Increased Transendothelial Channel Transport of Cerebral Capillary Endothelium in Stroke-Prone SHR

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SUMMARY Permeability of brain capillaries of stroke-prone spontaneously hypertensive rats (SHRSP) was studied using labelling (horseradish peroxidase) and cytochemical techniques at the cellular level.

In the cerebral capillary endothelium the tracer molecules were quickly transported by abundant transendothelial channels which directly connected the capillary lumen to the subendothelial space. Transendothelial channels are abundant and should be postulated as structural formations engaged in the increased transport of proteins across the capillary endothelium.

Ultracytochemical studies revealed that the channels, bounded by indistinct delimiting membranes, initially had no acid phosphatase activity. With the passage of time, however, the channels showed acid phosphatase activity and were lined with distinct membranes. These observations suggested that the lysosomes might fuse with the transendothelial channels and might play an important part in the transport of macromolecules.

THE PATHOGENETIC MECHANISMS related to stroke have been well understood since the establishment of stroke-prone spontaneously hypertensive rat (SHRSP).¹

The penetration of blood-born macromolecules is highly restricted, because the endothelial cells of small vessels in the brain are connected by tight junctions. In natural diseases or in experimentally induced lesions of the central nervous system injected protein tracers may be observed traversing plasmalemmal vesicles or tubulo-channels.³⁻¹⁰

In addition, ultracytochemical studies of endothelial cells have revealed that lysosomes fuse with microcystotic vesicles.¹¹ Another study has shown that the lysosomes are directly connected to the endothelial tubulo-channels.¹²

In the present experiments, we have used labelling and cytochemical techniques to study the tubulo-channels in the cerebral capillary endothelium of SHRSP with symptoms of stroke. The ultrastructural and ultracytochemical findings clarify the characteristics of the endothelial tubulo-channels. These data are reported herein.

Materials and Methods

Nineteen SHRSP, from 32 to 40 weeks of age, with symptoms of stroke caused by cerebral infarction and edema, were used. Sixteen Wistar Kyoto rats (WKY) from 32 to 35 weeks of age served as controls. Horseradish peroxidase (Type II, Sigma Chemical Co., St. Louis, Missouri) were used as tracer molecules.

All rats in each group were anesthetized with pentobarbital. Either 10 mg/100 g body weight of peroxidase dissolved in physiological saline or physiological saline alone was injected into the femoral vein. The cerebral artery was perfused with a fixative through the left ventricle of the heart ½, 1 ½, 3, 5 and 15 min after the peroxidase injection. The perfusion fixation of 17 SHRSP was carried out at 180 mm Hg pressure for 4–5 min with a 2% formaldehyde — 2.5% glutaraldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) (Karnovsky, 1965),¹³ at room temperature. The perfusion fixation of 10 WKY was performed at 100 mm Hg pressure. The perfusion fixation of 3 WKY was performed at 180 mm Hg pressure. After the perfusion fixation, the brains of SHRSP were carefully removed and fixed with the same preparation for 1 hr at 4°C. The intracranial extracerebral arteries were then dissected as far as possible up to the periphery. Moreover the cerebral cortices from both the hemisphere with cerebral infarction and the normal (contralateral) hemisphere were collected. The dissected arteries and the cerebral cortices were fixed again with a 2% formaldehyde — 2.5% glutaraldehyde mixture buffered with 0.1 M cacodylate buffer for 1 hr at 4°C, and then washed overnight at 4°C in 0.1 M cacodylate buffer. Afterwards, the dissected arteries and the cerebral cortices were cut into small pieces.

Two SHRSP and 3 WKY were fixed by vascular perfusion with aldehydes. Forty-five min after fixation, 10 mg/100 g body weight of peroxidase were injected into the arterial system through the aorta. The brain tissues obtained from this experiment were treated as described above.

These semi-thin sections, obtained from the rats injected with either peroxidase or physiological saline, were washed briefly in cold, distilled water. The sections were then incubated for visualization of either peroxidase activity using a buffered solution containing diaminobenzidine and hydrogen peroxide (Karnovsky, 1967)¹⁴ or acid phosphatase activity according

Stroke, Vol 14, No 4, 1983
to a modified Gomori method (Barka, 1962)\textsuperscript{15} using a buffered solution containing $\beta$-glycerophosphate as a substrate. After incubation, the sections were thoroughly washed in four changes of distilled water over a period of about 10 min.

After post-fixation with 2\% OsO$_4$ buffered with 0.1 M cacodylate buffer for 2 hr, tissue sections were stained with 2\% uranyl acetate in 50\% ethanol for 1 hr at 4$^\circ$C,\textsuperscript{16} dehydrated in graded ethanol and embedded in Epon 812.

