Inhibition of Retrograde Axoplasmic Transport in Rat Optic Nerve by Increased IOP In Vitro

Jan-Olof Johansson

Horseradish peroxidase (HRP) injected into one lateral geniculate nucleus of hooded rats, is taken up by terminals of the optic nerve and transported retrogradely towards the opposite retina. Four hours after injection, a small portion of HRP has reached the retina. After excision of the bulb and optic nerve and another 4 hours of in vitro transport during which different levels of intraocular pressure (IOP) were set, more HRP accumulated in the retina. The amounts entering in vitro at IOPs of 35 and 50 mmHg were 29% and 76% less, respectively, than at 20 mmHg. The lamina cribrosa of the rat strain used was studied by scanning electron microscopy. It consisted of only one complete laminar sheet, a fact that minimizes optic nerve fiber strangulation by laminar shearing in raised IOP. Thus, moderately increased IOP can inhibit fast retrograde transport of HRP in the optic nerve of the rat by direct mechanical pressure, and does not involve either the blood circulation or a multilayered structure of the lamina cribrosa. Invest Ophthalmol Vis Sci 24:1552-1558, 1983

The possibility exists that inhibition of axoplasmic transport plays a role in glaucomatous nerve damage. It has been found that axoplasmic transport can be blocked by anoxia1 and by graded mechanical pressure on a nerve.2 In the optic nerve, the inhibition of axoplasmic flow produced by an increase in IOP is localized to the region of the scleral lamina cribrosa.3 The pathogenetic explanations for this blockage are closely related to those discussed in the formation of disc cupping and visual field defect.

One hypothesis is that a misalignment of the holes in succeeding layers of the lamina cribrosa occurs when the IOP is elevated, resulting in local nerve fiber strangulation.* To minimize this effect, we used the rat as an experimental animal because it has a poorly developed lamina cribrosa.4

Another long-held view is that optic nerve damage is caused by interference with the blood circulation. By observing transport in vitro, pressure effects on the blood circulation are avoided. The rat optic nerve is thin and its nutritive requirements should be satisfiable at least partly in vitro.

Materials and Methods

Animals

Inbred, hooded male rats (PVG/Mol from Møllegaard, Denmark) weight 165-245 g, were used. Methohexital sodium, 50 mg/ml, 0.35 ml ip usually provided sufficient anesthesia.

Stereotaxic HRP Injection

The animals were fixed in a stereotactic instrument. One microliter of 20% aqueous solution of horseradish peroxidase (HRP) type VI (Sigma) was injected into the right lateral geniculate nucleus through a vertical cannula, outside diameter (od) 0.40 mm. The cannula was left in place for a total of 10 minutes after the start of the injection.

Dissection

At the end of the in vivo phase, four hours after the HRP injection, the animals were given 35 mg methohexital sodium ip. The apex of the heart and the tip of the right auricle were cut off and a cannula introduced into the aorta. About 500 ml of 0.9% saline were perfused for 10 minutes at a pressure of 500 mmH2O. The entire animal, including its head, was cooled in ice water while the perfusate was kept at room temperature.

After the optic nerves had been cut close to the chiasm, the brain was lifted out from the skull cavity and put in 4% formaldehyde for later division and control of injection site. If the cannula track was outside the nucleus the experiment was discontinued.

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The anterior part of the orbital roof was removed and the bulb and optic nerve gently dissected free under ×10 magnification, avoiding more than 90° rotation around the optic nerve. The dural sheath was incised close to the posterior pole and cut circumferentially. Thereafter, the optic nerve could be pulled easily out through the dural canal since the nerve is not adherent to the dura. Immediately before removing the bulb and nerve from their orbital position, a 0.5-mm od cannula with closed tip and a side-opening was introduced through the anterior chamber in a double puncture with the side-opening facing the cornea. The cannula was connected to a 2-ml horizontal graduated pipette that could be placed at different levels above the eye. The entire dissection up to this point was performed with the tissues on a bed of crushed ice and was completed within 15 minutes. The pressure system was filled with degassed buffer solution and set at the pressure to be used during incubation. The experimental and the opposite (control) eye were treated identically.

**Incubation**

Each bulb-nerve preparation was suspended in a separate 50-ml flask with buffer solution pH 7.4. The composition of the solution was (w/v): 1,000 ml 0.9% NaCl; 40 ml 1.15% KCl; 12.7 ml 1.22% CaCl₂; 10 ml 2.11% KH₂PO₄; 10 ml 3.13% MgCl₂·6 H₂O; 210 ml 0.1 M Na₂HPO₄; and 2.57 g anhydrous D-glucose. The solution was gassed with 100% oxygen during the in vitro time of 4 hours. The temperature as a rule was kept at 38 °C with a water bath if not otherwise stated.

