An Adenosine Agonist and Prostaglandin E₁ Cause Breakdown of the Blood-Retinal Barrier by Opening Tight Junctions Between Vascular Endothelial Cells

Macular edema occurs in several disease processes, but little is known about the mechanisms by which it occurs in any disease process. Previously, the authors showed that intravitreous injection of adenosine agonists, prostaglandin E₁ (PGE₁), or epinephrine in rabbits, causes breakdown of the blood-retinal barrier (BRB) measured by vitreous fluorophotometry. N-ethylcarboxamidoadenosine (NECA), a nonspecific adenosine agonist, and PGE₁ cause much greater breakdown of the BRB than the other agents tested. In this study, rabbit eyes were examined ultrastructurally and electron immunocytochemically for extravascular albumin as an indicator of BRB failure after intravitreous injection of these agents or vehicle alone to investigate potential mechanisms involved in BRB compromise. Six hours after injection, there were significantly more open tight junctions between retinal vascular endothelial cells in NECA-, PGE₁-, and adenosine-injected eyes than in vehicle-injected eyes. Immunocytochemical staining for serum albumin showed that many of the junctions that appeared open were functionally open. Forty-eight hours after injection of PGE₁ (10⁻⁴ mol/l), the percentage of open vascular endothelial cell tight junctions had returned to that of the control specimens, but the opening of tight junctions by NECA (10⁻³ mol/l) did not appear to be reversed after 48 hr. Pinocytotic vesicular transport was prominent in all eyes, and no difference was found between vehicle- and drug-injected eyes. These data suggest that NECA and PGE₁ cause breakdown of the BRB, at least in part, by opening tight junctions between retinal vascular endothelial cells. Invest Ophthalmol Vis Sci 33:1870-1878, 1992

Macular edema is a major cause of visual morbidity that occurs in several different disease processes. In some settings, the cause of macular edema can be deduced from the presence of identifiable structural abnormalities, such as telangiectasia or microaneurysms. However, in other settings, such as the aphakic or pseudophakic cystoid macular edema or diffuse macular edema that occurs in the ischemic retinopathies, structural abnormalities are either not present or appear insufficient to account for the edema. This suggests the possibility that chemical mediators play a role in these types of macular edema.

Adenosine has been postulated to be a mediator in the macular edema of ischemic retinopathies, and prostaglandins may play a role in aphakic macular edema. Intravitreous injection of adenosine agonists or prostaglandin E₁ (PGE₁) in rabbits causes breakdown of the blood-retinal barrier (BRB) as assessed by vitreous fluorophotometry. In both instances, the induced vitreous fluorescein leakage is dose dependent and reversible. In this study, we used immunocytochemical staining for albumin at the ultrastructural level to investigate the mechanisms of the adenosine agonist- and PGE₁-induced breakdown of the BRB.

Materials and Methods

Concentrated stock solutions of drugs were made in dimethyl sulfoxide, filter sterilized, and diluted to desired concentrations in sterile phosphate-buffered saline solution. Control injections were made using vehicle alone.

Pigmented rabbits were used in a manner that conformed to the ARVO Resolution on the Use of Animals in Research. They were anesthetized with xylazine hydrochloride 5 mg/kg and ketamine hydrochloride 25 mg/kg, and their pupils were dilated with phenylephrine hydrochloride 2.5% eyedrops. A 30 G needle was inserted 3 mm posterior to the limbus, and 0.1 ml of vehicle or test drug was injected slowly into the vitreous cavity under visualization by indirect oph-
Fig. 1. Retinal vascular endothelial cell tight junctions. (A) The junction from a control rabbit eye 6 hr after intravitreous injection of vehicle appears closed, with the membranes of adjacent endothelial cells in apposition (original magnification ×50,000). (B) The junction from an eye 6 hr after intravitreous injection of 0.1 ml of $10^{-5}$ M NECA appears open, as a distinct space is visible between adjacent endothelial cells for the entire length of the junction (original magnification ×50,000). (C) The junction from a rabbit 6 hr after intravitreous injection of $10^{-4}$ M PGE, also appears open (original magnification ×62,500). (D) This junction from a rabbit eye 6 hr after injection of $10^{-4}$ M PGE, is considered closed, since a distinct space is not present over the entire length of the junction, but it is likely to be in the process of opening since such a space is evident in the abluminal portion of the junction (bottom) (original magnification ×62,500). Counterstained with uranyl acetate.
thalmoscopy. Injection concentrations of test drugs that previously were shown to cause breakdown of the BRB were used\(^7,8\) (10\(^{-4}\) mol/l N-ethylcarboxamido-adenosine [NECA], 10\(^{-4}\) mol/l PGE\(_1\), 10\(^{-3}\) mol/l adenosine, and 10\(^{-4}\) mol/l epinephrine). The rabbits were killed 6 or 48 hr after injection. Their eyes were removed, opened at the equator, and placed in freshly made paraformaldehyde 4% with sucrose 8.5% and 1 mmol/l CaCl\(_2\) in 0.1 mol/l phosphate buffer, pH 7.4, for 1 hr at room temperature. After this, they were transferred to freshly made paraformaldehyde 4% with 1 mmol/l CaCl\(_2\) in 0.1 mol/l sodium bicarbonate buffer, pH 10.4, and incubated overnight at 4°C. The tissue strips were dehydrated through a series of graded alcohols and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut perpendicular to the retinal layers, examined, and photographed on a Hitachi HU-12A electron microscope at 75 kV, using the high-resolution mode without a counterstain to optimize visualization of the immunoperoxidase reaction product and for electron immunocytochemical staining for albumin.\(^9\)