Then semi-thin sections were cut on a Sorvall Porter-blum MT-2B Ultratome. The ultra-thin sections were examined under a Hitachi HS-9 electron microscope. Staining of ultra-thin sections with lead citrate was not done in our experiments.

\section*{Results}

\subsection*{I. The Cerebral Capillary Endothelium in WKY}

\subsubsection*{a) Horseradish peroxidase (HRP)}

The cerebral capillaries, incubated in the medium containing diaminobenzidine after the injection of physiological saline only, had no reaction products either in the endothelium or in the pericyte.

In the cerebral capillaries at 3 min after injection, HRP was evident on the luminal surface and within some of the vesicles of the endothelium. Most of the vesicles, which trapped HRP, looked spherical in both transverse and oblique sections of the capillaries. Some of the vesicles, however, were fused, showing raindrop-like formations or chains.

HRP was slowly transported by the vesicles to the subendothelial surface. HRP was detected in small quantities in the subendothelial space at 15 min after HRP injection. Most of the HRP, however, was found within the dense bodies of the endothelial cells or the pericytes.

All the normal organelles were observed within the endothelial cells including mitochondria, rough endoplasmic reticulum and Golgi apparatus. These findings were common to the intracranial extracerebral capillaries and the cortical capillaries.

The same findings described above were also observed in WKY in which perfusion fixation was performed at 180 mm Hg pressure.

In the capillaries fixed for 45 min before the intravascular administration of peroxidase, enormous amounts of HRP were observed within the capillary lumen. HRP was rarely evident on the luminal surface of the endothelium. Luminal pits and plasmalemmal vesicles were free of it. The tracer did not penetrate the endothelial junctions or reach the subendothelial space (fig. 1).

\subsubsection*{b) Acid phosphatase}

In the majority of sections a few acid phosphatase reaction products in the endothelial cells were found. These findings were present in the rats injected with HRP and also in the rats injected with physiologic saline only.

\subsection*{II. The Cerebral Capillary Endothelium in SHRSP}

\subsubsection*{a) Horseradish peroxidase (HRP)}

In the endothelial cells of SHRSP with symptoms of stroke a great number of HRP-filled structures were observed at 30 sec after HRP injection. A great amount of HRP was already present in the subendothelial space and within the dense bodies of the pericytes, and the phagocytes which lay scattered around the capillaries.

In the endothelium of transversely sectioned capillaries HRP was evident in the randomly distributed spherical vesicles. Some of HRP-filled vesicles showed a raindrop-like formation. Serial thin sections revealed that HRP-filled vesicles, arranged like raindrops, were frequently connected.

In the endothelium of obliquely sectioned capillaries HRP was frequently found within the channels which directly connected the capillary lumen to the subendothelial space. In other words these channels spanned the entire width of the endothelium (transendothelial channel). Serial thin sections demonstrated that these channels had a lot of curves and branches and might be described as snake-like in form (fig. 2, 3).

It was noteworthy that abundant transendothelial channels existed near the interendothelial junctions. The transendothelial channels paralleled the interendothelial junctions (fig. 4). Furthermore in the capillaries near the cerebral infarction numerous channels were evident within every endothelial cell (fig. 5).
The plasma membranes of the transendothelial channels, which fixed the boundaries, were frequently obscure, especially in the capillaries at 30 sec after HRP injection. The plasma membranes of the endothelial luminal and abluminal surface were also obscure at the entrance and exit of the transendothelial channels containing HRP. Most of the transendothelial channels, however, were lined by clear membranes at 15 min after injection. The membranes of the plasmalemmal vesicles adjacent to the channels were always clear. Most of the plasmalemmal vesicles adjacent to the channels were free of HRP.

The transendothelial channel measured about 500–1000 Å in width and up to 1.0 μ in length.

In the endothelial cells of SHRSP all the normal cell organelles had decreased in number. A small number of mitochondria, endoplasmic reticulum and vesicles were observed around the transendothelial channels. However, the interendothelial junctions were well-preserved. There was no leakage of HRP through the interendothelial junctions.

In the capillaries fixed for 45 min before the injection of peroxidase, the tracer was consistently present only within the lumen. The tracer was rarely found on the luminal surface of the endothelium. Luminal pits and plasmalemmal vesicles were free of HRP. Transendothelial channels were not present at all.

b) Acid phosphatase

Positive reaction for acid phosphatase activity was limited to the lysosomes of some endothelial cells, pericytes, phagocytes and neurons. Apparently more lysosomes were observed in the endothelium of SHRSP than in the endothelium of WKY, especially at 15 min after HRP injection. Most of the lysosomes appeared spherical and were lined with membranes. Acid phosphatase activity, however, was also observed within the channel-like structures bounded by distinct delimiting membranes. Direct connections were found between the spherical lysosomes and the channel-like structures with acid phosphatase activity (fig. 6). The channel-like structures with acid phosphatase activity were mostly observed in SHRSP injected with HRP.