**Retinal Preparation**

Special care was taken to prevent the retina from being contaminated by extracellular HRP. At the end of the in vitro period, the optic nerve was separated from the bulb by a microscissors cut, close to the posterior bulb wall. The bulb was opened and divided immediately behind the limbus. The retina was separated carefully from the choroid and the retinal nerve fibers were cut close to their exit, leaving about half a millimeter of papillary tissue. Part of the pigment epithelium often adhered to the retina. Much of this was removed by gentle scraping, followed by rinsing in the buffer solution. The retina was then put into a 25-ml stoppered vessel containing 10 ml of the same solution and air. These bottles, one for each retina, were placed on a mechanical shaker, 80 turns/minute, at 4 °C, overnight.

**Homogenization and Centrifugation**

The retinas were homogenized separately in micro-tissue grinders (glass/glass, 0.2 ml, Kontes), containing 0.2 ml of stock ABTS (see Biochemical assay). The homogenization was done for 90 seconds using a rotation speed of 60 per minute and chilling with ice. No difference in HRP extraction could be found if the time of grinding was doubled or reduced by half. Each retinal homogenate then was washed down into a Beckman 1.5 ml polyallomer microcentrifuge tube, using four portions of 0.2-ml stock ABTS and spun in a Beckman microfuge B for 5 minutes at 4 °C. The supernatant was transferred as completely as possible into a 2-ml glass cuvette (1 cm light path) and another 1 ml of stock ABTS was added. The reaction was started by adding 50 µl of 0.2 M H₂O₂.

**Biochemical Assay**

HRP was quantified by the use of a chromogenic substrate, 2,2’-azino-di-(3-ethyl-benzthiazoline sulfonic acid) (ABTS, Sigma) dissolved in a buffer solution consisting of 0.1 M sodium acetate and 0.05 M sodium dihydrogen phosphate to which concentrated acetic acid is added to make the pH 4.2. ABTS has three levels of oxidation, i.e., reduced, semi-oxidized, and fully oxidized, each having a specific absorbance curve. It is the reduced and semi-oxidized forms that are used in the HRP assay by reading at 420 nm.

In the reduced stock ABTS there is about 1% semi-oxidized ABTS which gives a green color to a millimolar solution. We used 3 mM ABTS. A weaker solution is decolorized by tissue homogenate and there may be a time lag after addition of the H₂O₂ before color appears. Thus, if there is an excess of reducing power in the sample solution, it is impossible to visualize the start of the oxidation reaction caused by HRP. Three millimolar ABTS is well above the Kₘ value of 0.5 mM (pH 4.2; 5.0 mM H₂O₂) which was reported by Gallati. In the present experiments, the reaction velocity was constant between 1 and 5 mM ABTS.

A Beckman DBG recording spectrophotometer equipped with a R 213 photomultiplier was used, with the sample at 25 °C. The reaction was allowed to progress for at least 200 seconds, over which period the graph was essentially linear. The first 200 seconds were used. H₂O served as a reference. A blank control was set for each series of samples. The blank was composed of the stock ABTS solution with H₂O₂, both in the same concentrations as in sample solutions but lacking tissue extract. A small spontaneous increase in absorbance was always noted in the blank, of the order of 0.01 cm⁻¹ per 200 seconds. This was subtracted when net absorbance increase was calculated for each individual sample in the series preceding the blank. The spontaneous increase in absorbance was unaltered when HRP-free retinal extract was added to the blank.
Table 1. Variation of different parameters in the in vitro bath. Oxygen vs nitrogen, uncut optic nerve vs cut, 38 vs 4 C. Different intraocular pressures 20, 35, and 50 mmHg.