Immunocytochemical staining was done in covered glass vials using rabbit anti-rat albumin (Nordic, Capistrano Beach, CA) and a peroxidase-antiperoxidase technique adapted for electron microscopy.\(^10\) After washing the tissue with phosphate-buffered saline and CaCl\(_2\), the excess fluid was removed, and the tissue was incubated in powdered nonfat dry milk 2% (Carnation) in 0.05 mol/l Tris-buffered saline, pH 7.6, for 30 min at room temperature. Excess fluid was removed, and the sections were incubated overnight at 4°C with a 1:500 dilution of rabbit anti-rat albumin antiserum and powdered milk 1% in Tris-buffered saline. Control samples were incubated with an equal dilution of normal rabbit serum.

The retinal strips were warmed to room temperature for 1 hr and washed with powdered milk 1% in Tris-buffered saline. All subsequent reactions were done at room temperature. The tissues were incubated in a 1:40 dilution of goat anti-rabbit globulins (Arnel, Brooklyn, NY) in milk 1% solution for 30 min followed by washing with milk 1% in Tris-buffered saline. The sections were incubated in a 1:100 dilution of peroxidase-antiperoxidase conjugated to rabbit immunoglobulins in milk 1% in Tris-buffered saline for 30 min, after which they were washed twice for 10 min each with 0.05 mol/l Tris buffer, pH 7.6. The tissues were incubated in the dark with freshly made 0.07% 3'-diaminobenzidine · 4HCl in 0.05 mol/l Tris, pH 7.6, containing hydrogen peroxide 0.0185% for 15 min, at which time a brown reaction product appeared on the sections reacting with the antiserum. The solution was removed, and the tissues were washed three times for 10 min each with 0.05 mol/l Tris buffer, pH 7.6. The tissues were incubated for 1 hr at 4°C with osmium tetroxide 2% in 0.1 mol/l cacodylate buffer, pH 7.2, and washed twice for 10 min each with the same buffer.

The tissue strips were dehydrated through a series of graded alcohols and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut perpendicular to the retinal layers, examined, and photographed on a Hitachi HU-12A electron microscope at 75 kV, using the high-resolution mode without a counterstain to optimize visualization of the immunoperoxidase reaction product and with a uranyl acetate counterstain for better resolution of the cell junctions. The percentages of open

### Table 1. The effect of intravitreous drugs on vascular endothelial cell tight junctions

<table>
<thead>
<tr>
<th>Agent injected</th>
<th>Time after injection (h)</th>
<th>Number of eyes examined</th>
<th>Number of blocks examined</th>
<th>Total number of junctions examined</th>
<th>Open junctions (mean % ± SEM)</th>
<th>Significance (t-test) (P &lt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>287</td>
<td>5.1 ± 0.94</td>
<td></td>
</tr>
<tr>
<td>N-ethylcarboxamido-adenosine (10(^{-4}) M)</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>186</td>
<td>30.7 ± 6.7</td>
<td>0.001</td>
</tr>
<tr>
<td>N-ethylcarboxamido-adenosine (10(^{-3}) M)</td>
<td>48</td>
<td>3</td>
<td>4</td>
<td>179</td>
<td>32.4 ± 7.7*</td>
<td>0.005</td>
</tr>
<tr>
<td>Prostaglandin E(_1) (10(^{-4}) M)</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>288</td>
<td>27.6 ± 1.5</td>
<td>0.0005</td>
</tr>
<tr>
<td>Prostaglandin E(_1) (10(^{-4}) M)</td>
<td>48</td>
<td>2</td>
<td>5</td>
<td>131</td>
<td>9.7 ± 3.8†</td>
<td>NS</td>
</tr>
<tr>
<td>Adenosine (10(^{-2}) M)</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>73</td>
<td>16.8 ± 4.7</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Rabbit eyes were injected with the specified agents, and then the eyes were removed at the specified times and processed for electron microscopy. Tissue blocks were dissected transversely across the retinal vessels 1 mm from the optic nerve head. Tight junctions were counted in each tissue block and divided into those that appeared open (space present along the entire length of the junction) and those that appeared closed (apposition of the adjacent endothelial cell membranes in at least one area). The results are reported as the mean percentage of open junctions per block (± SEM).

