anti-MAP1 monoclonal antibody for 1 hour, and then with 10 nm colloidal gold-conjugated antimouse IgG antibody. Immu-
FIGURE 3. Conventional electron micrographs of optic nerve axons with increased intraocular pressure. (a) In a nerve bundle at the level of the lamina cribrosa, some axons (arrowheads) are indistinguishable from those described under normal intraocular pressure; others contain many organelles (large arrows). (b, c) Many membranous organelles such as multilamellar bodies (large arrows), mitochondria (M), and vesicles (V) have accumulated within the axoplasm, in which some microtubules (arrows) are still observed. (a) Scale bar, 0.5 μM; (b, c) Scale bar, 0.2 μM; G, glia cell; C, collagen beam.

Morphometric Analysis

Morphometric analyses were performed by using an image analysis system (National Institutes of Health, Bethesda, MD). The numbers of microtubules or gold particles were measured on the electron micrographs at a magnification ×40,000. Ten optic nerves from each group were selected. The numbers of microtubules per 1 μm² axoplasmic area were counted in 100 axons from each cross-sectioned nerve embedded in Epon 812. The numbers of gold particles per 1 μm² axoplasmic area were counted in 20 axons from each cross-sectioned nerve embedded in LR Gold. The method of analysis reported by Furuta et al. was adopted for the measurement of cross-bridges. All values are expressed as mean ± standard deviation (SD), and statistically analyzed with Student’s t-test.

RESULTS

Conventional Electron Microscopy

Optic Nerves at Normal Intraocular Pressure. In cross or longitudinal sections through the level of the lamina cribrosa, the axoplasm of normal optic nerves usually contained microtubules, neurofilaments and membranous organelles, such as mitochondria and vesicles (Figs. 1a, 1b). Some axons appeared to have such membranous organelles, whereas other axons had none. At high magnification, some fuzzy structures, which may have been axonal cross-bridges, were seen on the electron micrograph, among the cytoskeletal elements (Fig. 1a, inset); but their dimensions were difficult to measure because of the low contrast of their images.

Optic Nerves at Increased Intraocular Pressure. The ultrastructural appearance in prelaminar or postlaminar portions of optic nerves was entirely normal in all specimens (Fig. 2b, 2f). Abnormal changes in axons were observed only at the level of the lamina cribrosa (Fig. 2d). At that level, some axons were indistinguishable from those seen in conditions of normal IOP, but others contained many accumulated organelles (Figs. 2d, 3a). Among the membranous organelles, microtubules and neurofilaments were still observed (Figs. 3b, 3c). However, morphometric data (see Table 1) show that the number of microtubules in such axons was significantly decreased, compared with those in normal axons.

Quick-Freezing and Deep-Etching Method

Optic Nerves at Normal Intraocular Pressure. In replica electron micrographs, ultrastructural cytoskeletons were observed in axoplasm (Fig. 4). The axoplasm was filled with longitudinally oriented neurofilaments, approximately 11 nm in diameters, which were cross-linked by numerous short filaments (Fig. 4a). Microtubules, approximately 25 nm in diameter, were linked by cross-bridges. Membranous organelles, which were interconnected by cross-bridges with microtubules, neurofilaments, or both, were scattered in the axoplasm (Fig. 4a, inset).

Optic Nerves at Increased Intraocular Pressure. Among accumulated membranous organelles, a few microtubules were observed (Fig. 4b). The number of cross-bridges along microtubules appeared to be dramatically decreased (Fig. 4b, inset). When we measured the frequency of cross-bridges on microtubules (number of cross-bridges per 100 nm of microtubule), we noted a statistically significant reduction (Table 2). The distance between microtubules appeared to be shorter than that in the normal IOP group. Granular material (approximately 4 nm in diameter) were frequently observed on the microtubules (Fig. 4b, inset).

