Unidirectional Transport Mechanism of Horseradish Peroxidase in the Vessels of the Iris

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When horseradish peroxidase (HRP) is introduced into the bloodstream it is retained in the lumen of the iridial vessels. In this paper, we report that when the same tracer is perfused into the anterior chamber of macaque monkeys, it permeates the stroma of the iris and penetrates the lumen of iridial vessels by transcellular vesicular transport. This unidirectional movement of HRP out of the eye is not inhibited by ouabain or fluoroacetate. Invest Ophthalmol Vis Sci 25:827–836, 1984

It is well known that when an electron opaque tracer such as HRP (MW 40,000; radius of an equivalent hydrodynamic sphere 3 nm) is injected into the bloodstream it is retained in the lumen of the closed vessels of the iris.1 The aim of this study was to establish the permeability properties of the iridial vessels when the same tracer is perfused, at controlled intraocular pressure, into the anterior chamber. We have demonstrated that iridial vessels, like retinal vessels,2 are capable of unidirectional vesicular transport of this tracer out of the eye.

Materials and Methods

Animals

A total of four Macaca mulatta and three Macaca fascicularis monkeys were used for these experiments. The animals were young adults of either sex.

Intravenous Injection of HRP

One M. mulatta and one M. fascicularis were injected intravenously with HRP. With the animals under general pentobarbital anesthesia, HRP (Type II; Sigma Chemical Co.; St. Louis, MO) dissolved in phosphate buffered saline at pH 7.3 was injected slowly into the small saphenous vein (0.5 gm/kg body weight). After 30 min and 1 hr, the globes were removed from the animals under deep pentobarbital anesthesia and opened with an equatorial incision. The anterior segment was immersed and trimmed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 0.2% CaCl2. After 4 hr fixation at room temperature the ciliary body and iris were dissected free from the sclera and radial sections, 200 μm in thickness, were prepared with a Smith and Farquhar tissue chopper. These sections were processed for the histochemical demonstration of HRP,3 postfixed in a mixture of equal volumes of 2% osmium tetroxide and 3% potassium ferrocyanide in distilled water and stained en bloc with uranyl acetate. Tissue blocks were finally embedded flat in Epon-Araldite. Thin sections were stained with uranyl acetate and lead citrate. Micrographs were taken with a JEOL 100CX electron microscope at 60 KV (JEOL USA, Electron Optics Div.; Peabody, MA).

Perfusion of the Anterior Chamber with HRP

We perfused the anterior chamber of both eyes of four monkeys (two M. mulatta and two M. fascicularis) under pentobarbital anesthesia. In order to introduce the perfusate and other substances into the anterior chamber, the cornea was transfixed with a single 23-gauge curved needle, which had two holes drilled in the barrel some 3 mm apart and placed about 26 mm from the tip. Between the holes, the needle was crimped to obstruct the bore completely so that with the holes within the anterior chamber, one acted as an inflow opening for the perfusate, while the other formed the exit.4 The needle was incorporated into a continuous circuit through which the perfusion fluid (Bárány’s mock aqueous humor5) was propelled with a delta flow inducer from one chamber of a divided reservoir to the other, the two being separated by a latex membrane. The divided reservoir (separating chamber) closed the
perfusion circuit, preventing mixing of influent and
effluent perfusate and allowed, with a calibration col-
umn containing perfusate, substances to be introduced
to the anterior chamber with no corresponding change
in volume.6 Also incorporated in the circuit was a
strain gauge pressure transducer, which allowed the
intrinsic IOP to be measured continuously or to be
maintained artificially at any desired level. In these
experiments, the separating chamber and tubing were
filled with perfusate alone, while the solution of HRP
was contained in a separate tube, joined in parallel
with the main circuit. The anterior chamber was perfu-
sed with mock aqueous humor for 20 min at 20
mmHg at a rate of 25 µl/min, after which the solution
of HRP (100 mg of HRP in 1.0 ml of mock aqueous
humor) was introduced to the anterior chamber at the
same rate. The exposure to HRP lasted 40 min. In-
troduction of HRP into the anterior chamber of these
macaque monkey eyes did not cause any appreciable
change in the intraocular pressure, which indicated
that the tracer had little direct or indirect pharma-
cologic effect on the uveal vasculature. This was in
contrast to findings of our preliminary experiments in
the rabbit in which intracameral administration of
HRP frequently had caused an increase of intraocular
pressure.

Perfusion of the Anterior Chamber with Ouabain or
Fluoroacetate Followed by Perfusion with HRP

In these experiments, one eye of M. mulatta was
perfused for 20 min with ouabain (10.0 mmol/l in
mock aqueous humor) and the other eye with a solution
of fluoroacetate (5.0 mmol/l in mock aqueous humor).
The rate of perfusion was 25 µl/min. Finally the an-
terior chambers were perfused with HRP as described
in the control experiments.