NS = not significant.

* Not significantly different from the 6-hr value.

† Difference from 6-hr value is statistically significant (P < 0.005).
retinal vascular endothelial cell tight junctions were determined by scanning uranyl acetate-stained sections at 17,000x. The entire circumference of all retinal vessels was examined, and the endothelial cell junctions were counted. A tight junction was considered open if a space was seen between endothelial cells for the entire length of the junction as illustrated in Figure 1B. Otherwise, it was considered closed (Fig. 1A). Electron micrographs were taken with SO-163 electron image film (Eastman Kodak, Rochester, NY).

Results

Ultrastructural examination of eyes 6 hr after intravitreous injection of adenosine, NECA, or PGE showed no evidence of cellular damage or toxicity...
that could account for breakdown of the BRB. The retinal pigment epithelium and vascular endothelium appeared normal, but in the eyes injected with NECA or PGE\(_1\), several of the tight junctions between vascular endothelial cells appeared opened (Figs. 1B–C). We quantified this apparent opening of vascular tight junctions, and the results are shown in Table 1. Apparently open tight junctions occasionally occurred in control animals but occurred with a significantly greater frequency after injection of adenosine, NECA, or PGE\(_1\). The increased frequency was particularly striking after injection of NECA or PGE\(_1\). The distribution of apparently open vascular endothelial cell tight junctions was not uniform in rabbit retinas exposed to different agents. In some vessels, nearly all junctions appeared open, but in other vessels, the in-
Integrity of the junctions appeared to be maintained. In addition to a higher percentage of endothelial cell tight junctions appearing open after treatment with NECA or PGE$_1$, many of the tight junctions that were considered closed had only one small contact point between cells that appeared intact. The cell junction in one or both directions from this contact point appeared opened (Fig. 1D). Forty-eight hours after intravitreous injections, the vascular endothelial cell tight junctions that opened in PGE$_1$-treated eyes appeared to have reclosed; those in NECA-treated eyes did not. The frequency of open tight junctions did not appear to be greater in epinephrine-injected eyes than in control eyes.

To determine if the junctions that appeared open were functionally open, we used immunocytochemical staining for serum albumin. An unstained section of a retinal vessel 6 hr after injection of vehicle alone is shown in Figure 2. The dark areas represent the reaction product and show albumin accumulation. A surprising and interesting finding was that, in the rabbit, there is albumin accumulation in the extracellular matrix on the abluminal side of endothelial cells. This is not seen in rats or humans. The probable cause of this extravascular deposition of albumin is illustrated in Figure 3, which shows retinal vessels with albumin in the lumen and along the abluminal surface of endothelial cells. It also shows vesicles at the plasma membrane along the lumen, in the cytoplasm, and along the membrane on the abluminal surface, suggesting the possibility of vesicular transport across the endothelial cells. Examples consistent with vesicular transport were seen in almost all albumin-stained sections examined from both control and injected eyes. Large albumin-filled vesicles (Fig. 4), however, were observed more frequently in the retinal vascular endothelial cells of rabbits treated with NECA, PGE$_1$, or adenosine than in those treated only with vehicle.

Despite the rare occurrence in control eyes of vascular tight junctions that appear open by morphologic criteria (Table 1), we found no evidence of functionally open tight junctions in vehicle-injected eyes. By contrast, eyes examined 6 hr after injection of NECA contained many functionally open tight junctions in
which albumin could be seen throughout their entire length from the luminal to the abluminal surface (Fig. 5A). Functionally open tight junctions were also numerous in eyes 6 hr after PGE$_2$ injection (Figs. 5B–C), but they were rare 48 hr after PGE$_2$ injection, suggesting that the opening of tight junctions was reversible (Fig. 3B). Examination of eyes 6 hr after adenosine (Fig. 5D) or epinephrine injection showed occasional functionally open tight junctions but fewer than those in NECA- or PGE$_2$-injected eyes.