Immunofluorescence Microscopy

The double immunolabeling with antitubulin and anti-MAP 1 antibodies demonstrated colocalization of MAP 1 with tubulin in the optic axons (Figs. 5a, 5b, 5c). The specimens in the normal IOP group were diffusely immunostained (Fig. 5e), and their fluorescence intensity was higher than in immunocontrol specimens (Fig. 5d). On the contrary, the immunostaining patches of tubulin and MAP 1 became scattered in the specimens with increased IOP (Fig. 5f).

Electron Microscopic Immunocytochemistry

Immunogold particles in specimens incubated with anti-MAP 1 at normal IOP were seen mostly on microtubule domains in the ultrathin sections. Most immunogold particles tended to localize near microtubules, whereas others were localized on microtubules (Fig. 6a, 6b). In the specimens with increased IOP, immunogold particles were found in axoplasm (Fig. 6c). Morphometric data (Table 3) show that the number of gold particles in axons in the presence of increased IOP was significantly decreased, compared with those in the presence of normal IOP.

DISCUSSION

Morphologic changes including accumulation of various membranous organelles and damage of microtubules and neurofila...
FIGURE 4. Replica electron micrographs of optic nerve axons with normal (a) or increased (b) intraocular pressure. The ultrastructures in the axoplasm are clearly observed. (a) The spaces among the neurofilaments vary between 20 and 50 nm. Some cross-bridges (large arrows) interconnect adjacent neurofilaments. Other cross-bridges (small arrows) that connect adjacent microtubules are fewer and shorter than those detected between neurofilaments. Inset: Mitochondria and microtubules, neurofilaments, or both are often interconnected by cross-bridges (arrows'). (b) A few microtubules (arrows) are observed in these areas, where many organelles (asterisks) have accumulated. Inset: The number of cross-bridges on microtubules appears to be dramatically reduced. Arrows indicate granular material on the microtubules. Scale bars, 0.2 μM.

ments in axons were detected in the region of the lamina cribrosa in specimens in which IOP was maintained at 60 mm Hg for 4 hours. Such changes were observed in some parts of the axons. These findings suggest that the present model can induce damage in only part of the axons in the region of the lamina cribrosa. Similar findings have been reported in primate eyes under the same conditions by several investigators.45 It has therefore been concluded that the lamina cribrosa is a site...
TABLE 2. Frequency of Cross-bridges on Microtubules

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<tr>
<th>Group</th>
<th>Number of Cross-bridges per 100 nm</th>
<th>P</th>
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<tr>
<td>Control (n = 30)</td>
<td>16.35 ± 4.04</td>
<td></td>
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<tr>
<td>Increased IOP (n = 30)</td>
<td>4 ± 2.85</td>
<td>&lt;0.001*</td>
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* Compared with the control group using t-test. n: number of microtubules selected. Values are mean ± standard deviation.

IOP, intraocular pressure.

at which IOP may exert its strongest effect on the optic nerve. The lamina cribrosa of guinea pigs consists of three lamellar sheets in which pores are aligned to form approximately 80 to 100 channels, which transmit the axons of retinal ganglion cells grouped into optic nerve fiber bundles. The lamina also forms a tunnel for optic nerve fibers and provides structural support along the tunnel, which contains the vascular supply in that portion of the optic nerve. It has been reported that high pressure can distort the lamina cribrosa in vitro. The lamina cribrosa in vivo studies have suggested that ultrastructural changes result from pressure-induced distortion of the lamina cribrosa, with direct mechanical compression of axonal cylinders kinked by connective tissues and other structural elements at the optic nerve head. However, it is possible that ischemia induces such a morphologic distortion. The axonal damage may have been caused by a mechanical mechanism in the present study. Although the mechanism of this damage cannot be clearly determined, the QF-DE method confirmed morphologic changes in cytoskeletons in the affected axons. Damage to their axonal cross-bridges was scarcely detectable by electron microscopic in conventional ultrathin sections (Fig. 3a). A previous study has demonstrated that this is probably because their electron-scattering properties are similar to those of the embedding resin.

