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Permeability of Horseradish Peroxidase in the Area of Cold Injury-induced Brain Edema — Time-sequence observation of cerebral micro-blood vessels —

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Abstract A cold-lesion injury of mild degree was induced in the parietal cortex of adult rats by application of a cooled metal plate. Twenty four hours after injury, the ultrastructure of the micro-blood vessels in the edema area was observed, following intravenous administration of horseradish peroxidase (HRP). The circulation time of HRP was restricted to a maximum of 40 min and the transit time of HRP across the cerebral endothelium of the edema area was investigated morphologically. At least 5 min was required for intravenously injected HRP to cross the endothelium in the area of vasogenic brain edema following cold-lesion injury. In addition to the capillaries, the arterioles appeared to play an important role in the increased permeability of cerebral blood vessels after cold-lesion injury. The increased permeability was thought to be due to increased vesicular transport and damage to endothelial plasma membranes, but the priority of these factors could not be determined using this method.

Key Words : Vasogenic brain edema, Cold-lesion injury, Horseradish peroxidase, Endothelial cell

Introduction

Vasogenic brain edema is caused by increased vascular permeability associated with many brain disorders. Experimentally, vasogenic brain edema can be created by cold-lesion injury (1-3). The permeability can be accelerated by (a) opening of tight junctions, (b) increase of vesicular transport, (c) damage of endothelial plasma membranes and (d) creation of transendothelial channels.

In most of previous reports with horseradish peroxidase (HRP) as a tracer, opening of tight junctions and increase of vesicular transport in capillaries have been stressed (4-8). But it has not been established how long the tracer takes to cross the cerebral vascular endothelium in vasogenic brain edema. Also, in the previous studies, observation was often restricted to certain periods, thus failing to reveal the exact movement of the tracers (6,9).

A purpose of this study was to reveal mechanism of brain edema due to investigation of transit time of HRP tracer to across blood-brain barrier and of sequential ultrastructural changes of the micro-blood vessels in the region of vasogenic brain edema.

Materials and Methods

Twenty four male Wistar rats, each weighing 200 g, were anesthetized by intraperitoneal injection of pentobarbital sodium. A metal plate, 3 mm in diameter, cooled to -70 ℃ with a mixture of dry ice and acetone, was applied to the parietal bone revealed by scalp incision, without craniotomy, for 20 seconds, and then the skull was warmed immediately with saline at room temperature. In this condition, the brain lesion was maximal in extent 24 hours after the surgery and its diameter was about 2 mm (10,11). Horseradish peroxidase (Sigma type II) (12), dissolved in saline, was injected intravenously (80 mg/2 ml/rat) 24 hours after induction of cold-lesion injury and allowed to circulate for 3, 5, 10 and 40 min before sacrifice. The circulation time was measured from injection of HRP to the beginning of infusion of the fixative. The animals were perfused through the aorta, first with heparinized saline and thereafter with glutaraldehyde (2.5%) in phosphate buffer (0.1 M, pH 7.4) for 10 min at a perfusion pressure of 120 cm H_2O. Two mm -thick coronal sections including the lesion, were obtained and immersed for 1 hour in the same fixative. The tissues were then washed with sucrose-added phosphate-buffered saline in order to minimize freezing artifacts. Immediately after washing, the tissues were frozen and cut into thin sections (6.0 μm thick). To visualize HRP activity, the sections were incubated with 3,3' -diaminobenzidine tetrahydrochloride; the period of incubation at room temperature was 45 min (13). For electron microscopy, the incubated sections were fixed with 1 hour with O_sO_4 (1%) in phosphate buffer (0.1M, pH 7.4), embedded in Epock 812 and observed with JEM 200 CX electron microscope at 80 kV. If necessary, the ultrathin sections were stained with lead citrate.

For control study, two animals with scalp incision and intact periosteum were observed without HRP injection (sham-treated rats). Another two animals were observed without surgery 5 and 10 min after intravenous administration of HRP in the same dose.

Results

1) HRP circulation at 3 min (n=5):

The arterioles were differentiated from capillaries by the presence of the muscle in their walls.

