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Leakage of blood-retinal barrier due to damaging effect of protamine sulfate on the endothelium*

W.-L. Lin

Kresge Eye Institute, Wayne State University School of Medicine, 3994 John R, Detroit, MI 48201, USA

Summary. The effect of the polycations, protamine sulfate and poly-L-lysine, on the blood-retinal barrier of rat retinal vessels were studied by retrograde perfusion through the aorta or by intracarotid perfusion of the polycation followed by the protein tracer, hemoglobin. Protamine sulfate induced swelling of cytoplasmic organelles and diffuse staining of many endothelial cells by tracer molecules which subsequently entered the subendothelial and perivascular areas. Polylysine caused some diffuse staining but no leakage of tracer through the endothelial cell. Occasionally, tracer was found in the interendothelial junction after protamine perfusion. The results indicate that surface charge is important for maintaining membrane integrity of the endothelial cells and that breakdown of the blood-retinal barrier may be due to the cytotoxic effect of protamine on the endothelial cell.

Key words: Blood-retinal barrier – Negative surface charge – Endothelial damage – Protamine sulfate – Hemoglobin

The endothelium of retinal vessels has morphological features similar to the barrier-type endothelium of brain vessels. It is a continuous lining with tight junctions between the endothelial cells and a minimal amount of vesicles or channels for transcellular transport [11, 20, 21]. In addition, the luminal surface of the retinal endothelial cell, like that of other endothelia, bears a net negative charge due to the presence of a glycocalyx coat [11, 17]. Recent studies of brain vessels have shown that alteration of the endothelial surface charge by polycations can affect blood-brain barrier function [3, 7–9, 12, 14, 15, 22, 25]. Little is known about this aspect in the retinal vessels. Pino [16] have shown that removal of heparan sulfate proteoglycan from the luminal surface of the retinal endothelium increases the permeability of the blood-retinal barrier. In this study we examine how polycations affect the permeability of the blood-retinal barrier.

Materials and methods

Wistar rats were maintained in our animal facility on a 12-h on/off light cycle and given food and water ad libitum. Protamine sulfate (from salmon, grade X), poly-L-lysine (hydrobromide, mol. wt. 3,000 and 8,400) and hemoglobin (from bovine, type II) were purchased from Sigma Chemical Co. (St. Louis, Mo). Dulbecco's phosphate-buffered saline (PBS) was obtained from GIBCO Lab (Grand Island, NY).

Anesthetized rats were laparotomized and kept on a warm table at 37° C. The aorta was ligated just above the renal artery. An infusion needle was inserted above the ligation and retrograde perfusion was performed as described previously [10]. The vasculature was flushed with PBS (25 ml) followed by 25 ml of protamine sulfate (500 µg/ml PBS) or poly-L-lysine (1 mg/ml PBS) and 25 ml of hemoglobin (150 mg/ml PBS). Hemoglobin was chosen as the tracer because it does not cause diffuse staining of the retinal endothelium [10]. For intracarotid perfusion, the procedures of Nagy et al. [13] were followed. Briefly, the rats were anesthetized and the right common carotid artery was catheterized after ligation of its proximal part and the external branch at the bifurcation. The perfusion sequence was as follows: PBS, 30 s; protamine sulfate, 2 min; hemoglobin, 2 min. In both procedures all solutions were perfused at 37° C. Control animals were perfused with PBS in place of polycations. In the intracarotid perfused animals, the contralateral eye also served as a control.

After the hemoglobin perfusion, the eyes were enucleated and dissected at the limbus to obtain the posterior eyecup consisting of the retina, choroid and sclera. The posterior eyecup was immersed in 1.25% glutaraldehyde-1% paraformaldehyde-0.1 M cacodylate buffer, pH 7.4, for 1.5 h at 4° C, rinsed several times and kept in cold buffer overnight. The mid-posterior region of the retina was cut into 50-µm-thick sections on a Sorvall TC-2 tissue sectioner and incubated for 1 h at 37° C to reveal hemoglobin activity. The incubation medium contained 30 mg