In the present study, the significant decrease of axonal cross-bridges on microtubules could be related to the significant reduction of MAP 1 protein in axons. Microtubule-associated protein 1, a major MAP in the axons, consists of multiple distinct polypeptides that form flexible and rod-like structures 100 to 200 nm long. Some cross-bridges between microtubules were also formed through MAP 1 projection domains. In previous studies, the colocalization of...
### TABLE 3. Gold Particles of MAP 1 Per Unit Area in Cross Sections of Axons in the Region of Lamina Cribrosa

<table>
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<tr>
<th>Group</th>
<th>Number of Gold Particles/μm²</th>
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<tbody>
<tr>
<td>Control (n = 200)</td>
<td>16.685 ± 2.65</td>
<td></td>
</tr>
<tr>
<td>Increased IOP (n = 200)</td>
<td>5.615 ± 2.19</td>
<td>&lt;0.0001*</td>
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* Compared with the control group using t-test. n: different areas of axons selected. Values are mean ± standard deviation.

MAP, microtubule-associated protein; IOP, intraocular pressure.

MAP 1 with tubulin was demonstrated at the light microscopic level, and an electron microscopic immunocytochemical study showed that the anti-MAP 1 antibody immunostained microtubules or fuzzy structures associated with them. Accordingly, it has been suggested that MAP 1 is part of the elaborate cross-bridge system that is observed in microtubule domains of the axon. The Tau protein is another major MAP in axons that can form short cross-bridges between microtubules. The amount of Tau protein was far less than that of MAP 1 in optic nerve axons. This suggests that the decrease of some axonal cross-bridges on microtubules may also be caused by alteration of Tau protein in the optic nerve after acutely increased IOP.

The morphometric data have demonstrated a significant reduction of microtubules in the axons in which membranous organelles have accumulated. Although the disorganization of microtubules probably involves various unknown factors, MAP 1 and Tau protein are thought to be important factors. They have two domains, one of which binds to microtubules. This domain simultaneously binds to several unpolymerized tubulin molecules, and it is thought that they speed up the nucleation step of tubulin polymerization in vitro. Furthermore, their ability to cross-link microtubules into thicker bundles may also contribute to the stability of microtubules. Without coating the outer walls of microtubules, tubulin subunits are easily dissociated at the middle or end of microtubules. Granular material observed on microtubules in replica micrographs (Fig. 4b), seems to be tubulin subunits dissociating from the microtubules. Based on their sizes, it cannot be completely ruled out that they are cross-bridge structures that have been disrupted. Shimomura and Hirokawa and Sato-Yoshitake et al. have reported that the amount of Tau protein is relatively less than that of MAP 1 in the optic nerve axons. A study on the axons of mice that have no Tau protein has demonstrated that their microtubule stability do not differ significantly in the optic axons; it has been concluded that MAP 1 may compensate for the loss of Tau protein in these axons. Accordingly, it is possible that the disorganization of microtubules in the present study could have been partially caused by the decrease of MAP 1 protein in some parts.

In conclusion, the present study confirmed that ultrastructural changes in the optic nerve axoplasm of guinea pigs result from increased IOP. The sites of this damage were similar to those described in previous studies of primate eyes. After acutely increased IOP, the amount of MAP 1 protein and the number of axonal cross-bridges between microtubules were dramatically decreased.

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**FIGURE 6.** Electron micrographs of immunolocalization of MAP 1 in optic nerve axons with normal (a, b) or increased intraocular pressure (c). Arrows indicate immunogold particles. (a) Longitudinal section of the axon. Inset: immunoneutral section. (b) Cross-section of the axon in conditions of normal intraocular pressure. (c) Cross-section of the axon in conditions of increased intraocular pressure. Scale bars, 0.2 μM.
References