The capillaries in the edema area showed a slightly thickened endothelium with increased numbers of cytoplasmic vesicles (Fig. 1A). Horseradish peroxidase-positive materials (reaction products) were washed away from the opened vesicles fusing the luminal plasma membrane during perfusion fixation. In some capillaries and arterioles, the reaction products were found in the vesicles but they were not recognized in the perivascular basement membrane.

2) HRP circulation at 5 min (n=5):

HRP reaction products were scantly seen in the basement membrane of both capillaries and arterioles (Fig. 1B, Fig. 2A). Many vesicles containing HRP-reaction products were observed. In both capillaries and arterioles, the reaction products were sometimes observed in the tight junctions, but they were often confined to a limited area around the junctions (Fig. 1B, Fig. 2A). In some capillaries and arterioles, diffuse endothelial cytoplasmic staining with reaction products as well as increased numbers of filled vesicles were also observed (Fig. 3A, B).

3) HRP circulation at 10 min (n=5):

The reaction products were concentrated in the basement membrane and abluminal vesicles. Compared with the findings at 5 min circulation, more extravasated reaction products were recognized (not shown).

4) HRP circulation at 40 min (n=5):

Reaction products had accumulated in the vesicles and basement membrane (Fig. 1C, Fig. 2B), but were observed predominantly in the tight junctions on the abluminal side (Fig. 1C). In arterioles, vesicles containing reaction products were recognized on the abluminal side (Fig. 2B).

5) Control study (n=4):

No apparent extravasation of the HRP reaction products was noticed in the capillaries and arterioles of the rats in the control group. HRP reaction products were seldom recognized in the cytoplasmic vesicles.

Discussion

At least 5 min was required for intravenously injected HRP to cross the endothelium in the area of vasogenic brain edema following cold-lesion injury in rats,

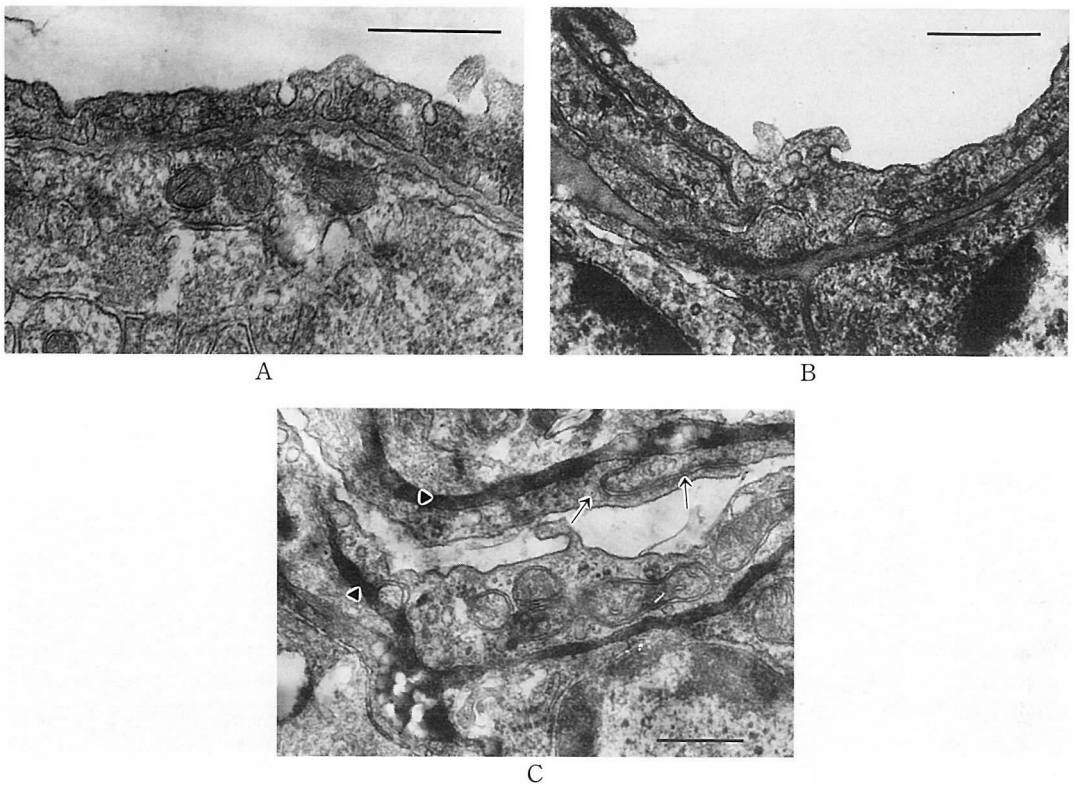
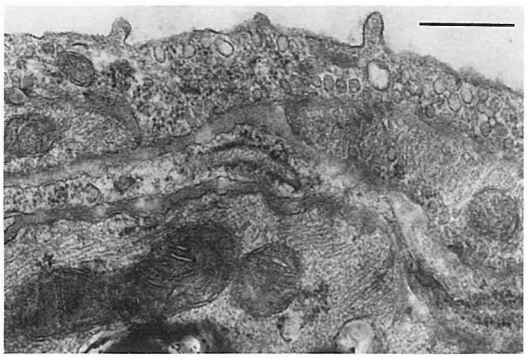