<table>
<thead>
<tr>
<th>Gas</th>
<th>N2</th>
<th>N2</th>
<th>O2</th>
<th>O2</th>
<th>O2</th>
<th>O2</th>
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</thead>
<tbody>
<tr>
<td>Nerve</td>
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<td>uncut</td>
<td>cut</td>
<td>uncut</td>
<td>uncut</td>
<td>uncut</td>
</tr>
<tr>
<td>Temperature (C)</td>
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<td>4</td>
<td>38</td>
<td>38</td>
<td>38</td>
<td>38</td>
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<tr>
<td>IOP (mmHg)</td>
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<td>20</td>
<td>20/50</td>
<td>20</td>
<td>35</td>
<td>50</td>
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<td>8</td>
<td>17</td>
<td>20</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Absorbance</td>
<td>Mean</td>
<td>± SEM</td>
<td>Mean</td>
<td>± SEM</td>
<td>Mean</td>
<td>± SEM</td>
</tr>
<tr>
<td>0.0162 (a)</td>
<td>0.0019</td>
<td></td>
<td>0.0233 (b)</td>
<td>0.0025</td>
<td>0.0239 (c)</td>
<td>0.0029</td>
</tr>
<tr>
<td>0.0239 (e)</td>
<td>0.0368 (d)</td>
<td>0.0029</td>
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<td>0.0368 (d)</td>
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Probability: a vs b < 0.025; a vs c < 0.025; b vs c = NS; c vs d < 0.0025; c vs e < 0.05; c vs f = NS; d vs f < 0.01; d vs e = NS; e vs f = NS.

solution. The assay measures down to 10 pg of the HRP used which is five times less than that found in the average retina in the in vitro experiment. Results are expressed in absorbance units which are linearly proportional to the HRP amount at the low concentrations used.

Scanning Electron Microscopy

Small pieces containing the optic nerve head were cut out and treated according to Quigley and Addicks. After critical point drying and gold sputtering they were examined in a Jeol scanning electron microscope type U3 at 20 KV.

Results

HRP Accumulation in the Retina during the in Vivo Phase

To determine the amount of HRP that reaches the retina already during the in vivo phase of the experiment, two procedures were performed. In the first, the optic nerve was cut close to the posterior bulb wall, just prior to cannulation of the anterior chamber. The experiment then followed the same steps as an ordinary test for HRP transport in vitro. The resulting mean absorbance after subtraction of the blank was 0.0239 (Table 1c). This figure is based on a series of nine eyes with an IOP of 50 mmHg and eight eyes with a pressure of 20 mmHg. The pressure difference did not affect the absorbance values. In the second procedure, incubation was under N2 but with the optic nerve connected to the bulb. If the temperature was 38 C, the absorbance values were significantly less than the values with oxygen and cut nerve (Table 1, a vs c). If however, the temperature was lowered to 4 C in the same oxygen-free solution, the absorbance values were approximately the same as found for the eyes in oxygen at 38 C and with cut nerve (Table 1, b vs c).

It is reasonable to believe that the loss of HRP after warm oxygen-free incubation is caused by leakage from damaged nerve fibers in the retina. Much of this probably occurs during the retinal wash phase (in air at 4 C) that follows the in vitro period.

HRP Transport into the Retina against Different Levels of Intraocular Pressure in Vitro

Different IOPs were set during the in vitro phase and the absorbance values of these experiments are listed in Table 1 d, e, and f. The HRP transport that occurs during the in vitro phase is specified by subtracting the average absorbance, obtained during the 4-hour in vivo period, 0.0239, from those listed. The value for 20 mmHg shows that an in vitro transport of HRP (Table 1, c vs d) really exists. There was a significantly higher absorbance in eyes with an IOP of 20 mmHg compared with those with an IOP of 50 mmHg (d vs f). The value for IOP 35 mmHg falls between the other two with no significant difference to either (d vs e and e vs f). Thus, HRP transport in vitro at an IOP of 50 mmHg is only about 25% of that transported at 20 mmHg (f-c)/(d-c).

Scanning Electron Microscopy of the Rat Lamina Cribrosa (Preliminary Observations)

Four eyes were examined with a scanning electron microscope using 300–3,000 times magnification. The rat lamina cribrosa was studied only from the anterior aspect, looking at it from the inside of the bulb. One rather well-developed laminar sheet can be seen in which 20–30 holes with diameters of 5–50 microns are present. Outside this sheet one can see a few fibrous strands that run parallel to the anterior sheet (Figs. 1 and 2).

Discussion

The possibility for HRP to reach the retina through a nonaxoplasmic route after intracerebral injection must be considered. A part of the injected HRP is taken up by the blood and the cerebrospinal fluid (CSF). The blood HRP can penetrate into the optic nerve head by way of the choroid circulation and circumpapillary scleral tissue. The CSF HRP can
migrate through the pia-glial membrane into the optic nerve\textsuperscript{11} and perhaps diffuse along perivascular and perineural channels. We have checked both these non-axonal pathways by: (1) sparing half a millimeter of prepapillary retina during retinal excision and thus excluding the unprotected part of the optic nerve fibers. (2) A control eye, running parallel to each sample eye, was used throughout the experiment. The resulting HRP measured in these control retinas showed consistently low values, at 20 mmHg it was 0.0014 ± 0.0011 (n = 19). This is 3.8\% of the opposite eye. Crossed fibers in the rat\textsuperscript{12} are about 4\%. (3) A second control experiment was performed by injecting 50 \( \mu \)g colchicine intravitreally in a sample eye four days prior to the intracerebral HRP injection. No reaction was found in the sample retina 12 hours after HRP injection. With saline instead of colchicine, a strong retinal HRP reaction was seen. Thus, we also have excluded a possible para-axonal path, that would follow the nerve fibers proportionally to the two eyes from the injection site.