**Discussion**

Although BRB breakdown occurs in several different disease processes, its mechanism in any disease process is not understood. Diffuse leakage across the cytoplasm of BRB-forming cells has been found in experimental diabetes and hypotony. Experimentally induced osmotic stress causes reversible opening of tight junctions, but it has not been determined that it is related to the BRB breakdown leading to macular edema. There is also evidence for opening of endothelial cell tight junctions in diabetic dogs. There is good reason to believe that diffusible factors may play a role in the occurrence of BRB breakdown in some disease processes, but there is no information concerning which factors may be involved in each disease process and how they may produce such an effect.

In previous studies, we found that several agents are capable of causing reversible fluorescein leakage into the vitreous after intravitreous injection of the agent in rabbits. The two most potent agents in this regard were NECA, a nonspecific adenosine agonist, and PGE$_2$.

In this study, we used ultrastructural and electron immunocytochemical techniques to examine rabbit eyes after intravitreous injection of NECA, PGE$_2$, adenosine, epinephrine, or vehicle alone. Six hours after injection, we found morphologic evidence of open tight junctions between vascular endothelial cells, and quantitation of this finding showed it to be significantly more frequent in NECA-, PGE$_2$-, and adenosine-injected eyes. Functional evidence of open tight junctions is much more valuable than morphologic evidence. Usually, such functional evidence is sought by injection of tracer molecules. However, we recently showed that immunohistochemical staining for serum albumin is a useful technique for localizing
BRB breakdown at both light17,18 and electron microscopic5,11 levels. Immunocytochemical staining for albumin showed that many of the junctions that appeared open were, in fact, functionally open. None of the experimental or control eyes showed evidence of open junctions between retinal pigment epithelial cells. No functionally open vascular junctions were seen in vehicle-injected eyes, and only a small number were seen in adenosine- and epinephrine-injected eyes. Forty-eight hours after injection of PGE_2, the number of open junctions was significantly less than 6 hr after injection, but in those injected with NECA, the injected dose may have been too high or 48 hr may have been too brief a period to demonstrate reversibility. These data suggest that NECA and PGE_2 cause breakdown of the BRB, at least in part, by opening tight junctions between vascular endothelial cells.

An interesting ancillary finding of our study was the deposition of albumin along the abluminal surface of retinal blood vessels in the rabbit, a striking difference from rats9 and humans.11 This appears to be caused by a high basal rate of pinocytotic vesicular transport across rabbit retinal vascular endothelial cells that is not seen in rats9 or humans.11 We were unable to detect an increase in endothelial cell vesicular transport in rabbit eyes that had received intravitreous drug injections, but it is possible that such an increase could be masked by the high basal rate of vesicular transport. We also observed the presence of larger pinocytotic vesicles in eyes injected with NECA, PGE_2, and adenosine compared with vehicle-injected eyes, but whether this observation was anything more than a sampling difference could not be determined without evaluating many more eyes using morphometric analysis (this was beyond the scope of our study). Therefore, we cannot exclude the possibility that enhanced vesicular transport contributed to the breakdown of the BRB that occurred after intravitreous injection of the various test agents, but especially for NECA and PGE_2, such a contribution is likely to be minimal.

Previous studies19,20 show that topical administration of PGE_2 to rabbit eyes causes breakdown of the blood-aqueous barrier by opening tight junctions between nonpigmented ciliary epithelium. Thus, the opening of tight junctions may be a mechanism by which prostaglandins cause leakage at various sites in the eye.

Mechanisms of blood-brain barrier (BBB) breakdown have been investigated in several disease processes. Enhanced cerebral endothelial cell pinocytosis has been suggested in the barrier compromise that occurs with seizures21 and, acutely, after cerebral infarction.22 Late leakage into infarcted brain occurs by diffuse spread through damaged endothelium.22 Opening of endothelial tight junctions and enhanced pinocytosis have been observed in acute cerebral ischemia after occlusion and reperfusion in the rat.23 In experimental models of meningitis, opening of vascular tight junctions appears to be the major mechanism of BBB breakdown.24 Diffusible factors have been implicated in the vascular tight junction opening that occurs in meningitis,25 and therefore, these findings may have particular relevance to our study.

Macular edema occurs in several different settings and is a major cause of visual morbidity. Our study supports the concept that diffusible factors may play a role in the development of BRB breakdown in some types of macular edema. It also suggests that reversible opening of tight junctions is one mechanism by which BRB compromise can occur. There is information suggesting that PGE_2 and PGE_2,5,6 are potential mediators in macular edema associated with inflammatory eye disease, but additional studies are needed to determine if other mediators are involved in other disease processes and whether they have common mechanisms of BRB disruption.

References