Fixation of the Perfused Eyes

However rapidly tissue fixation is achieved, changes
in ocular fluid distribution inevitably occur on death.
It was considered desirable to lessen these changes by
maintaining the IOP at its experimental level during
fixation. Fixation by perfusion through the anterior
chamber in vivo is impracticable, because of the im-
mediate irritant effect of the chemicals, causing gross
reflex uveal vasodilatation,7 thereby creating an even
more abnormal physiologic state, irrespective of
whether the IOP is allowed to increase concomitantly
or is kept artificially normal. Moreover, intravascular
or intracameral perfusion of fixative will tend to wash
away the tracer, so fixation by immersion is preferable.
Therefore, the eyes were enucleated rapidly after death
with the needles left in situ, and the IOP was set to
the desired level. In this condition, the entire eyeball
was immersed in fixative for 1 hr and 30 min with the
pressure maintained during this period, after which
the needle was disconnected from the tubing, and the
globe was cut at the equator and reimmersed. We used
the same fixative as had been used for eyes of animals
injected intravenously with HRP. The subsequent steps
for histochemical demonstration of HRP, postfixation
with osmium and preparation of the tissue blocks for
transmission electron microscopy were identical to
those described above.

The investigations utilizing animals, as described in
this manuscript, conform to the ARVO Resolution on
the Use of Animals in Research.

Results

Intravenous Injection of HRP

The iris vessels of M. mulatta and M. fascicularis
are arranged to compose an unusual microcirculatory
network in which all vessels possess a very simple wall
comprising an endothelium and a discontinuous cov-
ering of pericytes. No smooth muscle cells are found
in the walls of iris vessels and no arteriole–capillary–
venule units are present in the iris circulatory pattern.
Vessels of quite different diameters possess morpho-
logically identical walls.8 After intravenous injection
of HRP, the interendothelial clefts of iridal vessels
appeared closed by junctions impermeable to the cir-
culating tracer. Furthermore, the plasmalemmal ves-
cicles of the endothelial cells seemed to be incapable
of transporting any significant amount of HRP across
the endothelium. As a result, even 1 hr after intra-
venous injection, the tracer was retained in the lumen
of the iridial vessels and no reaction product was found
in the basal laminae or the iridial stroma.

Perfusion of the Anterior Chamber with HRP

Light microscopic examination of the iris of eyes to
which HRP had been administered intracameral revealed
that the tracer had readily penetrated the an-
terior surface of the iris and was distributed throughout
the stroma. The brown peroxidase reaction product
was concentrated noticeably around the vessels and
was seen clearly inside their lumina. Furthermore, the
tracer had diffused from the root of the iris into the
ciliary body where it was found both under the ciliary
epithelium and between the fibers of the ciliary muscle.
In these regions HRP also was present in the lumen
of the iridial vessels and no reaction product was found
in the basal laminae or the iridial stroma.

With the electron microscope, HRP was seen to be
heavily bound to the collagen fibrils and to the basal
lamina of both melanocytes and Schwann cells of the
iridal stroma. Its progression toward the posterior
chamber was blocked by the tight junctions that con-
nect the lateral aspect of the cells of the posterior epithelium near their apices. HRP reaction product, however, was especially dense in the basal lamina of the iridial vessels and reaction product was abundant in the vessels’ lumen (Fig. 1). At high magnification, the clefts between adjacent endothelial cells appeared closed by typical tight junctions represented by multiple fusion points between the outer leaflets of adjacent plasma membranes (Fig. 2). A great number of plasmalemml vesicles filled with reaction product was present in the cytoplasm of the endothelial cells. Most of these vesicles were 70–80 nm in diameter and were located toward the abluminal surface of these cells (Fig. 3). Their number could be better appreciated in grazing or tangential sections that included the basal portion of the vascular endothelium rather than in perpendicular sections (Fig. 4). On the adluminal aspect of the endothelium, only a few vesicles filled with reaction product could be seen fused with the cell membrane, presumably in the process of discharging their contents into the lumen (Fig. 5). Frequently, in the cytoplasm of the endothelial cells, two or more plasmalemml vesicles containing HRP could be seen fusing with one another. Vesicles were seen near the Golgi apparatus but reaction product was never found in the cisterns of this organelle (Fig. 6). Vessels of different diameters had basal laminae that were equally stained by the reaction product, and the number and the distribution of tracer-labeled vesicles were similar in all segments of the iridial vascular tree.

Perfusion of the Anterior Chamber with Ouabain or Fluoroacetate Followed by HRP

Perfusion of the anterior chamber with these metabolic inhibitors had no apparent short-term effect on IOP. Neither ouabain nor fluoroacetate affected the transcellular transport of HRP across the walls of iridial vessels. In both cases, reaction product was present in the vessels’ lumen where it was detected easily with both the light and the electron microscopes.