Discussion

Endothelial vesicles, caveolae and flaps have long been discussed by many authors in relation to vascular permeability. Tubular vesicles, which are formed by vesicular fusion, connecting luminal and abluminal surface have been described in numerous studies.

Arguments, however, against vesicular transport include the following: the mechanism is a relatively slow process requiring energy and the ferrying of independent vesicles across the endothelium is not entirely suf-
Several investigators have searched for an energy independent transport mechanism for blood-born materials. An energy independent system could rapidly transport macromolecules. As a result experiments were performed and transendothelial tubular structures were observed in the brains of hypertensive rabbits. Moreover the tubular structures were found in either unilateral carotid-artery ligation or intracarotid air embolism in Mongolian gerbils and in mechanically injured mouse brains. Hansson et al. suggest that only a fairly limited number of endothelial cells might contain tubulo-channels and that proof of their existence requires careful screening. Lossinsky et al. proposed that channel structures were more readily seen in obliquely sectioned cylinders. Our SHRSP, suffering from spontaneous stroke and cerebral edema, revealed that HRP was frequently found within the tubular structures in the cerebral capillary endothelium. Abundant transendothelial channels containing HRP were evident in the endothelium of obliquely sectioned capillaries. Furthermore HRP was already present in the subendothelial space at 30 sec after injection. Therefore it should be postulated that these channels were structural formations engaged in the increased transport of proteins across the capillary endothelium. Our data also suggests that transendothelial channel trans-
Transport may play an important role in the genesis of cerebral edema. Yamori et al.\textsuperscript{21, 22} demonstrated that the regional cerebral blood flow in the frontal cortex in SHRSP was markedly decreased at the age of 5 and 10 months. Ischemia, resulting from the cerebral blood flow reduction, might be the trigger of the transendothelial channel formations. Cerebral edema, possibly caused by the transendothelial channels, might further reduce the blood flow. The reduction of the blood flow and the channel formation might result in a chain reaction, which could be considered as an important process for causing arterionecrosis, the basic cerebrovascular lesions in SHRSP. These observations, however, are preliminary and need further study to clarify the relationship between the blood flow reductions and the channel formations.

The transendothelial channels described here were frequently observed close to the interendothelial junctions. It is unclear why so many channels are formed near the junctions. The plasma membranes close to the junctions may be the sites where the receptors of the macromolecules are redistributed. At these sites, the caps of the receptors are formed and the deep invaginations develop.\textsuperscript{23-25}

Westergaard et al.\textsuperscript{9, 26} reported that the tracer was consistently observed on the luminal surface of the endothelium and filled some luminal pits in the mice arteriole fixed for 45 min before the injection of peroxidase.

In the capillary of our WKY and SHRSP fixed for 45 min before the injection of peroxidase, the tracer was consistently present within the lumen and was rarely observed on the luminal surface. Transendothelial channels were not found at all. These results indicate that the active, energy-requiring formation of cell organelles is stopped by aldehyde fixation. And this suggests that the active receptors which trap the macromolecules are essential for the transendothelial channel formation.

Ultracytochemical studies revealed that the endothelial cells of SHRSP had larger number of lysosomes than the ones present in WKY. We observed both spherical and channel-like lysosomes which were bounded by membranes.

In general lysosomal content in the endothelium of cerebral microvessels is either sparse\textsuperscript{27} or non-existent.\textsuperscript{28} Following a variety of brain damage, however, the acid phosphatase activity of the brain endothelium has been shown to increase in experimental ani-
mals. Our studies also suggested an increase of lysosomes in the cerebral capillary endothelium of SHRSP.

Increased lysosomal acid phosphatase activity is generally accepted as an enhanced intracytoplasmic digestion of exogenous or endogenous material. Van Deurs demonstrated uncoated micropinocytotic vesicles fused with lysosomes in intraendothelial transport of exogenous material. Lossinsky et al. showed that lysosomes were directly connected to the endothelial channels and that this channel-lysosome connection could play a part in intralysosomal modification of blood-borne material. Our studies showed that both spherical and channel-like structures, bounded by clear membranes, contained apparent acid phosphatase activity. We noted that the delimiting membranes of transendothelial channels were initially unclear, and that the membranes became distinct with the passage of time. These observations suggested that the lysosomes might fuse with the native channels and make the delimiting membrane distinct. In other words the lysosomes might change the energy dependent transendothelial channels into channels of a different nature. The transport of large molecules in the affected channels might be regulated by a lysosomal enzyme.

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