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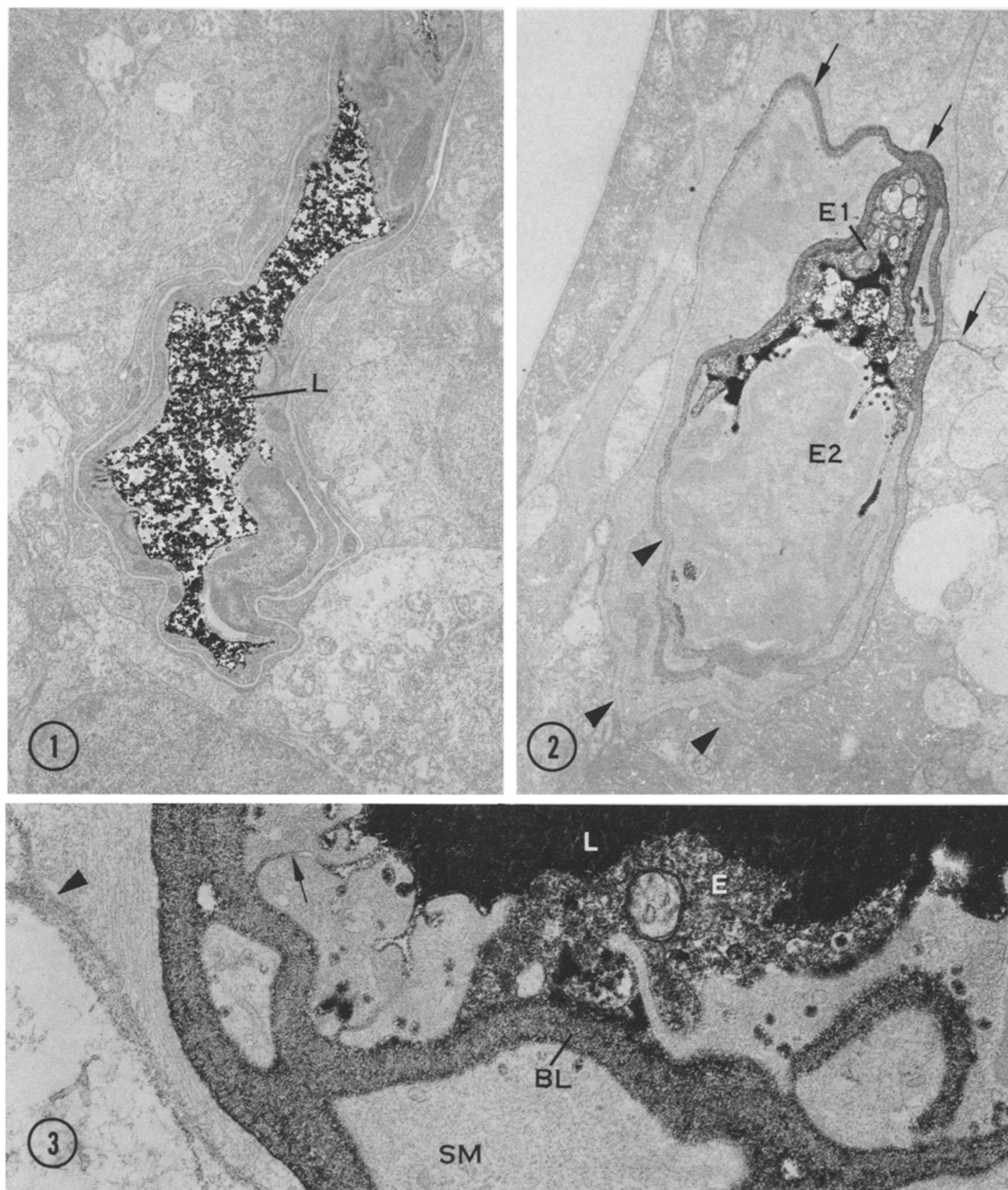

Fig. 1. Retinal capillary after buffer perfusion showing tracer reaction product confined to the lumen (*L*). ×8,400

Fig. 2. Retinal capillary or precapillary arteriole after protamine perfusion. Note deposits of tracer reaction product in the cytoplasm of one endothelial cell (*E1*) but not the other (*E2*). Cytoplasmic organelles in E1 are swollen but impermeable to the tracer. The density of reaction product in the basal lamina and perivascular space subjacent to E1 (*arrows*) is greater than the density in comparable areas subjacent to E2 (*arrowheads*). ×13,100