Discussion

The blood–aqueous barrier, which excludes unwanted blood solutes and macromolecules from the aqueous humor, has two components. One is an epithelial barrier localized in the ciliary and iridial epithelia surrounding the posterior chamber; the other is an endothelial barrier that controls the movement of materials from the lumen of the vessels of the iris into the iridial stroma and, hence, into the anterior chamber.1 From the experiments in which HRP was injected intravenously, we can confirm previous observations made in monkeys and mice that the interendothelial clefts of the iridial vessels are closed by junctions that are impermeable to circulating HRP.5,10 This result is also in keeping with the morphological appearance of these junctions in freeze-fractured replicas of the iris where they exhibit a rather high number of branching and anastomosing strands, sealing the intercellular clefts.11 Moreover, as has been reported previously, we have observed that the plasmalemml vesicles of iridial vessels seem to be incapable of transporting any significant amount of blood-borne tracer across the endothelium, and it would be reasonable to suppose that there is little or no movement of blood-borne macromolecules between the vascular bed and the iris stroma.1

However, contrary to that which one might expect, in the experiments in which HRP was perfused into the anterior chamber, not only is it seen to have rapidly permeated the iris stroma, a finding which had been reported previously for a variety of tracers of different sizes and molecular weight,12–14 but it also is seen clearly by light and electron microscopy to be present in the lumina of the iridial vessels in considerable concentration. The penetration into the lumen of these vessels of a variety of tracers including indigocarmine dye, ferritin, carbon, thorotrast, inulin, and dextrans up to 80,000 daltons had been reported previously,18–26 but the present experiments have clarified for the first time the mechanism of this transport. We have established that this tracer crosses the vessel walls contained in plasmalemml vesicles that traverse the cytoplasm, while the interendothelial clefts appear to remain closed by intact tight junctions. Thus, this transport was transcellular rather than paracellular.

Both the unidirectionality and the modality of transport of HRP across the walls of the iridial vessels are not unique to this vascular tree. In a previous report, we described an identical phenomenon in the retinal vessels of monkey and rabbit in which the tracer, injected into the vitreous body, was found in the vessels’ lumen.2 The vessels of the brain, too, can be crossed by interstitially or intraventricularly injected tracer while a blood–brain barrier blocks the passage of intravenously injected HRP in the opposite direction.27,28 Thus, the vessels of the iris, retina, and brain share a common behavior in relation to intravenously or interstitially injected HRP, and in this respect, they are different from other closed vessels of the body. For instance, in the closed vessels of striated muscles, plasmalemml vesicles transport electron dense macromolecules from the interstitium to the blood but, at the same time, they shuttle blood borne macromolecules from the lumen to the basal lamina and, thus, are capable of a bidirectional transport.29–31 We have observed that the number of plasmalemml vesicles labelled with HRP was consistently higher in
Fig. 1. *M. mulatta* iris. HRP perfused into the anterior chamber has penetrated the iridial stroma, is found in the intercellular spaces and is selectively bound to the collagen fibrils (arrow in the upper right part of the figure). The figure shows a capillary located near the anterior surface of the iris. Reaction product is heavily bound to the vessel's basal lamina and is found in its lumen. P is a pericyte. In its cytoplasm, plasmalemmal vesicles loaded with tracer are located on the abluminal front of the cell (×12,000).

The abluminal regions of the cytoplasm than in the luminal regions. This may be explained by the fact that the number of vesicles attached to the abluminal cell membrane usually exceeds that of the luminal membrane.32,33

In addition, HRP was present in the iris stroma and, thus, the first vesicles labeled were the abluminal ones. More difficult to explain was the observation that the concentration of HRP in the plasmalemmal vesicles appeared to decrease from the abluminal to the luminal front of the endothelial cells and only seldom were tracer-labeled plasmalemmal vesicles seen in the process of discharging their content into the lumen. Such a modality of HRP movement is inconsistent with the commonly accepted idea of vesicle transport by the complete translocation of individual vesicles and their contents from one front of the endothelium to the other one. It agrees, however, with an alternative model for vesicle transport in which the steady state transfer of macromolecules occurs during transient fusions between adjacent vesicles in the cytoplasm of the endothelial cells.34

Fig. 2. *M. fascicularis* iris. HRP permeates the basal lamina that surrounds the pericyte and the space between pericyte and endothelial cells. The progression of the tracer toward the vessel's lumen is blocked by a zonula occludens connecting adjacent endothelial cells and represented by fusion points between the outer leaflets of the facing plasma membranes (arrows). The cytoplasm of the endothelial cells contains plasmalemmal vesicles loaded with a moderate amount of HRP (×135,000).
Although plasmalemmal vesicles loaded with tracer could be found occasionally in proximity to the Golgi apparatus, HRP never was observed in the cisterns of this organelle. While in secretory cells, the membranes of discharged secretory granules are recovered by the Golgi apparatus and reutilized in the packing of successive generations of secretory granules, plasmalemmal vesicles of endothelial cells appear to traverse the cytoplasm without interacting with other cell organelles or compartments. They carry substances through the