A most realistic problem has been HRP contamination of the retina from dissecting instruments and tissues. This was coped with by an extensive retinal wash as described in the Methods section. Figure 3 shows a light micrograph of a rat retina fixed 20 hrs after contralateral intrageniculate HRP injection. Reaction product is seen in some ganglion cells and portions of the nerve fiber layer. The uneven staining indicates that the intrageniculate HRP is unevenly taken up. Contamination could not give this type of distribution.
On the whole, it is very likely that the listed absorbance values in Table 1 are based on axoplasmically transported HRP with negligible contamination from nonaxoplasmatic paths.

Earlier studies of IOP and axoplasmic transport in the optic nerve have all been done in vivo. The first experiment in this direction was done by Hansson in 1973 who reports an inhibition in the optic nerve of cats and rabbits during increased IOP. Since then, many experiments of similar character have been presented using mainly primates. It has been shown that elevated IOP causes inhibition of axonal flow, ortho- and retrograde at the lamina cribrosa. Many experiments have used quite high IOPs, often close to the mean arterial blood pressure. In a few experiments, a moderately raised IOP (approximately 40 mmHg) has been used. Inhibition of axonal flow has been observed also at this pressure. The present experiments in vitro have demonstrated that fast retrograde axoplasmic transport in the optic nerve fibers of the rat is affected when IOP is elevated. An inhibitory effect was seen at a moderately increased IOP (35–50 mmHg). The effect at 35 mmHg was not in itself statistically significant, but the fact that the value is intermediate between those at 50 and 20 mmHg speaks in favor of a graded effect already at 35 mmHg.

What is the mechanism by which elevated IOP impedes the retrograde axonal transport in the present experiments? Scanning electron microscopic examination ad modum Quigley and Addicks, showed only one perforated sheet crossing the scleral opening in this strain of rat. The size of the laminar holes is about the same for primates and rats. The number of holes is also comparable per unit area. The difference is
primarily in the number of layers. In the rat, the risk for nerve fiber compression caused by laminar hole misalignment in raised IOP must be very small indeed.

As a result of the presence of at least one laminar layer in the rat lamina cribrosa, we can not totally exclude a "pinching off" mechanism as a factor in axoplasmic transport inhibition. The holes through the lamina can perhaps change form upon raised IOP and thereby put pressure on exposed parts of individual fiber bundles.16

If there is no pinching off effect, what physical cause(s) remains to explain the blockage of axoplasmic flow by IOP? There is the pressure as such and the pressure gradient along the nerve as it traverses the lamina cribrosa. The pressure as such could conceivably collapse intraxonal structures such as the smooth endoplasmatic reticulum. Such a collapse can only take place if there is an escape for the content of the collapsed structure. Collapse therefore can occur only at the exit from the high-pressure compartment, where a sufficient pressure gradient exists. The same is true for collapse of the whole axon. These structural changes do not exclude another possibility, viz, that retrograde flow is unable to ascend a steep enough pressure gradient, independent of absolute pressure difference caused by a lack of either force or energy.

In our situation, the "orbital" pressure is zero and the intraneural pressure of the optic nerve must be very low.17 The lamina cribrosa of the rat eye is thin, less than 0.1 mm (own observation). With an IOP of 20 mmHg, the pressure step is close to 20 mmHg but the steepness of the gradient in mmHg/mm length is not known with certainty. In an anisotropic gel as the axoplasm,18 the pressure distribution cannot be easily foreseen. The fact that a defect in the lamina causes a retinal prolapse in rats19 shows, however, that the lamina carries the IOP and that the main pressure change most probably occurs over the laminar layer. This would make the pressure gradient to be of the order of 200 mmHg/mm length already at 20 mmHg.

At the present stage of the investigation, it is still not possible to decide to what extent block of axoplasmic flow is due to structural changes caused by pressure and functional changes caused by the pressure gradient.

Key words: retrograde axoplasmic transport, intraocular pressure, lamina cribrosa, horseradish peroxidase, rat
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References