Fig. 3. Portion of a retinal arteriole after protamine perfusion. Tracer reaction product is found in the lumen (*L*), the cytoplasm of an endothelial cell (*E*), a channel/junction (*arrow*) of a healthy endothelial cell, the basal lamina (*BL*) and perivascular space (*arrowhead*). *SM:* Smooth muscle cell. ×32,500

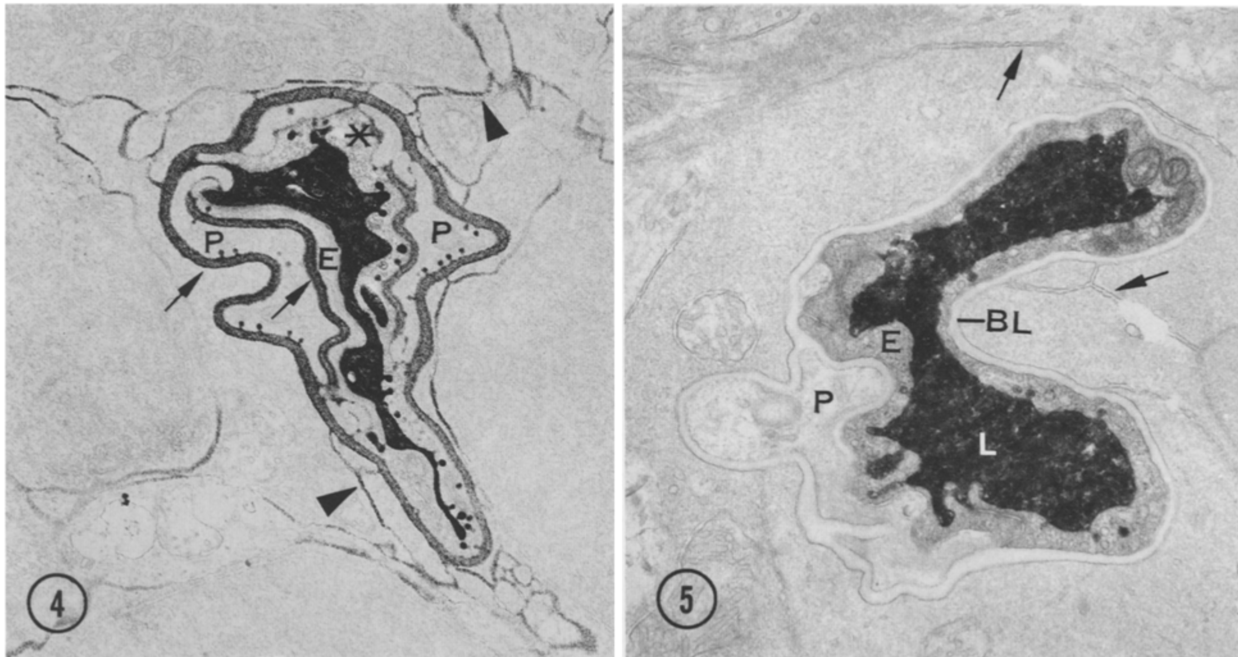

Fig. 4. Retinal capillary from the same animal as shown in Fig. 2, showing more extensive deposits of reaction product in the basal lamina (*arrows*) of the endothelium (*E*) and pericytes (*P*), as well as the perivascular space (*arrowheads*). Note partial staining of the endothelial cell (*asterisk*) by tracer reaction product. ×13,900

Fig. 5. Retinal capillary after polylysine perfusion, showing diffuse staining of the endothelial cell (*E*) but not the pericyte (*P*). The basal lamina (*BL*) and perivascular space (*arrows*) are also free of tracer. ×19,000

of 3,3′-diaminobenzidine tetrachloride in 10 ml of 0.05 M Tris-HCl, pH 7.0, and 0.03% H_2O_2 (final concentration [5]. After incubation the sections were rinsed in Tris buffer, postfixed in 2% O_sO_4 for 1 h, dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812. Thin sections, either stained briefly with lead citrate or unstained, were examined in a Philips 301 electron microscope.