Fig. 1 The walls of capillaries.
A: HRP circulation at 3 min. HRP-positive materials are not present in the vesicles and opened vesicles fused to luminal plasma membrane. The vesicles are also attached to the abluminal plasma membrane. Bar=0.5 μm.
B: HRP circulation at 5 min. Scant reaction products for HRP are present in the basement membrane. Bar=0.5 μm.
C: HRP circulation at 40 min. Dense reaction products for HRP are shown in the basement membrane (arrow head). No reaction products were recognized in the tight junction (arrows). Stained with lead citrate. Bar=0.5 μm.

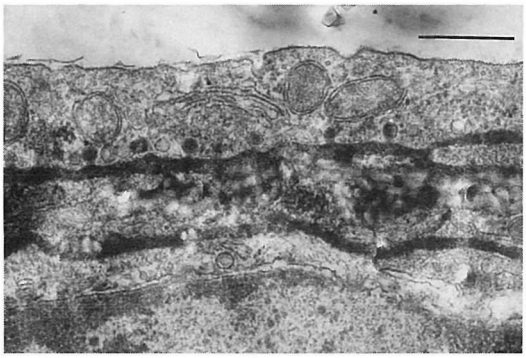
and vesicles in the arterioles as well as capillaries and damage to the endothelial plasma membranes played an important role in increasing the permeability. However, such a time-sequence study has many limitations, which have already been mentioned in the previous report (6).

Under our experimental conditions, a small area of edema was produced (10,11). As the vessels in the center of the lesion were thrombosed or severely damaged, making exact evaluation impossible (14), we studied the cortex of at a depth of approximately 3 mm from the brain surface, i.e. area of pure edema. At such a distance form the center of the lesion, perivascular diffusion from areas of more leaky blood-brain barrier other than the endothelium can be ruled out. And we randomly obtained several 6 μm-thick sections from each cortical section, to certify that the HRP did not come from the distant lesion to enter the basal lamina.

Perfusion fixation was used in the present study for good preservation of the tissue (3). In some experiments, the blood remained in the vessels, suggesting that the perfusion fixation was incomplete. In this situation, it is not clear how long it takes for the fixative to reach a given area, and thus the transit time may be different. For this reason the vessels containing blood were eliminated from the evaluation of the transit time. After comple-

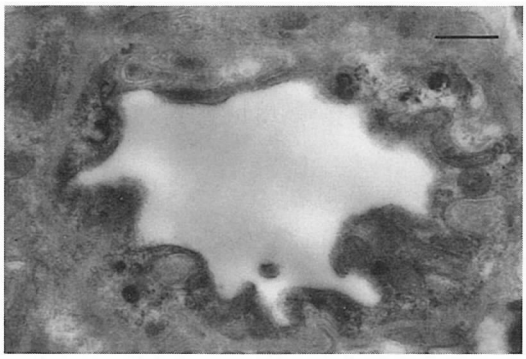


A



B

Fig. 2 The walls of arteriole.
A: HRP circulation at 5 min. Scant reaction products are present in the basement membrane. Numerous vesicles are also visible. Bar=0.5 μm.
B: HRP circulation at 40 min. HRP-containing vesicles are fused to the abluminal plasma membrane. Strong reaction for HRP is visible in the basement membrane and the extracellular space. Stained with lead citrate. Bar=0.5 μm.