Fig. 3. *M. mulatta* iris. The transport of HRP from the basal lamina to the lumen of the iridal vessels is carried out by endothelial plasmalemmal vesicles. These are mostly concentrated on the abluminal aspect of the endothelial cell where they appear loaded with a variable amount of reaction product (×69,000).
endothelium by a shortcut between endocytosis and exocytosis, a process which has been termed transcytosis.36

Vesicular transport by endothelial cells is not energy dependent37,38 and, thus, it was not surprising to find that metabolic inhibitors such as ouabain or fluoracetate did not affect the movement of HRP into the iridial vessels' lumina. Recent studies on the topographical distribution of electric charges on capillary endothelium and on its components involved in transport (plasmalemmal vesicles, transendothelial channels, and fenestrae) have demonstrated that on the cell surface of endothelial cells biochemically differentiated microdomains are present.39 These microdomains are characterized by the preferential distribution of anionic sites, some glycoproteins and proteoglycans and correspond to the structures involved in endocytosis and transcytosis. These new findings suggest that the capillary endothelium has differentiated pathways and that the electric charge and chemical composition of macromolecules may be important for their interaction with the endothelial features responsible for their uptake and transport across the capillary lining. In the fenestrated capillaries of the pancreas and intestinal mucosa, plasmalemmal vesicles have the same distribution of chemical residues on the luminal and abluminal fronts of the endothelial cells,40 and this symmetry strongly suggests that a two-way traffic of some macromolecules across the walls is possible in this kind of vessel. By the same reasoning, we might expect that in the vessels of the iris the unidirectional transport of HRP by plasmalemmal vesicles is generated by an asymmetric distribution of chemical residues and electric charges in the plasmalemmal vesicles on the luminal and abluminal fronts of the endothelial cells. This hypothesis, however, awaits further experimental evidence. Additional factors also may contribute to the asymmetry of the iris vessels. The luminal and abluminal aspects of vessels are exposed to two different environments, both with respect to potential chemical mediators and to the direction of hydrostatic and osmotic pressure gradients. The importance of these en-
Fig. 5. *M. fascicularis* iris. Plasmalemmal vesicles loaded with tracer, and discharging their content into the lumen, represent an occasional finding. In this case, the content of the plasmalemmal vesicle adjacent to the luminal plasma membrane of the endothelial cell is very electron opaque (arrow in the lumen). It is not different from the content of the two plasmalemmal vesicles located at the basal aspect of the endothelial cell (arrows in the basal lamina) (×150,000).

Environental factors is unknown, but we cannot exclude the possibility that, as a result of their control, an otherwise symmetrical exchange process may result in asymmetric transport of specific solutes.

In addition to the conventional route, two alternative pathways had been demonstrated for the exit of aqueous humor out of the eye, the uveo-scleral route and the uveo-vortex route. By the term uveo-scleral pathway, we mean the diffusion of fluids from the anterior chamber into the uvea, into the suprachoroidal space, and, hence, across the sclera into the orbital tissues. In the uveo-vortex route, tracers had been seen to penetrate the vessels of the iris, those of the ciliary muscle and the choroidal blood supply concerned with drainage of the anterior uvea. The blood of all these vessels eventually reaches the vortex veins. Clearly, iris vessels belong to the latter system and a few experiments had been done to quantify the passage of tracers from the anterior chamber into the vortex veins. McMaster and Macri, using 14C-inulin, found that 8% of aqueous emerged via the vortex veins in the arterially and intracameraly perfused rabbit eyes. In experiments on Rhesus monkeys in which the anterior chamber was perfused with fluorescein and 125I human serum albumin, the vortex vein outflow accounted for about 10% of the total outflow from the eye. Our experiments do not allow us to establish the amount of fluid that leave the anterior chamber via the iridial vessels. Our main finding is that a one-way traffic of macromolecules takes place between
aqueous humor and the blood in iridial vessels. Since HRP is dissolved in the fluid of the anterior chamber, this result strongly suggests that a continuous, unidirectional bulk flow of aqueous humor takes place across the endothelial cells of iridial vessels by the way of plasmalemmal vesicles (representing the large pore system). It is quite probable, however, that a parallel transport of fluid and ions takes place across the interendothelial clefts of the same vessels. The relative importance of these two pathways, and the role of iris vessels in aqueous humor dynamics, remains to be clarified.

Key words: monkey, iris, electron microscopy, horseradish peroxidase, blood-aqueous barrier, vessels, plasmalemmal vesicles.
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References