Results and discussion

The ultrastructure of retinal vessels perfused with buffer alone was normal, and reaction product of the tracer was confined to the vascular lumen (Fig. 1). In contrast, protamine sulfate-perfused vessels showed the presence of tracer molecules in the lumen as well as in the vascular basal lamina and the perivascular space of the neuroretina (Figs. 2–4). In these vessels, however, many endothelial cells were diffusely stained by the tracer and the cytoplasmic organelles, although impervious to tracer, were often swollen. Furthermore, tracer molecules appeared to have leaked through the diffusely stained cells and entered the subendothelial area (Figs. 2 and 3). Figure 2 shows differential densities of tracer reaction product in areas subjacent to diffusely stained and unstained endothelial cells. This indicates that the "damaged" cell is the site of barrier breakdown. This finding is further supported by the following observations (data not shown): (1) there is a gradual decrease of reaction product in the extravascular space with increasing distance from the "leaky" vessels, and (2) tracer extravasation is not found at/near the retinal pigment epithelium, which also serves as a barrier tissue for the retina.

Diffuse staining of the endothelial cell was also found in polylysine-perfused vessels. However, the cytoplasmic organelles appeared normal, and there was no leakage of tracer molecules from the vessels (Fig. 5).

Because of their positive charge, polycations such as protamine sulfate can interact with the anionic components of glycoproteins on the surface of the endothelial cells. Several studies of brain microvessels have shown that alteration of the endothelial surface charge by polycations can increase vascular permeability of the blood-brain barrier (see paragraph before "Materials and methods"). In retinal vessels, surface glycoproteins of the endothelial cells were stained by ruthenium red [10], and the presence of sialic acid and heparan sulfate proteoglycan were implicated by lectin-binding and enzyme-digestion studies [16, 17]. Therefore, it is feasible that perturbation of these surface molecules may affect the function of the blood-retinal barrier.

Pino [16] showed that removal of heparan sulfate proteoglycan by vascular perfusion of heparitinase resulted in breakdown of the blood-retinal barrier in retinal capillaries. In his enzyme-treatment model, vesicle/channel formation was the major route of tracer extravasation. In contrast, the present results indicate that neutralization of the luminal surface charge by polycations is insufficient to completely disrupt the blood-retinal barrier. This is indicated by the inability of cationic polylysine to cause tracer extravasation or swelling of endothelial cell organelles. Another difference between the protamine-treatment and enzyme-treatment model is that tracer extravasation occurs mainly through "damaged" endothelial cells of retinal vessels. Similar disruption of the blood-retinal barrier has also been found in monkeys that had lens extraction with vitreous loss and those that had cyclocryotherapy and ocular hypotony, and in kittens with retrolental fibroplasia [23, 24].

Nagy et al. [14, 15] also showed endothelial swelling and diffuse staining of brain endothelial cells after 1 – 2 min of protamine perfusion. They suggested that protamine-induced cell death may be an additional mechanism of tracer extravasation besides opening of the inter-endothelial junctions [15]. Hardebo and Kåhrström [7] suggested that a direct, cytotoxic effect and the positive charge of protamine contributed to opening of the blood-brain barrier. On the other hand, Vorbrodt et al. [25] reported vesicle/channel formation as evidence of increased permeability in spinal cord leptomeningeal vessels after protamine treatment. In the present study tracer reaction product was occasionally found in junction-like structures after protamine perfusion (Fig. 3). Therefore, one cannot exclude the possibility of junctional alteration by protamine. In addition, tracer material was found in cytoplasmic vesicles and plasmalemmal invaginations on both luminal and abluminal fronts of the endothelial cells (Figs. 3 and 4). It has been shown previously that in the rat retina the majority of these vesicles/invaginations are continuous with the plasma membrane and are probably not involved in the transport of macromolecules [2, 4, 6, 11]. On the other hand, Raviola and Butler [18, 19] showed that in monkeys plasmalemmal vesicles are involved in the transport of tracers from the abluminal to the luminal front of the endothelial cells. The role(s) of endothelial vesicles in protamine-treated animals remains to be determined.

Other effects, besides surface charge alteration, may induce barrier opening. For instance, it has been shown that hyperosmolar solutions can open the blood-brain barrier [1]. According to Pino [16], the osmolarity of hemoglobin used in this study is not high enough to cause osmotic opening of the barrier in retinal vessels. Perfusion time is not a major factor either, since both short (intracarotid) and long (aortic) perfusion experiments showed similar results.

The present study suggests that surface charge plays a role in membrane integrity of the endothelial cells in retinal vessels. The nature of the charge-related alteration of the endothelial plasma membrane remains unclear.

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