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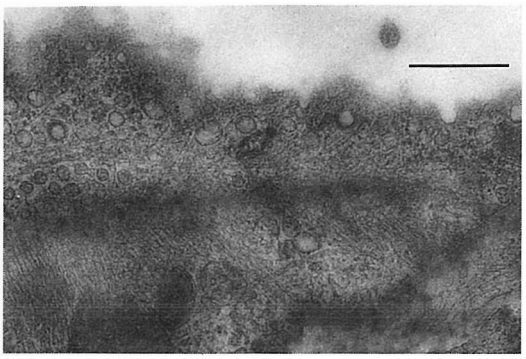

B

Fig. 3 A: capillary, HRP circulation at 5 min. Diffuse endothelial cytoplasmic staining with reaction products is shown. Increased numbers of vesicles are seen in the endothelium. Bar=0.5 μm.
B: arteriole, HRP circulation at 5 min. HRP-positive materials are present in the basement membrane and increased numbers of vesicles are also shown.
Bar=0.5 μm.

tion of perfusion fixation, the injected tracer never penetrates the endothelial junctions nor reaches the basement membrane (15). The washing of blood vessels with saline may withdraw the attached tracer from the opened vesicles fusing with the luminal plasma membrane.

Administered tracers (HRP) circulate in the lumen of the arterioles and capillaries and are then transferred from the luminal side to the abluminal side of the endothelium (normograde transport). In the very short period of time immediately after intravenous injection of tracer, the transfer must be unidirectional. However, once the tracer has crossed the endothelium, it may be transferred from the abluminal side to the luminal side (reverse transport), although in a previous study, this was found not to occur in capillaries (9). A few reports have suggested that the endothelial transit time is about 5 min in acute freeze-injury in mice (4,1). Consequently, most observations have been performed at around 5 min after injection of HRP, although no attempts have been made to the actual transit time itself. In this study, "extravasation" was judged from the staining of the basement membrane and perivascular

space with reaction products, and it was difficult to know very scant staining even without electron staining. In addition, the transfer time for a vesicle across the endothelium must be measured in seconds using the different methods (6) and, in this respect, our method was limited in its sensitivity. However, until 5 min after intravenous injection of HRP, the "extravasated" reaction products were able to recognize morphologically.

In rats, the circulation time for a bolus of solution injected into the carotid artery is approximately 15 seconds (16,17), thus, our observation time was sufficient for HRP to perfuse the entire vascular tree. Indeed, the transit time observed in this experiment included the circulation time of HRP before entering the carotid artery, and may, therefore, have been affected by several factors, such as cardiac output, cerebral blood flow and the anesthetic agents used (17,18).

In vasogenic brain edema, the capillaries are thought to play a major role, but our study confirmed that the arterioles also played an important role in increasing the permeability. In mice, extravasation of HRP can occur in normal cerebral endothelium (15), but no such findings were observed in our study.

It was not determined conclusively which factors were predominant in increasing the permeability because the reaction products were not found in the tight junctions, continuously from the first luminal to first abluminal end. The tight junction is a complex structure, and only limited aspects can be observed in an ultrathin section. So we should have observed them with continuous sections. However, with respect to vasogenic brain edema after cold-lesion injury, vesicular transport is thought to play a more important role.

In our experiments, increased vesicular transport caused by hypertension during the procedure cannot be ruled out, as blood pressure has not been monitored in each experiment (5,15).

In addition to vesicular transport, diffuse endothelial cytoplasmic staining by reaction products, which indicates direct transmembrane and transcytoplasmic passage, was noticed (7,17). In such a state, endothelial injury is severe and the transit time may be altered. But extravasation did not occur when the abluminal plasma membrane was maintained. The transcytoplasmic passage should be taken into consideration when discussing the permeability of the cerebral endothelium after cold-lesion injury.

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